UNIVERSITY OF SOUTHAMPTON

THE FUNCTION AND REGULATION OF RETINOIDS AND THEIR RECEPTORS IN HEPATIC STELLATE CELLS

BY

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ABSTRACT

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Hepatic stellate cells (HSC) are the major body stores of vitamin A which is stored as retinyl palmitate. Following liver injury, HSC proliferate and transform to fibrogenic myofibroblasts as retinyl palmitate is simultaneously hydrolysed to produce free retinoids. These retinoids might influence HSC activation since retinoids effect the proliferation and phenotypical transformation of a variety of other cell types. Consequently, the effects of the retinoid all-trans retinoic acid (RA) on HSC proliferation and expression of HSC activation markers were investigated in vitro. Despite effectively inhibiting HSC proliferation, all-trans RA had no effects on expression of the activation markers gelatinase A, pro-collagen I, tissue inhibitor of matrix metalloproteinases-1 and -2, β_1-integrin and α-smooth muscle actin (α-SMA). However, other retinoids produced by HSC may modulate genes that regulate their fibrogenic and fibrolytic properties. Therefore, reverse-phase high performance liquid chromatography was used to examine retinoids produced by HSC during progressive activation. It was demonstrated that, although retinyl palmitate decreased with activation, HSC generated all-trans RA and other retinoic acid derivatives, including 13-cis RA.

Retinoids mediate their responses via nuclear retinoid receptors. However, little is known about expression of these receptors in HSC. As different retinoid receptors control different functions, the phenotypic response of HSC to retinoids may change during transformation due to different or altered levels of expression of these receptors. The expression of one of these receptors, retinoic acid receptor beta (RARβ), was therefore examined during the transformation of HSC from a quiescent to an activated phenotype. Whilst RARβ mRNA was consistently detected by northern hybridisation in freshly isolated HSC, expression dropped rapidly during HSC activation such that the mRNA became undetectable over 2-12 days of culture activation. In contrast, western blotting data showed that synthesis of RARβ protein actually increased during this time. Immunocytochemistry and immunoprecipitation studies also detected RARβ protein in activated (7 day) HSC. To analyse the DNA binding activity of RARβ, electromobility shift assays were performed. These supported the protein expression data; compared with freshly isolated HSC, RAR-DNA binding activity increased with HSC activation. The results demonstrate that in activated HSC, expression of RARβ protein is dissociated from that of steady state mRNA and RARβ may be subject to extensive post-transcriptional regulation in these cells.

The potential role of RARβ in regulating growth and differentiation of HSC was examined using a RARβ selective antagonist. This antagonist was found to decrease HSC proliferation and expression of α-SMA. This suggests RARβ may be one of the major factors responsible for the progression of HSC activation and a potential, novel HSC directed therapeutic target for liver fibrosis.

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"A PhD is about finding out more and more about less and less until one eventually knows everything about nothing."

(Anon.)

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Emma Jones
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ABBREVIATIONS

AA	Ascorbic acid
Ab	Antibody
AEBSF	4-(2-aminoethyl) benzene sulphonyl fluoride
AM580	Selective RARα agonist
AP-1	Activator protein-1
APS	Ammonium persulphate
ATP	Adenosine triphosphate
AT	All-*trans*
BDEC	Bile duct epithelial cell
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
bp	Base-pair
C	Centigrade
CCl_4	Carbon tetrachloride
CD437	Selective RARγ agonist
cDNA	Complementary deoxyribonucleic acid
cm	Centimetre
CO_2	Carbon dioxide
cpm	Counts per minute
CRABP	Cellular retinoic acid binding protein
CRBP	Cellular retinol binding protein
DAB	3,3'-diaminobenzidine tetrahydrochloride
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DEPC	Diethylpyrocarbonate
dGTP	Deoxyguanine triphosphate
DMEM	Dulbeccos modified essential medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphates
DS	Double stranded
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
ECL	Enhanced chemiluminescence
E.coli	*Escherichia coli*
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EHS	Englebreth-Holm-Swarm
EMSA	Electromobililty shift assay
Expt	Experiment
FCS	Foetal calf serum
FGF	Fibroblast growth factor
g	Gram
g	Gravity
GFAP	Glial fibrillary acidic protein
GIT	Guanidinium isothiocyanate
G-phase	Gap-phase

^{3}H	Tritium
HBS	Hydroxyethyl piperazineethane-sulphonic acid buffered saline
HBSS	Hanks buffered salt solution
HCl	Hydrochloric acid
HEPES	Hydroxyethyl piperazine ethane sulphonic acid
HPLC	High-performance liquid chromatography
14-HRR	14-Hydroxy 4,14 retro-retinol
HSC	Hepatic stellate cell
Ig	Immunoglobulin
IGBMC	Institut de Génétique et de Biologie Moléculaire et Cellulaire
IGF	Insulin like growth factor
IL	Interleukin
I.S.	Internal standard
kb	Kilobase
KC	Kupffer cell
kDa	Kilodalton
lac	Lactose
LB	Luria bertani
LE135	Selective RARβ antagonist
LRAT	Lecithin:retinol acyl transferase
M	Molar
µCi	MicroCurie
med^{-}AA	Medium lacking methionine and cysteine
µg	Microgram
mg	Milligram
µl	Microlitre
ml	Millilitre
µm	Micrometre
µM	MicroMolar
mm	Millimetre
mM	MilliMolar
MMP	Matrix metalloproteinase
M-MuLV	Moloney murine leukaemia virus reverse transcriptase
MOPS	3-(N-morpholino)propanesulphonic acid
M-phase	Mitosis-phase
mRNA	Messenger ribonucleic acid
MT-MMP	Membrane-type matrix metalloproteinase
MW	Molecular weight
N-CAM	Neural cell adhesion molecule
ng	Nanogram
nm	Nanometre
nM	Nanomolar
NP-40	Nonidet P-40
OD	Optical density
ODS-2	Octadecylsilane-2
P	Probability
^{32}P	Phosphorus-32
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction

PDGF	Platelet derived growth factor
pg	Picogram
pH	Negative log of hydrogen ion
pmoles	Picomoles
PMSF	Phenylmethylsulphonyl fluoride
pol	Polymerase
Poly(dI-dC)	Polydeoxyinosinic-deoxycytidylic acid
PSG	Penicillin/streptomycin/gentomycin
PVDF	Polyvinyldifluoride membrane
R	Retinol
r^2	Correlation coefficient
RA	Retinoic acid
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP	Retinol binding protein
RE	Response element
REH	Retinyl ester hydrolase
R-FA	Retinyl ester
RNA	Ribonucleic acid
RNase	Ribonuclease
RNasin	Ribonuclease inhibitor
Ro 28-2249	Selective RARβ agonist
Ro 41-5253	Selective RARα antagonist
RP	Retinyl palmitate
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase-polymerase chain reaction
RXR	Retinoid X receptor
RXRE	Retinoid X response element
^{35}S	Sulphur-35
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate
S.E.M.	Standard error of the mean
α-SMA	α-Smooth muscle actin
S-phase	Synthesis-phase
SS	Single stranded
SSC	Salt sodium citrate
SV40	Simian Virus 40
TAE	Tris(hydroxymethyl)aminomethane acetate ethylenediaminetetraacetic acid
TAF	TBP associated factor
Taq	*Thermus aquatics*
TBE	Tris(hydroxymethyl)aminomethane borate ethylenediaminetetraacetic acid
TBME	Methyl-tert-butyl-ether
TBP	TATA binding protein
TBS	Tris buffered saline
TE	Tris-ethylenediaminetetraacetic acid
TEDTA	Trypsin ethylenediaminetetraacetic acid
TEMED	N, N, N', N'-tetramethylenediamine
TGF	Transforming growth factor
TIMP	Tissue inhibitors of MMPs
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator

Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer ribonucleic acid
TTBS	Tween, tris, buffered saline
TTR	Transthyretin
TUNEL	Terminal deoxy-transferase mediated dUTP-biotin nick end labelling
UK	United Kingdom
uPA	Urokinase plasminogen activator
UV	Ultraviolet
V	Volt
v/v	Volume/volume ratio
w/v	Weight/volume ratio
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

CHAPTER 1

Introduction

1.1. INTRODUCTION TO LIVER FIBROSIS AND LIVER CIRRHOSIS

Hepatic fibrosis is a disease with world wide increasing incidence (Gressner, 1991) and in the United Kingdom (UK) alone accounts for approximately 6000 deaths annually (World Health Organisation, 1989). Fibrosis is characterised as an imbalance in the physiological homeostasis of the synthesis (fibrogenesis), degradation (fibrolysis) and deposition of extracellular matrix molecules (Clement *et al.,* 1993). As the final pathological pathway in the development of many chronic liver diseases, liver fibrosis is caused by a variety of aetiologies including alcohol, persistent viral and helminthic infections, and metabolic liver disorders such as genetic haemochromatosis. Regardless of the target of injury, which varies with the nature of the insult, fibrosis develops only after chronic not acute injury (Friedman, 1993). A single and transient liver tissue injury eventually leads to an almost complete resolution. In contrast, prolonged or recurrent hepatocellular damage leads to enhanced and persistent inflammation and repair with deposition of excessive extracellular matrix molecules resulting in hepatic fibrosis (Friedman, 1993). This may ultimately lead to cirrhosis in which there is replacement of the normal hepatic architecture by fibrous septa and nodules of regenerative parenchyma.

The mechanisms responsible for the increased hepatic connective tissue deposition in cirrhosis still remain unclear, and to date there are few therapeutic strategies for the treatment of liver fibrosis or liver cirrhosis, the stage at which fibrotic liver disease is generally considered to be irreversible.

1.2. NORMAL HEPATIC STRUCTURE

Structurally the liver is subdivided into roughly hexagonal lobules, 1-2 mm in diameter, oriented about a central hepatic vein with portal tracts at the periphery of the lobule (Figure 1.1.). Each portal tract contains a venule (a branch of the portal vein), an arteriole (a branch of the hepatic artery), a duct (part of the bile duct system) and lymphatic vessels.

On the basis of microcirculatory studies, the basic functional unit has been defined as the acinus (Rappaport *et al.,* 1954), a central portal tract surrounded by parenchyma with efferent veins now at the periphery. Blood flows from the hepatic arteriole and portal venule into the sinusoids and then drains into two or three terminal branches of the central vein. Arbitrarily, the liver acinus is divided into three zones; zone 1 (periportal zone) is closest to the arterial

and portal supply, zone 3 (perivenular zone) abuts the central vein and zone 2 (mid-zone) includes the intermediate hepatic parenchyma. Since cells in zones 2 and 3 receive blood that is progressively depleted of oxygen and nutrients, cells in different zones of liver acini may exhibit structural and functional heterogeneity (Gumucio and Miller, 1981).

Figure 1.1: Diagrammatic illustration of the hepatic lobule arranged around a single central (hepatic) vein into which its blood flows

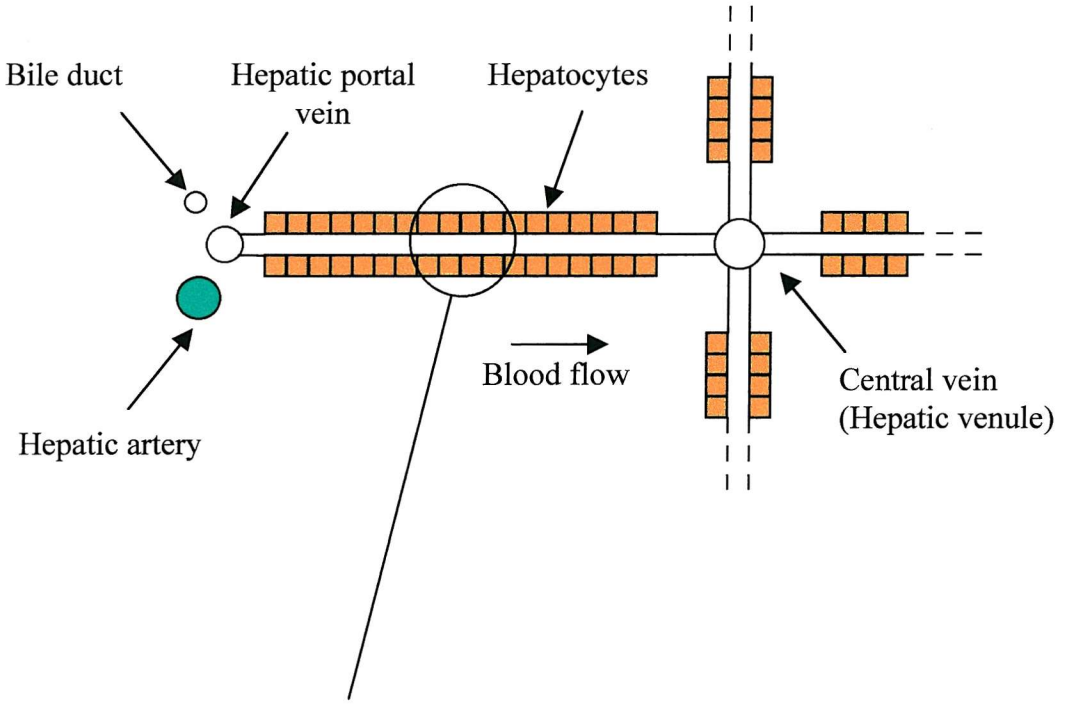
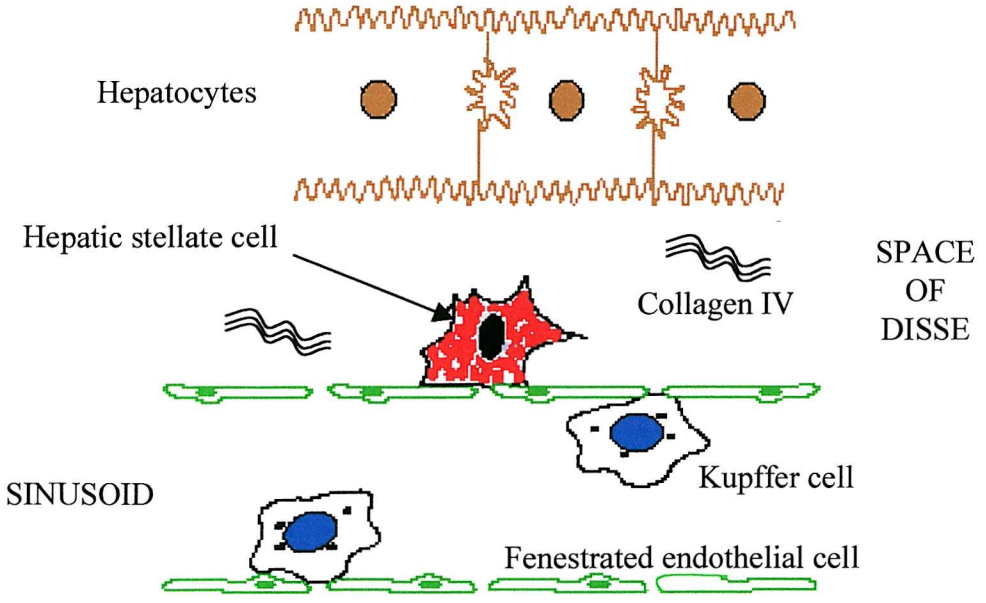


Figure 1.2: Cells in the space of Disse in normal liver



Approximately 80% of the volume of human liver consists of parenchymal cells or hepatocytes which carry out almost all liver synthetic and degradative functions. These polyhedral, epithelial cells are grouped in interconnected plates which pass radially from the portal tract to the efferent vein along hepatic sinusoids. The sinusoids which are larger than ordinary capillaries and more irregular in shape, are lined with endothelial cells which have multiple fenestrae and a low-density basement membrane (Figure 1.2.). This facilitates an efficient exchange of metabolites between the sinusoidal blood and the hepatocytes (Bouwens *et al.,* 1992). Adhering to the endothelial lining, preferentially in the periportal region of the sinusoid, are a large stable population of resident liver specific macrophages, the Kupffer cells. The Kupffer cells have a number of functions including endocytosis, antigen processing, secretion of bioactive factors, cytotoxicity and the regulation of metabolic and proliferative activities of hepatocytes and other non-parenchymal cells (Bouwens *et al.,* 1992). Large granular lymphocytes (termed pit cells) also lie on the sinusoidal domain of the endothelial cells and play a major role in clearing opsonized particles and tumour cells from the blood. The fourth type of sinusoidal cell is the hepatic stellate cell (also called perisinusoidal cell, Ito cell, lipocyte or fat-storing cell). Hepatic stellate cells (HSC) are located within the space of Disse, the space between the endothelial cells and the hepatocytes and have cytoplasmic extensions encircling the adjacent sinusoid. These cells represent approximately 5-8% of all human liver cells in a ratio 3.6-6 cells/100 hepatocytes (Gressner, 1994) and have a central role in the development of liver fibrosis (Figure 1.2.). They are discussed in more detail in section 1.7.

1.3. THE EXTRACELLULAR MATRIX IN NORMAL LIVER

The extracellular matrix is an active determinant of normal hepatocellular function (Bissell *et al.,* 1987) and is in a state of continual turnover; in normal tissues there is a net balance between fibrogenesis and fibrolysis so that the quantity and quality of extracellular matrix remains in balance. The extracellular matrix not only provides structural support but also induces polarisation of cells, and regulates cellular morphology and a diversity of cellular activities including proliferation, motility, gene expression and differentiation (Senoo and Hata, 1994). This specialised tissue component can be divided into the pericellular matrix interacting with integral cell membrane components, the classic interstitial matrix structuring the intercellular spaces, and the basement membrane, supporting epithelial, endothelial and certain mesenchymal cells.

In normal liver, there is a relatively small amount of extracellular matrix, constituting less than 0.6% of liver wet weight (Gressner and Bachem, 1990). However, it has a prominent role in liver fibrosis and cirrhosis. The extracellular matrix components of the liver can be divided into three main categories: proteins, glycoconjugates and glycosaminoglycans (Table 1.1.). The major constituents of the extracellular matrix are collagens, a family of proteins characterised by a triple helical structure and comprising of at least 19 types. The fibril forming collagens types I and III are the predominant collagen types (approximately 60% of total collagen) and are found in portal tracts and the space of Disse (Rauterberg *et al.,* 1981). The collagenous matrix serves as a scaffolding that supports the parenchyma and maintains hepatic integrity. Collagens types II and VII have not been found in the liver. In normal liver collagen synthesis and turnover are balanced, keeping the collagen content fairly stable at 5-10% of the protein of normal liver (Schuppan, 1990).

Although blood vessels and bile ducts within the liver have a basement membrane, only an attenuated extracellular matrix can be detected in the space of Disse, the site where liver fibrosis is initiated (Bissell, 1990). Perlecan and some collagen type IV and laminin can be detected in the space of Disse but entactin has not been detected, suggesting that this component may be needed for the assembly of a basement membrane (Martinez-Hernandez and Amenta, 1995).

Table 1.1: Main components of normal hepatic extracellular matrix
Adapted from Burt (1993).

Proteins
• *Collagens* 'Interstitial' collagens - types I, III and V Basement membrane collagen - type IV 'Short chain' or microfilamentous collagen - type VI
• *Elastin*
• *Fibrillin*
Glycoconjugates
• *Structural glycoproteins* Fibronectin Vitronectin Laminin Thrombospondin Tenascin Undulin Nidogen (entactin) SPARC (osteonectin)
• *Proteoglycans*
Glycosaminoglycans
• *Hyaluronic acid*

1.4. THE EXTRACELLULAR MATRIX IN FIBROTIC LIVER

Liver fibrosis results from matrix synthesis overwhelming the normal matrix-degrading capacity of the liver. The extracellular matrix components are increased in quantity, redistributed in different anatomical areas and altered in their relative ratios. Although the distribution of the extracellular matrix molecules varies according to the site and aetiology of the injury, the order of deposition of the matrix proteins are similar with different injuries. Initially, there is a marked accumulation of fibronectin in the space of Disse which precedes increased deposition of type I and III collagen (Hahn *et al.*, 1980; Martinez-Hernandez, 1985). This suggests it may provide a scaffold for subsequent deposition of collagen. With progressive disease the various types of collagens increase disproportionately. The predominant early change is an increase in collagen III followed later by a shift towards collagen type I, which can comprise up to 70% of the extracellular matrix [Table 1.2. (Schuppan, 1990)]. Later there is an associated deposition of elastin and undulin (Bedossa *et*

al., 1990; Schuppan, 1990). The deposition of the macromolecules, in particular the interstitial collagens (types I, III and V), may lead to constriction of the sinusoidal lamina and disruption of intrasinusoidal blood flow causing the development of portal hypertension.

In chronic liver disease, there is also an increase in the deposition of collagen type IV and laminin (Burt *et al.,* 1990). This coincides with the development of an identifiable basement membrane in the space of Disse (Hahn *et al.,* 1980). The size and number of endothelial fenestrae decrease, a state termed 'capillarization of the sinusoids' because the altered structure of the sinusoids resembles that of capillaries (Schaffner and Popper, 1963). This produces an increased diffusional barrier and may, by interfering with the transport of macromolecules between sinusoids and hepatocytes, contribute to deteriorating liver function.

The changes in extracellular matrix composition lead to formation of a new biochemical microenvironment. The presence and persistence of particular extracellular matrix components may modulate cell growth, migration and gene expression through interaction with cell adhesion molecules/integrins and indirectly through binding of certain mitogens in their active and inactive forms (Ruoslahti and Pierschbacher, 1987; Friedman *et al.,* 1989).

Table 1.2: Percentage of principal collagen subtypes in normal and cirrhotic human liver * *Taken from Schuppan (1990).*

Collagen Type	Normal	Cirrhosis
I	40-50	60-70
III	40-50	20-30
IV	1	1-2
V	2-5(?)	5-10(?)
VI	0.1	0.2

**Pathologic values are predominantly from livers with alcoholic cirrhosis.*

The nature of the liver cell type or types responsible for the excessive synthesis of extracellular matrix components in hepatic fibrosis is controversial. Hepatocytes, sinusoidal endothelial cells and HSC are all capable of synthesis and secretion of extracellular matrix components (Table 1.3.). However, a consensus view at present is that the HSC is the predominant cell type responsible for deposition of extracellular matrix molecules in normal and damaged liver tissue. This conclusion however, must be taken cautiously since it is based mainly on *in vitro*

studies, which of course do not allow a simple extrapolation to the *in situ* function of the cells. Furthermore, in the liver the hepatocyte cytoplasm volume surpasses HSC cytoplasm volume by several orders of magnitude (Martinez-Hernandez and Amenta, 1995). Therefore even a small contribution from hepatocytes could represent the major contribution. Additionally, the extracellular matrix molecules may be synthesised by other liver myofibroblasts that are not derived from HSC (Knittel *et al.,* 1999).

Table 1.3: Profile of extracellular matrix synthesis by liver cells
Adapted from Martinez-Hernanadez and Amenta (1995).

Extracellular matrix protein	Hepatocytes	Hepatic stellate cell	Endothelial cells	Kupffer cells
Collagen type I	+	+	-	-
Collagen type III	+	+	-	-
Collagen type IV	+	+	+	-
Laminin	+	+	+	-
Fibronectin	+	-	-	-
Entactin	+	+	-	-

1.5. MATRIX METALLOPROTEINASES

While synthesis and assimilation of matrix proteins is important, accumulating evidence indicates the pathogenesis of liver fibrosis is characterised by changes in the pattern of extracellular matrix degradation in addition to synthesis.

The physiologically relevant mediators of extracellular matrix turnover are the matrix degrading proteinases (metalloproteinases or MMPs). The MMPs which degrade matrix proteins, have been implicated in a wide variety of normal physiological processes such as morphogenesis and embryonic development and in pathological conditions such as tumour invasion, wound healing and arthritis (Murphy and Hembry, 1992). The MMP family are zinc and calcium dependent endopeptidases (Murphy *et al.,* 1991). These enzymes function at neutral pH and have the combined ability to degrade various components of the extracellular matrix, including fibrillar and non-fibrillar collagens. To date 21 members of the MMP family have been cloned and sequenced and their biochemical properties described. These have been grouped into four broad categories according to their major substrates (Table 1.4.).

Table 1.4: Nomenclature and substrate specificity of metalloproteinases
Adapted from Arthur (1995).

Nomenclature	MMP Number	Cellular Origin in liver	Substrate Profile
Collagenases			
Interstitial collagenase	MMP-1	HSC	Collagens III > I, II, VII, X
Neutrophil collagenase	MMP-8		Collagens I > III, II
Collagenase 3	MMP-13	HSC (in rodent)	Collagens III > I , II, VII, X
Gelatinases			
Gelatinase A (72 kDa type IV collagenase)	MMP-2	HSC	Collagens IV, V, VII, X, XI, Denatured collagens (gelatins), elastin
Gelatinase B (92 kDa type IV collagenase)	MMP-9	KC	As for MMP-2
Stromelysins			
Stromelysin-1 (Proteoglycanase, transin in rodent)	MMP-3	HSC	Collagens II, IV, IX, X, XI, laminin, gelatins, proteoglycans, fibronectin, casein, pro-collagenase, pro-gelatinase B
Stromelysin-2	MMP-10		As for MMP-3 but lower activity
Matrilysin (PUMP-1)	MMP-7		As for MMP-3, also elastin
Stromelysin-3	MMP-11		Not known
Others			
Metalloelastase	MMP-12		Elastin, fibronectin
Membrane-type (MT) MMPs, e.g. MT1-MMP	MMP-14	HSC, hepatocytes (?)	Activation of pro-gelatinase A

Key to abbreviations: HSC = hepatic stellate cells, KC = Kupffer cells

The interstitial collagenases have the most specific substrate profile of the MMP family. These enzymes are central to the process of remodelling of fibrotic tissue as they cleave the fibrillar collagens at a single site, rendering the collagen susceptible to degradation by other

proteases, including MMPs to which they were previously resistant (Nagase *et al.*, 1991). The gelatinases are significant for their important role in degrading basement membrane, type IV collagen in the space of Disse. In addition, they may act synergistically with interstitial collagenases to degrade denatured collagen/gelatin substrates (Nagase *et al.*, 1991). The final major group of the MMP family is the stromelysins. These have a broad substrate specificity including proteoglycans and glycoproteins such as laminin and fibronectin (Nagase *et al.*, 1991). They also activate both pro-collagenase (Murphy *et al.*, 1987) and pro-gelatinase B (Nagase *et al.*, 1991).

Since active MMPs degrade a plethora of substrates, their activity is stringently regulated at several levels: by regulation of gene transcription, by activation of pro-enzymes to catalytic forms and by inhibition of active or pro-MMPs by various types of serum and tissue specific inhibitors, including α_2-macroglobulin and members of the family of tissue inhibitors of MMPs (TIMPs). Regulation of MMP gene transcription occurs via a combination of growth factors, cytokines and retinoids (Table 1.5.). In fibrotic liver, MMP genes prove to be differentially expressed, providing the possibility of preferential degradation of certain matrix molecules (Milani *et al.*, 1994; Benyon *et al.*, 1996).

A second level of control resides where all MMPs are secreted as latent pro-enzymes which must be processed extracellularly to active forms to initiate matrix degradation, either through removal of the pro-peptide by proteolytic enzymes or by autocatalytic cleavage. The best characterised mechanism of MMP activation involves urokinase- or tissue-plasminogen activator (uPA or tPA) which converts plasminogen to plasmin. Plasmin can directly degrade matrix proteins but an additional role for plasmin in matrix degradation is mediated via the activation of pro-stromelysin-1 to active stromelysin (MMP-3) and pro-collagenase to active collagenase (MMP-1) (He *et al.*, 1989). Plasminogen activator inhibitor-1 (PAI-1) may also be produced by cells that synthesise MMPs and this plays an important regulatory role by inhibiting this cascade mechanism (He *et al.*, 1989). Other proteolytic enzymes which are involved in promoting MMP activation include neutrophil elastase, cathespin G (Okada and Nakanishi, 1989) and mast cell tryptase (Gruber *et al.*, 1989).

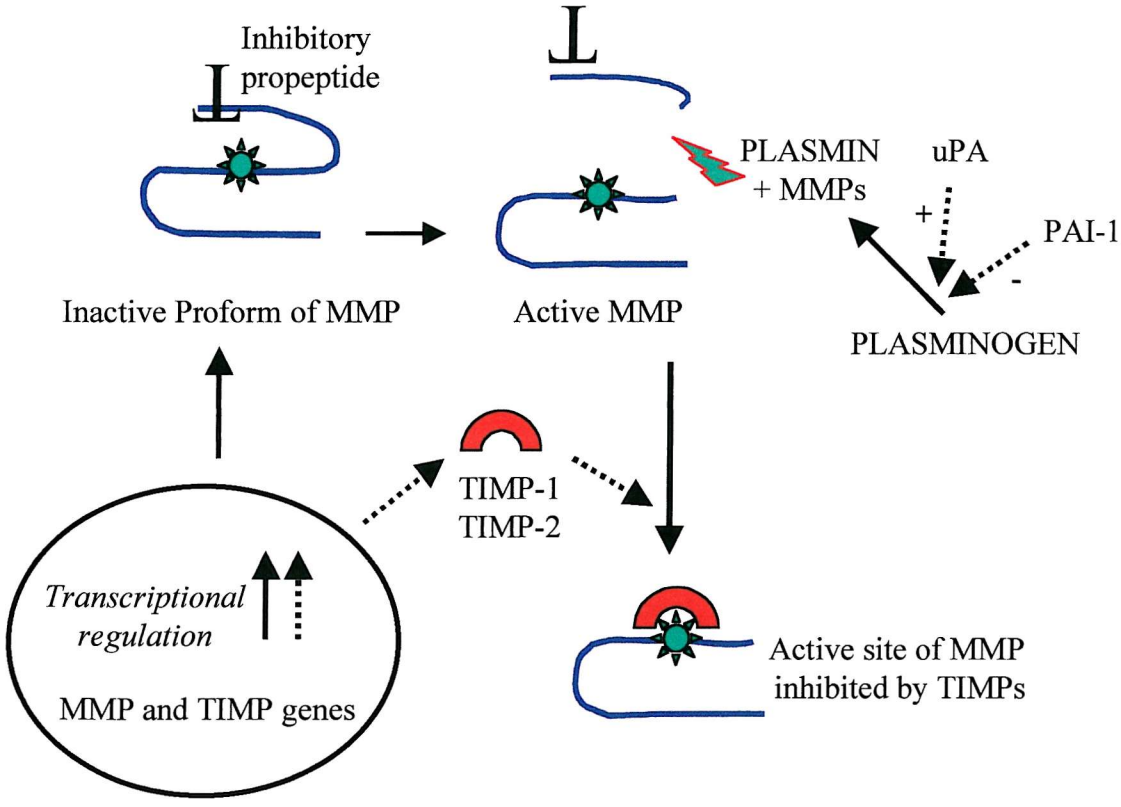
Pro-gelatinase A appears to have a unique mechanism of activation which involves binding to membrane-type matrix metalloproteinases (MT-MMP), a novel group of cell surface MMPs of which 5 members have been described to date. This mechanism of activation is discussed in

further detail in section 1.6. In addition to activating pro-gelatinase A, MT1-MMP also digests interstitial collagens (Ohuchi, 1997), suggesting that MT1-MMP has a dual role in pathophysiological digestion of extracellular matrix.

1.6. TISSUE INHIBITORS OF METALLOPROTEINASES AND LIVER FIBROSIS

Inhibiting active MMPs is a further mechanism of regulating the activity of MMPs. Four TIMPs have been identified to date (TIMP-1, -2, -3, -4). Binding of TIMPs to MMPs occurs in a stoichiometric manner and is irreversible under physiological conditions. The TIMP exerts its effect by binding to the catalytic site of MMP in a non-covalent manner and effectively inhibiting their degradative activity, although the precise mechanism for this effect is poorly understood (Figure 1.3.). Tissue inhibitor of MMP-1 and TIMP-2 inhibit the active forms of many MMPs and TIMP-3 and TIMP-4 probably have a similar spectrum of activity. However, some of the TIMPs have specific properties or specific relationships with individual pro-MMPs, for example pro-gelatinase A only interacts with TIMP-2.

Figure 1.3: Scheme of factors regulating metalloproteinase activation



The TIMPs are regulated at the level of transcription by cytokines and growth factors, several of which mediate MMP expression (Table 1.5.). Expression can be co-regulated, for example basic fibroblast growth factor (FGF) and epidermal growth factor (EGF) increase both interstitial collagenase and TIMP-1 gene expression, or inversely co-regulated, for example transforming growth factor-β_1 (TGF-β_1) decreases TIMP-2, interstitial collagenase and stromelysin gene expression but increases expression of TIMP-1 and gelatinase A (Arthur, 1994). Retinoids also inhibit expression of interstitial collagenase and stromelysin but increase expression of TIMP-1 (Wright *et al.*, 1991; Bigg and Cawston, 1995).

Table 1.5: Factors involved in regulating expression of metalloproteinases and their inhibitors *Taken from Arthur (1994).*

	Metalloproteinases	Metalloproteinase Inhibitors
TGF-β_1	Interstitial collagenase ↓ Stromelysin ↓ Gelatinase A ↑	TIMP-1 ↑ TIMP-2 ↓
Retinoic acid	Interstitial collagenase ↓	TIMP-1 ↑
Interleukin-1 (IL-1), tumour necrosis factor-α (TNFα), platelet derived growth factor (PDGF)	Interstitial collagenase ↑	-
EGF, basic FGF	Interstitial collagenase ↑	TIMP-1 ↑

In addition to direct binding to the catalytic site, it is now evident that TIMPs may also bind to pro-MMPs. This was first described for pro-gelatinase A which binds TIMP-2 and is often secreted from cells as a pro-enzyme-complex (Howard and Banda, 1991). This then interacts with MT1-MMP and the tri-molecular complex causes pro-gelatinase A activation (Howard *et al.*, 1991). In excess, TIMP-2 inhibits pro-gelatinase A activation but the presence of some TIMP-2 appears to be important for promoting activation via the formation of a trimolecular complex. Similar interactions have been described for other MMPs and TIMPs; pro-gelatinase B interacts with TIMP-1 and this cannot then be activated by stromelysin or plasmin (Goldberg *et al.*, 1992). Thus TIMPs have a dual function in both preventing pro-MMP activation and inhibiting active MMPs.

In combination with enhanced *de novo* production of virtually all extracellular matrix

components, the specific regulation of MMPs and of their respective inhibitors (TIMPs) may explain the qualitative and histological rearrangements of the fibrotic extracellular matrix.

1.7. THE HEPATIC STELLATE CELL

The HSC is an important cell type in normal liver metabolism and is responsible for fibrogenesis, and since it expresses MMPs it also participates in fibrolysis. Hepatic stellate cells are distributed rather regularly in the space of Disse with the perikaryon embedded in recesses between adjacent hepatocytes and long, contractile cytoplasmic processes encircling the endothelial lining of the sinusoids. This suggests a possible role of HSC in the regulation of hepatic blood flow (Rockey, 1997). In normal adult liver the most conspicuous feature of HSC is intracytoplasmic lipid droplets, ranging in diameter from 1-2 μm. As discussed in section 1.8.1.1., these lipid droplets are important in the hepatic storage of retinyl esters. This characteristic however, does not appear to be unique to HSC. Extrahepatic stellate cells that store vitamin A in their cytoplasm are present in other organs including the lung, digestive tract, spleen, adrenal gland, ductus deferens and uterus (Wake, 1980).

1.7.1. Markers of Hepatic Stellate Cells

Hepatic stellate cells express a heterogeneous pattern of cytoskeletal markers depending on their lobular location, the species examined and whether the tissue is normal or injured. Although desmin is considered to be a marker for the identification of HSC in normal rat or mouse liver (Yokoi *et al.,* 1984), up to 50% of centrolobular HSC are desmin negative (Ballardini *et al.,* 1994). In normal adult human liver however, HSC are desmin negative (Yamaoka *et al.,* 1993). The smooth muscle isoform of α-actin may represent an additional cytoskeletal marker for the identification of HSC. Although this protein is expressed in HSC of normal and fibrotic human liver, it is only expressed in rat HSC from injured liver or after several days in culture. Consequently, α-smooth muscle actin has acquired significance as a marker for rat HSC activation (Ramadori *et al.,* 1990; Rockey *et al.,* 1992a) despite its expression in vascular smooth muscle cells in normal rat liver (Nouchi *et al.,* 1991). Vimentin can always be identified in HSC regardless of species but since vimentin is also found in Kupffer cells and endothelial cells it is a less useful marker (Ahmed *et al.,* 1991). In normal rat liver HSC contain glial fibrillary acidic protein (GFAP), an intermediate filament protein normally expressed by astrocytes (Gard *et al.,* 1985) but in human adult liver GFAP

expression is limited to a small subpopulation of HSC. Since there is no definitive marker yet available for identification of HSC under physiological and pathological conditions, several techniques need to be used together. Hepatic stellate cells can also be verified by the characteristic autofluorescent properties of vitamin A in the lipid droplets. This causes a rapidly fading blue-green fluorescence at a wavelength of 328 nanometres (nm) and provides a useful endogenous marker for identification of HSC in culture since it does not require sacrificing the cells. Gold chloride staining can also be used to detect the characteristic lipid droplets in HSC (Wake and Sato, 1993), although a minority of HSC possess no lipid droplets (Sztark *et al.,* 1986).

1.7.2. Intralobular Heterogeneity

There is evidence for heterogeneity of HSC depending on their position in the lobule (Wake and Sato, 1993; Ballardini *et al.,* 1994). Structure, size and probably function of HSC differ from zone to zone. Both in rat and in human liver the number of HSC containing lipid droplets range from approximately 25% to 75% (Wake, 1980; Sztark *et al.,* 1986). A minority of HSC possess no lipid droplets (Sztark *et al.,* 1986). Hepatic stellate cells located in zone 1 appear small, contain minute vitamin A lipid droplets and possess short, smoothly contoured perisinusoidal branching processes which only occasionally contact hepatocytes. Desmin immunoreactivity is present but not particularly intense. Hepatic stellate cells located in zone 2 are larger, store abundant vitamin A lipid droplets and are endowed with better-developed, longer, hepatocyte-contacting processes that show intense desmin immunoreaction. Proceeding toward the centrllobular vein, Hepatic stellate cells become more elongated, assuming a dendritic appearance and their desmin immunoreactivity and vitamin A storage are progressively reduced, becoming virtually absent around the centre of the lobule (Wake and Sato, 1993; Ballardini *et al.,* 1994). Other intermediate filaments including GFAP (Niki *et al.,* 1996), neural cell adhesion molecule [N-CAM (Knittel *et al.,* 1996a; Nakatani *et al.,* 1995)] and α-smooth muscle actin (Enzan *et al.,* 1994) also illustrate the existence of considerable heterogeneity within the HSC population, both in rat and human.

The cause and functional significance of the heterogeneity is unclear but it may be related to the exertion of specific functions by subpopulations of HSC. It has been reported that the administration of excess vitamin A to rats induces a progressive increase in the number and size of vitamin A lipid droplets in HSC located in the central zone (Wake, 1980). Therefore,

the intralobular heterogeneity of HSC may reflect differences in the metabolic handling of retinoids. Another hypothesis relates HSC heterogeneity to the gradient in matrix composition which is believed to exist from portal tracts to central veins (Reid *et al.,* 1992).

The recognition that HSC are heterogeneous may have important implications for studies in which isolated cells are analysed either directly after purification or after culture.

1.7.3. Hepatic Stellate Cell Activation

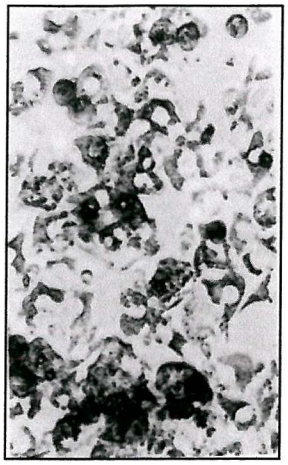
The pathogenetic significance of the HSC relies on its ability to undergo activation with transformation from a quiescent to a myofibroblast-like phenotype, the key event in the initiation of fibrogenesis (Figure 1.4.). In normal liver HSC show a quiescent phenotype. They have a compact shape, contain vitamin A rich lipid droplets and show a low proliferation rate. In response to acute or chronic liver injury quiescent HSC proliferate in areas of injury. For example, in rat carbon tetrachloride (CCl_4) liver injury HSC proliferation occurs around the necrotic centrilobular areas (Johnson *et al.,* 1992), whereas bile duct ligation leads to HSC proliferation radiating out from the periportal zones (Hines *et al.,* 1993). Similar findings are reported in human liver disease; in alcoholic liver fibrosis HSC proliferation occurs in the space of Disse (Minato *et al.,* 1983). Proliferation is accompanied by the differentiation of HSC into transitional cells, characterised by a reduction in the number of vitamin A lipid droplets, hypertrophied endoplasmic reticulum, cell enlargement, increased synthesis of extracellular matrix proteins and pronounced smooth muscle α-actin expression. In the absence of a persistent stimulus, for example in acute liver injury due to a single intraperitoneal CCl_4 injection in rats, the phenotypic changes revert and liver histology returns to normal. It is not clear to what extent the disappearance of the cells is caused by retrograde transition to quiescent cells or loss of activated cells through apoptosis (Iredale *et al.,* 1998).

By contrast, in chronic liver injury HSC differentiate into transitional cells and transform further into cells that acquire a morphology similar to myofibroblasts. In response to a variety of proliferative and fibrogenic stimuli, HSC exhibit complete loss of vitamin A containing lipid droplets, extensive microtubules, gene expression of a wide spectrum of matrix proteins, glycoproteins and glycosaminoglycans, and the acquisition of contractility following stimulation with endothelin-1 (Rockey and Weisiger, 1996).

Figure 1.4: Transformation of hepatic stellate cells to myofibroblasts

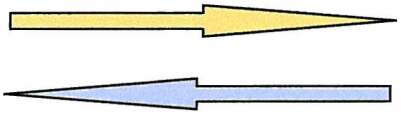
Quiescent phenotype
Compact shape, non-proliferative, high retinoid content, low matrix synthesis (collagen III>collagen I).

Activated phenotype
Cell spreading, loss of retinoids, enhanced production of matrix (collagen I>III>IV), expression of PDGF and TGFβ_1 receptors.



Proliferative: PDGF, EGF, basic FGF, TGFα Kupffer cell factors, matrix components e.g. collagen I, metallo- and serine proteases.

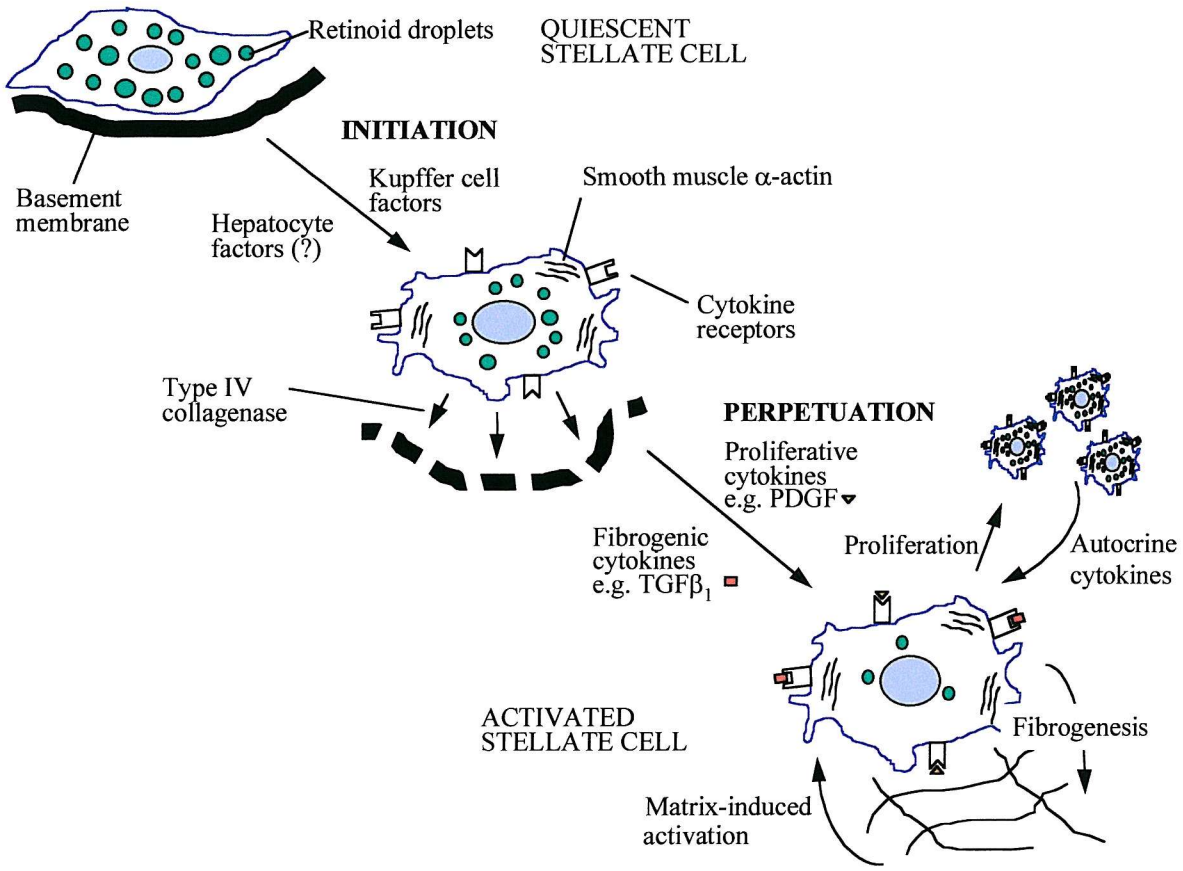
Fibrogenic: TGFβ_1 (+++), TGFα (+), EGF (+), matrix components (?).



retinoids, γ-interferon, basement membrane matrix, apoptotic stimuli (?).

The activation step occurs in two major phases, described as initiation and perpetuation of HSC activation [Figure 1.5. (Friedman, 1993)]. Initiation refers to early changes in gene expression and phenotype that render the cells responsive to proliferative and fibrogenic stimuli, whereas perpetuation results from the effects of these stimuli on maintaining the activated phenotype and generating fibrosis.

Figure 1.5: Model of hepatic stellate cell activation
Adapted from Friedman (1993).



1.7.4. Regulation of Hepatic Stellate Cell Activation

There is little information about how HSC become activated following liver injury. However, *in vitro* studies suggest HSC activation may be induced by both the extracellular matrix and soluble factors. Hepatic stellate cells can be maintained on a variety of extracellular matrices that can up-regulate or down-regulate HSC activation. A quiescent phenotype can be preserved when HSC are maintained on a gel whose composition resembles the subendothelial matrix of normal liver such as a basement membrane extract from mouse sarcoma, Englebreth-Hol-Swarm [EHS (Friedman *et al.*, 1989)]. The cells become non-proliferative, maintain a compact shape with abundant cytoplasmic lipid droplets, produce only small amounts of type III collagen and lack smooth muscle markers (Friedman *et al.*, 1989). The importance of matrix in regulating HSC activation is demonstrated when activated HSC, induced by culture on plastic, are reversed to a quiescent phenotype after re-plating the HSC onto a basement membrane-like matrix (Khan *et al.*, 1995; Gaça *et al.*, 2000). This suggests that damage to the normal liver matrix in early hepatic inflammation may disrupt the normal cell-matrix

interactions and induce HSC activation (Friedman *et al.,* 1989). Furthermore, hepatocyte function may also be perturbed by virtue of altered cell matrix interactions (Iredale and Arthur, 1994).

A second mechanism of HSC activation is influenced by soluble factors released by activated Kupffer cells (Friedman and Arthur, 1989), damaged hepatocytes (Hoffmann *et al.,* 1994), platelets (Bachem *et al.,* 1989), endothelial cells (Rieder *et al.,* 1993) and inflammatory cells. This occurs mainly through secretion of a wide variety of growth factors and cytokines, although non-peptide mediators such as reactive oxygen species, eicosanoids, iron and acetaldehyde probably also play a role (Gressner, 1995). Exposure to these mediators may be time-limited or they may be chronically present according to the nature, extent and reiteration of parenchymal damage.

Although the precise role of factors and timing of events in HSC activation remain to be determined, a hypothetical three step cascade mechanism has been proposed (Gressner, 1996). In a pre-inflammatory phase, complete or minor hepatocytic damage is frequently the primary event in initiation of liver fibrogenesis. This leads to a loss of membrane contact inhibition and facilitates the release of paracrine mitogenic factors which initiate proliferation of HSC (Gressner *et al.,* 1995). Hepatocytes produce a wide spectrum of important pro-fibrogenic and pro-mitogenic cytokines including FGF, TGF-α, insulin like growth factor 1 (IGF-1), TNF-α, IL-1α, IL-8 and IL-6. The hepatocyte derived mitogens act in concert with growth factors provided by activated Kupffer cells (Gressner *et al.,* 1993). In addition, hepatocytes metabolise profibrogenic xenobiotics to fibrogenic substances. For example ethanol (which is oxidised to acetaldehyde) and lipid peroxides formed from damaged hepatocyte membranes, stimulate HSC to synthesise certain collagen types, fibronectin and initiate proliferation of HSC (Bedossa *et al.*, 1994; Neuschwander-tetri, 1990; Casini *et al.,* 1991).

During the subsequent inflammatory phase, cytokines synthesised and secreted by activated Kupffer cells and damaged platelets stimulate HSC to proliferate and transform to myofibroblasts. Other potential paracrine sources of cytokines in the injured liver include lymphocytes, endothelial cells and mast cells (Levi-Schaffer and Rubinchik, 1995). It has also been suggested that thrombocytes may be involved in paracrine modulation of HSC (Bachem *et al.,* 1989).

Kupffer cell infiltration and activation play a prominent role in initiation of HSC activation. Activation of resident Kupffer cells is initiated partially by phagocytosis of cell debris at the site of necrosis. These activated cells might also induce hepatocyte damage via release of proteases, toxic cytokines and oxygen radicals. The role of Kupffer cells has been emphasised by culture studies which demonstrate that Kupffer cell conditioned medium promotes transition of the quiescent HSC into the myofibroblast-like phenotype. This is characterised by enhanced proliferation, increased extracellular matrix production, and responsiveness to the potent HSC mitogen, PDGF due to *de novo* expression of the PDGF-β receptor (Friedman and Arthur, 1989). The up-regulation of receptors for proliferative and fibrogenic cytokines is a major early event in HSC activation, stimulating and sustaining proliferation and fibrogenesis by the relevant cytokines. Although Kupffer cells secrete a number of cytokines including the HSC mitogens TNF-α, IL-1, and TGF-α (Matsuoka *et al.,* 1989), they are also an important source of TGF-β_1, the most potent fibrogenic mediator with multiple, pleiotrophic actions (Meyer *et al.,* 1990). This protein enhances fibrogenesis and inhibits extracellular matrix degradation. Transforming growth factor-β_1 induces the synthesis of fibronectin, laminin, collagen I, III, IV, proteoglycans and TIMP in HSC (Casini *et al.,* 1993; Matsuoka *et al.,* 1989; Matsuoka and Tsukamoto, 1990). In addition, TGF-β_1 down regulates the expression of stromelysin and interstitial collagenase thereby inhibiting matrix degradation. Although TGF-β inhibits the proliferation of rat HSC (Meyer *et al.,* 1990; Matsuoka *et al.,* 1989), it promotes the PDGF mediated proliferation of human HSC (Win *et al.,* 1993). Transforming growth factor-β_1 is one of a family of related peptides (TGF-β_1, β_2, β_3, β_4, β_5) of which only TGF-β_1, -β_2 and -β_3 have been detected in rat liver (Jakowlew *et al.,* 1991). In addition to Kupffer cells, TGF-β_1 is produced by a wide variety of liver cell types including activated HSC, endothelial cells, bile duct epithelial cells, platelets and hepatocytes (George *et al.,* 1999; Qi *et al.,* 1999).

In the post-inflammatory phase, myofibroblast-like cells are stimulated through autocrine and paracrine loops (Gressner, 1995). The response of activated HSC to the repetitive generation of soluble growth factors in chronic liver disease or recurrent injury, is enhanced by their increased expression of mitogenic receptors and profibrogenic mediators, stimulating proliferation, transformation and enhanced proteoglycan synthesis of quiescent HSC. This potentially contributes to self-perpetuation of fibrogenesis, even after cessation of the initiating event (Bachem *et al.,* 1992).

Not all cytokines however, promote fibrogenesis. Interferon-γ, secreted by inflammatory cells, in particular T lymphocytes and natural killer cells, results in reversion of HSC to a more quiescent phenotype in which proliferation is inhibited and the cell regains its lipid droplets (Rockey *et al.,* 1992b). It also reduces expression of fibronectin and collagen types I and IV by HSC. Addition of retinoids to HSC in culture can also maintain a quiescent phenotype. When added to activated HSC, retinoids can cause a reversion of phenotypic changes together with a decrease in the rate of cell proliferation (Pinzani *et al.,* 1992b). This is discussed in further detail in section 1.8.6.1.

1.7.5. Hepatic Stellate Cell Isolation and Culture

The most widely used method of HSC isolation is by perfusion of an adult rat liver through the portal vein with a combination of the digestive enzymes collagenase (type IV) and pronase. Further purification of HSC from other non-parenchymal populations is by density gradient separation which is based on the low buoyant density of HSC due to their large fat content. Thus, only those HSC that are relatively vitamin A rich and therefore less activated will be isolated. Furthermore, the vitamin A content and therefore possibly the density of the HSC varies according to age, body weight and nutritional status of the animal (Hendriks *et al.,* 1988; Blomhoff *et al.,* 1988b). Since some non-parenchymal cells can also take up fat during the isolation procedure, the cell suspension can be contaminated with Kupffer cells and endothelial cells.

The activation or myofibroblastic transformation of HSC which occurs *in vivo* in humans and experimental animals can be stimulated *in vitro* by culture of isolated HSC on a substratum of uncoated plastic or type I collagen, the predominant matrix component of hepatic fibrosis (Friedman *et al.,* 1989). Unlike the *in vivo* process which develops gradually over months and years, *in vitro* transformation occurs within 1-2 weeks following isolation. Freshly plated HSC form small clumps which attach to culture plates within 18 hours. After 48-72 hours of culture, the HSC spread, change their shape from dendritic to membranous and the vitamin A lipid droplets become smaller and disseminated. Hepatic stellate cells express pro-collagens I and III, collagen IV and laminin. After 4 days, the HSC show a fibroblast-like appearance as a result of losing their microprojections and lipid droplets. After culture on plastic for more than 7 days, the HSC acquire an activated, myofibroblast-like phenotype with increased spreading, diminished retinoid content and proliferation (Friedman *et al.,* 1992a). This

spontaneous activation *in vitro* can thus be exploited to investigate the role of HSC during the progression of liver fibrosis.

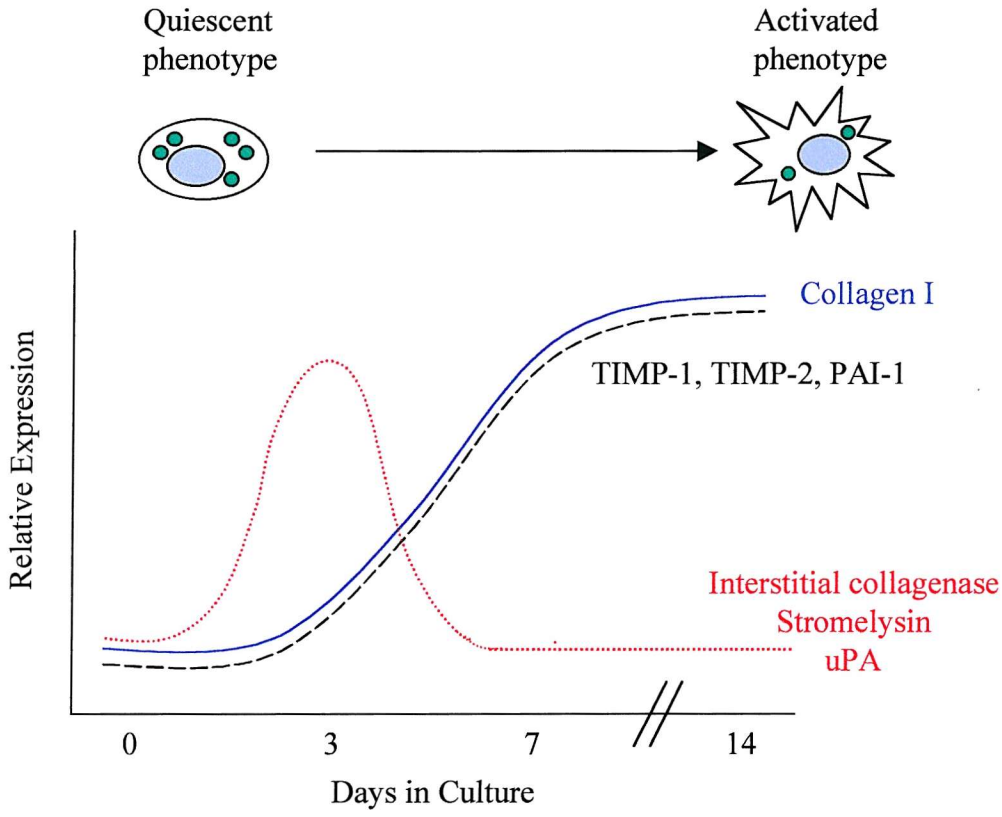
1.7.6. Hepatic Stellate Cells and Extracellular Matrix Degradation

In addition to their role in fibrogenesis and extracellular matrix synthesis (section 1.4.), HSC are involved in degradation of the matrix within the liver (Milani *et al.*, 1992; Herbst *et al.*, 1991). These cells are able to release a variety of MMPs and are also able to regulate the extracellular activity of these enzymes via simultaneous release of TIMPs. The sequence of MMP expression is very specific. In early primary culture (1-3 days) there is transient expression of interstitial collagenase [MMP-1 in humans, MMP-13 in rats (Iredale *et al.*, 1996)] and stromelysin-1 [MMP-3 (Vyas *et al.*, 1995)] which are then down-regulated (days 3-5), and in fully activated HSC (days 7-21) are not detectable. However, later cultures can express interstitial collagenase upon induction by cytokines such as TNFα (Iredale *et al.*, 1994). Although HSC can express MMPs under defined conditions in cell culture, current evidence suggests that MMP-1 is expressed infrequently *in vivo* in either normal or fibrotic liver (Milani *et al.*, 1994; Iredale *et al.*, 1993). In marked contrast to interstitial collagenase and stromelysin-1, gelatinase A (MMP-2) expression is absent in early culture but increases from days 3-5 and is a continuous prominent feature of activated HSC (Arthur *et al.*, 1992; Benyon *et al.*, 1999). This is accompanied by a similar pattern of expression of MT1-MMP, the system of pro-gelatinase A activation (Benyon *et al.*, 1999). The data obtained in cell culture have been complemented by studies in whole liver and indicates that HSC are capable of degrading extracellular matrix proteins, particularly during the early phase of HSC activation. As active gelatinase A can degrade type IV collagen, a paradox is apparent as fibrotic liver contains increased, not decreased, amounts of type IV collagen. The explanation for this is unclear but gelatinase A may not only be involved in matrix degradation. For example, active gelatinase A promotes proliferation of HSC (Benyon *et al.*, 1999) suggesting that it has profibrogenic effects despite its ability to degrade matrix proteins. In addition, in fibrotic liver the catalytic activity of gelatinase A may be constrained by the simultaneous increase in the TIMPs.

Expression of TIMP-1 and TIMP-2 by HSC, both in models of activation and during fibrotic liver injury *in vivo,* have also been investigated. In early primary culture (day 3) HSC do not express TIMPs but do transiently express interstitial collagenase. When HSC are activated to

a myofibroblast-like phenotype with duration of culture the reverse pattern is observed, there is a striking increase in TIMP-1 expression, synthesis and extracellular release, while interstitial collagenase expression becomes undetectable [Figure 1.6. (Iredale *et al.,* 1992)]. These studies have been complemented with a chronological analysis of TIMP expression in the rat models of bile duct ligation and CCl_4-induced liver injury and fibrosis (Roeb *et al.,* 1997). The observations suggest that TIMP-1 secretion by activated HSC may, by preventing degradation of excess fibrillar collagens, promote progression of liver fibrosis. Tissue inhibitor of MMP-2 expression by activated HSC and in models of liver injury parallels that observed for TIMP-1. In early primary HSC culture TIMP-2 is not detectable but it is evident in activated HSC (Benyon *et al.,* 1996). Hepatic stellate cells can also express uPA which initiates metalloproteinase activation together with its receptor and its inhibitor (Leyland *et al.,* 1994). As activation of HSC becomes more advanced, expression of uPA decreases and is replaced by PAI-1 with a resulting decrease in uPA activity (Leyland *et al.,* 1996; Knittel *et al.,* 1996b). This may further inhibit collagen breakdown by reducing production of plasmin, an enzyme which activates interstitial collagenase. Hepatocytes can also express TIMP-1, particularly after exposure to IL-6 and other inflammatory cytokines (Roeb *et al.,* 1993; Roeb *et al.,* 1994). However, the majority of TIMP-1 and TIMP-2 expression is confined to non-parenchymal cells and in particular activated HSC.

Figure 1.6: Changes in expression of regulators of the extracellular matrix during activation of the hepatic stellate cell *in vitro*



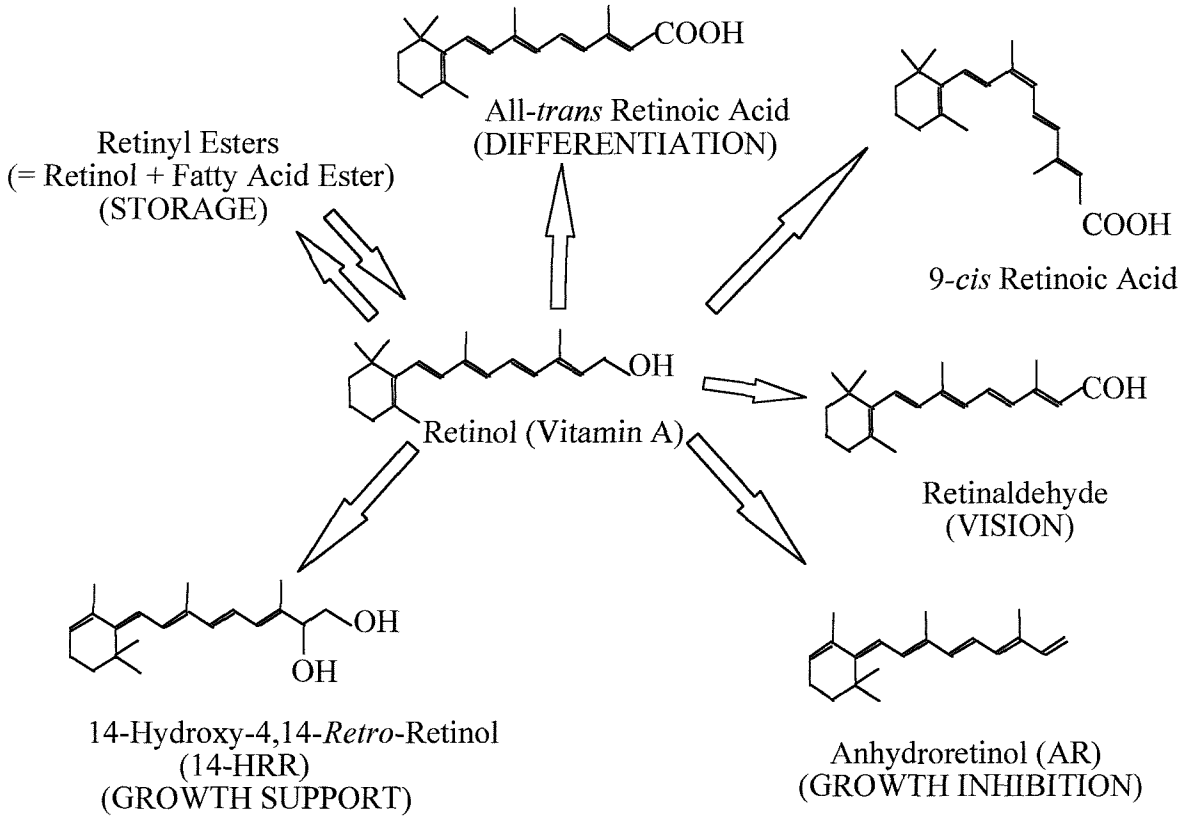
In summary, HSC are able to synthesise pro-MMPs, together with appropriate enzyme systems for their activation (uPA/PAI-1) and specific MMP inhibitors (TIMPs). The precise role of these enzymes in liver fibrosis is unclear but the net effect, caused by fine changes in the relative levels of TIMPs and MMPs, may be to accelerate the degradation of normal subendothelial matrix and hasten its replacement with interstitial or fibril forming matrix. This disruption of HSC-normal liver matrix interaction could perpetuate HSC activation.

1.8. RETINOIDS

Retinoids comprise a family of polyisoprenoid lipids that include all natural and synthetic compounds that are structurally related to retinol [vitamin A (IUPAC-IUB, 1982)]. Retinoids are known to regulate diverse cellular activities such as cell proliferation, differentiation, morphogenesis and tumorigenesis both during embryogenesis and in the adult animal. They are also required for vision, mucus secretion and for the maintenance of differentiated epithelia (Goodman, 1984).

Retinol is not known to be incorporated into the structural components of cells and does not bind to any of the nuclear retinoid receptors tested so far, thus retinol can be regarded as a pro-hormone from which the biologically active metabolites such as retinal, the aldehyde form of retinol (biologically active in the retina), retinoic acid (the carboxylic form of retinol) present in most tissues at low concentrations and their *cis/trans* isomers are derived (Figure 1.7.).

Figure 1.7: The structure of retinol and some of its derivatives



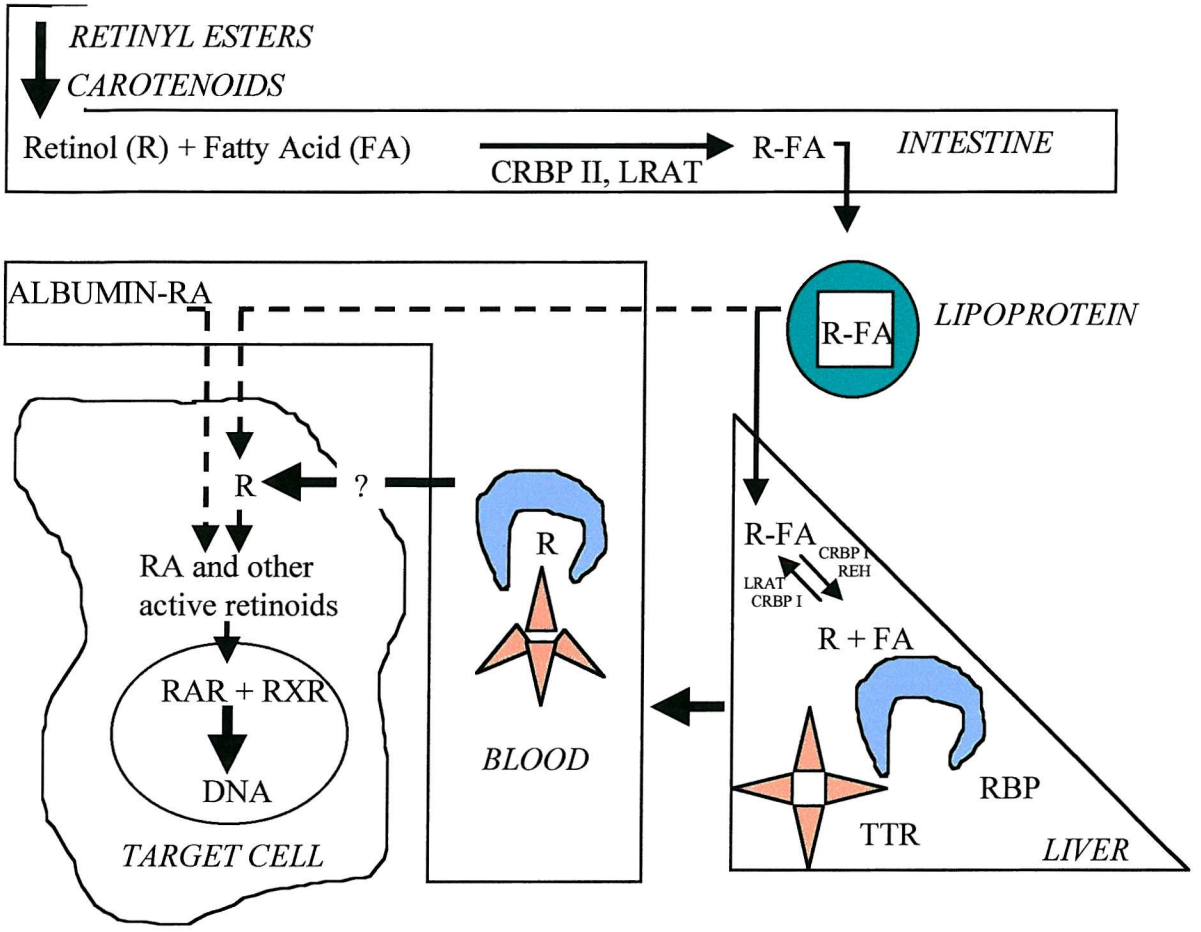
1.8.1. Retinoid Metabolism and Storage in the Liver

It is well established that the liver plays a central role in uptake, storage and mobilisation of retinoids in the body. The average retinoid concentration in human liver is 100-200 micrograms (μg) retinol per gram of liver (Hendriks *et al.*, 1993). In humans and rats between 50-80% of the total body retinoid under normal circumstances appears to be stored in the liver. In individuals with a low total body retinoid content however the percentage present in the liver may be considerably lower. The normal reserve is adequate for several months in adult rats and in humans. Smaller amounts of retinoid are stored in other tissues such as the kidneys, lungs and adipose tissue. In the liver, both the hepatocytes and HSC play essential

roles in retinoid storage and metabolism. Uptake and processing of retinoids occurs in hepatocytes, whereas HSC are the major site of storage of retinoids. The ability of HSC to store and mobilise retinoids ensures that the retinol plasma concentration is maintained within a narrow range [1-1.5 x 10^{-6} Molar (M) retinol in human and bovine plasma] in spite of fluctuations in daily vitamin A intake (Blomhoff *et al.*, 1990). Thus retinol is continuously available to cells to take up and metabolise for their own regulation or possibly secrete to regulate other cells in a paracrine mechanism. In addition to retinol, there is a 5-10 nanoMolar (nM) plasma concentration of retinoic acid (Goodman, 1984; Goodman and Blaner, 1984) presumably bound to albumin which can be spontaneously transferred to cells to elicit biological activity. There is also some evidence for the involvement of Kupffer cells in retinoid metabolism; retinoids have been shown to increase Kupffer cell endocytic activity (Earnest *et al.*, 1986; Hendriks *et al.*, 1987a) and mediators produced by Kupffer cells, including PDGF can modulate retinoid metabolism (Friedman *et al.*, 1993; Shiratori *et al.*, 1986).

Body stores of vitamin A are maintained entirely by dietary intake. Pro-vitamin A carotenoids (e.g. β-carotene) produced by plants and other photosynthetic micro-organisms, along with retinyl esters from animal tissues are the main dietary sources of retinol (Blomhoff *et al.*, 1992). Retinyl esters are hydrolysed in the intestinal lumen to free retinol before absorption by the enterocytes of the gut (Blomhoff *et al.*, 1992); carotenoids are partially converted into retinol in the enterocytes. The retinol in the enterocytes is bound by cellular retinol binding protein II (CRBP II, section 1.8.2.), re-esterified to retinyl ester by the enzyme lecithin:retinol acyl transferase (LRAT) and together with triacylglycerol is incorporated into chylomicrons, the main intestinal lipoproteins (Hendriks *et al.*, 1985). The chylomicra are metabolised to chylomicron remnants and are transported to the hepatocytes via the lymphatic route [Goodman and Blaner, 1984 (Figure 1.8.)].

Figure 1.8: Transport and metabolism of retinol
Adapted from Vieira et al. (1995).



RBP: Retinol binding protein
TTR: Transthyretin
R-FA: Retinyl esters
RA: Retinoic acid
CRBP I and II: Cytosolic retinol binding proteins

RXR: Retinoid X receptor
RAR: Retinoic acid receptor
REH: Retinyl ester hydrolase
LRAT: Lecithin retinol acyl transferase

Several studies have shown that hepatocytes take up a major fraction (about 70-80%) of chylomicron retinyl esters from the circulation and hydrolyse the esters to retinol by retinyl ester hydrolase which interacts with CRBP I (Ross, 1993). The hepatocytes store only a minor portion of the newly endocytosed retinol. Some of the retinol may be further converted into a number of different metabolites such as retinoic acid. The majority of the retinol (70-80%) either becomes bound to the plasma retinol binding protein (RBP) in the endoplasmic reticulum and secreted into the blood for distribution to peripheral tissues, or is transferred to neighbouring HSC for storage as retinyl esters. The transfer is quite specific as other components of chylomicron remnants such as cholesterol and vitamin D are not transferred (Blomhoff *et al.*, 1982). When retinol is required to meet body needs, the stored retinyl ester in HSC is hydrolysed by retinyl ester hydrolase to free retinol. Retinol is then bound to the

serum RBP and secreted into the circulation.

The fate of hepatocyte retinol is determined by the vitamin A status of the animal. In vitamin A sufficient rats, retinol is transferred from hepatocytes to HSC but when vitamin A stores are low, retinol is retained by hepatocytes and less transferred to HSC; retinol retained by hepatocytes is bound to CRBP I and in the presence of LRAT esterified and stored as retinyl esters. In vitamin A deficient animals, dietary retinoids transported to the liver by chylomicron remnants are immediately bound to RBP in hepatocytes and exported to the circulation. It is not known how hepatocytes detect peripheral levels of vitamin A.

1.8.1.1. The Role of Hepatic Stellate Cells in Retinoid Storage and Metabolism

Much evidence has accumulated which suggests HSC play an important role in hepatic storage and metabolism of retinoids. The involvement of HSC in retinol storage was initially suggested by Wake (1980) who reported that the gold chloride staining in these cells is based on a reduction of chloride by vitamin A. After HSC are stained with gold chloride, the reduced gold is associated with the lipid droplets, indicating that retinoid is specifically stored in these droplets. Gold chloride staining is absent when retinoids are destroyed by fluorescence and is also absent in hepatocytes.

The retinol in hepatocytes that is not secreted into the circulation is transferred to HSC, bound to CRBP I and esterified in the presence of LRAT. The resulting retinyl esters are stored in large cytoplasmic lipid droplets. Up to 90% of rat hepatic retinoids are present in the lipid droplets of HSC (Hendriks *et al.*, 1985). Only when the retinol storage becomes very low will the quantitative contribution of hepatocytes increase. The number and size of the lipid droplets seems to be related to the amount of vitamin A contained in the diet rather than to the amount of fat (Moriwaki *et al.*, 1988; Wake, 1974). Biochemical analysis of lipid droplets purified from isolated HSC has demonstrated that the major components of these droplets are triglycerides and retinyl esters, predominantly retinyl palmitate but at least seven other retinyl esters are present (Hendriks *et al.*, 1993). Minor amounts of un-esterified retinol, cholesteryl esters, cholesterol, free fatty acids and phospholipids are also found in the lipid droplets in HSC (Moriwaki *et al.*, 1988). Hepatocyte lipid droplets however mainly consist of triglycerides and cholesteryl esters (Hendriks *et al.*, 1987b).

Two types of lipid droplets have been identified in HSC: membrane bound (type I) and non-membrane bound [type II (Wake, 1980)]. Both types of lipid droplets contain retinoids. Type II lipid droplets are usually larger than type I lipid droplets. Type II lipid droplets are present in normal vitamin A status, whereas type I lipid droplets occur when large quantities of retinoids are administered and actively metabolised (Wake, 1980). In vitamin A deficient rats HSC do not contain lipid droplets. However, during vitamin A deficiency lipid droplet formation in HSC can be induced by the administration of retinoic acid (Yumoto *et al.,* 1989). This is unexpected since retinoic acid cannot be converted into retinol or retinyl esters. The droplets induced by retinoic acid are void of retinyl esters but contain triglycerides and minor quantities of other lipids, similar to retinoid-containing lipid droplets. One possible explanation could be that retinoic acid induces the expression of the genes responsible for lipid droplet formation.

1.8.1.2. Retinol Exchange between Liver Cells

Several hypotheses have been proposed as mechanisms for retinol transfer between hepatocytes and HSC. The first hypothesis proposes retinol transfer via desmosomes. The cytoplasmic extensions of HSC insure that a large proportion of the cell membrane is in close contact with the hepatocyte cell membrane. These cell contacts have been described in the baboon and the human but not in the liver of the rat (Mak *et al.,* 1984).

The second hypothesis proposes that retinol release from the liver is solely mediated by RBP synthesised in hepatocytes and retinol uptake by HSC does not involve internalisation of RBP by HSC (Fex and Johannesson, 1987). Instead retinol uptake depends on dissociation of retinol from RBP, its transfer over the cell membrane and internalisation within the cell by CRBPs. This mechanism is consistent with the constants determined for dissociation and membrane crossing, and the kinetics of retinol uptake observed in *in vitro* studies.

The third hypothesis proposes that RBP is solely responsible for the release of retinol from both the hepatocyte and HSC and that retinol uptake in HSC is mediated by the internalisation of RBP through RBP receptors. This hypothesis is supported by the observations that 1) holo-RBP can be taken up by HSC, 2) antibodies to RBP inhibit retinol transfer from hepatocytes to HSC, 3) RBP and its messenger ribonucleic acid (mRNA) have been identified in HSC and 4) HSC *in vitro* can release holo-RBP. Others however, have not detected

significant amounts of RBP or its mRNA in HSC (Blaner *et al.*, 1985; Hendriks *et al.*, 1988; Suhara *et al.*, 1990).

Any physiological condition which requires increased retinoids at the periphery will result in the mobilisation of these compounds from the primary liver storage site, the HSC. Essentially all circulating retinoids are bound to RBP. Serum RBP (Blaner, 1989) is a small single polypeptide chain molecular weight of approximately 21 kilodaltons (kDa) which has one binding site for one molecule of retinol. Whether utilised as retinol, retinal or retinoic acid, RBP only transports all-*trans* retinol to target cells and only retinol triggers secretion of RBP (Muto *et al.*, 1972). The third hypothetical mechanism for retinol transfer between hepatocytes and HSC, together with the supporting data, suggest retinol does not necessarily have to be transferred from HSC to hepatocytes for its secretion into the bloodstream bound to RBP. Instead HSC may synthesise RBP and mobilise retinol-RBP directly into the plasma, without prior transfer of retinol to hepatocytes. The existence of extrahepatic retinol storing cells also supports the possibility that HSC secrete retinol-RBP directly into the blood.

Once in the blood, retinol-RBP associates with a 55 kDa homotetrameric plasma transthyretin (TTR, previously called prealbumin). This prevents glomular filtration and renal catabolism of the small RBP molecule. After delivering retinol to target tissues, RBP unbound to retinol has less binding affinity for TTR and is filtered through the kidney.

1.8.2. Cellular Retinoid Binding Proteins

In addition to the extracellular RBP, many cell types contain cellular (cytosolic) RBPs. Four such proteins have been well characterised, two bind retinol or retinal (CRBP I and CRBP II) and two bind retinoic acid (cellular retinoic acid binding protein, CRABP I and CRABP II). The role of these proteins is uncertain, but they may be involved in retinoid storage, metabolism and the regulation of the free level of biologically active retinoids within a given cell. The predominant intracellular RBPs in most tissues are CRBP I and CRABP I. These two proteins are often concentrated in different cell types within a given organ (Ong *et al.*, 1982).

CRBP I and II have similar primary and tertiary structures. It is therefore not surprising that they have some common properties including binding of retinol and retinal (Li *et al.*, 1991),

lack of affinity for retinoic acid (MacDonald and Ong, 1987) and involvement in retinol esterification by the enzyme LRAT (Ross, 1993). However, they also have several differences: CRBP II has greater affinity for retinal and intestinal LRAT (Li *et al.*, 1991; MacDonald and Ong, 1987). These properties render it specialised for assimilation of dietary retinol and carotenoid-derived retinal, reflecting the restriction of CRBP II to the small intestine. In contrast, CRBP I is widely expressed in adult tissues, has a higher affinity for retinol and is involved in the conversion of retinol via a retinaldehyde intermediate to retinoic acid (Sundelin *et al.*, 1985).

The precise roles of CRABP I and CRABP II are not entirely understood. Their importance remains elusive because recent genetic studies have shown that double mutant mice null for both CRABP I and CRABP II are apparently normal (Lampron *et al.*, 1995). In addition, CRABPs are present in only low concentrations in the liver, both in hepatocytes and in HSC. Since the intracellular concentration of free retinoic acid is probably critical in determining the extent of activation of retinoic acid nuclear receptors, it is noteworthy that CRBP I and CRABP II gene expression is induced by retinoic acid. Furthermore, since CRABP I and CRBAP II can bind retinoic acid, it has been proposed to fine tune the intracellular concentration of free retinoic acid. This could be achieved either by sequestering it in the cytoplasm or by facilitating its catabolism, thereby preventing the retinoid from reaching the nucleus and subsequently reducing the expression of retinoic acid response genes. To complement this proposal, there is evidence that the higher the level of the CRABP I in the cytoplasm, the less sensitive the cell is to a given external concentration of all-*trans* retinoic acid (Boylan and Gudas, 1991). Since the CRABP I protein can enhance the enzymatic conversion of retinoic acid to more polar oxidised derivatives, it appears that the CRABP I protein reduces the amount of retinoic acid available to regulate gene expression in the nucleus. Interestingly, neither CRABP I nor CRABP II bind 9-*cis* retinoic acid which suggests the possible existence of additional CRABPs specific for 9-*cis* retinoic acid.

1.8.3. Retinoid Metabolism

The hypotheses mentioned in section 1.8.1.2. on retinol exchange between liver cells is not only relevant for the transfer of retinol between liver cells but also for understanding the mechanism of retinol uptake by other organs and tissues. After cellular uptake of all-*trans* retinol, retinol serves as the metabolic precursor of biologically active retinoids. For example,

it may be metabolised according to the following classical pathway: retinol is oxidised in a reversible step to retinal and subsequently in an irreversible step to all-*trans* retinoic acid by alcohol dehydrogenase. An isomerase activity reversibly converts all-*trans* retinoic acid to 9-*cis* retinoic acid. However, the complexity of the biological actions of vitamin A suggests that retinol derivatives other than retinoic acid mediate some of these actions. Consistent with this idea, other biologically active metabolites have been isolated including 14-hydroxy-4,14-*retro*-retinol [14-HRR (Buck *et al.*, 1991)], anhydroretinol (Buck *et al.*, 1993), 4-oxoretinol (Achkar *et al.*, 1996), 3,4-didehydroretinoic acid (Thaller and Eichele, 1990) and various retinyl esters (Eppinger *et al.*, 1993). It is uncertain whether all of these derivatives act by binding to the retinoid nuclear receptors.

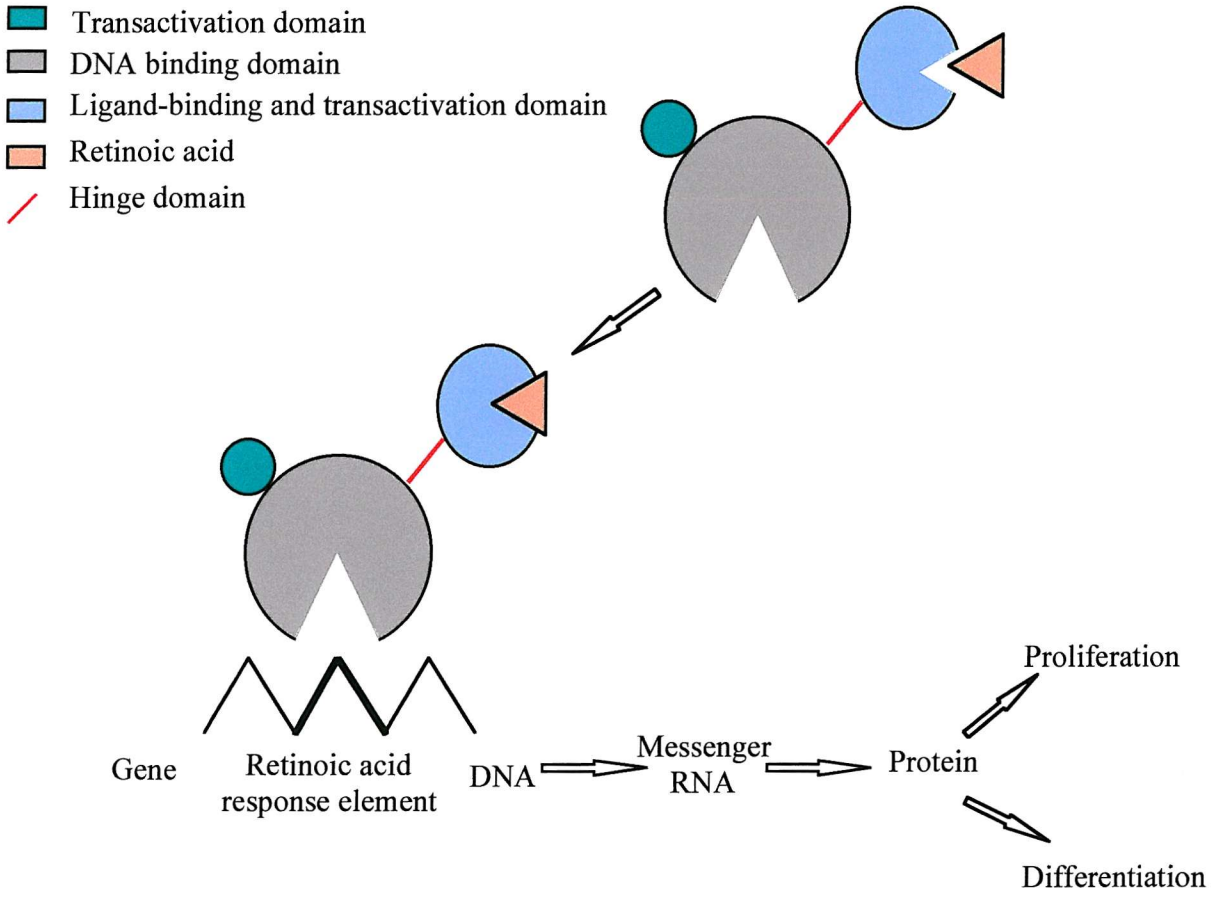
1.8.4. Nuclear Retinoid Receptors

Retinoids do not exert their actions on gene transcription directly but through binding to nuclear retinoid receptors that are members of the steroid/thryroid/retinoid hormone receptor superfamily. Upon binding their cognate ligand, these receptors act as ligand-inducible transcription factors which activate or repress expression of target genes by binding to specific *cis*-acting deoxyribonucleic acid (DNA) sequences termed retinoic acid response elements (RAREs) and retinoid X response elements (RXREs) located in the promoter/enhancer regions of target genes (Figure 1.9.).

At least two classes of these nuclear receptors have been identified and cloned, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [for review see Chambon (1996)]. The classification into RAR and RXR subfamilies is based on the difference in primary structure, sensitivity to synthetic retinoid ligands and ability to regulate expression of different target genes. Each receptor class consists of three receptor subtypes (α, β, γ), each with several isoforms (RARα 1 and 2, RARβ 1-4, RARγ 1 and 2). These isoforms are derived as a result of alternative splicing and induction by two different promoters. The expression of the second of each RAR isoform (i.e. RARα_2, RARβ_2, RARγ_2) is modulated by retinoic acid through RAREs present in the promoter regions of each of these genes (de The *et al.*, 1990; Sucov *et al.*, 1990; Leroy *et al.*, 1991; Lehmann *et al.*, 1992). Comparison of the amino acid sequences of the three human receptors with the mouse receptors shows that the interspecies conservation of a member of the RAR subfamily is much higher than the conservation of all three receptors within a given species, suggesting that RARα, RARβ and RARγ each have

their own specific function (Krust *et al.*, 1989). In addition, the subtypes are expressed in distinct patterns throughout development and in the mature organism, also indicative of different functions (Dolle *et al.*, 1989). The multiplicity of nuclear retinoid receptors and gene pathways may in part explain the pleiotrophic effects of retinoids in controlling a wide variety of cellular processes.

Figure 1.9: The mechanism of action of retinoids, showing the intracellular signalling system of retinoid receptors



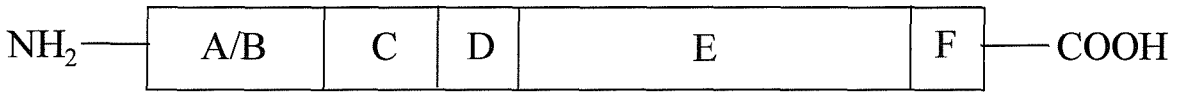
The RARs and RXRs have overlapping ligand specificity even though they have low homology (only 29%) in the primary amino acid sequence ligand binding domain (Leid *et al.*, 1992). Both subfamilies are high affinity (nM range) receptors for 9-*cis* retinoic acid. However, all-*trans* retinoic acid is a ligand only for the RARs, activating this subfamily with similar potency as 9-*cis* retinoic acid (nanomolar concentration). Although RARs appear to bind 9-*cis* retinoic acid, all-*trans* retinoic acid and other differentiation retinoids with high affinity, they bind retinol and retinal with lower (1/100) affinity. However, the concentration of retinol is about 400 times that of retinoic acid in the body fluids (Goodman, 1984;

Goodman and Blaner, 1984). Therefore, retinol is probably also a ligand for RAR *in vivo*. All-*trans* retinoic acid is not a RXR ligand. This derives from the observation that 9-*cis* retinoic acid is up to 40 times more potent than all-*trans* retinoic acid on RXRα (Heyman *et al.*, 1992). *In vivo* all-*trans* retinoic acid is converted to 9-*cis* retinoic acid, the RXR specific ligand. The identification of different ligand specificity provides the capacity for independent control of physiological processes mediated by the RARs and RXRs. For example a response element in the CRBP II gene can be selectively activated by the RXRs but not the RARs (Suruga *et al.*, 1997), providing functional discrimination between these two signalling pathways.

Diversity has also been generated by the heterodimeric interaction of RAR and RXR. The RARs require heterodimerization with any of the three RXRs for appreciable DNA binding and regulation of gene transcription. The RXRs are able to bind not only as heterodimers but also as homodimers in the presence of their ligand 9-*cis* retinoic acid. The RXRs can also heterodimerize *in vitro* with certain other members of the nuclear hormone receptor family such as the thyroid hormone receptor and the vitamin D receptor. Recent studies have shown that most retinoid-responsive genes appear to be regulated by RAR:RXR heterodimers, at least in tissue culture models.

The nuclear receptors have a characteristic modular structure and their amino acid sequence has been divided into six functional domains (A-F) for RARs and five functional domains (A-E) for RXRs, based on homology among themselves and with other members of the nuclear receptor superfamily (Figure 1.10.).

Figure 1.10: Functional domain structure of the retinoic acid receptors (A-F) and retinoid X receptors (A-E)



The N-terminal (A/B) region modulates target gene transcription and has been shown to contain a ligand independent transcriptional activation function called AF-1 (Nagpal *et al.*, 1993). This can efficiently synergize with the corresponding ligand-dependent transactivation function (AF-2) located in the E region of these receptors (Nagpal *et al.*, 1993). The central region C, which contains two zinc fingers each co-ordinated by four cysteines, corresponds to

the core of the DNA binding domain responsible for the specific binding to *cis*-acting response elements. The homology in this region is very high between different members of the receptor family [94-97% between the three RAR types; 91-97% between the three RXR types (Chambon, 1996)]. It is the most conserved region among the receptors of this superfamily. Region E is also highly conserved between the three RAR types (84-90%) and the three RXR types [88-95% (Chambon, 1996)]. This region is multifunctional and mediates dimerization, ligand-binding, transactivation (AF-2) and transcriptional repression through protein-protein interactions. The functions of regions D and F are relatively unknown.

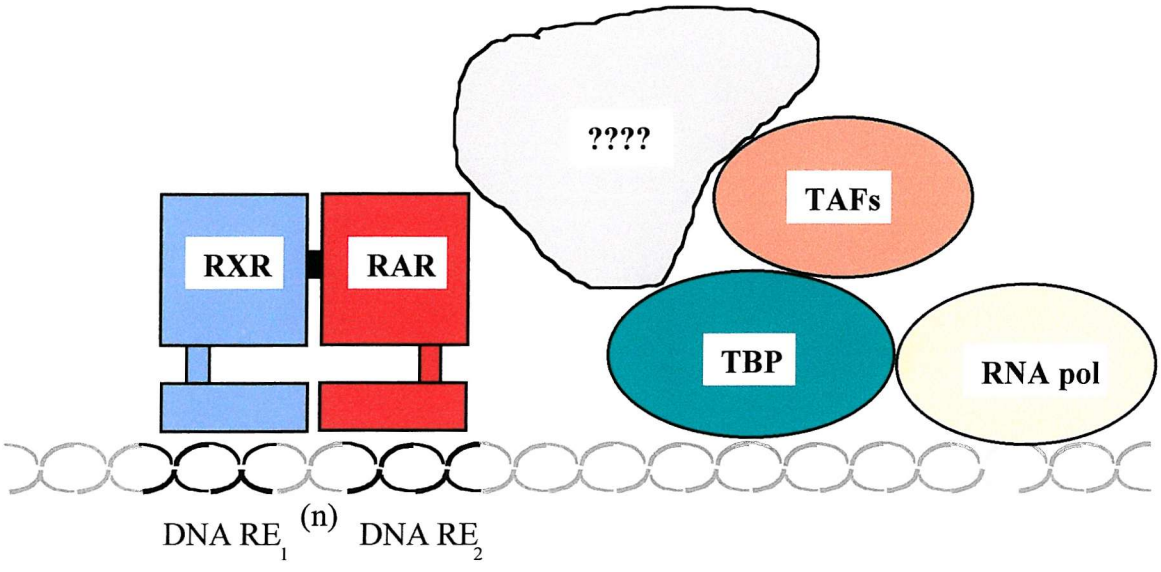
As most receptors bind DNA as dimers, the response elements are usually comprised of two 'half sites' each representing the binding site of one monomer. The half sites can be oriented with respect to each other either as direct repeats, palindromes, or everted repeats (inverted palindrome). Specificity of a response element for a particular hormone receptor complex is derived from the orientation of the half sites, the sequence of the half sites and the spacing between half sites. With reference to the retinoid receptors, there are sequences that are uniquely RXR or RAR response elements and those that are common to both. The three most common RAREs are 6-base pair direct repeats separated by 1, 2 and 5 base pairs (DR1, DR2 and DR5). In the presence of 9-*cis* retinoic acid, DR1 is known to interact preferentially with RXR homodimers and RAR-RXR heterodimers interact preferentially with DR2 and DR5 elements (Mader *et al.*, 1993). The RARs also recognise RAREs composed of everted repeats spaced by eight nucleotides (Tini *et al.*, 1993).

1.8.5. Retinoids and Transcription

The effects of retinoids on the transcription of a particular gene are dependent on a number of factors. These include the concentration of different receptor subtypes, the efficiency with which different heterodimers are formed, competition with homodimers, interaction with other transcription factors, and the availability of specific isomers of retinoic acid which is dependent on enzymatic activities and on cellular ratios of CRBP and CRABP. Little information is available on how RARs interact with basal transcription factors and RNA polymerase II. On some promoters and in certain cell types an E1A-like protein is required as a co-activator for the TATA box binding protein (TBP), which co-operates with RAR to effect retinoic acid-dependent transcriptional activation by RNA polymerase II (Figure 1.11.).

Figure 1.11: Diagram showing how RARs may interact with basal transcription factors

RXR-RAR	Retinoid receptor dimer
DNA RE	DNA response element
TBP	TATA binding protein
TAFs	TBP associated factors
RNA pol	RNA polymerase
????	Linkage protein



1.8.5.1. Negative Regulation

While primary target genes i.e. those directly activated by retinoic acid and its receptors contain RAREs to which RARs and RXRs bind directly (positive regulation), negative regulation of gene expression by retinoic acid can occur via several different mechanisms. In analogy to the positive elements for transcriptional activation, negative response elements that mediate retinoid induced repression have been reported. Receptors binding to such negative response elements in the presence of ligand presumably prevent the receptor from functioning as a transcriptional activator and allow negative interference with adjacent or overlapping binding sites that bind other transcription factors.

It is apparent that an additional negative regulatory mechanism exists that does not require DNA binding by the receptor to exert its action but functions by complexing with a protein which is prevented from binding to DNA. For example retinoids have been proposed as molecules that can down-regulate a broad spectrum of genes transcriptionally controlled by activator protein-1 (AP-1), a complex composed of the jun and fos oncoproteins, including several members of the MMP family.

All-*trans* retinoic acid modulates expression of the gene for collagenase by decreasing levels of collagenase mRNA without effecting mRNA half-life. The promoter region of the collagenase gene has an AP-1 responsive element that can be activated by the DNA binding protein, AP-1 (Lafyatis *et al.*, 1990). This causes stimulation of collagenase transcription and increased collagenase synthesis. Some models of anti-AP-1 activity predict that RAR becomes 'tethered' to the promoter through direct protein-protein interactions with the AP-1 complex bound to DNA, thereby disrupting AP-1 interactions with the basal transcription apparatus. However, it has also been proposed that all-*trans* retinoic acid represses expression of collagenase by forming an inactive protein-protein complex between the liganded RAR and c-jun (Schüle *et al.*, 1991). Whatever the mechanism, retinoic acid down-regulates expression of the collagenase gene. This result explains the destructive action of collagenase in vitamin A deficiency. For example severe vitamin A deficiency in humans and in rats leads to destruction of the cornea of the eye through the action of collagenase and neutral proteases (Pirie *et al.*, 1975). Retinoic acid can also influence collagenase gene expression by regulating post-transcriptional modification of c-jun through regulation of c-fos transcription.

Studies on stromelysin, a MMP related to collagenase that is also transcriptionally controlled by AP-1, have demonstrated that all-*trans* retinoic acid can also inhibit expression of this enzyme via a similar mechanism of forming an inactive protein-protein interaction (Nicholson *et al.*, 1990). It has also been demonstrated that RARs and RXRα can down regulate the $TGF\beta_1$ promoter by antagonising AP-1 activity by a mechanism which involves protein-protein interaction (Salbert *et al.*, 1993).

1.8.6. Retinoids and Hepatic Stellate Cell Activation

The relationship between vitamin A and liver fibrosis is complex. The loss of cytoplasmic lipid droplets in which vitamin A is stored is a dramatic feature of HSC activation, both in culture and in advanced liver fibrosis and cirrhosis. However, it is unknown whether the release of retinoids is required for activation. Loss of retinoid likely involves hydrolysis of intracellular retinyl esters to retinol because in culture models of activation extracellular retinoid is predominantly retinol, not retinyl ester and hydrolase activity can be detected in cell extracts (Friedman *et al.*, 1993).

The levels of different retinoid metabolites present in HSC before and after activation are disputed. When retinoids present in HSC from bile duct ligated rats were measured by high-performance liquid chromatography (HPLC), Ohata *et al.* (1997) found lower levels of retinol, 13-*cis* retinoic acid, 9-*cis* retinoic acid and all-*trans* retinoic acid compared to sham-operated rats. However, these results have been contradicted by Okuno *et al.* (1999) who found the levels of all-*trans* retinoic acid and 13-*cis* retinoic acid to be increased in cultured primary rat HSC; 9-*cis* retinoic acid was not detectable.

1.8.6.1. Retinoids and Hepatic Stellate Cell Proliferation

It has been demonstrated that the addition of 10^{-5} M retinol to passaged rat cultures of myofibroblasts for 10 days caused a marked inhibitory effect of proliferation of these cells (Davis and Vucic, 1988). The study by Davis and Vucic (1988) has been complemented by Pinzani *et al.* (1992b) who studied the effect of retinoids on the phenotypical and functional changes of passaged rat HSC in culture. They found that addition of 5×10^{-6} M retinol and all-*trans* retinoic acid to the culture medium, induced the appearance of vitamin A droplets in the cytoplasm and inhibited unstimulated and growth factor (PDGF and EGF) induced HSC proliferation [also noted by Davis *et al.* (1990; 1991)]. The observation that retinol or retinoic acid can induce discrete alterations of the morphology of HSC suggests that the spontaneous differentiation of isolated HSC in culture is a reversible process. The medically used and related 13-*cis* retinoic acid caused similar inhibition of HSC proliferation at 10^{-6} M and 10^{-8} M (Davis *et al.*, 1990). Thus while some work has indicated that the intracellular effects of the 13-*cis* isomer may differ from the all-*trans* isomer (Ball and Olson, 1988), it appears that HSC proliferation responds similarly to both compounds.

Despite these observations, a recent report by Hellemans *et al.* (1999) found that all-*trans* retinoic acid and 9-*cis* retinoic acid exerted different effects on proliferation. In primary rat HSC both retinoids inhibited proliferation but in passaged rat HSC all-*trans* retinoic acid did not influence proliferation, and 9-*cis* retinoic acid inhibited proliferation only after 72 hours. More importantly, Tanaka *et al.* (1991) have demonstrated that *in vivo* in regenerating rat liver no association is apparent between the proliferative activity of HSC and the levels of hepatic vitamin A.

1.8.6.2. Retinoids and Collagen Synthesis

Several studies have indicated that retinoids have a modulatory effect on the deposition of extracellular matrix components by HSC. Conflicting results have been reported concerning the effect of retinoids on collagen synthesis. Davis *et al.* (1987) found that the collagen expressed by HSC in response to retinol was to some extent dependent upon the underlying matrix. When passaged rat HSC were grown on type I collagen matrix, addition of retinol to HSC (10^{-5} M retinol for 18 hours) resulted in a significant increase in synthesis of collagen types I and III. However, when HSC were subcultured on a type IV matrix, retinol caused a decrease in type I collagen production and an increase in type III.

The effect of the related retinoid, all-*trans* retinoic acid on collagen gene expression has also been studied in HSC *in vitro* and in rat and mouse liver *in vivo* (Davis *et al.,* 1990). All-*trans* retinoic acid (10^{-6} M) caused a marked decrease in collagen type I and III in passaged rat HSC cultured on type I collagen over a 48 hour period. The steady state levels of type I collagen mRNA however were not altered in the presence of 10^{-6} M retinoic acid suggesting *in vitro*, retinoic acid may alter collagen production at a translational or post-translational level. *In vivo,* administration of retinoic acid to *mice* markedly decreased total hepatic and HSC type I collagen mRNA abundance. In contrast, type I collagen mRNA was undetectable in samples of total hepatic RNA from either retinoic acid treated or control *rats*. However, retinoic acid treatment induced a decrease in collagen type I mRNA in freshly isolated HSC from retinoic acid treated rats compared with controls. Okuno *et al.* (1997) found that not only does retinoic acid enhance collagen expression by HSC but also suppresses the production of collagenase by HSC. Hellemans *et al.* (1999) found the effect depended on the isomer of retinoic acid. Primary rat HSC treated for 48 hours with all-*trans* retinoic acid (10^{-6} M) exerted an inhibitory effect on the synthesis of pro-collagens type I, III and IV, whereas 9-*cis* retinoic acid (10^{-6} M) increased pro-collagen I mRNA but decreased the corresponding level of protein.

Contrary to the above data, other studies have found only a minor effect of 10^{-6} M retinol on collagen synthesis or secretion in passaged cultures of myofibroblasts. Geerts *et al.* (1989) found that although the addition of retinol was accompanied by a dose dependent inhibition of synthesis and secretion of proteins, an inhibitory effect on collagen synthesis could not be demonstrated. The absence of modifications on collagen synthesis by retinol after 24-48

hours, is consistent with Margis *et al.* (1992) who showed a decrease in collagen synthesis in a HSC line only after 6 days of culture. Thus although Davis *et al.* (1987) demonstrated retinol modulates collagen synthesis in HSC, it was not mentioned whether this modulating effect resulted in an overall increase or decrease of synthesis and secretion of collagenous proteins.

The above data imply there is an intimate relationship between vitamin A and collagen metabolism. Vitamin A clearly has effect on collagen phenotype and the extracellular collagen matrix appears to alter both collagen and vitamin A metabolism. The published data report that collagen is either increased, unchanged or reduced by exposure of rat HSC to retinoids, implying that the difference in the experimental conditions, especially the different stages in transformation of HSC used for experiments, caused the opposing effects of retinoids on HSC.

1.8.6.3. Retinoids and Transforming Growth Factor Beta

It has been found that addition of either all-*trans* retinoic acid, 9-*cis* retinoic acid, or 9,13 di-*cis* retinoic acid (generated *in vivo* from isomerisation of 9-*cis* retinoic acid) to rat HSC cultures induces the plasmin-mediated activation of latent TGF-β (Okuno *et al.*, 1997; Imai *et al.*, 1997). The TGF-β generated enhances the production of fibrogenic components such as collagen (Okuno *et al.*, 1999) causing the progression of fibrogenesis. Furthermore, it has been demonstrated that RARα is responsible for the retinoic acid induced up regulation of plasminogen activated TGF-β in HSC cultures (Imai *et al.*, 1997).

After stimulation with all-*trans* retinoic acid, the TGF-β produced endogenously from HSC suppresses the production and secretion of albumin from hepatocytes at the translational level (Koda *et al.*, 1996). The down-regulation of proteins from hepatocytes via TGF-β generated from retinoic acid stimulated HSC, demonstrates an additional mechanism by which retinoic acid is involved in the progression of liver cirrhosis. According to Okuno *et al.* (1999), the hepatic concentrations of retinoic acids, especially 9,13 di-*cis* retinoic acid, increase in fibrotic livers of rats and in activated rat HSC in culture. Since 9,13 di-*cis* retinoic acid is biologically active in inducing TGF-β synthesis in cultured HSC (Imai *et al.*, 1997), it is conceivable that retinoic acid suppresses the secretion of albumin *in vivo* through the same TGF-β mediated mechanism demonstrated *in vitro*.

1.8.7. Retro-Retinoids

Although it is currently unclear whether the physiologic effector molecule is actually retinol or retinoic acid, a potential role for retinoic acid in regulating HSC activation is attractive based on the compound's profound effects as a morphogen, and numerous studies which document various effects of exogenous retinoic acid on a wide variety of cell types. Although retinoic acid has been reported to be 2 to 3 orders more potent as an inhibitor of cell growth compared to retinol (Davis *et al.,* 1990), retinoic acid has been detected at much lower concentrations than retinol in HSC (Okuno *et al.,* 1999; Ohata *et al.,* 1997). This difference in sensitivity to retinol versus retinoic acid has been previously noted in other cell systems (Oikarinen *et al.,* 1985). Since HSC contain both CRBP and CRABP, it suggests HSC have the capability to metabolise or respond to both forms of the vitamin. In addition, it is possible that failure to detect retinoic acid may reflect insensitivity of current detection methods or perhaps that retinoic acid exists as a metabolite not recognised by current procedures. Preliminary studies for example, have identified a novel class of retinol metabolites in HSC, termed retro-retinoids (the position of conjugated double bonds are shifted by one carbon).

14-Hydroxy-4,14-*retro*-retinol (14-HRR) was the first bioactive retro-retinoid to be discovered, isolated from cultures of lymphoblastoid 5/2 and Hela cells (Buck *et al.,* 1991). It is also produced by various cell lines including fibroblast, leukaemia and *Drosophila* cells (Derguini *et al.,* 1994). The growth of B cells and the activation of T cells is sustained with 14-HRR at 10-30 fold lower concentration than with its precursor retinol (Derguini *et al.,* 1994). Although the mechanism of action of 14-HRR is still unknown, it may be analogous to retinoic acid and act as a ligand for a cytoplasmic or nuclear receptor. Anhydroretinol, the second retro-retinoid to be discovered, was identified in activated human B cells and also extracted from *Drosophila* and other insect cells (Buck *et al.,* 1993). Anhydroretinol functions as a reversible inhibitor of retinol and 14-HRR dependent effects, blocking B lymphocyte proliferation as well as the activation of resting T lymphocytes (Buck *et al.,* 1993). 14-Hydroxy-4,14-*retro*-retinol and anhydroretinol constitute the first naturally occurring agonist/antagonist pair of retinoids and Anhydroretinol could bind to the same hypothetical receptor as 14-HRR.

A previous study has detected 14-HRR in activated HSC in CCl_4 and bile duct ligated models of liver injury, as well as during culture induced activation (Friedman *et al.,* 1992b). Since 14-

HRR appears early in HSC activation, retro-retinoids may have regulatory roles in the development of HSC activation and hepatic fibrosis. They may also provide an explanation for the intracellular events associated with the release of retinoids during the transformation process. Following the detection of 14-HRR in the media (Friedman *et al.*, 1992b), consistent with the ability of these compounds to pass through cell membranes, retro-retinoids may also have autocrine effects on HSC.

1.8.8. Hyper and Hypovitaminosis A and the Relationship with Liver Fibrosis

In most forms of experimental and human liver injury the levels of vitamin A in the liver diminish. Yet there is an important clinical paradox in the literature, both hyper- and hypo-vitaminosis have been associated with liver fibrosis and cirrhosis. Senoo and Wake (1985) reported that the administration of retinyl palmitate increased retinoid content in HSC and suppressed the induction of experimental hepatic fibrosis produced by CCl_4 or by porcine serum in rats. Additionally, pre-treatment of rats with retinyl palmitate prior to isolation of HSC considerably decreases the proliferative capacity and the protein synthesis of these cells in early primary culture (Shiratori *et al.*, 1987). In support of this, it is reported that vitamin A deficiency promotes CCl_4-induced liver fibrosis (Seifert *et al.*, 1994). A lowered vitamin A status, hypovitaminosis was induced by feeding rats a vitamin A deficient diet until the liver retinoid content was strongly reduced but before clinical symptoms of vitamin A deficiency occurred. After the induction of hypovitaminosis, CCl_4 was given as a liver-fibrosis inducing agent. Administration of CCl_4 to vitamin A deficient rats induced the same degree of hepatocellular damage and mortality, but accelerated and intensified liver fibrogenesis compared to rats with normal retinoid status.

On the other hand, feeding excess retinoid in combination with CCl_4 has been shown to have dramatic effects on the liver. Excess retinoid feeding previous to or simultaneous with CCl_4 addition induces more extensive hepatocyte cell damage and increases mortality compared with CCl_4 treatment alone. However, feeding excess retinoid after liver fibrosis has been established significantly reduces liver fibrosis (Seifert *et al.*, 1989). Furthermore, it has been documented that the incidence of human hepatic fibrosis is correlated with the amount of vitamin A intake by patients with vitamin A hepatotoxicity (Geubel *et al.*, 1991). The suppression of stromelysin and collagenase gene promoters by retinoic acid (Nicholson *et al.*, 1990) could partly account for this observed fibrosis-enhancing effect.

Retinoids may potentiate the toxic effects of alcohol and selected drugs (Mclean *et al.*, 1969) on the liver. Vitamin A content of the diet has also been reported to influence the degree of alcohol induced liver fibrosis. In rats fed diets supplemented with vitamin A and alcohol, Leo and Lieber (1983) observed necrosis, inflammation and fibrosis of the liver [although this was later contradicted by Seifert *et al.* (1991)]. This is not the case when either vitamin A or alcohol alone are given. One explanation between these differing effects is competition between different pathways of retinol metabolites within HSC.

1.8.9. Hepatic Stellate Cells and Nuclear Retinoid Receptors

Some groups have considered the possibility that changes in nuclear retinoid receptors accompany retinoid release by HSC. Two groups in particular have shown that the expression of RARs and RXRs change during HSC activation. Expression of RARs in HSC was first demonstrated by Weiner *et al.* (1992). Using northern hybridisation, they verified the presence of RARα, RARβ and RARγ in freshly isolated HSC from normal rat liver. However, they also found that although freshly isolated rat HSC contain RARβ mRNA and the nuclear RARβ protein, rat HSC cultured for 7 days showed no detectable mRNA or nuclear staining for RARβ. The loss of this receptor was shown to be a reversible process; when the cultures were treated for 48 hours with either retinoic acid or retinyl acetate the expression of RARβ mRNA was restored.

Weiner *et al.* (1992) suggest an increase in HSC proliferation and collagen synthesis during cell culture or hepatic fibrosis may result in part from decreased HSC retinoid responsiveness, reflected by loss or decrease in nuclear RARβ gene expression. Furthermore, this would be exacerbated by loss of the cellular retinoids during activation. Depleted of retinoids, HSC could then proliferate and increase their collagen synthesis.

Ohata *et al.* (1997) complemented the findings by Weiner *et al.* (1992). Using the competitive polymerase chain reaction (PCR) they found a reduction in the expression of RARβ mRNA in HSC from rats with cholestatic liver fibrosis compared to sham-operated rats. A suppression in the level of RXRα mRNA was also detected. In agreement with the loss of retinoid receptors in HSC from rats with cholestatic liver fibrosis, electromobility shift assays (EMSA) showed a reduction in the amount of protein bound to RAREs in HSC nuclear extracts from these animals.

1.9. HYPOTHESIS AND AIMS

During the activation of HSC following liver injury, retinyl palmitate contained in lipid droplets is metabolised to other retinoids. These retinoids could influence HSC activation. Although the effect of exogenous retinoids on HSC has been studied, their role in HSC activation and in liver fibrogenesis is still unclear. This may be because reported studies have examined the effects of retinoids on cells that were already activated by prolonged primary culture or subculture on substrates that promote the injury response. Both *in vitro* and *in vivo,* retinoids have been shown to have varying effects on HSC. However, an understanding of retinoid metabolism and signalling in the injured liver could help to address the apparent paradox between the published data. Consequently, this study aims to examine the retinoids present during activation of HSC in primary culture and determine their effects on HSC phenotype and function. Additionally, it has been hypothesised that the response of HSC to retinoids is dependent on the temporal relationship between retinoid synthesis and retinoid receptor expression. To pursue this hypothesis, a further aim of this study was therefore to examine expression of the nuclear retinoid receptors during HSC activation, and to investigate the contribution of individual RAR subtypes to this process. Since so many retinoid receptors have been cloned and sequenced the study will concentrate only on those receptors previously implicated in HSC activation, in particular the nuclear retinoid receptor, RARβ.

CHAPTER 2

Materials and Methods

2. MATERIALS AND METHODS

Unless otherwise stated, all chemical reagents were supplied by Sigma Chemical Company, Poole, UK. Reagents denoted with an asterisk are defined in appendix 1.

2.1. CELL CULTURE

All solutions were pre-warmed to 37 °C and throughout the procedures sterile techniques were employed.

2.1.1. Cell Isolation

Several factors aid the isolation of HSC. Firstly, HSC are more resistant to enzymic digestion than other liver cell types. Therefore careful use of the digestive enzymes selects against hepatocyte contamination. Secondly, since freshly isolated HSC contain abundant lipid droplets, they have a greater buoyancy than other liver cells and can therefore be isolated by a density gradient. Finally, the method of differential adhesion can enhance the purity of the culture. Hepatocytes, unlike HSC, do not adhere very efficiently to plastic. Additionally, although Kupffer cells take only approximately 8 hours to adhere (HSC take approximately 2 days), they cannot proliferate and soon undergo necrosis. Therefore, once HSC have adhered to the plastic, hepatocytes and Kupffer cells can be washed away.

Materials
Collagenase, Roche Diagnostics, Lewes, UK
Deoxyribonuclease (DNase), Roche Diagnostics, Lewes, UK
Dulbeccos Modified Essential Medium (DMEM), Life Technologies, Paisley, UK
Foetal calf serum (FCS), Life Technologies, Paisley, UK
Hanks buffered salt solution (HBSS), Roche Diagnostics, Lewes, UK
Optiprep, Life Technologies, Paisley, UK
Pronase, Roche Diagnostics, Lewes, UK
Penicillin/streptomycin/gentamycin (PSG) *

Method

Based on the method by Pinzani *et al.* (1992a), liver cells were isolated from Sprague-Dawley rats [500 grams (g) plus] by digestion with pronase/collagenase followed by density gradient centrifugation (Arthur *et al.*, 1989). Isolations were kindly performed by Mrs J Gentry.

The procedure involved three steps; firstly pre-digestion and perfusion of the liver with calcium free physiological salts (HBSS) containing heparin [250 units/millilitre (ml)]. This aids digestion by disrupting calcium dependent intercellular interactions and removes blood from liver vessels, thus enabling digestive enzymes to reach all areas of the organ. The second step involved perfusion with the digestive enzymes collagenase [0.2 milligrams (mg)/ml HBSS containing calcium] and pronase (2 mg/ml HBSS containing calcium). Collagenase catabolises the liver extracellular matrix leaving unanchored non-parenchymal cells and hepatocytes. The latter were subsequently digested by pronase. Deoxyribonuclease (0.1 mg/ml HBSS containing calcium) was also included to prevent clumping of cells by DNA released from ruptured cells. The digested liver was filtered through a nylon mesh and the filtrate purified by isopyknic centrifugation with an Optiprep solution (11% upper gradient and 17% lower gradient). Isopyknic centrifugation separates cells by sedimentation to a point in a gradient equivalent to their own density. The cells at the top interface represented HSC and the cells immediately beneath this layer were Kupffer cells. Hepatocytes were isolated through the use of a modification of the HSC isolation procedure. Each cell type was additionally purified by centrifugal elutriation, a method which separates cells according to their size and resulting in a highly purified preparation (> 95%). Cell viability was determined by the trypan blue exclusion test. Human HSC were obtained from a normal liver unsuitable for transplantation, by the same procedure used to isolate rat HSC, with the exception that the human HSC were not purified by centrifugal elutriation.

The purified cells were re-suspended in DMEM supplemented with 16% FCS and 4% PSG. Cells were either used immediately in experiments (freshly isolated HSC, Kupffer cells and hepatocytes), or HSC were cultured on uncoated plastic at a plating density of 1×10^6 HSC/ml (unless otherwise stated) to allow the HSC to become activated. Cells were cultured in a humidified atmosphere of 95% air and 5% carbon dioxide (CO_2) and maintained at 37 ° centigrade (C). The culture medium was replaced every 48 hours.

2.1.2. Trypsinisation of Adherent Cells

Materials
Cultured rat HSC or COS-1 cells
DMEM, Life Technologies, Paisley, UK
FCS, Life Technologies, Paisley, UK
HBSS (not containing calcium), Roche Diagnostics, Lewes, UK
PSG *

Trypsin ethylenediaminetetraacetic acid [10 x (TEDTA)], Santa Cruz, Calne, UK

Method

Harvesting cells by trypsinisation was performed by washing the monolayer three times with HBSS (not containing calcium) and detaching the cells by a brief (5 minutes) exposure to TEDTA (1 x) in HBSS at 37 °C. Once detached, the cells were immediately centrifuged in a Fisons MSE chilspin rotor (1,500 rpm, 5 minutes, room temperature). The pellet was then washed with HBSS to neutralise the harvesting reagent and re-centrifuged. After re-suspending the cells in DMEM containing 16% FCS and 4% PSG, the concentration of cells was determined using a haemocytometer and the cells re-plated at the desired concentration.

2.2. PREPARATION OF RETINOIDS

To prevent oxidation by ultraviolet (UV) light, all procedures involving retinoids, including the culture and collection of HSC and their culture media was performed with minimal exposure to ambient light, and where possible in the dark with the aid of a sodium lamp (Griffin and George).

Materials

DMEM (Life Technologies, Paisley, UK)
Ethanol
FCS (Life Technologies, Paisley, UK)
PSG *
Retinoids: All-*trans* retinol, all-*trans* retinoic acid, 9-*cis* retinoic acid and 13-*cis* retinoic acid were purchased from Sigma Chemical Company, Poole, UK. 9,13-di-*cis* retinoic acid was a kind gift from Dr Kojima, the Institute of Physical and Chemical Research, Koyadai, Tsukuba, Ibaraki 305, Japan. Ro 41-5253 (selective RARα antangonist) was a kind gift from Dr Michael Klaus, F. Hoffmann-La Roche Limited, CH-4002 Basel, Switzerland. LE135 (selective RARβ antagonist) was a kind gift from Dr Hiroyuki Kagechika, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan.

Method

Immediately before addition to cell cultures, retinoids were prepared as 5 x 10^{-6} M stock solutions in ethanol. The desired concentration of retinoid was then obtained by dilution with culture medium (DMEM supplemented with 16% FCS and 4% PSG). The final concentration of ethanol was always < 0.2%. Stocks of retinoids were stored under nitrogen atmosphere at -80 °C.

2.3. PROLIFERATION ASSAY

The effect of retinoids on the rate of HSC proliferation was determined by [*methyl*-tritium (^{3}H)] thymidine incorporation into newly synthesised deoxyribonucleic acid (DNA).

Materials

Ethanol
Freshly isolated rat HSC suspended in DMEM containing 16% FCS and 4% PSG
HBSS (containing calcium), Roche Diagnostics, Lewes, UK
Methanol:acetic acid [19:1 volume/volume (v/v)]
Retinoids: All-*trans* retinol, all-*trans* retinoic acid, 9-*cis* retinoic acid and 13-*cis* retinoic acid were purchased from Sigma Chemical Company, Poole, UK. Ro 41-5253 (selective RARα antangonist) was a kind gift from Dr Michael Klaus, F. Hoffmann-La Roche Limited, CH-4002 Basel, Switzerland. LE135 (selective RARβ antagonist) was a kind gift from Dr Hiroyuki Kagechika, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan.
Sodium hydroxide (0.25 M)/Sodium dodecyl sulphate [0.2% (SDS)]
[*Methyl*-^{3}H] Thymidine, Amersham, Little Chalfont, UK

Method

To each of 24 plastic coated wells on a culture plate (Greiner, Stonehouse, UK), 2.5 x 10^5 freshly isolated rat HSC were added in 16% FCS and 4% PSG. On day 4, day 5 or day 6, retinoids were added to appropriate wells at various concentrations. The plates were then incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for a further 72, 48 or 24 hours respectively (until day 7 of culture), replacing the media with freshly prepared retinoid containing media after 48 hours.

[*methyl*-^{3}H] Thymidine [1 microCuries (μCi)/ml] was added to cultures 16 hours before experiments were terminated by washing the cells twice with HBSS containing calcium (room temperature, 15 minutes) followed by fixing the cells by incubation at -20 °C for 30 minutes in pre-cooled methanol/acetic acid (19:1 v/v). The amount of radioactively labelled DNA was analysed using a modification of the method by McGowan *et al.* (1981). Monolayers were washed three times on ice with HBSS containing calcium and then solubilised by incubation at 37 °C for 45 minutes with sodium hydroxide (0.25 M)/SDS (0.2%). The plates were then shaken thoroughly at room temperature for 15 minutes. After precipitation and neutralisation of the sodium hydroxide with hydrochloric acid (5 M), the contents of each well were assayed for radioactivity in a Wallac 1217 Rackbeta liquid scintillation counter.

Control groups included cells exposed to 0.5% FCS, reducing [*methyl*-^{3}H] thymidine incorporation to a baseline level and hence representing the minimal rate of HSC proliferation.

Due to different degrees of absolute stimulation between separate experiments, data were expressed as a percentage of control cells maintained in 16% FCS. Data were diagrammatically represented by use of the program Microsoft Excel 97 (Microsoft corporation) and presented as the mean ± standard error of the mean (S.E.M.) when appropriate. Statistical analysis was by paired *t*-test using the program GraphPad Instat (GraphPad Software), with the probability (P) < 0.05 taken to indicate statistical significance.

2.4. PREPARATION OF AGAROSE GELS FOR A HORIZONTAL APPARATUS

Electrophoresis through agarose (or polyacrylamide) gels is the standard method used to separate, identify and purify DNA fragments. At neutral pH, the negatively charged DNA loaded into a sample well at the cathode end of a gel, migrates toward the anode. The electrophoretic mobility of DNA fragments is dependent on the molecular size of the DNA and fairly independent of base composition or sequence. DNA from 200 base-pairs (bp) to approximately 50 kilobases (kb) in length can be separated on agarose gels of various concentrations. Agarose gels are usually run in a horizontal configuration in an electric field of constant strength and direction.

Materials

Agarose/low gelling temperature agarose
Distilled water
DNA loading buffer (6 x) *
DNA molecular weight markers, Life Technologies, Paisley, UK
DNA samples
Ethidium bromide (1 mg/ml) in water
10 x Tris(hydroxymethyl)aminomethane (Tris)-borate ethylenediaminetetraacetic acid (EDTA) (TBE) buffer * or 10 x Tris-acetate EDTA (TAE) buffer *

Method

Agarose, added to a measured quantity of buffer (1 x TBE or 1 x TAE) to give a desired final concentration of agarose, was dissolved by heating in a microwave oven (Panasonic). When the solution had cooled to 50 °C ethidium bromide (final concentration 0.5 μg/ml) was added. The gel was cast and the comb (sample well former) placed into the molten agarose. After

setting (30-45 minutes, room temperature), enough buffer was added to the electrophoresis tank (Bio-Rad) to just submerge the gel (250 ml of 1 x TBE buffer or 250 ml of 1 x TAE buffer).

Each DNA sample (including the DNA molecular weight markers) was mixed with DNA loading buffer (final concentration 1 x DNA loading buffer) and loaded into a separate well on the gel. The gel was run at 90 Volts (V) at room temperature until the bromophenol blue had migrated approximately 8 centimetres (cm) from the wells. Since the fluorescent dye ethidium bromide intercalates between stacked base pairs of DNA, the DNA in the gel could be detected by UV illumination.

2.5. EXTRACTION, PURIFICATION AND ANALYSIS OF RNA

RNA molecules are much more susceptible to degradation than DNA. During extraction and purification, ribonucleases may degrade RNA. To minimise this problem sterile techniques were employed to prevent contamination from hands, glassware or airborne dust particles. Gloves were worn at all times and glassware was baked at 200 °C for at least 4 hours. Before use, non-disposable plasticware, including the electrophoresis apparatus was thoroughly rinsed with 0.5 M sodium hydroxide and then with diethylpyrocarbonate (DEPC, which inhibits RNases by modifying tyrosine residues) treated water. Pipette tips and microcentrifuge tubes were autoclaved, and water and stock aqueous solutions were pre-treated with DEPC.

Total RNA and mRNA were extracted, separated by electrophoresis on an agarose gel under denaturing conditions, transferred to the surface of a nylon membrane and a specific mRNA detected by hybridisation with a radioactively phosphorus-32 [^{32}P] labelled probe (northern blotting).

2.5.1. Total RNA Extraction

Total RNA was isolated from cultured cells using a modification of the method described by Chomczynski and Sacchi (1987); the acid guanidinium-phenol-chloroform method.

2.5.1.1. Guanidinium Isothiocyanate Lysate Preparation

Materials
COS-1 cells transfected with pSG5 plasmids containing either mouse RARα, RARβ$_2$ or RARγ full-length DNA sequences (section 2.17.)
Freshly isolated rat HSC, Kupffer cells or hepatocytes, or cultured rat HSC
Guanidinium isothiocyanate [4 M (GIT)] *
β-Mercaptoethanol

Method

To a pellet of freshly isolated HSC, Kupffer cells or hepatocytes, five volumes of GIT (4 M) containing β-mercaptoethanol (0.72%) were added. Alternatively, 1 ml of GIT was added directly to a monolayer of HSC in a 75 mm^2 tissue culture flask, or 3 ml of GIT was added to COS-1 cells (transfected with pSG5 plasmids containing an RAR full-length DNA sequence) in a 100 mm petri-dish (Greiner, Stonehouse, UK). The cells bathed in GIT were vigorously shaken for 15 minutes to cause cell lysis and to denature the proteins, including RNases subsequently liberated. The lysates were stored at -70 °C until required for RNA isolation.

2.5.1.2. Total RNA Isolation

Materials
Chloroform/isoamyl alcohol (49:1 v/v)
DEPC treated water *
Ethanol [75% (in DEPC treated water)]
GIT lysate
Isopropanol
Phenol
Phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v)
Sodium acetate (2 M, pH 4.0)
Sodium acetate (3 M pH 6.0)

Method

Sequentially, 0.1 ml of 2 M sodium acetate pH 4.0, 0.7 ml of phenol and 0.1 ml of chloroform-isoamyl alcohol mixture (49:1) were added to the defrosted GIT lysate. The final suspension was thoroughly mixed by inversion and incubated on ice for 15 minutes to aid precipitation of proteins. After centrifugation in an Eppendorf 5402 centrifuge (10,000 g, 10 minutes, 4 °C), the upper aqueous phase containing the RNA was transferred to a fresh tube, mixed by inversion with 0.7 volume of phenol/chloroform/isoamyl alcohol (25:24:1) and then cooled on ice for 10 minutes to re-extract the RNA. The samples were again centrifuged

(10,000 g, 10 minutes, 4 °C) and the upper aqueous phase was transferred to a clean tube. Nucleic acids are uniformly and strongly negatively charged because of their phosphate backbones so they will partition into an aqueous environment where these charges are solvated. Everything else (proteins, carbohydrates, lipids) contains charged and uncharged regions as well as hydrophobic and hydrophilic regions, therefore preferring either a hydrophobic (organic) environment or the interface between organic and aqueous phases. To precipitate the RNA, 0.1 volume of 3 M sodium acetate pH 6.0 and 1 volume of isopropanol were mixed with the aqueous phase and placed at -70 °C for at least 90 minutes.

The defrosted samples were sedimented (10,000 g, 10 minutes, 4 °C) and the resulting RNA pellet washed twice with 4 °C ethanol (75%). The pellet was dried at 65 °C and dissolved in DEPC treated water [20 microlitres (μl)] at 65 °C for 10 minutes.

The yield of total RNA obtained was determined spectrophotometrically using a Beckman DU 7500 spectrophotometer at a wavelength of 260 nm (UV light). The purity of RNA isolated was assessed by determining the optical density (OD) 260/280 (absorbance ratio). For pure RNA not contaminated with proteins this exhibits a ratio ranging from 1.7-2.0. The integrity of the RNA samples was determined by electrophoresis through denaturing agarose gels (section 2.5.3.). Visualisation by UV light of 28 S and 18 S ribosomal bands on the gel confirmed the integrity of the RNA.

2.5.2. Messenger RNA Extraction

In contrast to ribosomal RNA (rRNA) and transfer RNA (tRNA), a vast majority of mRNA of mammalian cells carry tracts of poly $(A)^+$ at their 3' termini. Messenger RNA can therefore be separated from rRNA and tRNA by affinity chromatography on oligo(dT) cellulose.

2.5.2.1. Preparation of Poly $(A)^+$ Messenger RNA

Materials
Phosphate buffered saline (PBS)
Freshly isolated rat HSC

Method

Freshly isolated rat HSC (5×10^6) were pelleted by centrifugation in a Fisons MSE chilspin

rotor (300 g, 5 minutes, 4 °C). The cell pellet was washed in sterile, 4 °C PBS to remove any FCS which could interfere with the isolation of the mRNA. The cells were re-pelleted and stored at -70 °C until isolation of the mRNA.

2.5.2.2. Messenger RNA Isolation

Messenger RNA was isolated according to the instructions supplied with the Micro-Fast Track™ kit (Invitrogen, Groningen, Netherlands).

Materials
Ethanol

Buffers and components supplied with the Micro-Fast Track™ kit:
Binding buffer *
Disposable spin columns and microcentrifuge tubes
Elution buffer *
Glycogen carrier *
Low salt wash buffer *
Oligo(dT)$_{20\text{-}30}$ cellulose tablets
Ribonuclease (RNase/protein degrader)
Sodium acetate (2 M)
Sodium chloride (5M)
Stock buffer *

Method

Cells resuspended in stock buffer (1 ml), containing a detergent and an RNase/protein degrader, were passed three times through a sterile plastic syringe (19 gauge needle) to disperse clumps of cells and ensure complete lysis. To digest proteins and ribonucleases the suspension was incubated at 45 °C for 15 minutes. The sodium chloride concentration of the lysate was adjusted to 0.5 M and by passing the lysate four times through a sterile plastic syringe (19 gauge needle) the DNA was sheared. An oligo(dT) cellulose tablet was gently mixed with the lysate for 15 minutes, enabling the poly $(A)^+$ mRNA to attach to the oligo(dT) cellulose. After centrifugation in an Eppendorf 5402 centrifuge (3,500 g, 5 minutes, room temperature), the pellet was washed three times in 1.3 ml of a high salt binding buffer to remove the DNA and degraded proteins, finally resuspending the pellet in 0.3 ml of binding buffer. The sample was spun in a spin-column (3,500 g, 10 seconds, room temperature) to remove any cell debris and precipitated proteins. Non-polyadenylated RNA was removed by washing the spin-column three times with binding buffer (200 µl) and twice with low salt wash

buffer (200 µl).

The poly $(A)^+$ mRNA was eluted by centrifuging twice with elution buffer (100 µl). To precipitate the poly $(A)^+$ mRNA, glycogen (10 µl), 2 M sodium acetate (30 µl) and ethanol (600 µl) were added to the elute and the sample placed at -70 °C for 90 minutes. The poly $(A)^+$ mRNA was recovered by centrifuging (10,000 g, 15 minutes, 4 °C) and aspirating off the ethanol before finally resupending the poly $(A)^+$ mRNA in elution buffer (10 µl).

2.5.3. Electrophoresis of RNA

Materials
Agarose
DEPC treated water *
Ethidium bromide in DEPC treated water (1 mg/ml)
Formaldehyde [37% (v/v) analytical grade]
Formamide
3-(N-morpholino)propanesulphonic acid [10 x (MOPS)] *
RNA loading buffer *
RNA samples
RNA size markers, Promega, Southampton, UK

Method
A horizontal agarose gel was prepared by dissolving the appropriate amount of agarose in a MOPS (1 x)/DEPC treated water mixture by heating in a microwave oven (Panasonic). When cooled to 50 °C, formaldehyde (final concentration 2.2 M) was added and the gel cast with the comb in position. Formaldehyde disrupts RNA secondary structure, greatly improving the resolution and enabling an accurate estimate of the length of RNA molecules to be determined. Ethidium bromide was not added to the gel as it decreases the efficiency of transfer of RNA to the nylon membrane. Once set, the gel was placed in the electrophoresis apparatus (Bio-Rad) and submerged in the electrophoresis buffer, MOPS (1 x).

RNA samples were mixed with an equal volume of RNA loading buffer (containing formaldehyde and formamide), incubated for 15 minutes at 65 °C to denature the RNA and then cooled to room temperature. The size markers were treated exactly as for the RNA samples. Immediately before loading onto the gel, ethidium bromide (1 µl of 1 mg/ml solution) was added to the samples to enable visualisation of rRNA under UV light. The amount of RNA loaded per lane depended on the abundance of the RNA species being

studied. For abundant mRNA species, 5-20 μg of total RNA was loaded per lane. For detection of rare mRNA 1-10 μg of purified poly $(A)^+$ mRNA was loaded per lane.

The gel was run at 4-5 V/cm until the bromophenol blue had migrated approximately 8 cm from the wells. The RNA was then transferred from the gel to a nylon membrane.

2.5.4. Transfer of Denatured RNA to a Nylon Membrane

RNA was transferred immediately after electrophoresis from an agarose gel to a nylon membrane (northern blot) by capillary elution.

Materials
Agarose/MOPS gel with RNA samples run on it (section 2.5.3.)
Aluminium Foil
Chromatography 3 mm filter paper, Whatman, Maidstone, UK
DEPC treated water *
Nylon membrane, Hybond N, Amersham, Little Chalfont, UK
Salt sodium citrate [20 x (SSC)] *
Sodium hydroxide [50 milliMolar (mM)]
Tissues

Method
Before transfer, the gel was rinsed in DEPC treated water for 10 minutes to remove the formaldehyde and then soaked in sodium hydroxide (50 mM) for 20 minutes to partially hydrolyse the RNA and improve the speed and efficiency of transfer. Following a second rinse in DEPC treated water for 10 minutes, the gel was soaked twice in SSC (10 x) for 10 minutes.

The northern transfer was set up in a conventional gel-blotting apparatus using SSC (10 x) as the transfer medium. A piece of 3 mm filter paper, soaked in SSC (10 x), was placed on a support inside a large dish filled with transfer medium. The gel was placed centrally in an inverted position on the wet 3 mm filter paper and any air bubbles were removed. Aluminium foil surrounded the gel to prevent short-circuiting of the transfer solution. A piece of nylon membrane, soaked in SSC (10 x) was placed on top of the gel and a second piece of 3 mm filter paper, pre-wet with SSC (10 x), placed on top of the hybridisation membrane. A stack of tissues on the filter paper and a weight on the top of the stack ensured a tight connection between the layers of material. The objective was to draw liquid from the reservoir through the gel and the nylon membrane so that RNA molecules were eluted from the gel and

deposited on the membrane. Transfer occurred for 16-24 hours.

After transfer, the membrane was dried and marked to indicate which surface had RNA deposited on it. To immobilise the nucleic acid the dried membrane was exposed to low doses of UV irradiation (254 nm) for exactly 5 minutes. This causes cross-links to form between a small fraction of the bases in the RNA and the amine groups on the surface of the membrane. Too much irradiation can result in the covalent attachment of a higher proportion of thymidines, with a consequent decrease in the hybridisation signal. The cross-linked membrane was stored at room temperature until pre-hybridisation was performed.

2.5.5. Hybridisation of Northern Blots with Radiolabelled DNA

Hybridisation of radioactively labelled nucleic acids to filter bound RNA involves three-stages; pre-hybridisation, hybridisation and stringency washing.

2.5.5.1. Pre-Hybridisation of Northern Blots

Pre-hybridisation saturates non-specific binding sites on the nylon filter that would otherwise give an unacceptable background.

Materials
Hybridisation buffer *
Northern blot (section 2.5.4.)

Method
The filter was pre-hybridised for 3-4 hours with hybridisation solution in a rotating hybridisation oven (Hybaid) at 42 °C. The volume of liquid was kept to a minimum so the kinetics of nucleic acid reassociation were faster and the amount of radiolabelled probe required was reduced. Non-specific binding sites were blocked by including Denhardt's reagent, in combination with blocking reagent and herring sperm DNA in the hybridisation solution. To maximise the rate of annealing of the probe with its target, the hybridisation solution was also of a high ionic strength (5 x SSC).

2.5.5.2. Hybridisation of Northern Blots

Materials
Hybridisation buffer *
Pre-hybridised northern blot (section 2.5.5.1.)
Radioactively [^{32}P]-labelled DNA probe (section 2.8.)

Method

Prior to hybridisation the double stranded, radioactively [^{32}P]-labelled DNA probe [3 x 10^6 counts per minute (cpm), section 2.8.] was denatured by heating to 95 °C for 5 minutes. The denatured probe was mixed with fresh hybridisation buffer, pre-heated to 42 °C and added to the pre-hybridised filter in the rotating hybridisation oven (Hybaid). The filter was incubated at 42 °C with the hybridisation solution containing the DNA probe for 16-24 hours, by which time the DNA probe would have re-annealed.

2.5.5.3. Stringency Washing of Northern Blots

After hybridisation, the filter was washed under stringent conditions to remove excess unbound probe. Unstable, non-specific hybrids with few hydrogen bonds can be disrupted by high temperature and low salt conditions.

Materials
Blue sensitive X-ray film, Genetic Research Instrumentation, Braintree, UK
DEPC treated water *
Hybridised northern blot (section 2.5.5.2.)
SSC (0.2 x) */SDS (0.2%)

Method

The filter was washed for 15 minutes with SSC (0.2 x)/SDS (0.2%) solution in a rotating hybridisation oven (Hybaid) at 50 °C, three times. The location of the hybrid was then determined by autoradiography. X-ray film was placed between the filter and an intensifying screen. If further intensification was required, for example when trying to detect a rare mRNA, the film was pre-flashed. After exposure at -70 °C for 24-72 hours, the film was developed in a Fuji X-ray film processor.

To enable hybridisation of the same filter sequentially to a number of different probes, the filter was kept moist, thus preventing the probe from becoming tightly bound. To remove the

probe, the filter was immersed in boiling DEPC treated water for 15 minutes. This was repeated with a fresh volume of boiling water. To verify that the original probe had been efficiently removed, the filter was exposed to X-ray film. The filter was then dried and stored at room temperature until required for hybridisation to another probe.

2.6. AMPLIFICATION, PURIFICATION AND ANALYSIS OF PLASMID DNA

To detect RARα, RARβ or RARγ mRNA on a northern blot, a template DNA sequence for each of these mRNAs was required. These templates were obtained by isolating the full-length sequences from pSG5 plasmids cloned either with mouse RARα, RARβ$_2$ or RARγ sequences, a gift from Professor Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Strasbourg, France (see appendix 2. for circle maps of these vectors and their sequences). Prior to digestion of these sequences from the plasmids, the plasmids were first amplified by transformation of *Escherichia coli* (*E.coli*) with the plasmids, followed by their purification. Finally, restriction enzyme digestion was performed to confirm amplification of the plasmid and successful cloning of the DNA sequence into the plasmid.

2.6.1. Transformation and Amplification of Competent *Escherichia Coli*

Since the transformation efficiency of bacteria is low, cells which have taken up DNA have to be identified. This is accomplished by transforming bacteria with a plasmid that contains a gene conferring antibiotic resistance. In this case the plasmid vectors confer resistance to ampicillin. The few cells that take up the plasmid and express the gene will be able to grow on agar containing this antibiotic.

Materials
Ampicillin (50 mg/ml)
Glycerol (30%)
Glycerol stock of *E.coli*, DH5α
Luria Bertani broth (LB broth)
pHook^{TM}-2*lac*(lactose)*Z*, Invitrogen, Groningen, Netherlands
pSG5 plasmids containing either mouse RARα, RARβ$_2$ or RARγ full-length DNA sequences were gifts from Professor Chambon, IGBMC, Strasbourg, France
pSG5 plasmid without insert
Terrific broth *

Method

Throughout the procedure sterile techniques were employed. Plasmid DNA [2 nanograms (ng)] was added and mixed gently on ice with a competent *E.coli* suspension (100 µl) for 30 minutes. This allowed the DNA to precipitate around the competent cells. The mixture was then heat-shocked at 42 °C for 2 minutes to promote the uptake of DNA into the bacteria. The cells were transferred back to ice for 10 minutes to induce enzymes involved in the repair of DNA and other cellular components, allowing the cells to recover from the transformation process and increase the efficiency. For each transformation reaction, 100 µl of LB broth was added to the cells, mixed and spread onto an LB agar plate containing ampicillin (50 µg/ml) and incubated at 37 °C overnight to allow single colonies of bacteria to grow.

A single colony from a transformation plate was transferred to terrific broth (5 ml) and grown overnight with vigorous shaking in a Luckham R300 incubator [200 revolutions per minute (rpm)]. The broth included ampicillin (50 µg/ml) to maintain the selection for the presence of the plasmid. Stocks of the culture were prepared by freezing a portion of the culture at -70 °C in the presence of glycerol (final concentration of 15%) to protect the cells from ice-crystal formation. After confirmation, with a mini preparation (section 2.6.2.) that the bacteria had been successfully transformed with the plasmid, the main plasmid amplification procedure was undertaken; 200 ml of terrific broth was inoculated with 0.5 ml of culture and grown at 37 °C in a shaker overnight (200 rpm).

2.6.2. Isolation and Purification of Plasmid DNA

The cleared lysate method of plasmid isolation is commonly used to extract relatively small plasmids (up to about 20 kb) from gram negative *E.coli*. This method relies on gently lysing the bacteria to release small molecules, including very compact supercoiled plasmids, into solution whereas larger molecules, such as chromosomal DNA fragments remain in the cell. It also makes use of the observation that there is a narrow pH range (12.0-12.5) within which denaturation of linear DNA but not covalently closed circular DNA occurs (Birnboim and Doly, 1979). High-speed centrifugation will pellet the cell debris and trapped chromosomal DNA to produce a cleared lysate enriched with plasmid.

Plasmids were isolated and purified using Promega's Wizard™ Minipreps DNA purification

system and Promega's Wizard™ Maxipreps DNA purification system which configure to the cleared lysate method. Similar protocols applied for both purification systems. Text in italic font indicates the additions or adjustments that were made for the larger scale, Maxiprep purification system.

Materials

Distilled water
E.coli cultures transformed with plasmid DNA (section 2.6.1.)
Ethanol (75% and 80%)
Isopropanol
Sodium acetate (3 M, pH 6.0)
Sterile water
Tris-EDTA [TE buffer (1 x)] *

Buffers and components supplied with the Wizard™ Minipreps DNA purification system and the Wizard™ Maxipreps DNA purification system , Promega, Southampton, UK:
Cell lysis solution *
Cell resuspension solution *
Column wash solution *
Neutralisation solution *
Wizard™ Minicolumns/Maxicolumns with reservoirs
Wizard™ Minipreps/Maxipreps DNA purification resin

Method

Plasmid DNA was purified from 1.5 ml (*5 x 50 ml*) of overnight cultures of *E.coli*. After centrifugation in an Eppendorf 5402 centrifuge at 10,000 g for 2 minutes at room temperature (*Beckman TJ6 centrifuge, 550 g, 10 minutes, room temperature*), the bacterial pellet was resuspended in 300 µl of cell resuspension solution (*7.5 ml of cell resuspension solution was added to the pooled pellets*). Cell lysis solution, 300 µl (*7.5 ml*), containing sodium hydroxide and SDS to disrupt the cell membrane, was gently mixed with the cell suspension by inversion until the suspension became clear and viscous. Gentle lysis causes the plasmid DNA to be released from the cell without contamination from chromosomal DNA. Any chromosomal DNA which is released is denatured due to the alkaline conditions. Upon neutralisation with 200 µl (*7.5 ml*) of acidic, pH 4.8, 1.32 M potassium acetate, the chromosomal DNA renatures and aggregates to form an insoluble network. Simultaneously, the high concentration of potassium acetate causes the precipitation of protein-SDS complexes and of high molecular weight RNA. The closed, circular plasmid DNA molecules remain in a native state. The precipitate was removed by centrifugation at 10,000 g for 5 minutes at room temperature (550 *g, 45 minutes, room temperature*) and the cleared supernatant containing the plasmid DNA

transferred to a clean centrifuge tube. *For Maxipreps, 1 volume of room temperature isopropanol was added to the cleared supernatant. After centrifugation at 550 g for 15 minutes at room temperature, the DNA pellet was resuspended in 2 ml of TE buffer.*

To purify the plasmid from the cleared lysate, a vacuum manifold (Promega) was used. To the solution containing the plasmid DNA, 1 ml (*10 ml*) of silica-based resin was added and the resin/DNA mix transferred to the syringe barrel of a Minicolumn (*Maxicolumn)*/syringe assembly inserted in a vacuum manifold. A vacuum was applied to draw the solution into the column which was subsequently washed with 2 ml (*25 ml*) of column wash solution. *To rinse the resin, 5 ml of ethanol (80%) was added to the Maxicolumn and a vacuum applied.* The resin was dried by continuing to draw a vacuum for an additional 30 seconds (*1 minute*). The Minicolumn, transferred to a microcentrifuge tube, was spun at 10,000 g for 2 minutes to remove any residual solution. *The Maxicolumn was inserted into a 50 ml centrifuge tube and spun at 550 g for 5 minutes. The resin in the Maxicolumn was dried to completion by drawing a vacuum for an additional 5 minutes.* Pre-heated (65-70 °C) water, 50 µl (*1.5 ml*) was applied to the column and after one minute the DNA was eluted into a clean centrifuge tube by centrifugation at 10,000 g for 20 seconds at room temperature (*550 g for 5 minutes, room temperature*). Resin fines in the elute were removed by centrifuging the sample (10,000 g, 5 minutes, room temperature) and collecting the supernatant. The plasmid DNA was stored at -20 °C until required.

If plasmid DNA was to be used to transfect mammalian cells (section 2.17.), the plasmid DNA was subsequently purified by precipitating the DNA in 0.1 volume of 3 M sodium acetate, pH 6.0 and 1 volume of isopropanol at -20 °C for 90 minutes. The defrosted sample was sedimented (10,000 g, 10 minutes, 4 °C) and the resulting pellet washed twice with sterile, 4 °C ethanol (75%). The pellet was air dried in a sterile environment and dissolved in sterile water (500 µl). The concentration of each DNA sample was determined using a Beckman DU 7500 spectrophotometer at absorbance 260 nm and the purified plasmid stored at -20 °C.

2.6.3. Restriction Enzyme Analysis of Plasmid DNA

Plasmid DNA isolated from culture (section 2.6.2.) was analysed by digestion with restriction enzymes and gel electrophoresis. When DNA was to be cleaved with 2 different restriction enzymes, the digestions were carried out simultaneously if both enzymes worked in the same

buffer. If the enzymes had different requirements, the DNA was digested first with one enzyme and then with the second enzyme following the cleaning of the linearised product from the first digestion (section 2.6.4.).

Materials
Distilled water
Isolated plasmid (section 2.6.2.)
Multicore buffer compatible with the restriction enzymes (10 x), Promega, Southampton, UK
Restriction enzymes, Promega, Southampton, UK

Method

For each microgram of plasmid, 5 units of the appropriate restriction enzyme and 0.1 volume of buffer were added. The volume was made up with distilled water so the restriction enzyme contributed less than 10% of the final reaction mixture. This ensured the enzyme activity would not be inhibited by glycerol. The mixture was incubated at 37 °C for 60 minutes. When large quantities of DNA were to be cleaved, the digestion time was often increased.

To ensure cutting had proceeded to completion, cut DNA (0.5 μg) was analysed on a 0.8% agarose/TBE gel (section 2.4.) together with uncut plasmid (0.5 μg). Uncut plasmid DNA underwent an identical reaction to the cut plasmid except the restriction enzyme was replaced with distilled water. The remaining cut DNA was purified (section 2.6.4.).

2.6.4. Purification of Restriction Enzyme Digests

Restriction enzyme digests were purified using the QIAquick gel extraction kit (Qiagen). In the presence of a high salt buffer, the DNA adsorbed to a silica-gel membrane whilst contaminants were efficiently washed away. The pure DNA was then eluted from the membrane with water.

Materials
Distilled water
Isopropanol
Restriction enzyme digest (section 2.6.3.)
Sodium acetate (3 M, pH 5.0)

Buffers and components required from the QIAquick gel extraction kit, Qiagen, Crawley, UK:
Buffer QG
Buffer PE
QIAquick spin column

Method

To separate the unwanted vector from the DNA insert, the completed restriction enzyme digest was electrophoresed on a 0.8% low gelling agarose/TAE gel using a wide sample well former (section 2.4.). Low gelling temperature agarose is a modified form of agarose that gels and melts at a low temperature without significant deterioration in the strength of the hardened gel. Using a sterile razor blade, the desired DNA band was excised from the gel, cut into 2 mm^3 pieces to facilitate gel dissolution and transferred to a microcentrifuge tube (< 300 mg agarose/tube). Three volumes of Buffer QG were added for each volume of agarose. The mixture was then incubated at 50 °C for 10 minutes, with vortexing every 2-3 minutes to help dissolve the agarose. If the colour of the mixture changed from yellow to orange or violet, 3 M sodium acetate pH 5.0 (10 µl) was added. To increase the yield of DNA fragments, one volume of isopropanol was added to the dissolved agarose. To allow the DNA to bind to the silica matrix, the sample was applied to a QIAquick column and centrifuged in an Eppendorf 5402 centrifuge (9,000 g, 60 seconds, room temperature). Traces of agarose were removed by adding Buffer QG (0.5 ml) to the column and centrifuging (9,000 g, 60 seconds, room temperature). The DNA was washed by adding Buffer PE (0.75 ml) to the column and allowing the column to stand for 5 minutes before centrifuging (9,000 g, 60 seconds, room temperature). After the flow-though had been removed, the column was spun for an additional 60 seconds to remove final traces of buffer. The column was placed into a clean microcentrifuge tube and the DNA eluted by addition of water (30 µl) to the centre of the column followed by centrifugation (9,000 g, 60 seconds, room temperature).

The quantity and quality of the DNA fragment was determined by loading a small aliquot (estimated to contain 50 ng) of the final preparation on an agarose gel with appropriate DNA molecular weight markers (section 2.4.). The gel was examined carefully for the presence of contaminating species of DNA. The purified DNA sample was stored at -20 °C until required for synthesis of a radioactive DNA probe (section 2.8.).

2.7. REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION OF MESSENGER RNA SEQUENCES

The extremely efficient reverse transcriptase-polymerase chain reaction [RT-PCR; (Saiki *et al.*, 1988)] was used to detect RNA, since RT-PCR can detect RNA that is present at less than one part per 10^8. A double stranded complementary DNA copy (cDNA) of the total

RNA in the sample was synthesised in a 5' to 3' direction using reverse transcriptase (RNA dependent DNA polymerase), together with oligo$(dT)_{15}$ primer to initiate the reaction. The cDNA was subjected to the PCR using two oligonucleotide primers (a sense and an antisense oligonucleotide) which flanked the specific DNA region to be amplified. DNA synthesis by heat stable DNA polymerase, *Thermus aquatics (Taq)* subsequently doubled the region between the primers. Since the extension products were complementary to the primers, repeated cycles of denaturation, priming and extension (PCR) enabled a rapid exponential accumulation of the specific target fragment.

To avoid contamination of a solution with a PCR product, PCR preparations and products were separated by isolating a laboratory, a set of micropipettes and storage containers exclusively for the setting up of the PCR.

2.7.1. Synthesis of a Double Stranded DNA Copy

To prevent possible contamination by ribonucleases which degrade RNA, throughout the cDNA synthesis step, gloves were worn and autoclaved tips, microcentrifuge tubes and DEPC treated water and DEPC treated aqueous solutions were used.

Materials

Deoxynucleotide triphosphate mixture (dNTP) of all four dNTPs (100 mM, diluted to 10 mM with DEPC treated water), Pharmacia Biotech, St Albans, UK
DEPC treated water *
Moloney murine leukaemia virus (M-MuLV) reverse transcriptase, Immunogen, Sunderland, UK
Oligo$(dT)_{15}$ primer, Promega, Southampton, UK
Reaction buffer (5 x), Immunogen, Sunderland, UK:
Tris-hydrochloric acid [250 mM, pH 8.3 (HCl)], potassium chloride (250 mM), magnesium chloride (20 mM), dithiothreitol [50 mM (DTT)]
Ribonuclease inhibitor (RNasin), Promega, Southampton, UK
Total cellular RNA (section 2.5.1.)

Method

To reverse transcribe 2 μg of total RNA, the following reagents were mixed with the RNA sample and the volume adjusted to 20 μl with DEPC treated water. In a negative control tube, DEPC treated water replaced the total RNA sample. The final concentrations of the reagents are shown in brackets.

10 mM dNTP mixture (1 mM of each dNTP)
Oligo(dT) primer [80 picograms (pg)/μl]
5 x Reaction buffer (1 x)
Reverse transcriptase (1 unit/μl)
RNasin (1 unit/μl)

The mixture was incubated at 37 °C for 60 minutes to reverse transcribe the RNA to cDNA. The reaction was terminated by increasing the temperature to 95 °C for 2 minutes and the mixture was then diluted 5-fold with DEPC treated water. The cDNA sample was stored at -20 °C until the PCR was performed.

2.7.2. The Polymerase Chain Reaction

Materials
Distilled water
Double stranded cDNA sample (section 2.7.1.)
DNA polymerase (*Taq),* Flowgen, Lichfield, UK
10 x DNA polymerase buffer, Flowgen, Lichfield, UK
1 x DNA polymerase buffer contains: Tris-HCl (10 mM, pH 8.8), Triton X-100 (0.1%), potassium chloride (50 mM)
dNTP mixture (100 mM, diluted to 10 mM with distilled water), Pharmacia Biotech, St Albans, UK
Magnesium chloride (50 mM), Flowgen, Lichfield, UK
PCR molecular weight markers, Promega, Southampton, UK
Specific PCR primers, Perkin Elmer, Warrington, UK

Method

For each PCR, 50 μl of 'master mix' was required containing DNA polymerase buffer (final concentration 1 x) and an optimum concentration of magnesium chloride. Distilled water constituted the remaining volume. Since the PCR is exquisitely sensitive to magnesium ions, the optimum concentration (0.8-2.0 mM) must be established for each pair of primers. If the magnesium concentration is too low, the primers will hybridise non-specifically and a ladder of fragments will be produced, which may or may not contain the required specific fragment. At too high a magnesium concentration, the primers will not form stable hybrids and no product will be seen.

In a Perkin Elmer PCR tube, 13 μl of master mix was added to 0.1 mM dNTP mix and 30-50 picomoles (pmoles) of each primer. This formed the lower layer. These tubes do not require the addition of wax to prevent evaporation of the contents. The reagents of the upper layer, 37

μl of master mix, 1.6 units of DNA polymerase and either 5 μl of diluted cDNA mix or 5 μl of distilled water (the negative control) were then added.

The tubes were placed in an automated PCR cycling apparatus (Perkin Elmer Gene Amp PCR system 2400) with programmable incubation blocks allowing temperature, incubation time and cycle number to be controlled. The cycling conditions were adjusted for each PCR to ensure highly efficient and accurate amplification of the desired template, without the appearance of non-specific products. Parameters taken into consideration included the optimum temperature at which *Taq* causes polymerisation, the annealing temperature of the primers, the temperature for the dissociation of the cDNA strands and the number of cycles of annealing, extension and strand dissociation required.

The PCR products were analysed on a 1-1.5% agarose/TBE gel (section 2.4.) with PCR molecular weight markers to ensure the amplified DNA band was of predicted size.

2.7.2.1. β-Actin Cycling Parameters and Primers

To act as a positive control, the PCR was performed using β-actin primers for every double stranded cDNA made from total RNA.

Table 2.1: Rat β-actin RT-PCR primers

Primer	Orientation	Sequence	Location	Product size
β-Actin	Sense	TGTACGTAGCCATCCCGGCT	475	319 bp
	Antisense	TTCTTCAGGGAGGAAGAGGA	774	

Primers were designed by Thompson et al. (1998)

The β-actin cycling conditions and parameters were as follows:

Magnesium chloride (1 mM)
Sense and antisense primers for rat β-actin (50 pmoles of each primer)

Cycling parameters:

1 minute at 95 °C to denature any existing contaminating enzymes (one cycle).

35 cycles of:
20 seconds disannealing at 95 °C
30 seconds annealing at 55 °C
1 minute extension at 72 °C

10 minutes at 72 °C to ensure that all the PCR products are fully elongated followed by cooling at 4 °C.

2.7.2.2. β_1-Integrin Cycling Parameters and Primers

Table 2.2: Rat β_1-integrin RT-PCR primers

Primer	Orientation	Sequence	Location	Product size
β_1-Integrin	Sense	TGCAGGTGTCGTGTCTGTGAATGC	475	380 bp
	Antisense	CAGCAGTCATCAATGTCCTTCTCC	774	

Primers were designed by Krzesicki et al. (1997)

The β_1-integrin cycling conditions and parameters were as follows:

Magnesium chloride (1.75 mM)
Sense and antisense primers for rat β1-integrin (20 pmoles of each primer)

Cycling parameters:

1 minute at 95 °C to denature any existing contaminating enzymes (one cycle).

30 cycles of:
45 seconds disannealing at 95 °C
1 minute annealing at 53 °C
1 minute extension at 72 °C

10 minutes at 72 °C to ensure that all the PCR products are fully elongated followed by cooling at 4 °C.

2.7.2.3. RARα and RARβ Cycling Parameters and Primers

Table 2.3: Mouse RARα and RARβ RT-PCR primers

Primer	Orientation	Sequence	Location	Product size
RARα	Sense	CAGATGCACAACGCTGGC	886-903	397 bp
	Antisense	CCGACTGTCCGCTTAGAG	1282-1265	
RARβ	Sense	ATGCTGGCTTCGGTCCTC	875-892	470 bp
	Antisense	CTGCAGCAGTGGTGACTG	1344-1327	

Primers were designed by Wan et al. (1992)

The PCR was performed using conditions and cycling parameters stated by Wan *et al.* (1992)

Magnesium chloride (1.5 mM)
Sense and antisense primers for mouse RARs (30 pmoles of each primer)

Cycling parameters:

1 minute at 95 °C to denature any existing contaminating enzymes (one cycle).

30 cycles of:
30 seconds disannealing at 95 °C
30 seconds annealing at 55 °C
45 seconds extension at 72 °C

10 minutes at 72 °C followed by cooling at 4 °C.

2.7.3. DNA Purification from Polymerase Chain Reactions

Applications such as cloning, labelling and sequencing of DNA often require the purification of DNA fragments from agarose gels or amplification reactions. Double-stranded PCR-amplified DNA was purified using Promega's Wizard™ PCR preps DNA purification system.

Materials
Distilled water
Isopropanol (80%)
PCR molecular weight markers, Promega, Southampton, UK

Components supplied with the Wizard™ PCR preps DNA purification system, Promega, Southampton, UK:

Wizard™ PCR preps DNA purification Minicolumns
Wizard™ PCR preps DNA purification resin

Method

To purify the PCR product away from non-specific amplification products, the reaction products were separated by electrophoresis in a TAE, low-gelling temperature agarose gel (section 2.4.). Using a sterile razor blade, the desired DNA band was excised quickly from the gel to minimise damage to the DNA by the UV light. The agarose slice (< 300 mg) was transferred to a microcentrifuge tube and incubated at 65 °C until the agarose had completely melted.

Wizard™ PCR preps DNA purification resin (1 ml), was mixed thoroughly with the melted agarose for 20 seconds. The resin/DNA mixture was added to the syringe barrel of a Minicolumn/syringe assembly, inserted in a vacuum manifold (Promega) and a vacuum applied. To wash the column, 80% isopropanol (2 ml) was added to the syringe barrel and a

vacuum re-applied. To dry the resin, a vacuum was drawn for an additional 2 minutes and residual isopropanol was removed by centrifugation in an Eppendorf 5402 centrifuge (400 g, 30 seconds at 25 °C). The Minicolumn was transferred to a microcentrifuge tube and distilled water (50 µl) applied to the Minicolumn for one minute. Centrifugation of the Minicolumn eluted the bound DNA fragment.

The quantity and quality of the DNA fragment was determined by loading a small aliquot (estimated to contain 50 ng) of the final preparation on an agarose gel with appropriate PCR molecular weight markers (section 2.4.). The gel was examined carefully for the presence of contaminating species of DNA. The purified DNA sample was stored at -20 °C until a radioactive DNA probe was synthesised (section 2.8.).

2.8. PREPARATION OF RADIOLABELLED PROBES

Radioactively labelled DNA was prepared using Amersham's Megaprime™ DNA labelling kit. This system uses random sequences of hexanucleotides to prime DNA synthesis on denatured template DNA (Feinberg and Volgelstein, 1983). A radiolabelled nucleotide is substituted for a non-radioactive equivalent during the synthesis of DNA, resulting in a radiolabelled probe.

Materials

[α-^{32}P] deoxyadenosine triphosphate (dATP), Amersham, Little Chalfont, UK
DEPC treated water *
Glass wool
Sephadex G-50
Template DNA: Rat gelatinase A [bases 1793-3231 of Genebank sequence, accession number X71466]
Rat TIMP-1 (Genebank sequence, accession number L29512)
Rat TIMP-2 (kindly supplied by Dr Nicola Partridge, St Louis University, St Louis, America)
α_1 Chain of procollagen-1
α-Smooth muscle actin
Template DNA from section 2.6.4. and section 2.7.3.

Buffers and components required from the Megaprime™ DNA labelling kit, Amersham, Little Chalfont, UK:
DNA polymerase I Klenow fragment
Nucleotide solutions: deoxycytidine triphosphate (dCTP), deoxyguanine triphosphate (dGTP), deoxythymidine triphosphate (dTTP)
Primer solution
Reaction buffer (10 x)

Method

Template DNA (100 ng) was added of the primer solution (5 µl) and an appropriate volume of DEPC treated water added to give a total volume of 50 µl in the final reaction mixture. The DNA was denatured by heating to 95 °C for 5 minutes. The following components were mixed with the denatured DNA: 4 µl of each unlabelled dNTP nucleotide (dCTP, dGTP, dTTP), 5 µl of radiolabelled [α-^{32}P] dATP, 2 µl of Klenow fragment lacking 5'-3' exonuclease activity and 5 µl of reaction buffer. The contents of the tube were incubated at 37 °C for 60 minutes.

To reduce background from the probe during hybridisation, unincorporated nucleotides were removed from the probe by sephadex chromatography. Sephadex G-50 was equilibrated in water at 95 °C for 15 minutes. Once equilibrated, a syringe (1 ml) plugged with glass wool was filled with sephadex. Centrifugation in a Beckman TJ6 centrifuge (550 g, 5 minutes, room temperature) removed any liquid from the sephadex column. To the reaction mixture, DEPC treated water (50 µl) was added. The sample was then applied to the sephadex column and centrifuged as before. As the probe passes through the column the free nucleotides are retained within the matrix. Between 10^5-10^6 cpm/ml of sample were generally obtained for use as a DNA probe to hybridise with a northern blot (section 2.5.5.2.).

2.9. SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis (PAGE) is a quick and sensitive method for analysing the composition of mixtures of proteins. PAGE utilises a cross-linked acrylamide support through which protein samples are electrophoresed.

Sodium dodecyl sulphate (SDS) PAGE separates proteins according to their size. The anionic SDS detergent binds and denatures the proteins. The amount of SDS bound is proportionate to the molecular weight of the polypeptide (ratio of approximately one SDS molecule for every two amino acid residues). Binding of SDS to the protein hides the native charge and a constant negative net charge per unit mass is obtained. This causes all protein-SDS complexes to migrate toward the anode of the polyacrylamide gel at a mobility dependent on the molecular weight of the protein. The method that has been used is based on the study described by Laemmli (1970).

Materials

Ammonium persulphate (APS) [10% weight/volume (w/v)]
Butanol
Distilled water
Ethanol
β-Mercaptoethanol
N, N, N', N'-Tetramethylenediamine (TEMED)
Running buffer (5 x) *
SDS (10% w/v)
SDS-PAGE broad range prestained molecular weight (MW) markers, New England BioLabs, Hitchin, UK
Stock acrylamide solution containing acrylamide monomer and bis-acrylamide (40%)
Tris-HCl (0.5 M, pH 6.8)
Tris-HCl (1.5 M, pH 8.8)
Whole cell lysates (section 2.11.)

Method

The percentage polyacrylamide in the resolving gel varied depending on the molecular weight range of the proteins to be separated.

<u>12% Resolving gel (enough for 2 gels)</u>
Distilled water (8.7 ml)
10% SDS (0.2 ml)
40% Stock acrylamide (6 ml)
Tris-HCl [1.5 M, pH 8.8 (5 ml)]

To initiate the polymerisation by a free radical chain reaction, TEMED (10 µl) and 10% APS (100 µl) were added. The separating gel mixture was immediately transferred to ethanol cleaned gel plates, assembled to form vertical gel cassettes in the electrophoresis apparatus (Atto). A layer of butanol over the separating gel mixture ensured the gel set with a flat surface and prevented the gel from drying out. When set (approximately 45 minutes), the butanol was replaced with a low percentage stacking gel (into which the well-forming combs were placed).

<u>4% Stacking solution (enough for 2 gels)</u>
10% APS (100 µl)
Distilled water (6.4 ml)
10% SDS (100 µl)
40% Stock acrylamide (1 ml)
TEMED (10 µl)
Tris-HCl [0.5 M pH 6.8 (2.5 ml)]

The combs were removed from the polymerised stacking gel (after approximately 45 minutes)

and the gel cassette assembled into the electrophoresis tank. Sufficient running buffer (1 x) to cover the tops of the gels filled the upper and lower electrophoresis reservoirs.

The protein samples to be separated, including the SDS-PAGE prestained molecular weight markers, were denatured by heating to 95 °C for 10 minutes and loaded into a well within the stacking gel. The loaded gel was run at room temperature at 0.3 milliamps/cm^2 gel [(constant voltage), Flowgen, Consort E424] until the bromophenol blue reached the bottom of the resolving gel (approximately 60 minutes).

2.10. ELECTROPHORETIC TRANSFER OF PROTEINS FROM SDS-POLYACRYLAMIDE GELS TO POLYVINYLDIFLUORIDE MEMBRANE (WESTERN BLOTTING)

Proteins were transferred from the SDS-polyacrylamide gel to an immobilising medium by semi-dry transfer electrophoresis. Once transferred, the proteins were detected using antibodies. This would have proved difficult, or impossible if the proteins were still in the gel.

Materials

Chromatography 3 mm filter paper, Whatman, Maidstone, UK; Six pieces each cut to the same size as the gel
Coomassie blue stain *
Destain *
Methanol
Polyvinyldifluoride membrane (PVDF), cut to the same size as the gel
SDS-polyacrylamide gel with protein samples run on it (section 2.9.)
Transfer buffer *

Method

The freshly run SDS-polyacrylamide gel was cut from the stacking gel and soaked in transfer buffer for 20 minutes to remove the SDS. Prior to assembly of the electroblotting apparatus (Bio-Rad trans-blot SD), the membrane was prewet in methanol and then the membrane and pieces of filter paper were saturated with transfer buffer. The gel in contact with the PVDF membrane, was sandwiched between the filter paper layers and placed between the graphite plate electrodes, with the membrane on the anode side. The transfer buffer on the graphite plate was soaked up and any trapped air bubbles were removed. An electric current of 0.8 milliamps/cm^2 of gel at constant voltage for 60 minutes was applied (Flowgen, Consort E424). During this time the protein bands transferred from the gel to become strongly bound on the

membrane. After blotting, the membrane was recovered and processed directly.

To ensure the proteins had completely transferred from the SDS-polyacrylamide gel to the PVDF membrane, after transfer the gel was stained with coomassie blue for 4 hours. Excess dye was removed from the gel by soaking in destain for 4-8 hours, replacing the solution with fresh destain 3-4 times.

2.10.1. Immunological Detection

Following western blotting (section 2.10.), the presence or absence of specific proteins on the PVDF membrane were detected using a double-antibody detection method.

Materials

Blocking solution (5% and 10%) *

Primary antibody:
- Non-immune mouse immunoglobulin G (IgG)
- Non-immune rabbit IgG
- Non-immune rabbit serum
- Rabbit polyclonal antibody to human RARα and RARβ, Santa Cruz, Calne, UK
- Rabbit polyclonal antibody to human RARβ, SP172, a kind gift from Professor Chambon, IGBMC, Strasbourg, France
- Monoclonal antibody to mouse RARβ, Affinity Bioreagents, Cambridge, UK
- Monoclonal antibody to mouse α-smooth muscle actin
- Monoclonal antibody to mouse proliferating cell nuclear antigen (PCNA), Santa Cruz, Calne, UK

Secondary antibody:
- Goat anti-rabbit IgG conjugated to horse-radish peroxidase
- Goat anti-mouse IgG conjugated to horse-radish peroxidase

Tween, tris, buffered saline (TTBS) *

Western blot (section 2.10.)

Method

The membrane was washed for 5 minutes in TTBS followed by gentle shaking in blocking solution (10%) for 30 minutes to block remaining binding sites on the membrane. All incubation steps were carried out at room temperature. The membrane was incubated with a solution of primary antibody (1 μg/ml, or 2,000-fold dilution with α-smooth muscle actin) in blocking solution (5%) overnight. When excess antibody had been removed by washing for 5 minutes in TTBS three times, the filter was incubated for 60 minutes in blocking solution (5%) with a secondary antibody (5,000-fold dilution) conjugated to horseradish peroxidase. This secondary antibody binds to any first antibody-protein complex present on the membrane.

Following repeated (three times) 5 minute washes in TTBS to remove the secondary antibody solution, specific proteins on the membrane were detected by an enhanced chemiluminescence (ECL) reaction (2.10.3.).

2.10.2. Peptide Neutralisation

Blocking peptides were used as controls for some of the primary antibodies used in western blotting.

Materials
Blocking peptide for Santa Cruz polyclonal antibody to human RARβ antibody (Sequence: SISPSSVENSGVSQSPLVQ), Santa Cruz, Calne, UK
Blocking peptide for SP172, a polyclonal antibody to human RARβ antibody from Professor Chambon (Sequence: PSISPSSVENSGVSQSPLVQ), Alta Bioscience, Birmingham University, UK
Blocking solution (5%) *
Non-immunogenic peptide (Sequence: SLIGRL), Alta Bioscience, Birmingham University, UK
Western blot, pre-blocked in 10% blocking solution (section 2.10.1.)

Method

The primary antibody was combined with an excess of peptide antigen (10-fold, by weight) and incubated for 2 hours at room temperature. Following neutralisation, the antibody/peptide mixture was added to blocking solution (5%) and the protocol for immunological detection of proteins on western blots continued (section 2.10.1.).

2.10.3. Enhanced Chemiluminescent Reaction

Materials
Blue sensitive X-ray film, Genetic Research Instrumentation, Braintree, UK
Clingfilm
ECL detection reagents (reagent 1 and reagent 2), Amersham, Little Chalfont, UK
Western blot with specific proteins attached to a primary and secondary antibody, conjugated with horseradish peroxidase (section 2.10.1.)

Method

Immediately before use, an equal volume of detection solution 1 was mixed with detection solution 2 to give a sufficient volume to cover the blot (final volume of 0.25 ml/cm^2 of membrane). The ECL reaction begins when reagent 1 decays to hydrogen peroxide, a

substrate for horseradish peroxidase. Reagent 2 contains luminol which simultaneously oxidises as horseradish peroxidase reduces hydrogen peroxide, resulting in the production of a blue light.

Excess TTBS on the membrane was drained off and the mixed detection reagents were added to the membrane face carrying the protein. After incubation for precisely one minute at room temperature, the excess detection buffer on the membrane was drained off and the blot wrapped in clingfilm. Any air pockets and creases were smoothed out. The blot was exposed to autoradiography film for one minute before development in a Fuji X-ray film processor. On the basis of the appearance of the first film, it was estimated how long to expose a second autoradiography film.

2.10.4. Stripping and Reprobing Western Blots

Western blots can be stripped of bound primary and secondary antibodies, allowing the same blot to be probed with different antibodies.

Materials
Stripping buffer *
TTBS *
Western blot probed with primary and secondary antibodies (section 2.10.1.)

Method
The membrane was submerged in stripping buffer and incubated at 50 °C in a rotating hybridisation oven (Hybaid) for 30 minutes. After washing the membrane twice for 10 minutes in TTBS at room temperature, the protocol for immunological detection of proteins on western blots was followed (section 2.10.1.).

2.11. PREPARATION OF WHOLE CELL LYSATES

Materials
DMEM, Life Technologies, Paisley, UK
HSC (freshly isolated and cultured cells) or COS-1 cells transfected with RARβ_2
PBS
expression vector
Sample buffer (2 x) *

Method

Freshly isolated HSC (10 x 10^6) suspended in DMEM containing 16% FCS and 4% PSG were pelleted at Fisons MSE chilspin rotor (300 g, 5 minutes, 4 °C). Alternatively, HSC or COS-1 cells cultured on 100 mm petri-dishes (Greiner, Stonehouse, UK) were washed twice in DMEM before the cells were removed from the substratum using a cell scraper. The suspension was then centrifuged as detailed for the freshly isolated HSC.

The cell pellet was resuspended in sterile, 4 °C PBS (1 ml) to remove any FCS which may interfere with the subsequent DNA assay. The cells were pelleted in an Eppendorf 5402 centrifuge (10,000 g, 10 seconds, 4 °C) and resuspended in exactly 200 µl of sterile, 4 °C PBS. A 5 µl sample of cell suspension was removed to measure the DNA concentration and the remaining (195 µl) cell suspension was centrifuged (10,000 g, 10 seconds, 4 °C). The pellet was stored at -20 °C until completion of the DNA assay (section 2.11.1.).

The cell pellet was resuspended in sterile PBS to a concentration of 0.15 µg DNA/µl and then diluted with an equal volume of 2 x sample buffer (with or without β-mercaptoethanol) to lyse the cells. The sample (final concentration 0.075 µg DNA/µl) was stored at -20 °C until required for SDS-PAGE (section 2.9.).

2.11.1. DNA Assays

Depending on the sensitivity of the assay required, the amount of DNA in whole cell lysates was measured either with bisbenzamidazole (Hoescht 33258) or with PicoGreen reagent.

2.11.1.1. Hoescht 33258-Based Assay

This assay was based on the enhanced fluorescence of Hoescht 33258 which occurs when it binds to an A=T base pair.

Materials

Herring sperm DNA, Promega, Southampton, UK
Hoescht 33258
PBS
Whole cell lysate samples (section 2.11. and section 2.19.1.)

Method

DNA whole cell lysate samples (5 µl) were sonicated in a water bath (Branson 2210, 60 sonications/minute) for 5 minutes in a total volume of 50 µl PBS. Each sample (50 µl) was then added to a separate well on a 96 well plate (Nunc Maxisorb). To quantitate the amount of DNA per sample, samples of herring sperm DNA (0-5 µg/well), diluted in PBS were added in triplicate to each well. To each DNA sample (including standards), 50 µl of Hoescht 33258 (1 µg/ml in PBS) was added. The fluorescence was measured on a cytofluor II microwell fluorescence spectrophotometer (Persephic Biosystems) at an excitation of 360 nm and emission of 460 nm.

2.11.1.2. PicoGreen-Based Assay

PicoGreen reagent exceeds the sensitivity achieved with Hoescht 33258 by 400-fold.

Materials

Herring sperm DNA, Promega, Southampton, UK
PicoGreen reagent, Cambridge Biosciences, Cambridge, UK
TE buffer (1 x) *
Whole cell lysate samples (section 2.11. and section 2.19.1.)

Method

DNA whole cell lysate samples (5 µl) were sonicated in a water bath (Branson 2210, 60 sonications/minute) for 5 minutes in a total volume of 100 µl TE buffer. Each sample (100 µl) was then added to a separate well on a 96 well plate (Nunc Maxisorb). To quantitate the amount of DNA per sample, samples of herring sperm DNA (0-300 ng/well), diluted in TE buffer were added in triplicate to each well. To each DNA sample (including standards), 100 µl of PicoGreen reagent (200-fold dilution of concentrated stock in TE buffer) was added. The fluorescence was measured on a cytofluor II microwell florescence spectrophotometer (Persephic Biosystems) at an excitation of 480 nm and emission of 530 nm.

2.12. GELATIN ZYMOGRAPHY

Gelatinases of different molecular weights can be easily distinguished by fractionation on an SDS-polyacrylamide gel containing gelatin. Clear lytic zones appear in specific regions of the acrylamide gel, reflecting the presence of localised activation of gelatin by gelatinase. It has

previously been shown that zymograms are a quantitative technique, with the degree of digestion being directly proportional to the amount of enzyme activity.

2.12.1. Collection of Cell-Conditioned Media

Materials
Concanavalin A (30 μg/ml)
Cultured rat HSC
DMEM, Life Technologies, Paisley, UK
Ethanol
FCS, Life Technologies, Paisley, UK
PSG *
Retinoids: All-*trans* retinoic acid, 9-*cis* retinoic acid and 13-*cis* retinoic acid

Method

Primary rat HSC cultured on a 6 well tissue culture plate (Greiner, Stonehouse, UK) for 5 days in serum-containing media, were washed three times with serum-free media. The cells were then incubated in fresh media containing 0.5% FCS and 4% PSG, and either different isomers of retinoic acid [10 microMolar (μM)] or the same amount of ethanol used to dissolve the isomer. After 48 hours, the media was harvested, clarified by centrifugation in an Eppendorf 5402 centrifuge (1,000 g, 5 minutes) and immediately frozen at –20 °C until the detection of gelatinase activity by gelatin zymography was performed (section 2.12.2.).

Cultured rat HSC were also treated for 24 hours with concanavalin A (30 μg/ml) in media containing 0.5% FCS and 4% PSG to stimulate gelatinase synthesis and thereby aid identification of gelatinolytic bands in the zymogram.

2.12.2. Gelatin Zymography

Materials
Cell conditioned media (section 2.12.1.)
Coomassie blue stain *
Destain *
Distilled water
Gelatin (10 mg/ml)
Incubation buffer *
Sample buffer (2 x) *
SDS-polyacrylamide gel (section 2.9.)
Triton X-100 (2.5% v/v)

Method

An SDS-polyacrylamide gel (8%) was cast from a mixture of the solutions indicated in section 2.9. together with gelatin (final concentration of 1 mg/ml). A 4% stacking gel with sample wells was cast on top of the resolving gel. Aliquots of conditioned media (7 µl) to be analysed (section 2.12.1.) were mixed with an equal volume of sample buffer (2 x) and, without denaturing the proteins were loaded onto the SDS-polyacrylamide gel. Media from HSC treated with concanavalin A were analysed in parallel with the media from HSC treated with retinoids. Electrophoresis of the samples in the SDS-polyacrylamide gel was performed as in section 2.9.

Although gelatinase is enzymatically inactive in SDS-containing solutions, activity can be recovered after removal or neutralisation of SDS with Triton X-100. After the samples had been fractionated in the separating gel and the stacking gel had been removed, the SDS-gel was washed in distilled water (10 minutes), before the gel was washed twice with Triton X-100 (2.5%) for 30 minutes to remove the SDS. After washing twice with distilled water (10 minutes), the gel was washed with incubation buffer (10 minutes) and maintained in this buffer for 16 hours at 37 °C to restore the protease activity.

The gel was fixed and stained for 30 minutes in coomassie blue stain and then rinsed in distilled water before the stain was removed from the gel by washing twice in destain (30 minutes). The gel was then photographed on a light box.

2.13. IMMUNOCYTOCHEMISTRY

Immunocytochemistry is commonly used for light and electron microscope studies to detect antigens within any position within a cell. Immunocytochemistry relies upon the specific binding of an antibody to the antigen in the tissue, the reaction being localised with respect to cell structure by attaching a microscopically dense marker to the antigen-antibody complex. In this method, the primary antibody was detected using a three stage streptavidin-biotin complex technique. A biotinylated secondary antibody, acting as a link antibody binds to an un-conjugated primary (first layer) antibody. A tertiary layer of preformed avidin-biotin-enzyme complex is then applied which binds to the biotin on the link antibody. The strong affinity of avidin for biotin and the mild biotinylation process makes the avidin-biotin method more sensitive than a direct method or a two step indirect method, since additional enzyme is

placed at the site of the primary antigen and thereby produces increased colour intensity.

Materials

Acetone (water-free), BDH, Poole, UK
Alcohol (70%, 100%)
3,3'-diaminobenzidine tetrahydrochloride (DAB), BioGenex, Wokingham, Berkshire, UK
DMEM, Life Technologies, Paisley, UK
DPX, Poole, UK
Freshly isolated rat HSC suspended in DMEM containing 16% FCS and 4% PSG
Harris haematoxylin *
Hydrochloric acid (1%)/alcohol (70%)
Primary antibody: Non-immune rabbit IgG
Non-immune rabbit serum
Rabbit polyclonal antibody to chicken gizzard desmin
Rabbit polyclonal antibody to human RARβ, Santa Cruz, Calne, UK
Rabbit polyclonal antibody to human RARβ, SP172, a kind gift from Professor Chambon, IGBMC, Strasbourg, France
Secondary antibody: Biotinylated swine anti-rabbit IgG, DAKO, High Wycombe, UK
Tertiary antibody: Streptavidin and biotinylated horse-radish peroxidase reagents, DAKO, High Wycombe, UK
Tris buffered saline (TBS) *
Xylene, Poole, UK

Method

Freshly isolated rat HSC were cultured on 2 well, glass chamber slides (Lab-Tech) at a density of 0.5 x 10^6 HSC/ml for 2 or 7 days. The HSC were washed three times with DMEM to remove any traces of FCS before the HSC were air dried and immediately stored at -20 °C until immunostaining was performed.

After removal of the condensation from the previously frozen slides by air drying (10 minutes), the HSC were fixed with water-free acetone (room temperature, 15 minutes) to denature the proteins and stabilise the cells against dehydration and staining. The acetone was allowed to evaporate for a minimum of 10 minutes before the primary antibody [1 μg/ml of RARβ (Santa Cruz) and non-immune rabbit IgG; 200-fold dilution of SP172 and non-immune rabbit serum; 20-fold dilution of desmin] in TBS was applied at room temperature for 30 minutes. The HSC were washed for 2 minutes in TBS three times and the secondary antibody, biotinylated swine anti-rabbit IgG (200-fold dilution in TBS) applied (30 minutes, room temperature).

Streptavidin and biotinylated horse-radish peroxidase were mixed (200-fold dilution in TBS) and allowed to stand for at least 30 minutes at room temperature for complexes to form.

After washing the slides for 2 minutes in TBS three times, the prepared streptavidin/biotin complexes were added to the slides (30 minutes, room temperature) which attach to the biotinylated secondary antibody. The antigen was then visualised by the addition of the substrate chromogen DAB (5 minutes, room temperature).

The slides were rinsed in TBS, followed by a wash in running tap water for 2 minutes before the water was removed from the slide by briefly (seconds) exposing the cells to alcohol (70%). The cells were counter-stained using Harris's haematoxylin followed by rinsing in running tap water. To prevent damage resulting from excessive changes in solvent concentrations, the cells were dehydrated through a graded series of solvents; hydrochloric acid/alcohol (seconds), a rinse in water (5 minutes), 70% alcohol (5 minutes), and three separate exposures to absolute alcohol (5 minutes per incubation). The slides were incubated twice for 5 minutes in the clearing agent xylene before mounting the slides, since the dehydrating agent (alcohol) was not miscible with the mounting medium, DPX.

2.14. PREPARATION OF NUCLEAR EXTRACTS

Nuclear extracts were prepared based on the method by Dignam *et al.* (1983).

Materials
4-(2-Aminoethyl) benzene sulphonyl fluoride [1 mM (AEBSF)]
Aprotinin (1 mM)
Buffer A *
Buffer C *
PBS
Propidium iodide
Rat HSC (freshly isolated and cultured cells)

Method

Rat HSC cultured on 100 mm petri-dishes (Greiner, Stonehouse, UK) were washed twice in DMEM before the cells were removed from the substratum using a cell scraper. Nuclear extracts were also prepared from freshly isolated rat HSC (10×10^6). Subsequent steps were all performed at 4 °C to minimise proteolytic activity during isolation and preparation of the extract protein.

Hepatic stellate cells were separated from cell culture media by centrifugation in a Fisons MSE chilspin rotor (300 g, 5 minutes). Pelleted cells were then resuspended in 5 volumes of sterile,

4 °C PBS and collected by centrifugation in an Eppendorf 5402 centrifuge (2,500 g, 10 seconds). This wash in PBS was repeated.

Immediately before use, the protease inhibitors aprotinin (1 mM) and AEBSF (1 mM) were added to buffer A and buffer C. The HSC were gently resuspended in 5 packed cell volumes of buffer A (approximately 100 µl), lysing the cell membranes but retaining intact nuclei. The nuclei were collected by centrifugation (9,000 g, 10 seconds) and the cytoplasmic debris removed. Buffer C (2 volumes) was added to the pellet and the suspension vortexed every 2 minutes for 10 minutes to break up the nuclei. Following centrifugation (9,000 g, 60 seconds), the supernatant designated the nuclear extract was carefully decanted. The protein concentration was determined using 1 µl of nuclear extract (section 2.14.1.) and the remaining extract was stored as aliquots at -70 °C until required for EMSA (section 2.15.).

To ensure nuclei were still intact after addition of buffer A but lysed following addition of buffer C, propidium iodide (1 µg/ml), which stains intact nuclei, was mixed with a small (10 µl) volume of extract before and after addition of buffer C. The absence, or presence of intact nuclei were subsequently examined using an inverted light Leica microscope.

2.14.1. Protein Assay

Based on the Lowry method (Lowry *et al.*, 1951), protein concentrations were determined using the Bio-Rad DC (detergent compatible) protein colorimetric assay.

Materials

Bio-Rad DC protein assay reagents: Reagent A, reagent B, reagent S, Bio-Rad, Hemmel Hempstead, UK
Bovine serum albumin
Buffer C *
Distilled water
Nuclear extract sample [1 µl (section 2.14.)]
PBS
SDS (20%)

Method

Prior to the determination of protein concentration in nuclear extract samples, a protein standard curve was established using bovine serum albumin (0-12 µg/20 µl), prepared in buffer C as the standard.

The nuclear extract sample (1 µl) was diluted with distilled water to a final volume of 20 µl. The control sample contained buffer C (1 µl) instead of the protein. To each sample, reagent A (100 µl) was added to induce a reaction in an alkaline medium between proteins present in the sample and copper present in reagent A. Addition of reagent B (800 µl), a dilute folin reagent, caused the production of a blue colour with a maximum absorbance of 750 nm. After 15 minutes the absorbance of each sample was read at 750 nm on a Beckman DU 7500 spectrophotometer. The concentration present in the nuclear extract sample (1 µl) was subsequently determined from the standard curve.

2.15. ELECTROMOBILITY SHIFT ASSAYS

The electromobility shift assay or gel retardation assay determines the binding interactions between DNA and DNA binding proteins, such as the interactions of transcription factors with regulatory sequences. A short radiolabelled nucleic acid, carrying a given regulatory sequence, is mixed with a cell nuclear extract expected to contain the binding protein. Samples of nucleic acid with or without extract are run on a non-denaturing polyacrylamide gel. Complexes of protein and DNA will migrate more slowly through the gel than unbound DNA fragments. Competition experiments and antibody detection methods provide evidence of binding specificity and protein identity. In competition experiments, unlabelled oligonucleotides which correspond to the same binding site or an unrelated binding site are added to the reactions. Interactions between the binding protein and the unlabelled, related sequences will decrease the band intensity of the previously shifted complexes, while unrelated binding sequences will not alter the intensity of the complex. Adding antibodies that recognise the binding protein to the reaction mixture, produce even slower migrating species than the original protein-DNA complex. This phenomenon is known as a supershift.

2.15.1. Preparation of Labelled or Unlabelled Oligonucleotide Probes

Materials
[γ-^{32}P] dATP, ICN, Thame, UK
Forward exchange buffer *

RARE consensus oligonucleotide [binding site for the retinoic acid receptor, (Ohata *et al.*, 1997)].
Sense strand: GAAGGGTTCACCGAAAGTTCACTCGCATA, Genosys, Pampisford, UK

RARE mutant oligonucleotide (identical to the consensus RAR binding motif, with the exception of two 'TT→AA' substitutions).
Sense strand: TCGAGGGTAGGGAACACCGAAAGAACACTCG, Santa Cruz, Calne, UK

T4 polynucleotide kinase, Promega, Southampton, UK
TE buffer (1 x)*

Buffers and components required from QIAquick nucleotide removal kit, Qiagen, Crawley, UK:
Buffer EB
Buffer PE
Buffer PN
QIAquick spin column

Method

To radiolabel the oligonucleotide, 30 ng of sense strand was added to 5 µl of [γ-^{32}P] dATP. Immediately after the addition of T4 polynucleotide kinase (5 units), which catalyses the transfer of the γ-phosphate from adenosine triphospshate (ATP) to the 5' terminus of polynucleotides, the reaction mixture was incubated at 37 °C. After 15 minutes, the mixture was made to a final volume of 50 µl with TE buffer.

To improve the quality of gel shifts, unincorporated nucleotides were removed from the DNA probe using the Qiagen nucleotide removal kit. To the reaction sample, Buffer PN (500 µl) was added before the sample was applied to a QIAquick spin column containing a silica column membrane and centrifuged in an Eppendorf 5402 centrifuge (2,000 g, 60 seconds, room temperature). The sample was washed with Buffer PE (500 µl) and centrifuged (2,000 g, 60 seconds, room temperature). After a second wash, the column was spun for an additional 60 seconds at 9,000 g to remove final traces of buffer. The column was placed in a clean microcentrifuge tube and the DNA eluted by applying Buffer EB [30 µl (10 mM Tris-HCl, pH 8.5)] to the centre of the column followed by centrifugation, (9,000 g, 60 seconds, room temperature). The final concentration of the probe was 1 ng/µl. Before use in a DNA binding reaction (section 2.15.3.), the probe was diluted 10-fold.

To anneal a sense labelled or unlabelled oligonucleotide with an antisense oligonucleotide, 150 ng of antisense oligonucleotide was incubated with 30 ng of sense oligonucleotide at 85 °C for 5 minutes, followed by cooling to room temperature. The double stranded oligonucleotide probe was stored at -20 °C until required.

To verify that the labelled sense and antisense strand had annealed, 1 µl of radiolabelled single stranded oligonucleotide (sense strand) was analysed on a polyacrylamide gel alongside the annealed, double stranded probe in the absence of nuclear extract. If the sense and antisense strand have annealed, the single stranded oligonucleotide will migrate faster through the gel than the corresponding, double stranded oligonucleotide.

2.15.2. Preparation of Non-Denaturing Polyacrylamide Gel

Materials
Agar (2% w/v)
APS (10% w/v)
Stock acrylamide solution containing acrylamide monomer and bis-acrylamide (40%)
TBE buffer (10 x) *
TEMED

Method

To initiate the polymerisation, 50 µl of TEMED and 500 µl of 10% APS were added to 50 ml of 5% acrylamide solution containing 0.25 x TBE. The polyacrylamide mixture was immediately transferred to ethanol cleaned gel plates assembled to form vertical gels and sealed with 2% molten agar. A well-forming comb was placed into the acrylamide and after polymerisation, the combs were removed and the gel assembled into a electrophoresis tank containing a cooling system (Cambridge Electrophoresis). Sufficient running buffer (0.5 x TBE) to cover the top of the gel filled the upper and lower electrophoresis reservoirs and the gel was pre-run for 30 minutes at 10 milliamps/gel (Flowgen, Consort E424).

2.15.3. DNA Binding Reaction

Materials
Blue sensitive X-ray film, Genetic Research Instrumentation, Braintree, UK
Buffer C *
Chromatography 3 mm filter paper, Whatman, Maidstone, UK
Consensus [^{32}P]-labelled RARE probe [0.1 ng/µl (section 2.15.1.)]
Gel-loading buffer (6 x) *
Mutant [^{32}P]-labelled RARE probe, Santa Cruz, Calne, UK
Nuclear protein extracts (section 2.14.)
Polydeoxyinosinic-deoxycytidylic acid [poly(dI-dC)]
Pre-run, non-denaturing polyacrylamide gel [5% (section 2.15.2.)]
Rabbit polyclonal antibody to human RARβ antibody, Santa Cruz, Calne, UK
TBE buffer (10 x) *
Unlabelled consensus RARE probe (section 2.15.1.)

Method

Nuclear protein extracts (10 μg) from freshly isolated or cultured rat HSC (made up to a total volume of 5 μl with buffer C) were incubated with [^{32}P]-labelled consensus RARE probe (0.2 ng) and poly(dI-dC) (1 μg). Poly(dI-dC) was added to reduce non-specific DNA binding. After incubation for 20 minutes at 4 °C, gel-loading buffer (1x) was added to each reaction. The reaction mixture was resolved on a pre-run, 5% non-denaturing polyacrylamide gel. The gel was run in TBE buffer (0.5 x) at 10 milliamps/gel (Flowgen, Consort E424) until the bromophenol blue dye had migrated through three-quarters of the gel (approximately 2.5 hours). The gel was dried on a piece of chromatography filter paper using a gel dryer (Bio-Rad model 483 slab dryer) before exposure to an X-ray film at -70 °C for 24-48 hours to detect the labelled DNA. The film was then developed with a Fuji X-ray film processor. Protein bound to radioactive DNA migrates more slowly through the gel than the 'free', radioactive probe.

Supershift assays were performed by incubating nuclear protein extract with RARβ antibody (0.2 μg or 5 μg) for 20 minutes at 4 °C, before incubation with the [^{32}P]-labelled RARE probe. A [^{32}P]-labelled double stranded mutant RARE probe was also incubated with nuclear protein extract, replacing the [^{32}P]-labelled consensus RARE probe. In controls for specificity, 10-fold or 100-fold Molar excess of unlabelled RARE was incubated with the nuclear protein extract (4 °C, 20 minutes), prior to incubation with the consensus [^{32}P]-labelled RARE probe.

2.16. IMMUNOPRECIPITATION

Immunoprecipitation is used to detect and quantitate target antigens in mixtures of proteins. The specificity of the immunoglobulin for its ligand is so high that the resulting antigen-antibody complexes can be purified from contaminating proteins. When coupled with SDS-PAGE, the technique is ideal for analysis on the rate of synthesis of a protein and its half-life. The target protein is usually immunoprecipitated from extracts of cells that have been radiolabelled. Immunoprecipitation of radiolabelled target proteins and their subsequent analysis consist of the following steps: radiolabelling of cells expressing the target protein, lysis of cells, formation of specific immune complexes, collection and purification of the immune complex, and analysis of the radiolabelled proteins in the immunoprecipitate.

2.16.1 Radiolabelling and Lysis of Cells Expressing the Target Protein

Metabolic radiolabelling of cultured mammalian cells is most commonly carried out by incubating the cells in medium containing sulphur-35 [^{35}S] labelled methionine and cysteine. Media used to grow mammalian cells in culture contains high concentrations of both methionine and cysteine. To increase the efficiency of incorporation of radiolabelled amino acids, it was necessary to incubate the cells in medium that was depleted of methionine and cysteine.

Materials
Cultured rat HSC or COS-1 cells transfected with RARβ_2 expression vector
DMEM without L-cystine, L-methionine and L-cysteine, ICN, Thame, UK
PBS
Single lysis buffer *
Tran[^{35}S]-label, ICN, Thame, UK
Triple lysis buffer *

Method
Cells cultured on 100 mm petri-dishes (Greiner, Stonehouse, UK) were washed twice with medium lacking both FCS and methionine and cysteine (med$^-$AA). The cells were incubated for 60 minutes at 37 °C in med$^-$AA to deplete the intracellular pools of sulphur containing amino acids. The medium was replaced with med$^-$AA (5 ml) containing [^{35}S]-labelled amino acids (100 μCi/ml) for 16 hours. The volume of medium was kept to a minimum to increase the concentration of radiolabelled amino acids.

The medium was aspirated and the cells were washed twice in 4 °C PBS before lysing the cells with single or triple lysis buffer (500 μl) for 20 minutes. To minimise proteolytic activity in the cell extracts, the extracts were kept cold and inhibitors of proteases were included in the lysis buffer. The cells were removed from the substratum using a cell scraper and the lysate centrifuged in an Eppendorf 5402 centrifuge (2,500 g, 15 minutes, 4 °C). The supernatant (total cell lysate) was stored at –20 °C until ready for pre-clearing.

2.16.2. Preclearing the Cell Lysate

Immunoprecipitation involves adding specific antibody, directed against the target protein to aliquots of cell lysates. Antigen-antibody complexes are then collected by adsorption to

staphylococcal protein A that is covalently attached to agarose beads. To reduce the background caused by adsorption of irrelevant cellular proteins to staphylcoccal protein A, the cell lysate was first pretreated with an antibody that had no activity against the target protein and which was drawn from the same animal that was later immunised against the target antigen (rabbit IgG).

Materials
Non-immune rabbit IgG
Protein A agarose, Santa Cruz, Calne, UK
Radiolabelled cell lysate (section 2.16.1.)

Method

To the sample of lysate, rabbit IgG (2 μg) was added and the mixture incubated for 60 minutes at 4 °C on a rocking platform. The cell lysate/antibody mixture was transferred to a microcentrifuge tube containing a pellet of protein A agarose that had been washed in the lysis buffer used to make the original cell extract. The mixture was incubated for 30 minutes at 4 °C before centrifugation in an Eppendorf 5402 centrifuge (2,500 g, 5 minutes, 4 °C). The supernant was transferred to a fresh microcentrifuge tube and precipitated with specific antiserum (section 2.16.3.).

2.16.3. Immunoprecipitation of the Target Protein

Materials
Amplify flurographic reagents, Amersham, Little Chalfont, UK
Chromatography 3 mm filter paper, Whatman, Maidstone, UK
Glacial acetic acid
Isopropanol
NET-gel buffer *
Non-immune rabbit IgG
Precleared cell lysate (section 2.16.2.)
Protein A agarose, Santa Cruz, Calne, UK
Rabbit polyclonal antibody to human RARβ, Santa Cruz, Calne, UK
Sample buffer (2 x) *
Wash buffer *

Method

The precleared cell lysate was divided into two equally sized aliquots and the volume of each aliquot adjusted to 500 μl with NET-gel buffer. Dilution of the cell extract with NET-gel buffer lowers the level of non-specific immunoprecipitation. To one aliquot, RARβ antibody

(1 μg) was added and to the other, the same concentration of rabbit IgG (a control antibody). The aliquots were gently rocked for 60 minutes at 4 °C. Protein A agarose (20 μl) was added to the antigen/antibody mixture and incubated again for 60 minutes at 4 °C. The tertiary protein A antigen-antibody complexes were collected by centrifugation in an Eppendorf 5402 centrifuge (2,500 g, 5 minutes, 4 °C). The supernatant was removed and the agarose beads were resuspended in NET-gel buffer (1 ml) by vortexing. The aim of this step was to displace proteins that were non-specifically adsorbed to the protein-A matrix, while leaving specific tertiary complexes intact. The resuspended beads were incubated for 20 minutes at 4 °C on a rocking platform to allow time for the buffer to equilibrate with the fluid within the beads. The agarose beads were washed a total of 3 times (twice with NET-gel buffer and once with wash buffer). To the final pellet, 30 μl of sample buffer (1x) was added and the proteins in the sample were denatured by heating to 100 °C for 3 minutes. The protein A agarose was removed by centrifugation (2,500 g, 20 seconds, room temperature) and the supernatant transferred to a fresh tube.

The sample (20 μl) was analysed by SDS-PAGE (section 2.9.). The SDS-polyacrylamide gel, containing the protein radiolabelled with [^{35}S]-labelled amino acids was fixed at room temperature in 5-10 volumes of glacial acetic acid:isopropanol:water (10:20:70) for 30 minutes. To amplify the radioactive signal the gel was then washed for 15 minutes in Amplify. The gel was dried on a piece of chromatography filter paper using a gel dryer (Bio-Rad model 483 slab dryer) before exposure to an X-ray film at –70 °C for 24-48 hours. The film was developed using a Fuji X-ray film processor.

2.17. TRANSFECTION OF CELLS WITH EXPRESSION VECTORS

An ideal method to transfer genes or macromolecules into eukaryotic cells exhibits the following features a) high efficiency of transfer, b) low toxicity, c) reproducibility and d) suitability for *in vitro* and *in vivo* applications. Each transfection method has advantages and disadvantages.

2.17.1. Transfection of COS-1 Cells with Expression Vectors

The calcium phosphate transfection protocol is the most widely used method for both transient and stable transfection of a variety of cell types. The exact mechanism remains unknown

although it is believed the DNA forms a co-precipitate with the calcium phosphate, enhancing the uptake of the DNA into the cytoplasm of cells by endocytosis. The DNA is then transferred to the nucleus.

Materials

Calcium chloride (2 M)
COS-1 cells
DMEM, Life Technologies, Paisley, UK
FCS, Life Technologies, Paisley, UK
Glycerol (15%) in 1 x N-(2-Hydroxyethyl)piperazine-N'-(2-ethane)sulphonic acid (HEPES) buffered saline (HBS) *
HBS (2 x) *
PBS
Plasmid DNA (section 2.6.2.)
PSG *
Sterile water

Method

COS-1 cells were harvested by trypsinisation (section 2.1.2.) and re-plated at a density of 1 x 10^6 cells/100 mm petri-dish (Greiner, Stonehouse, UK). The COS-1 cells were incubated for 24 hours in a humidified atmosphere of 95% air and 5% CO_2, maintained at 37 °C.

For each 100 mm monolayer of cells to be transfected, 31 µl of 2 M calcium chloride was added to 10 µg of sterile plasmid DNA and the final volume adjusted to 250 µl with sterile water. This solution was added drop-wise, without mixing to 250 µl of 2 x HBS and incubated for 10-15 minutes. During this time a fine precipitate formed. The calcium phosphate precipitated DNA was added dropwise onto the medium covering the cells.

Brief treatment of transfected cells with glycerol can increase the efficiency of expression of the introduced DNA. After the cells had been incubated with the calcium phosphate precipitated DNA for 4 hours, the cells were washed with sterile PBS and then shocked for 30 seconds with 15% glycerol (1 ml) in 1 x HBS. The glycerol/HBS mixture was removed and the cells washed again with sterile PBS before replacement with DMEM containing 16% FCS and 4% PSG. After 48 hours, whole cell lysates were prepared (section 2.11.).

2.17.2. Transfection of Hepatic Stellate Cells with Expression Vectors

The Qiagen Effectene transfection reagent is a non-liposomal lipid formulation combined with a special DNA-condensing enhancer. The enhancer first condenses the DNA molecules and Effectene subsequently coats the DNA with cationic lipids.

Materials
Cultured rat HSC
DMEM, Life Technologies, Paisley, UK
FCS, Life Technologies, Paisley, UK
PBS
pHook^{TM}-2*lacZ* plasmid containing a β-galactosidase expression vector (section 2.6.)
PSG *
Sterile water

Buffers supplied with the Effectene transfection reagent kit, Qiagen, Crawley, UK:
Buffer EC
Effectene reagent
Enhancer reagent

Method
The day before transfection, primary cultured rat HSC were harvested by trypsinisation (section 2.1.2.) and re-plated at a density of 1 x 10^6 HSC/ml into a 6 well culture plate (Greiner, Stonehouse, UK). For each well, either sterile plasmid DNA (0.4 μg) or the same volume of sterile water (negative control) was diluted with Buffer EC to give a total volume of 100 μl. Enhancer reagent (3.2 μl) was vortexed with the mixture for 1 second and the sample incubated for 15 minutes at room temperature. Effectene transfection reagent (10 μl) was added to the DNA-Enhancer mixture and mixed by vortexing for 10 seconds. The sample was then incubated at room temperature for 10 minutes to allow complex formation. Whilst complex formation was occurring, the HSC were washed with sterile PBS and then replaced with 1.6 ml of DMEM containing 16% FCS and 4% PSG. Medium (600 μl) containing 16% FCS and 4% PSG was also added to the transfection complexes before immediately adding the mixture, dropwise onto the monolayer of cells. The cells were incubated at 37 °C and 5% CO_2 for 48 hours to allow for gene expression.

2.17.2.1. X-gal Staining of Transfected Hepatic Stellate Cells

This protocol was used to measure the number of cells expressing a high level of β-

galactosidase following transfection with pHook^{TM}-2*lacZ* plasmid.

Materials
5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) solution *
Paraformaldehyde (2%)
PBS
Rat HSC cultured on a 6 well plate and transfected with pHook^{TM}-2*lacZ* plasmid

Method

The culture medium was discarded and replaced with 2% paraformaldehyde solution (2 ml) to fix the cells. The cells were incubated at 37 °C for 15 minutes. After fixation, the cells were washed three times in PBS at room temperature before a minimal volume of X-gal solution was added. After incubation at 37 °C for 16 hours, any positive cells were stained blue under an inverted light Leica microscope.

2.18. QUANTIFICATION OF APOPTOSIS IN CULTURED HEPATIC STELLATE CELLS

Induction and quantification of programmed cell death or apoptosis of HSC was detected by acridine orange staining as described by Baker *et al.* (1994).

Materials
Acridine orange (1 mg/ml)
Cultured rat HSC
Retinoids: All-*trans* retinoic acid, 9-*cis* retinoic acid and 13-*cis* retinoic acid were purchased from Sigma Chemical Company, Poole, UK. LE135 (a selective RARβ antagonist) was a kind gift from Hiroyuki Kagechika, University of Tokyo, Japan. Ro 41-5253 (selective RARα antagonist) was a kind gift from Dr Michael Klaus, F. Hoffmann-La Roche Limited, CH-4002 Basel, Switzerland. LE135 (selective RARβ antagonist) was a kind gift from Dr Hiroyuki Kagechika, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan.

Method

To each well of a 24-well tissue culture plate (Greiner, Stonehouse, UK), 2.5 x 10^5 freshly isolated rat HSC were added in 16% FCS and 4% PSG. On day 4 or day 5, retinoids (10 μM) were added to appropriate wells. The plates were then incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for a further 72 hours or 48 hours respectively, replacing the media with freshly prepared retinoid containing media after 48 hours. Control groups included cells which were incubated for periods of 72 hours in the presence of 16% FCS

containing the same amount of solvent used to dissolve the retinoid.

Acridine orange (final concentration 1 μg/ml) was added to the wells and after 10 minutes the number of cells undergoing apoptosis was determined using an inverted fluorescent microscope (Leica). Normal and apoptotic cells were counted in three random power fields (200x magnification), in each of two duplicate wells, based on characteristic morphology (Majno and Joris, 1995). Cells were initially counted in the adherent monolayer and subsequently in the supernatant to include any detached apoptotic cells. The mean number of small condensed particles, both adherent to the monolayer and detached in the media were expressed as a percentage of the total number of cells in each field.

Data were diagrammatically represented by use of the program Microsoft Excel 97 (Microsoft corporation) and presented as the mean ± S.E.M. Statistical analysis was by paired *t*-test using the program GraphPad Instat (GraphPad Software), with $P < 0.05$ taken to indicated statistical significance.

2.19. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High preformance liquid chromatography is highly sensitive and specific and is the method of choice to separate and quantify retinoids. In particular, reverse-phase HPLC is a well-established technique for the separation of retinyl esters. The principle of reverse-phase HPLC is that components of a mixture are separated by virtue of their differential distribution between a mobile phase and a non-polar stationary phase. This is in contrast to normal-phase chromatography which uses a non-polar mobile phase. Consequently, the order of elution of solutes from a reverse-phase column is the reverse of that of a normal phase column; highly lipophilic, non-polar solutes are better retained (and elute later) by the stationary phase and polar molecules are less well retained (and elute earlier).

To quantitate the amount of retinoids present in HSC and their culture media before and during their activation, their *in situ* production was monitored by modifications of the HPLC methodology used by Buss *et al.* (1994) and Dzerk *et al.* (1998). Since retinoids are photosensitive, the precautions mentioned in section 2.2. were applied throughout the analysis. Additionally, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were added to solutions and organic solvents to minimise the chemical oxidation of retinoids.

2.19.1. Preparation of Hepatic Stellate Cells for High Performance Liquid Chromatography Analysis

Materials
BHA
BHT
DMEM, Life Technologies, Paisley, UK
Freshly isolated rat HSC or cultured rat HSC
HBSS (not containing calcium), Roche Diagnostics, Lewes, UK
Sterile water

Method

Freshly isolated rat HSC (10×10^6) were pelleted in a Fisons MSE chilspin rotor (300 g, 5 minutes, 4 °C) and the pellet washed with calcium free HBSS to remove any DMEM containing FCS. After washing the pellet three times, it was resuspended in sterile water (1 ml), containing BHT (10 μg/ml) and BHA (20 μg/ml) and the cells were stored at -80 °C until analysis.

The remaining HSC from the isolation, were cultured on 75 mm^2 tissue culture flasks (Greiner, Stonehouse, UK) for 3, 5, 7 and 9 days. On days 2, 4, 6 and 8, prior to harvesting, the cells were washed three times with calcium free HBSS and cultured for a further 18-24 hours with serum-free media (DMEM). Following incubation, the serum-free media was collected and BHT (10 μg/ml) and BHA (20 μg/ml) were added before storing the media at – 80 °C. The monolayers of HSC were trypsinised (section 2.1.2.) and the cells resuspended in sterile water (1 ml) containing BHT (10 μg/ml) and BHA (20 μg/ml) and were also stored at -80 °C.

Prior to extraction and analysis by HPLC, the cell fractions were thawed and dispersed by sonication for 10 minutes (Camlab Transsonic T460) and an aliquot (5 μl) removed for DNA analysis (section 2.11.1).

2.19.2. Preparation of Retinoid Stocks for High Performance Liquid Chromatography

Materials
Amber glassware, Fisher Scientific, Loughborough, UK
Dimethyl sulphoxide (DMSO), BDH, Poole, UK
Ethanol, Hayman, Witham, UK; containing BHT (10 μg/ml) and BHA (20 μg/ml)
Retinoids: All-*trans* retinol, all-*trans* retinol acetate, all-*trans* retinyl palmitate, all-*trans*

retinoic acid, 9-*cis* retinoic acid and 13-*cis* retinoic acid were purchased from Sigma Chemical Company, Poole, UK. 9,13-di-*cis* retinoic acid was a kind gift from Dr Kojima, the Institute of Physical and Chemical Research, Koyadai, Tsukuba, Ibaraki 305, Japan and 13-*cis* acitretin (RO 13-7652) was a gift from Hoffmann-La Roche.

Method

Stock solutions of retinoids were prepared by dissolving all-*trans* retinol, all-*trans* retinol acetate, 13-*cis* acitretin and isomers of retinoic acids in ethanol (1 mg/ml). Retinyl palmitate was dissolved in DMSO (1 mg/ml). To minimise binding of the highly lipophilic retinoids to apparatus, glass rather than plastic was used whenever possible. Stocks of retinoids were stored at -80 °C. Combined, secondary working dilutions in ethanol, either of retinol and retinyl palmitate (100 μg/ml, 10 μg/ml, 1 μg/ml) or of the retinoic acid isomers (100 μg/ml, 10 μg/ml, 1 μg/ml) were prepared on the day. Working dilutions of the internal standards, all-*trans* retinol acetate (100 μg/ml) and 13-*cis* acitretin (100 μg/ml) were also freshly prepared.

2.19.3. *Extraction and Chromatography of Retinoids*

The partition of a solute between two immiscible liquids provides the basis for simple solvent extraction. A known amount of internal standard is always introduced into the sample before any extraction or purification procedures are undertaken. The internal standard, which is closely related to the compounds of interest but which is not found in the biological sample, will result in the appearance of an additional peak in the chromatogram of the modified sample. Any variation in the injection volume or losses during extraction and/or adsorption on the column will equally effect the internal standard and the test sample. Since the integrated amount of internal standard is always the same, a change in the internal standard response indicates a change in the chromatogram.

As the retinoids to be analysed vary in their physico-chemical properties, two separate reverse-phase HPLC methods were developed to enable all the retinoids to be adequately resolved. Method 1 separated retinol and the highly lipophilic compound retinyl palmitate, and method 2 resolved the more polar retinoic acid isomers.

2.19.3.1. Method 1

2.19.3.1.1. Extraction of Retinol and Retinyl Palmitate

Materials
Aluminium foil
DMEM, Life Technologies, Paisley, UK
Methyl-tert-butyl ether (TBME) HPLC grade, Romil, Cambridge, UK
Solvent A (section 2.19.3.1.2.)
Sonicated cell fractions and their culture media (section 2.19.1.)
Working solutions of retinol, retinyl palmitate and retinol acetate (section 2.19.2.)

Method

Different volumes for various fractions (10-100 µl for HSC fractions, and 1 ml for culture media) were added to foil wrapped, culture glass tubes (Corning) and the total volume in each tube was adjusted to 1 ml using serum-free media (DMEM). Aliquots (1 ml) of blank serum-free media, spiked with retinol and retinyl palmitate were extracted simultaneously to provide calibration curves with final concentrations of 0, 2, 5, 10, 25, 50, 100, 250, 500, 1000 and 1500 ng/ml.

Prior to extraction with TBME (5 ml), each sample (including the standards) was spiked with the internal standard, all-*trans* retinol acetate (500 ng). After mixing for 10 minutes, the organic layer was separated in a Jouan CR322 centrifuge (1,700 g, 10 minutes, 10 °C) and collected using a Tecan genesis RSP 150 robot. The extraction was repeated and the organic phases pooled and evaporated to dryness under a stream of nitrogen at 25 °C with a TurboVap LV evaporator (Zymark). The residue was reconstituted in solvent A (200 µl), the initial mobile phase solvent and mixed for 20 minutes before transfer into an HPLC vial (Chromacol).

2.19.3.1.2. Chromatography Conditions for Retinol and Retinyl Palmitate

Materials
Deionised water, Milli-Q-system, Millipore, Watford, UK
HPLC column, Phenomenex, Macclesfield, UK
Methanol, HPLC grade, Romil, Cambridge, UK
Propan-2-ol, HPLC grade, Romil, Cambridge, UK

Method

The extracts (180 µl per injection) were automatically injected, with a 200 µl injection loop (Merck-Hitachi AS-4000A Auto-sampler) into the reverse-phase HPLC system (LC-9A HPLC pump, Shimadzu) and chromatographed onto a 3 µm spherclone, octadecylsilane-2 (ODS-2) reverse-phase column (150 x 4.6 mm). To resolve retinol and retinyl palmitate, a linear gradient system was established, from 100% solvent A (36.25% methanol, 36.25% propan-2-ol and 27.5 % deionised water) to 100% solvent B (50% methanol and 50% propan-2-ol) over 25 minutes at a constant flow rate (1 ml/minute). Between each run, the column was re-equilibrated (cycle time 30 minutes). All the solvents were degassed before use with helium. The column was maintained at 75 °C (Jones chromatography model 7990) and the analytes monitored via UV absorption at 325 nm (Shimadzu UV spectrophotometric detector SPD-6A), the maximum absorption for retinol and retinyl palmitate (see appendix 3.).

2.19.3.2. Method 2

2.19.3.2.1. Extraction of Retinoic Acid Isomers

Materials

Aluminium foil
Anhydrous sodium sulphate, BDH, Poole, UK
DMEM, Life Technologies, Paisley, UK
Ethyl acetate, Romil, Cambridge, UK
n-Hexane, Romil, Cambridge, UK
Hydrochloric acid (2 M)
Initial mobile phase solvent (30% solvent C and 70% solvent D, section 2.19.3.2.2.)
Sodium hydroxide (2 M)
Sonicated cell fractions and their culture media (section 2.19.1.)
Working solutions of retinol, retinyl palmitate, isomers of retinoic acids and 13-*cis* acitretin (section 2.19.2.)

Method

Various fractions of sonicated HSC extracts (500-800 µl) and their culture media (3 ml) were added to foil wrapped, culture glass tubes (Corning) and the total volume in each tube adjusted to 3 ml using serum-free media (DMEM). To enable the samples to be quantified, a calibration curve was extracted from blank serum-free media (3 ml) spiked with all-*trans* retinoic acid, 9-*cis* retinoic acid, 13-*cis* retinoic acid and 9,13-di-*cis* retinoic acid with final concentrations of 0, 2, 5, 10, 25, 50, 100, 250, 500, 1000 and 1500 ng/ml. The standard samples were also spiked with retinol (100 ng) and retinyl palmitate (100 ng) to mimic the cell

culture samples that contain these retinoids at a much higher concentration than the retinoic acids. These metabolites were included in case they interfered with the measurement of the retinoic acid isomers. To each sample (including standards) the internal standard, 13-*cis* acitretin (500 ng) was added.

Applying the chromatography conditions stated in section 2.19.3.2.2. to the extracts, results in the resolution of retinol in close proximity to 9-*cis* retinoic acid (Figure 4.3.). A high concentration of retinol in a sample could potentially conceal a smaller 9-*cis* retinoic acid peak. Consequently, the extraction process was modified to eliminate retinol from the samples. To each sample, 300 µl of 2 M sodium hydroxide was added and after mixing for 10 minutes, neutral and basic lipophilic compounds were extracted twice with *n*-hexane (6 ml). The organic layers were separated in a Jouan CR322 centrifuge (1,700 g, 10 minutes, 4 °C) and collected using a Tecan genesis RSP 150 robot. The now ionised retinoic acid isomers remained in the aqueous phase. After the addition of 600 µl of 2 M hydrochloric acid to neutralise the retinoic acids and aid their movement into an organic fraction, the aqueous phase was extracted twice with *n*-hexane and ethyl acetate [6 ml (9:1 v/v)]. The organic layer was dehydrated with anhydrous sodium sulphate (200 mg) and then evaporated under nitrogen gas at 25 °C with a TurboVap LV evaporator (Zymark). The resulting residue was dissolved in the initial mobile phase buffer (200 µl) and transferred to an HPLC vial (Chromacol).

2.19.3.2.2. Chromatography Conditions for Retinoic Acid Isomers

Materials
Ammonium acetate buffer (5 mM, pH 2.7 with acetic acid)
Glacial acetic acid, Fisher Scientific, Loughborough, UK
HPLC column, Phenomenex, Macclesfield, UK
Methanol, HPLC grade, Romil, Cambridge, UK

Method
The HPLC system used for the acid metabolites of retinol was similar to that used for retinol and retinyl palmitate. However, to resolve the retinoic acid isomers, a multilinear gradient of solvent C (5 mM ammonium acetate, pH 2.7) and solvent D (1% acetic acid in methanol) at a constant flow rate (1 ml/minute) was required (Table 2.4.). Additionally, the column temperature was maintained at 36 °C and the absorbance monitored at a wavelength of 330 nm, the maximum absorption for retinoic acid isomers (see appendix 3.).

Table 2.4: Composition of the mobile phase used in the chromatography of retinoic acid isomers

Time (minutes)	Percentage of Solvent C (%)	Percentage of Solvent D (%)
0.01	30	70
6.5	30	70
7.0	20	80
21.5	11	89
22.0	30	70
32.0	30	70

2.19.4. Data Acquisition and Analysis

The acquisition of chromatograms and analysis of the results were performed using the data package EZChrom Elite Client (Scientific Software). The peak height ratio of a known concentration of authentic retinoid standard to the fixed concentration of the internal standard was plotted against retinoid concentration to produce a calibration curve. Quantification of retinol, retinyl palmitate and each of the acid metabolites present in HSC and their culture media was achieved by computer integration of the peak height ratio (unknown concentration of retinoid:internal standard) and reference to the calibration curve run with that set of samples. The final results were standardised to micrograms of DNA present in the HSC fraction from which the retinoids had been extracted. Data were diagrammatically represented by use of the program Microsoft Excel 97 (Microsoft Software) and reported as the mean ± S.E.M. when appropriate. Statistical analysis was by paired *t*-test using the program GraphPad Instat (GraphPad Software), with $P < 0.05$ considered to be statistically significant.

CHAPTER 3

The Effect of Retinoids on Hepatic Stellate Cells

3.1. INTRODUCTION

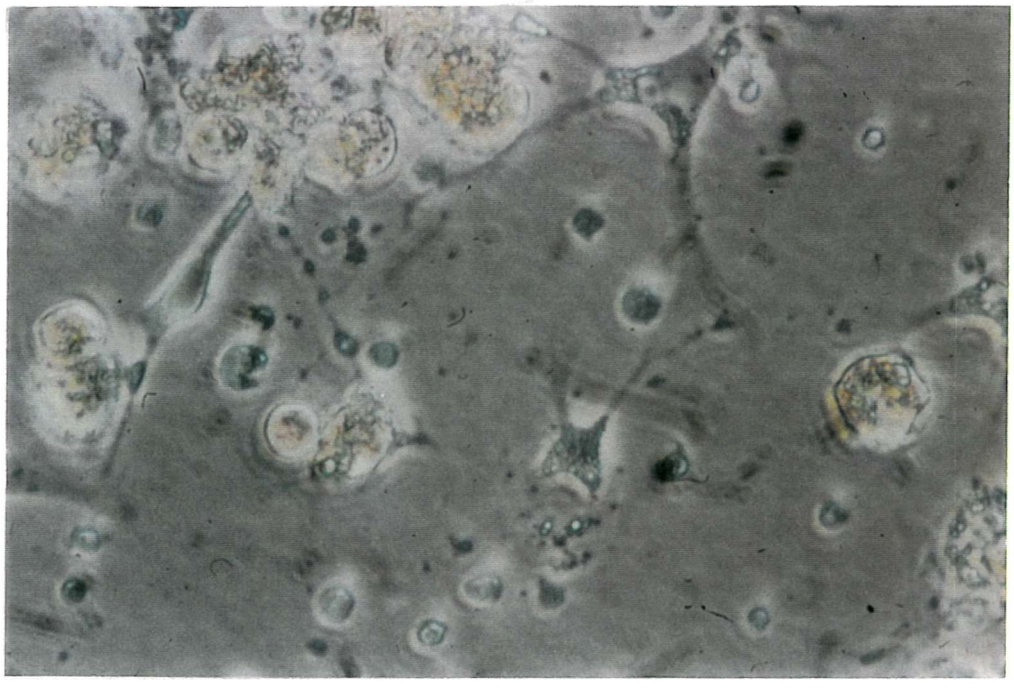
Despite the clear importance of HSC in storing and metabolising retinoids in normal liver, the role of these compounds in regulating HSC activation during liver injury is still not understood. Hepatic vitamin A levels are greatly reduced in livers of rats and baboons fed alcohol (Sato and Lieber, 1981) and in humans with alcoholic liver disease (Leo and Lieber, 1982). These observations have led to the key question whether the release and metabolism of retinoids is a necessary step in the activation of HSC and, as a corollary, whether the maintenance of high concentrations of retinoids in HSC can inhibit activation and fibrogenesis. Additionally, the release of retinoids during transformation of quiescent HSC to myofibroblasts may result in high local concentrations of metabolites around these cells. These metabolites of vitamin A may have autocrine effects and further regulate the transformation of HSC to myofibroblasts.

Retinoids have profound effects on morphogenesis and on the growth and differentiation of normal and transformed cells. Although a number of studies have examined the effect of exogenous retinol and all-*trans* retinoic acid on HSC proliferation, there are few data available on the effect of these retinoids on other markers of HSC activation. Different forms of retinoids may have varying effects on the phenotype and function of HSC. All-*trans* retinoic acid, 13-*cis* retinoic acid and 9-*cis* retinoic acid are biologically active metabolites of vitamin A with potent biological activities towards almost all types of cells. The purpose of this study was therefore to investigate the effect of exogenous retinol and other biologically active retinol metabolites on the transdifferentiation and activation of cultured rat HSC.

3.2. RAT HEPATIC STELLATE CELLS IN PRIMARY CULTURE

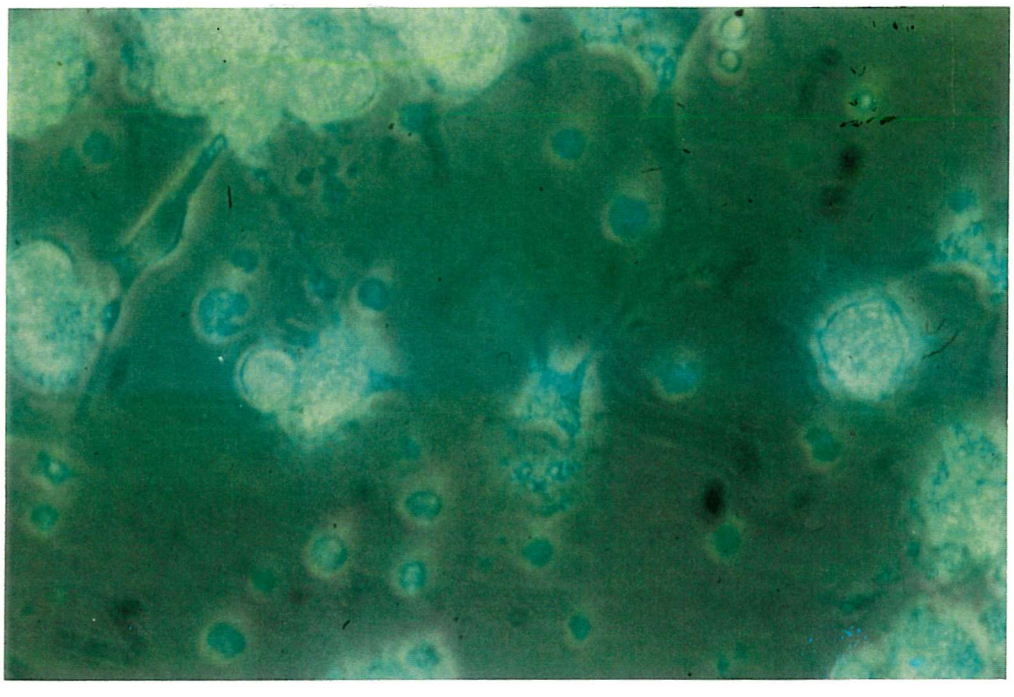
Within 4 days in culture, freshly isolated rat HSC had rapidly spread on a plastic surface, assuming a flattened and stellate-like appearance with branched cytoplasmic extensions (Figure 3.1.). Vitamin A fading green-blue fluorescence was easily detectable by exposing the cells to UV light at 330 nm (Figure 3.2.).

Figure 3.1: A light micrograph of cultured rat hepatic stellate cells



Primary cultured rat HSC 4 days after plating on plastic (400x magnification).

Figure 3.2: A light micrograph of cultured rat hepatic stellate cells exposed to ultraviolet light



Primary cultured rat HSC 4 days after plating on plastic (as in Figure 3.1.) and exposed to UV light (400x magnification).

These characteristic features of HSC are well documented. The photographs demonstrate that HSC store vitamin A and also show the phenotype of typical HSC used in subsequent experiments.

3.3. MODULATION OF HEPATIC STELLATE CELLS BY RETINOIDS

3.3.1. Modulation of Proliferation by Retinoids

Initially the effect of different retinoids on the growth of HSC was studied, to validate the findings that retinoids contribute to the regulation of cell proliferation and differentiation in a variety of systems.

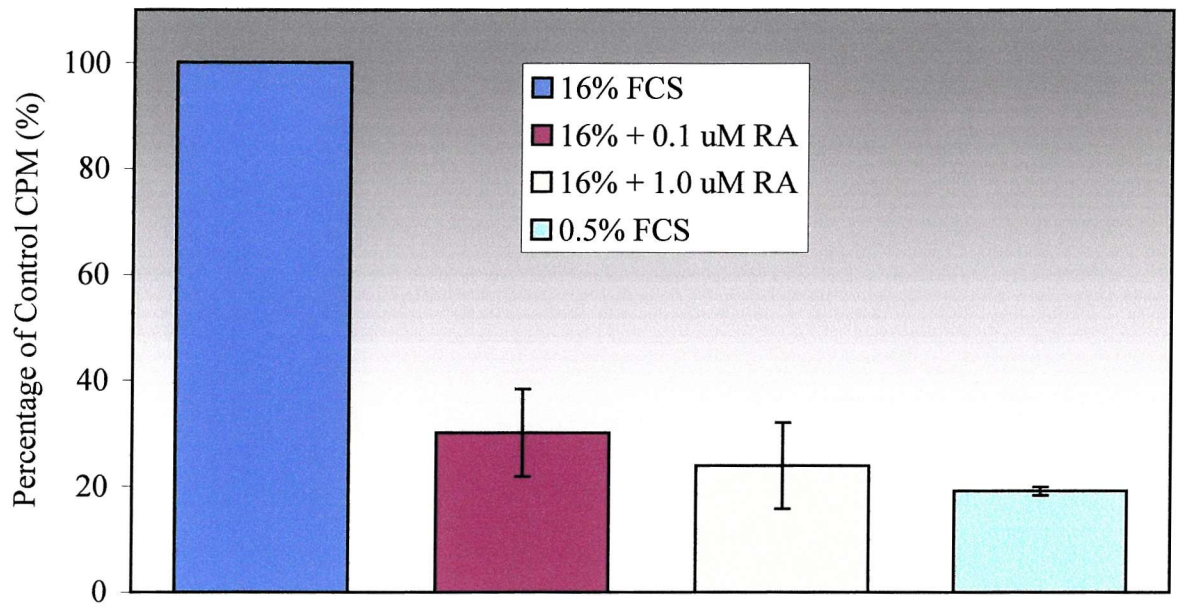
Cultures of primary rat HSC grown on plastic for 4 days, were supplemented with either retinol or isomers of retinoic acid (RA) for 72 hours and the effect on proliferation assessed by incorporation of [*methyl*-^{3}H]-thymidine into nuclear DNA (section 2.3.). Due to different degrees of absolute incorporation between separate experiments, data are expressed as a percentage of control cells maintained in 16% FCS. In contrast to HSC maintained in media containing 16% FCS, the proliferation rate of HSC decreased rapidly by 80.9% ± 1.4 when the serum concentration was lowered to 0.5% (Figure 3.3.). Subsequently cells cultured with 0.5% FCS are considered to be quiescent.

As shown in Figure 3.3., both all-*trans* retinoic acid and retinol caused a marked inhibition of HSC proliferation as assessed by [*methyl*-^{3}H]-thymidine incorporation. Inhibition of proliferation was statistically significant in all cases ($P < 0.05$) and [*methyl*-^{3}H]-thymidine incorporation was reduced to a quiescent level at the highest retinoid concentrations. Shorter exposure to all-*trans* retinoic acid (48 hours instead of 72 hours) resulted in less effective inhibition of proliferation (Figure 3.4.).

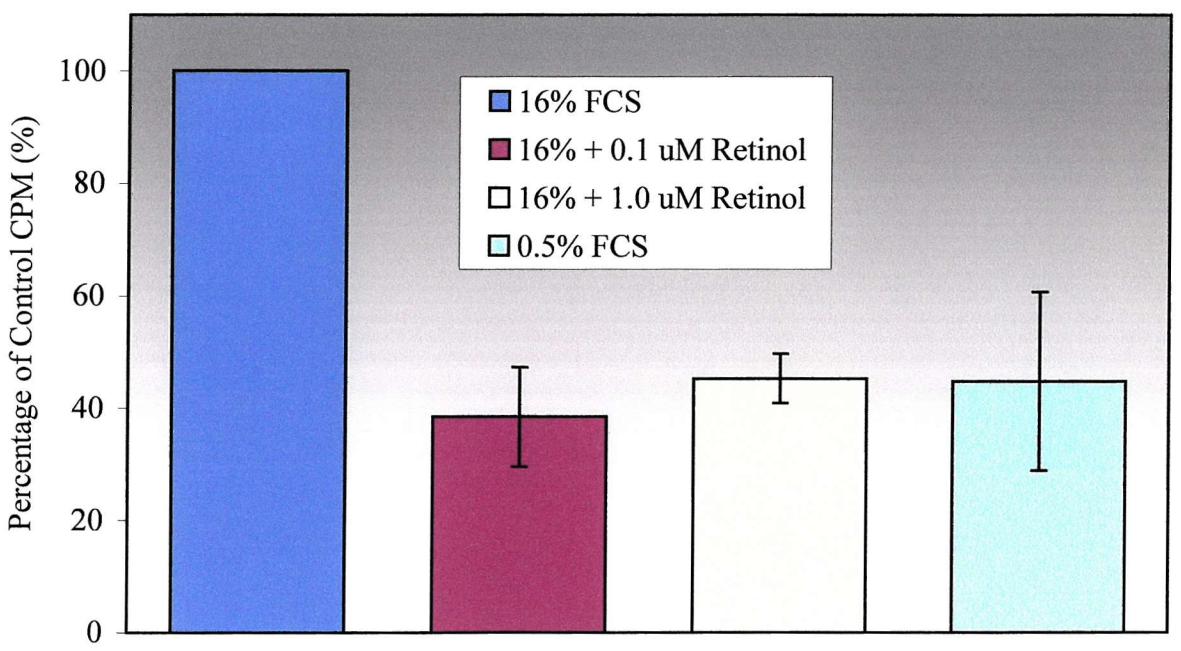
Although treatment with retinol yielded results similar to those obtained with all-*trans* retinoic acid (Figure 3.3.), retinol was less efficient (but not significantly different, $P > 0.05$) at inhibiting proliferation at both 0.1 μM concentrations (inhibition to 30.1% ± 14.3 with all-*trans* retinoic acid compared to inhibition to 38.4% ± 8.9 with retinol) and 1 μM concentrations (inhibition to 23.9% ± 14.1 with all-*trans* retinoic acid compared with inhibition to 45.2% ± 4.4 with retinol). Retinol had a greater significant inhibitory effect at 0.1 μM concentration (38.4% ± 8.9) than at 1 μM concentration (45.2% ± 4.4).

Figure 3.3: Effect of incubation of retinol or all-*trans* retinoic acid for 72 hours on hepatic stellate cell proliferation

A:

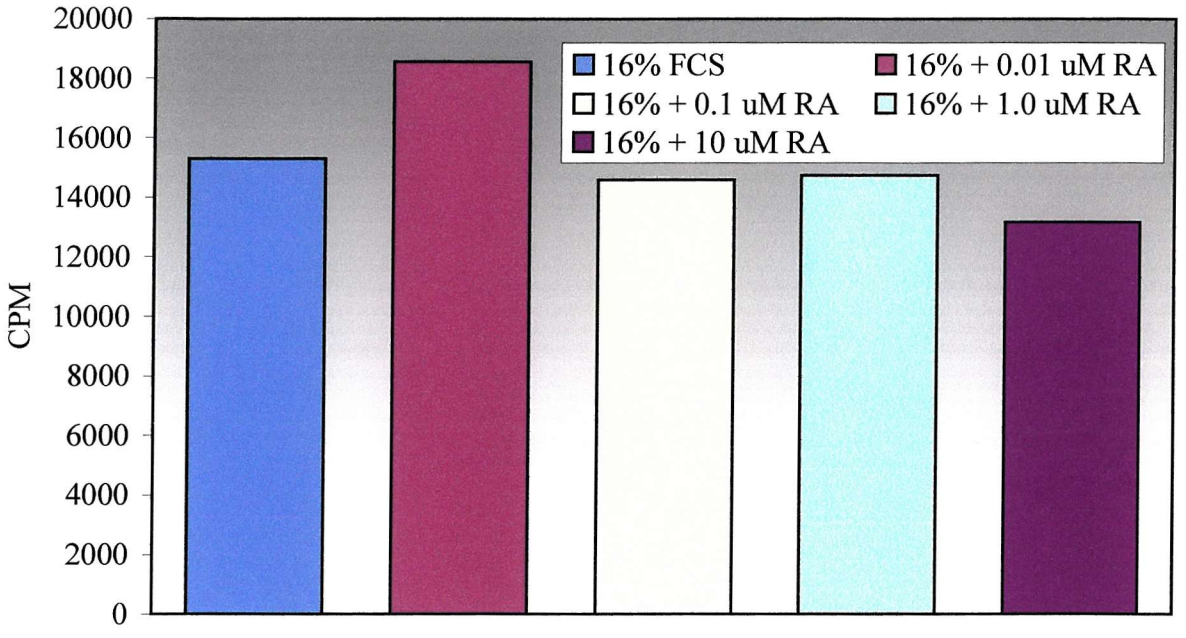


B:



Rat HSC cultured for 4 days, were incubated for 72 hours with different concentrations of either all-trans retinoic acid (RA, panel A) or retinol (panel B) and the rate of proliferation assessed by thymidine incorporation. Data are expressed as percent of control, 16% FCS. Data presented are mean ± S.E.M. from 3 separate experiments. $P < 0.05$ for difference between retinol or all-trans retinoic acid and control for all concentrations.

Figure 3.4: Effect of incubation of all-*trans* retinoic acid for 48 hours on hepatic stellate cell proliferation

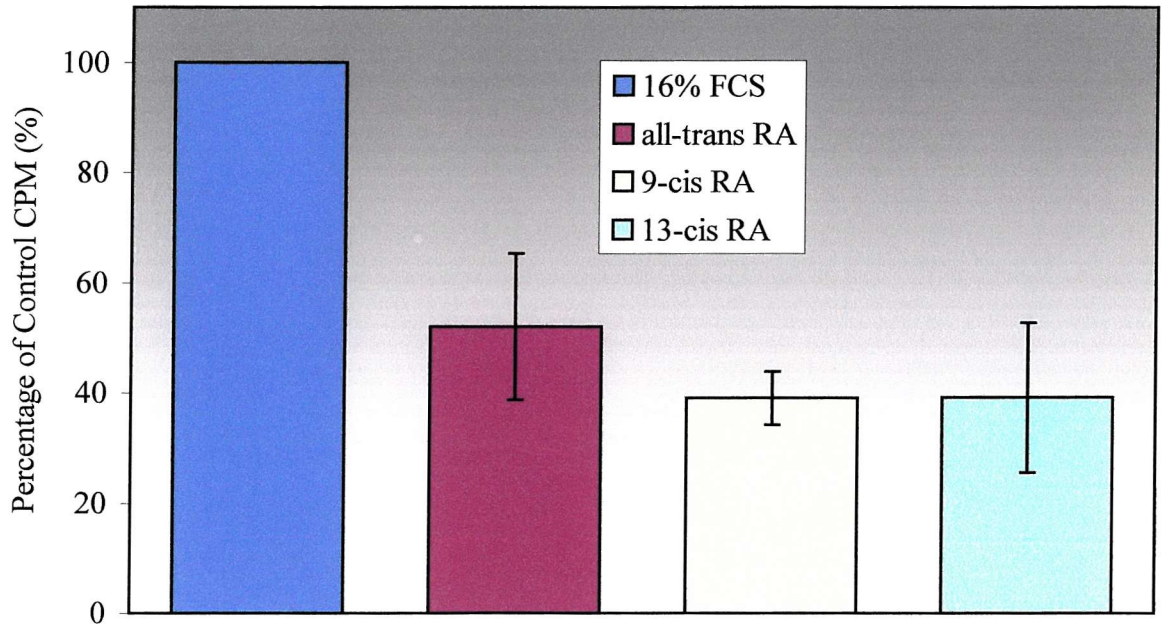


Rat HSC cultured for 5 days, were incubated for 48 hours with different concentrations of all-trans retinoic acid (RA) and the rate of proliferation assessed by thymidine incorporation. Data presented are from 1 experiment.

Different isomers of retinoic acid also showed a significant inhibitory effect ($P < 0.05$) on HSC growth (Figure 3.5.). Addition of either all-*trans*, 9-*cis* or 13-*cis* retinoic acid at a concentration of 10 μM, resulted in significant growth inhibition of HSC (inhibition to 52.0% ± 13.3 with all-*trans* retinoic acid, 39.0% ± 4.8 with 9-*cis* retinoic acid and 39.1% ± 13.6 with 13-*cis* retinoic acid). The extent of the inhibition varied with each isomer but was more pronounced with 9-*cis* retinoic acid (39.0% ± 4.8) and 13-*cis* retinoic acid (39.1% ± 13.6) than all-*trans* retinoic acid (50.0% ± 13.3).

Thus supplementation of primary cell culture media with a variety of retinoids which can be produced biosynthetically, can markedly diminish HSC proliferation.

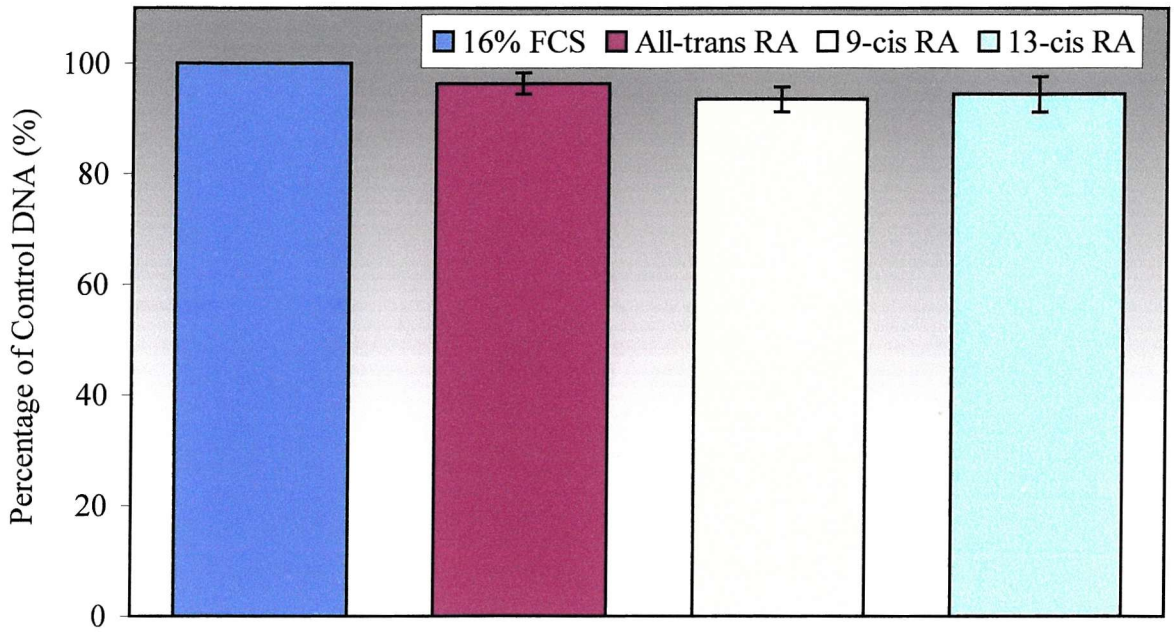
Figure 3.5: Effect of different isomers of retinoic acid on hepatic stellate cell proliferation



Rat HSC cultured for 4 days, were incubated for 72 hours with different isomers of retinoic acid (RA, 10 μM) and the rate of proliferation assessed by thymidine incorporation. Data are expressed as percent of control, 16% FCS. Data presented are mean ± S.E.M. from 3 separate experiments. $P < 0.05$ for difference between each retinoic acid isomer and control.

The effect of retinoic acid isomers on cell proliferation was not only assessed by incorporation of [*methyl*-^{3}H]-thymidine into nuclei but also by measuring DNA content in the cells (section 2.11.1.1.). Following plating of equal numbers of primary rat HSC on tissue culture plastic, HSC were harvested after 4 days of culture, and also after 7 days of culture following 72 hours treatment with either isomers of retinoic acid (10 μM) or vehicle (replacing the media with media containing fresh retinoids after 48 hours), and the DNA content assayed. The DNA in HSC did not change significantly ($P > 0.05$) from day 4 to day 7 (103.6% ± 0.9). Although mean DNA content was slightly lower in cultures treated for 72 hours with retinoic acid isomers (Reduction to 96.3% ± 1.9 with all-*trans* retinoic acid, 93.4% ± 2.3 with 9-*cis* retinoic acid and 94.4% ± 3.2 with 13-*cis* retinoic acid) compared with control [day 7 cultures treated with vehicle (Figure 3.6.)], the differences were not statistically significant ($P > 0.05$).

Figure 3.6: Effect of isomers of retinoic acid on hepatic stellate cell DNA content



Rat HSC cultured for 4 days, were incubated for 72 hours with different isomers of retinoic acid (RA, 10 μM) and the changes in DNA measured on Day 7. Data is expressed as percent of day 7 cultures treated with vehicle (16% FCS). Data presented is mean ± S.E.M. from 3 separate experiments. $P > 0.05$ for difference between each retinoic acid isomer and control.

3.3.2. Modulation of Apoptosis by Retinoids

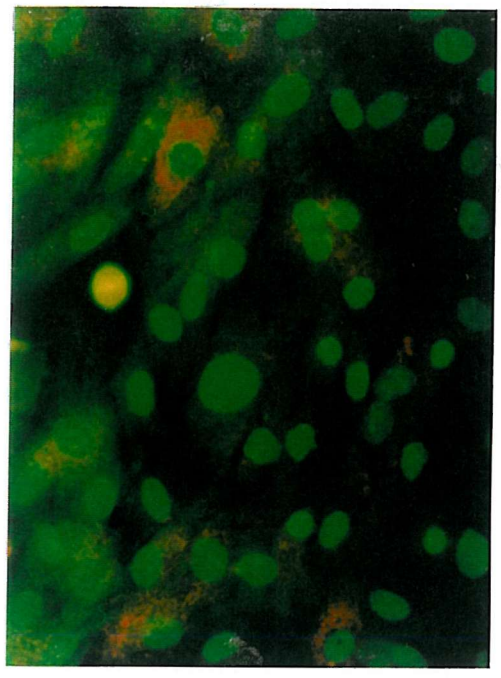
It is well documented that retinoids can cause rapid and extensive apoptosis in many culture cells. It is possible that programmed cell death or apoptosis contributes to retinoid mediated inhibition of proliferation in HSC. To test the specific effects of different isomers of retinoic acid on apoptosis, the technique of fluorescence microscopy was used after treatment with retinoic acid isomers for 72 hours (section 2.18.). In this experiment, primary rat HSC were plated on uncoated, 24-well tissue culture plates, 4 days before different isomers of retinoic acid (10 μM) were added to the medium. After a further 72 hours (replacing the media with media containing fresh retinoids after 48 hours), acridine orange was added to the wells and the cells were counted using an inverted fluorescent microscope.

Small, rounded condensed cells were observed on the surface of the monolayer (Similar to that observed in Figure 3.7.). The cells could be displaced by agitation of the monolayer and some were detached and floating in the culture supernatant. These features are compatible with HSC that have undergone apoptosis and distinguish programmed cell death from

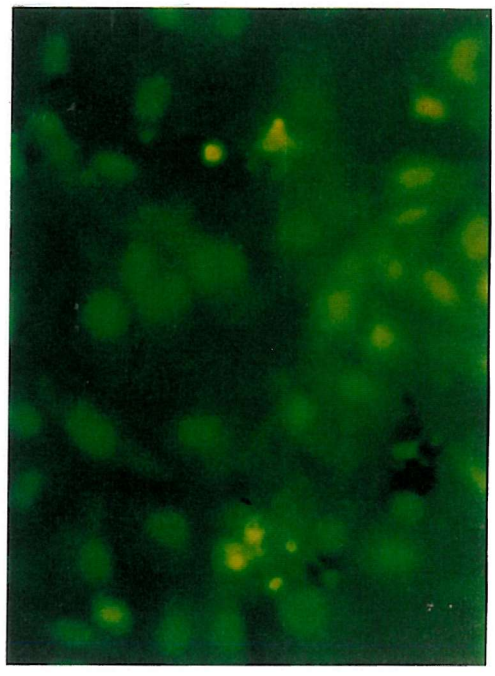
necrosis.

Figure 3.7: Micrographs of normal and apoptotic cultured rat hepatic stellate cells in the presence and absence of serum

A:



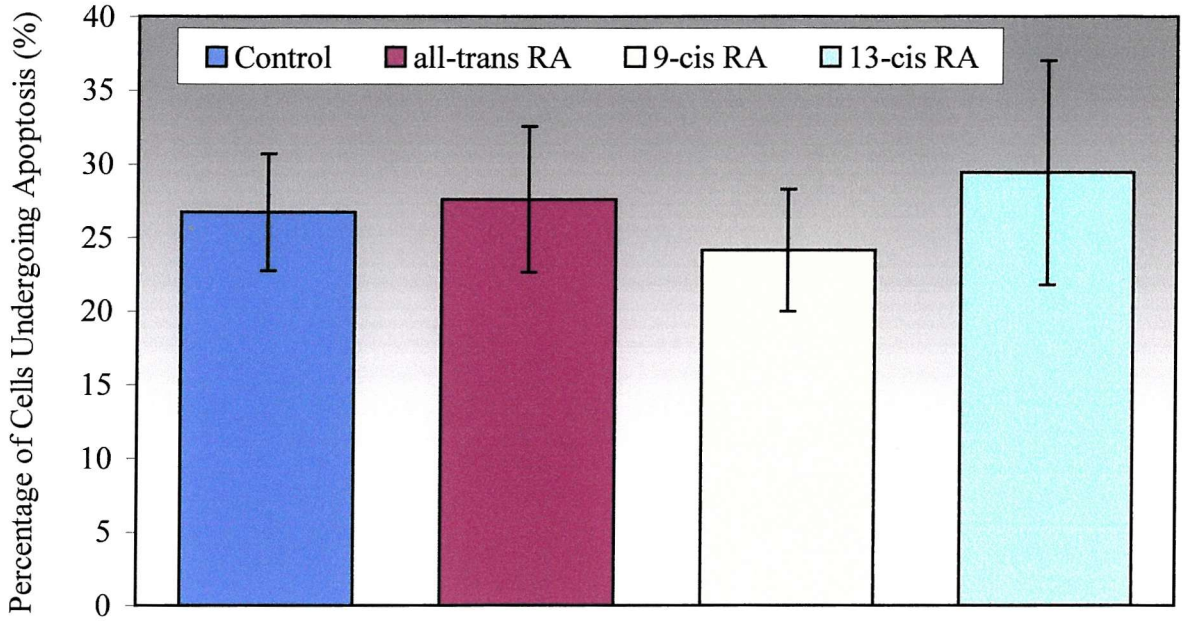
B:



Fluorescence microscopy of 4 day old passaged rat HSC in the presence (panel A) or absence (panel B) of 16% FCS and stained with acridine orange (400x magnification). Apoptotic HSC have brightly stained condensed chromatin, and shrunken or absent cytoplasm. Photographs obtained from Mr T. Kendall, the University of Southampton, Southampton, UK.

As shown graphically in Figure 3.8., the retinoic acid isomers did not induce an increase in apoptotic cells. Similar numbers of apoptotic nuclei appeared in HSC treated with 10 μM retinoic acid isomers for 72 hours compared with control cultures (27.6% ± 4.9 apoptosis with all-*trans* retinoic acid, 24.1% ± 4.2 apoptosis with 9-*cis* retinoic acid and 29.4% ± 7.6 apoptosis with 13-*cis* retinoic acid apoptosis, compared with 26.7% ± 4.0 apoptosis in control cultures), thus supporting the DNA assays (Figure 3.6.).

Figure 3.8: Effect of different isomers of retinoic acid on spontaneous apoptosis of hepatic stellate cells



Rat HSC cultured for 4 days, were incubated for 72 hours with different isomers of retinoic acid (RA, 10 μM) or 0.2% ethanol (control cultures) prior to adding acridine orange and determining the number of apoptotic cells on day 7. Data are expressed as percent of control, 16% FCS. Data presented are mean ± S.E.M. from 3 separate experiments. P > 0.05 for difference between each retinoic acid isomer and control.

3.3.3. Modulation of Messenger RNA for Hepatic Stellate Cell Activation Markers by Retinoids

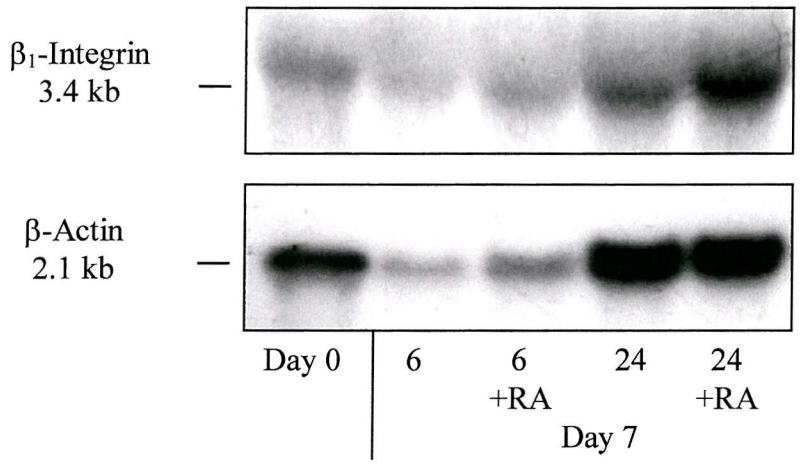
Published studies on a variety of cell types indicate that retinoic acids may be important in modulating the phenotype of cells. Since HSC contain retinoids and release them during activation, it was therefore logical to investigate the effect of exogenous retinoids on the transcription of a wide range of genes that are associated with the cellular phenotype [gelatinase A (MMP-2), procollagen, TIMP-1, TIMP-2, β_1-integrin and α-smooth muscle actin]. An extensive series of experiments were conducted to examine the effect of all-*trans* retinoic acid on expression of these genes by culturing HSC supplemented with all-*trans* retinoic acid at either an early or at a late stage of culture.

To determine how long HSC must be exposed to retinoic acid to effect mRNA synthesis, primary rat HSC cultured on plastic were treated with serum, or serum-free media containing all-*trans* retinoic acid (10 μM, 1 μM, 0.1 μM and 0.01 μM) for 6, 24, 48 or 72 hours before

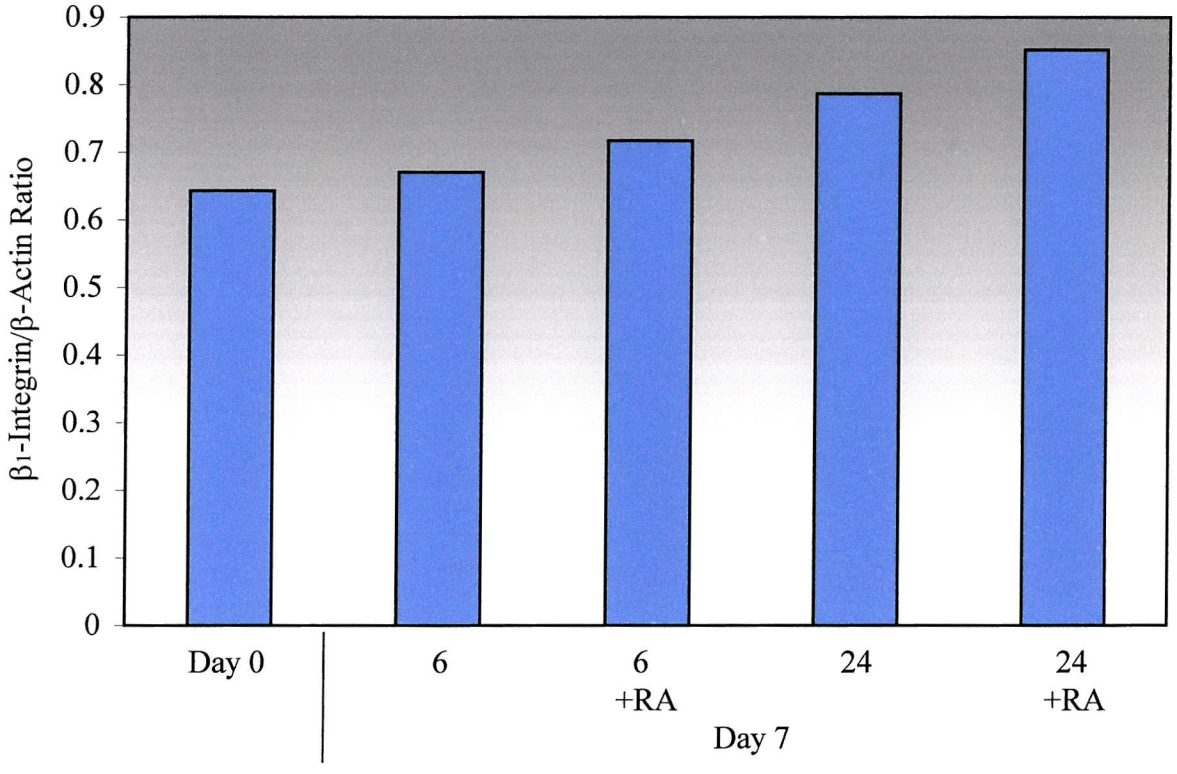
the cells were harvested at day 7. The changes in the mRNA levels of gelatinase A, procollagen I, TIMP-1, TIMP-2, β_1-integrin and α-smooth muscle actin in HSC were investigated by northern blot analyses using rat cDNA probes for each of these genes and compared to β-actin mRNA, a house keeping gene (section 2.5.). Figures 3.9., 3.10.(a and b) and 3.11. show representative autoradiographs of hybridisation experiments. All hybridisations provided specific signals. The HSC were demonstrated to contain mRNAs of approximately 3.1 kb for gelatinase A, 4.1 kb for collagen I, 0.9 kb for TIMP-1, 1.0 kb for TIMP-2, 3.4 kb for β_1-integrin and 1.8 kb for α-smooth muscle actin. Hybridisation signals were quantified by autoradiography and normalised to the signal obtained for β-actin mRNA. The densitometric β-actin ratios showed all-*trans* retinoic acid doses did not cause any apparent change in the mRNA of the various genes studied in primary rat HSC cultured for 7 days on plastic, compared with the corresponding controls containing the same amount of ethanol used as a diluent for the retinoid.

Figure 3.9: Effect of incubation of all-*trans* retinoic acid for 6 and 24 hours on mRNAs in hepatic stellate cells cultured for 7 days in serum-free media

A:

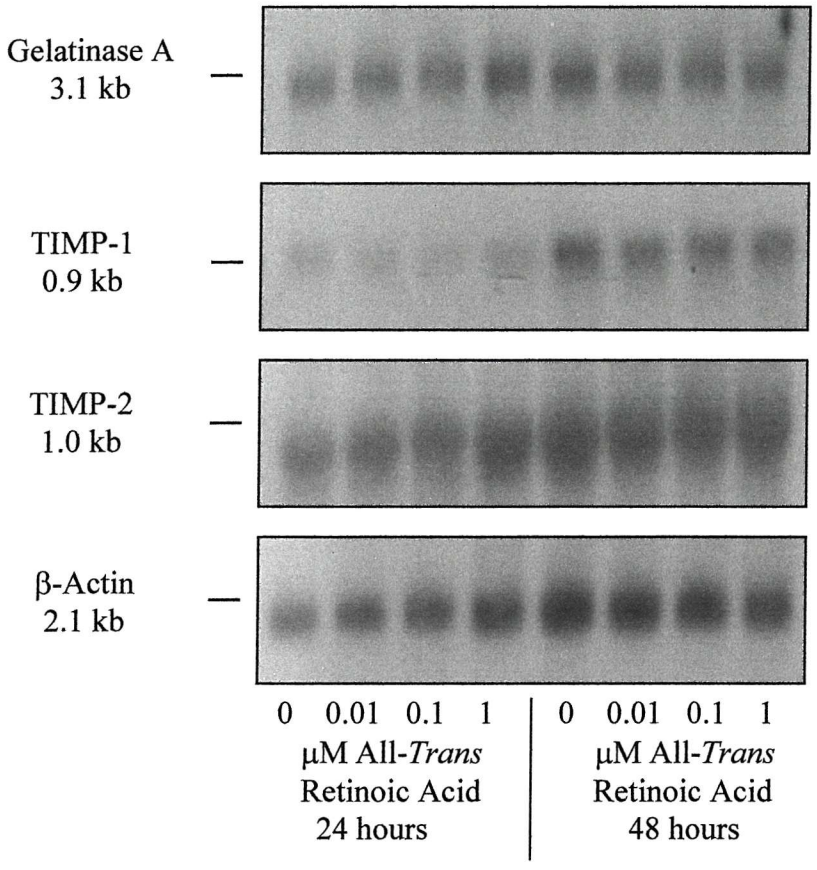


B:



Total RNA from rat HSC cultured on plastic for 7 days after incubation with exogenous all-trans retinoic acid for 6 and 24 hours in serum-free media, were analysed for expression of β_1 integrin and β-actin mRNA using northern hybridisation (panel A). The band intensity for β_1-integrin was expressed as a ratio to that for β-actin mRNA (panel B).

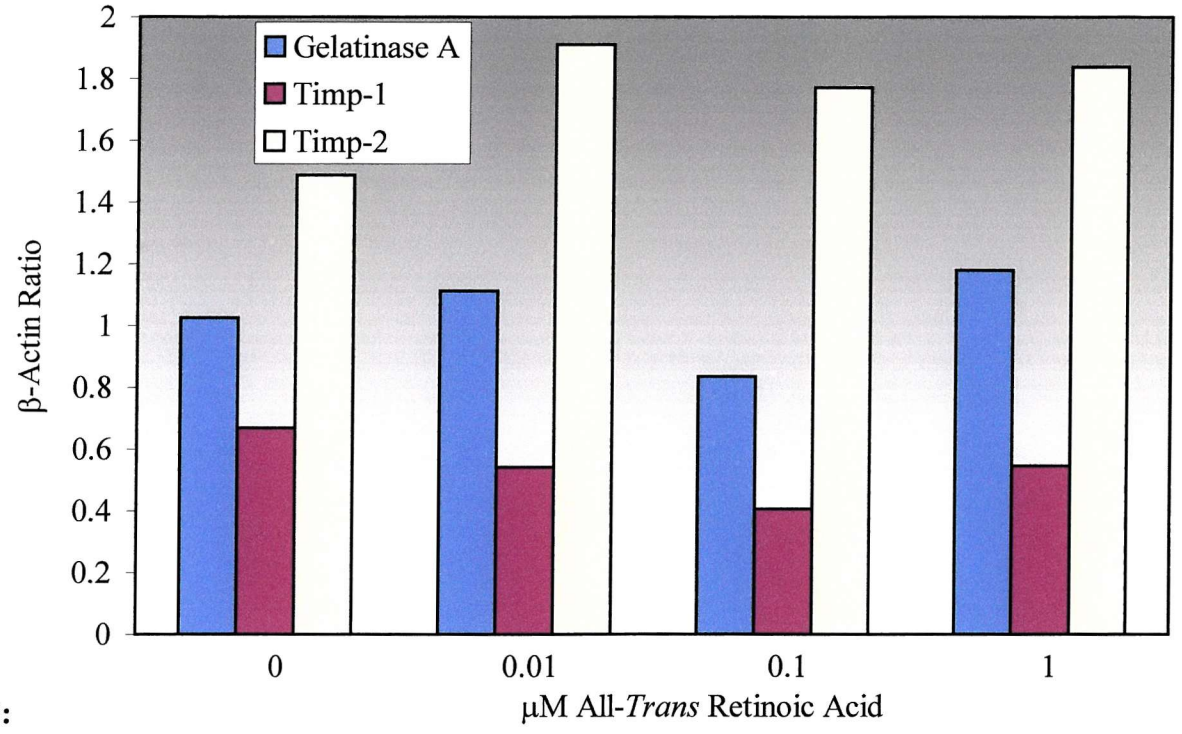
Figure 3.10.a: Effect of incubation of all-*trans* retinoic acid for 24 and 48 hours on mRNAs in hepatic stellate cells cultured for 7 days



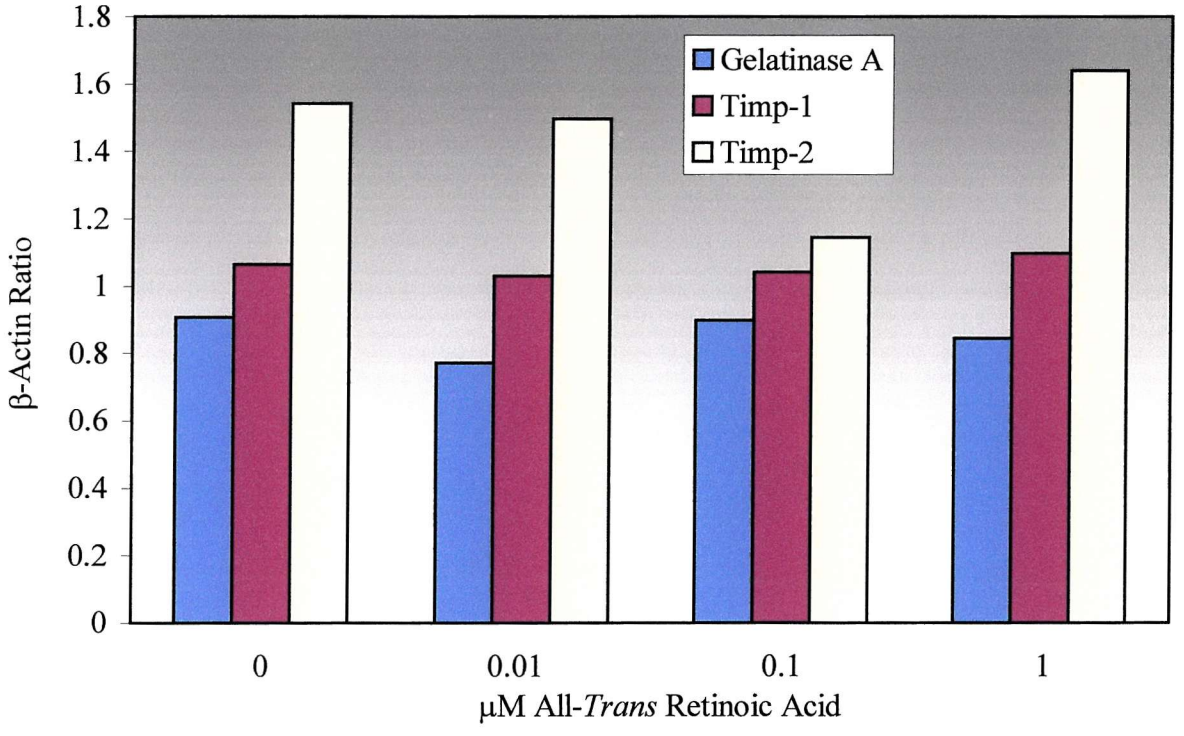
Total RNA from rat HSC cultured on plastic for 7 days after incubation with exogenous all-trans retinoic acid for 24 and 48 hours, were analysed for expression of gelatinase A, TIMP-1, TIMP-2 and β-actin mRNA using northern hybridisation. A further experiment using a separate cell culture gave a similar result.

Figure 3.10.b: β-Actin ratios for the mRNAs analysed in Figure 3.10.a following incubation of all-*trans* retinoic acid for 24 and 48 hours with hepatic stellate cells cultured for 7 days

A:



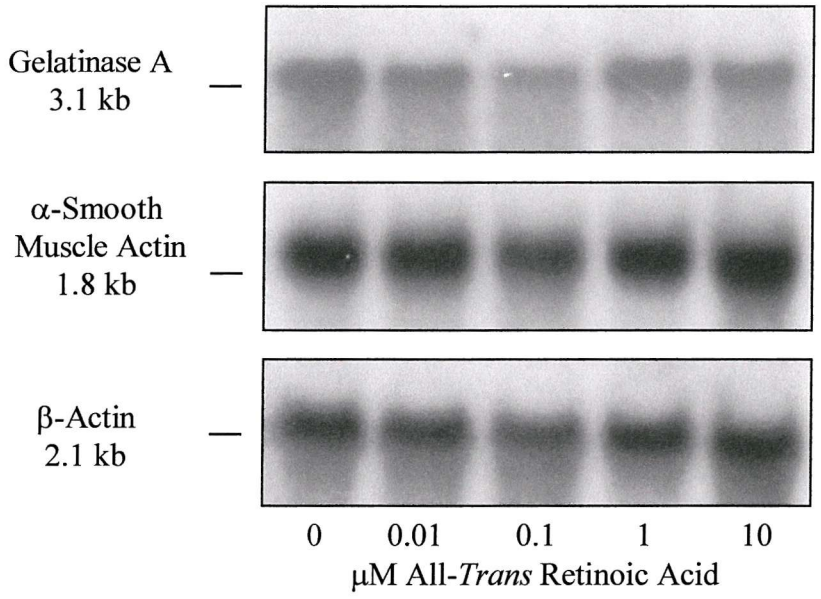
B:



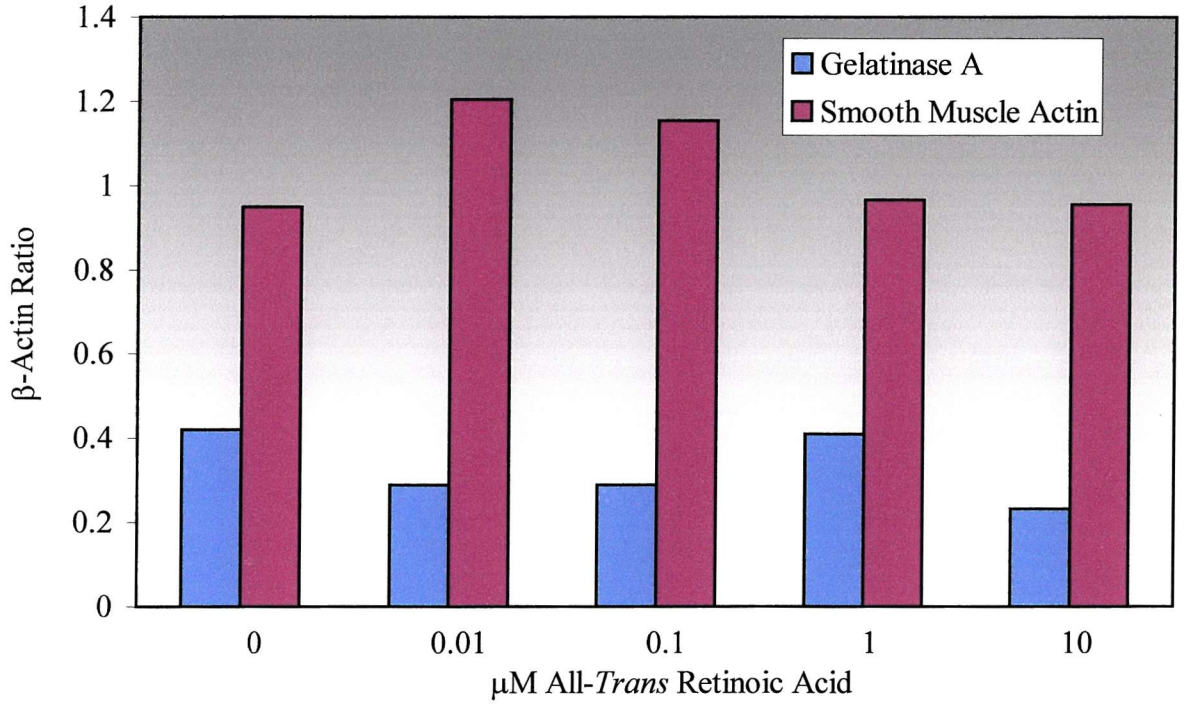
The band intensity for each of the mRNAs analysed in Figure 3.10.a was expressed as a ratio to that for β-actin mRNA. Panel A represents the β-actin ratios for 7-day old primary rat HSC following incubation for 24 hours with exogenous all-trans retinoic acid. Panel B represents the β-actin ratios for 7-day old primary rat HSC following incubation for 48 hours with exogenous all-trans retinoic acid. A further experiment using a separate cell culture gave a similar result.

Figure 3.11: Effect of incubation of all-*trans* retinoic acid for 72 hours on mRNAs in hepatic stellate cells cultured for 7 days

A:



B:



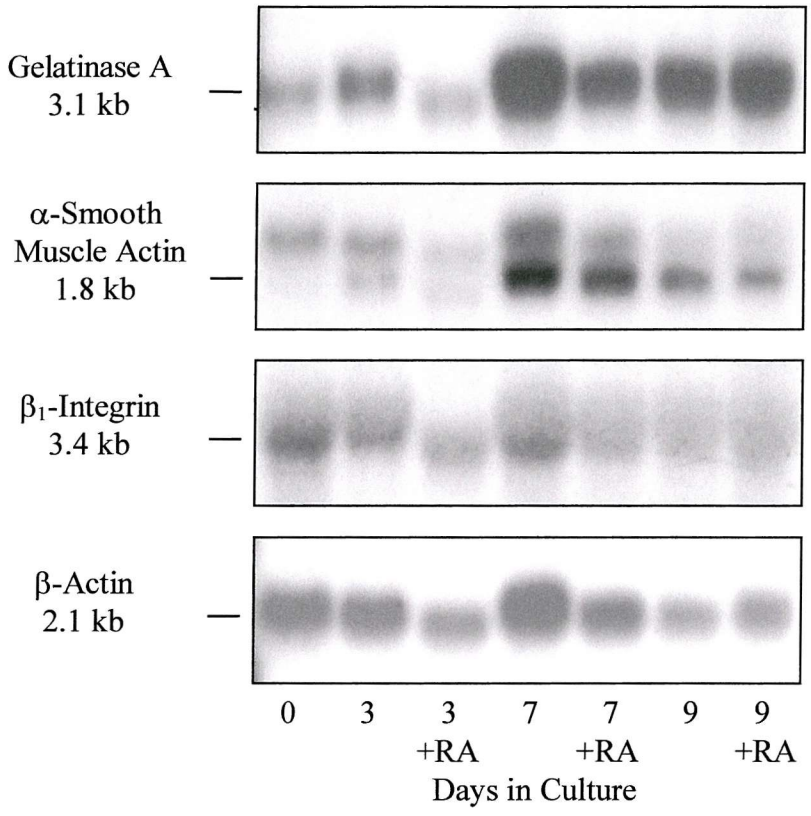
Total RNA from rat HSC cultured on plastic for 7 days after incubation with exogenous all-trans retinoic acid for 72 hours, were analysed for expression of gelatinase A and α-smooth muscle actin mRNA using northern hybridisation (panel A). The band intensity for each of these transcripts was expressed as a ratio to that for β-actin mRNA (panel B).

To examine whether chronic incubation with retinoic acid effected any changes in the mRNAs for activation induced markers, HSC were cultured in the continuous presence of all-*trans* retinoic acid from the first day of plating [Figures 3.12. (a and b) and 3.13.]. All-*trans* retinoic acid (1 μM or 10 μM) was supplemented into primary rat HSC cultures every 48 hours following their isolation and the incubations were continued into their myofibroblastic state (9 days of culture). Whereas untreated, freshly isolated HSC (day 0) produced either minimal or undetectable amounts of gelatinase A and α-smooth muscle actin, expression of these mRNAs increased from days 3-5 and became a continuous prominent feature as HSC adopted a myofibroblast like phenotype. This is in contrast to β_1-integrin mRNA which was strongly expressed at day 0 but decreased with culture. The expression of gelatinase A, β_1-integrin and α–smooth muscle actin at the mRNA level was not effected in HSC cultured in the presence of either 1 μM or 10 μM all-*trans* retinoic acid, relative to controls containing the equivalent concentration of ethanol. The mRNAs were essentially unaltered after incubation with all-*trans* retinoic acid, even when the HSC had become activated and depleted of their endogenous retinoids following culture on plastic for 9 days.

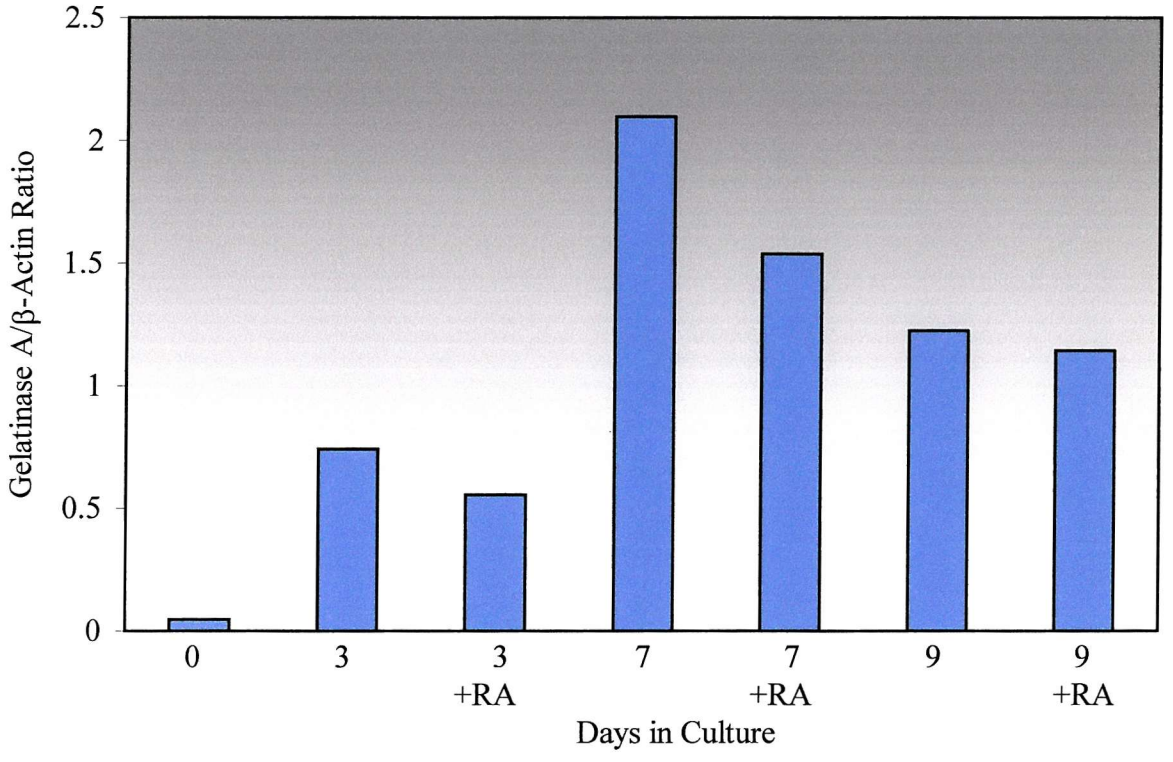
One exception, was the marked difference between HSC cultured for 6 days on plastic and HSC cultured for 6 days on plastic in the presence of 10 μM all-*trans* retinoic acid (Figure 3.13.). To explore this phenomenon further, cultured primary rat HSC were continuously exposed to 10, 1, 0.1 and 0.01 μM all-*trans* retinoic acid (replaced every 48 hours). A representative experiment showing the changes observed in gelatinase A and α-smooth muscle actin mRNA expression in 6-day old HSC cultures treated with these concentrations is shown in Figure 3.14. Northern blot analysis revealed no quantitative or qualitative differences between mRNAs isolated from control or all-*trans* retinoic acid treated cultures at each of the concentrations studied. This questions the reproducibility of the difference previously detected in Figure 3.13.

Figure 3.12.a: Effect of continuous incubation of 1 μM all-*trans* retinoic acid on mRNAs in cultured hepatic stellate cells

A:



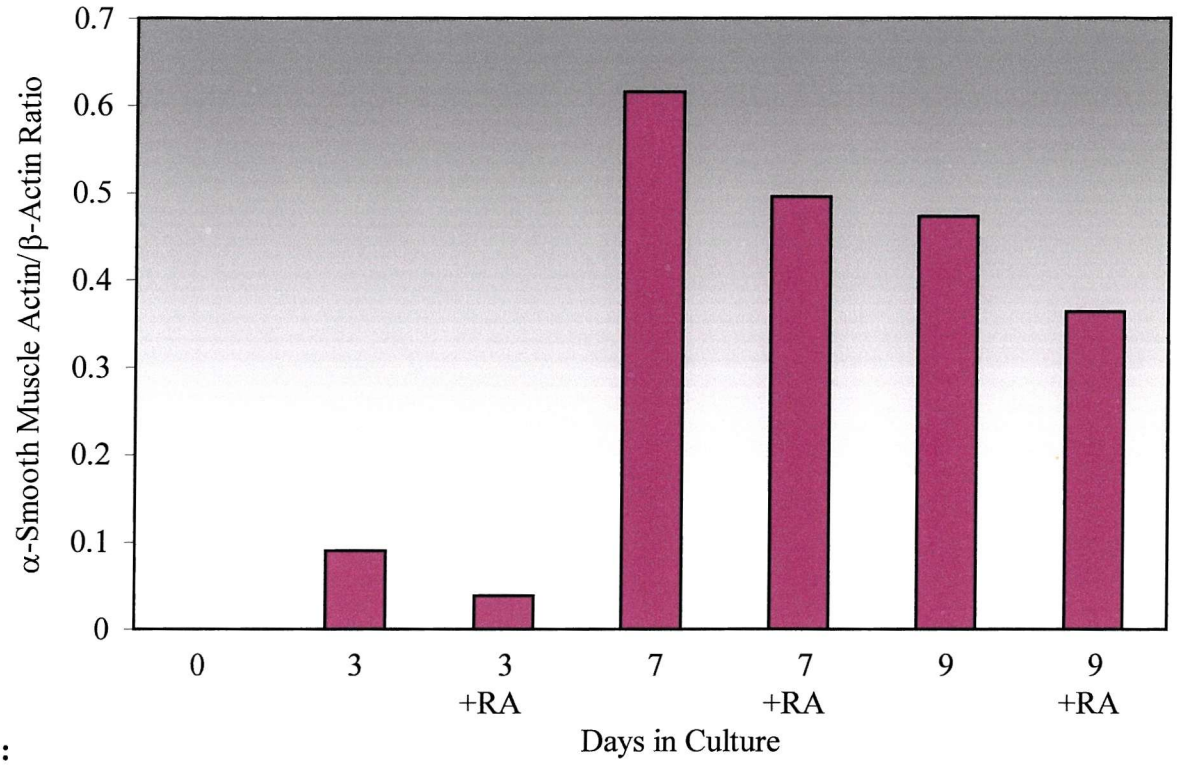
B:



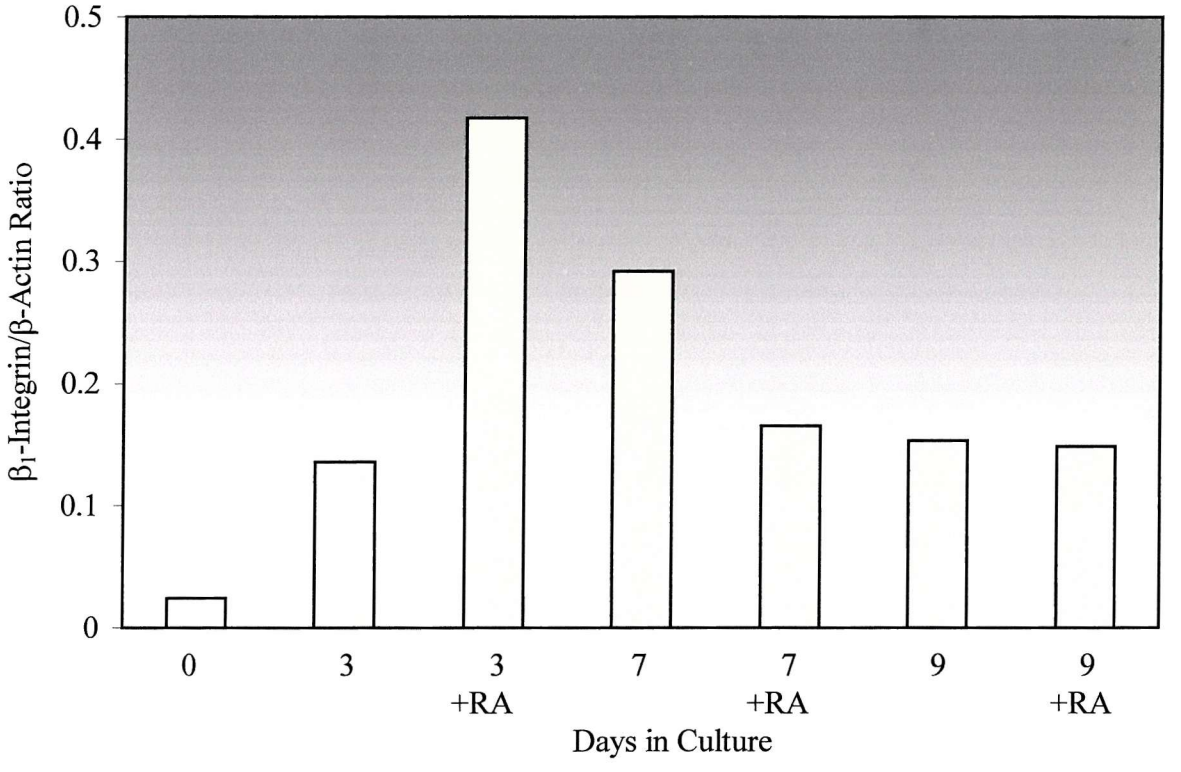
Total RNA from freshly isolated rat HSC (day 0) and rat HSC cultured on plastic in the continuous presence or absence of exogenous all-trans retinoic acid (1 μM), were analysed for expression of gelatinase A, α-smooth muscle actin and β_1-integrin mRNA using northern hybridisation (panel A). The band intensity for gelatinase A mRNA was expressed as a ratio to that for β-actin mRNA (panel B).

Figure 3.12.b: β-Actin ratios for mRNAs analysed in Figure 3.12.a following continuous incubation of 1 μM all-*trans* retinoic acid with cultured hepatic stellate cells

A:



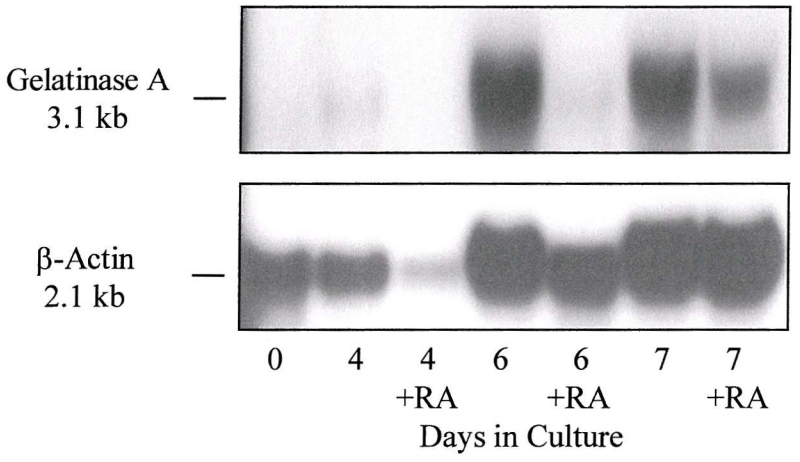
B:



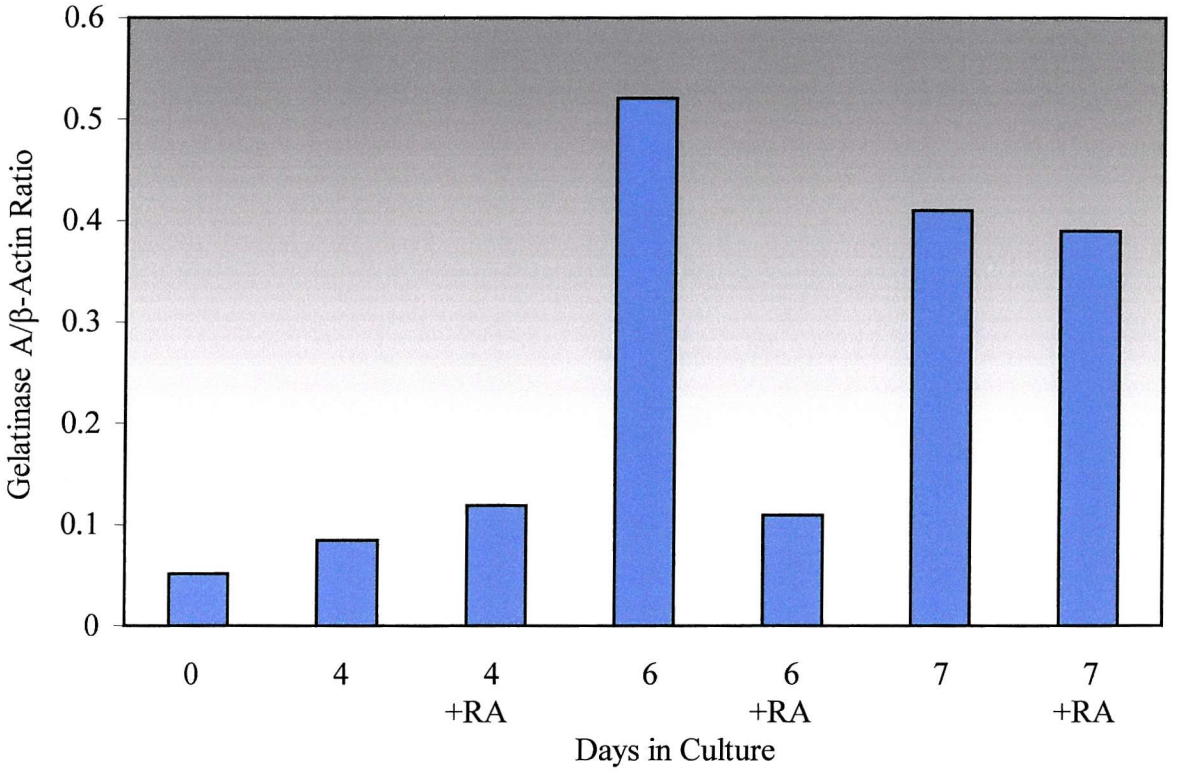
The band intensity for the α-smooth muscle actin and β_1-integrin mRNAs analysed in Figure 3.12.a, panel A was expressed as a ratio to that for β-actin mRNA. Panel A represents the β-actin ratio for α-smooth muscle actin mRNA. Panel B represents the β-actin ratio for β_1-integrin mRNA.

Figure 3.13: Effect of continuous incubation of 10 μM all-*trans* retinoic acid on mRNAs in cultured hepatic stellate cells

A:



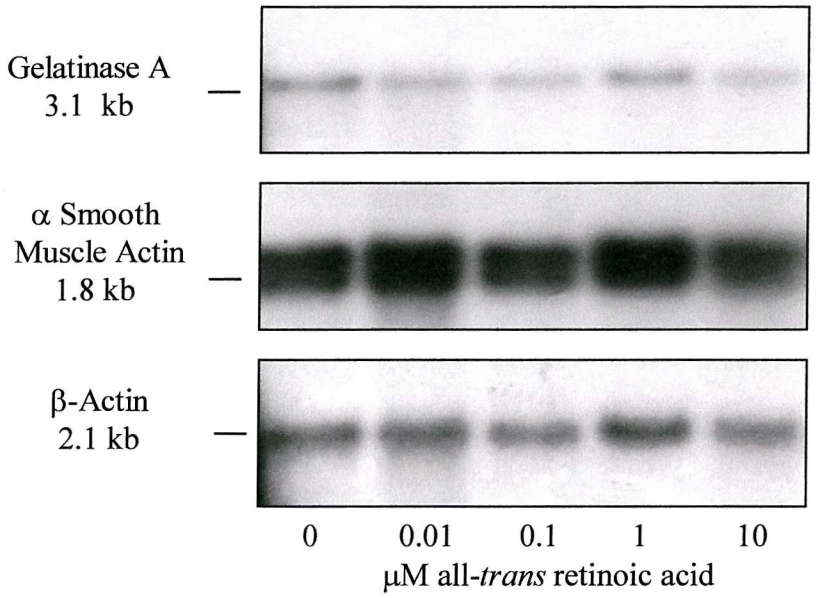
B:



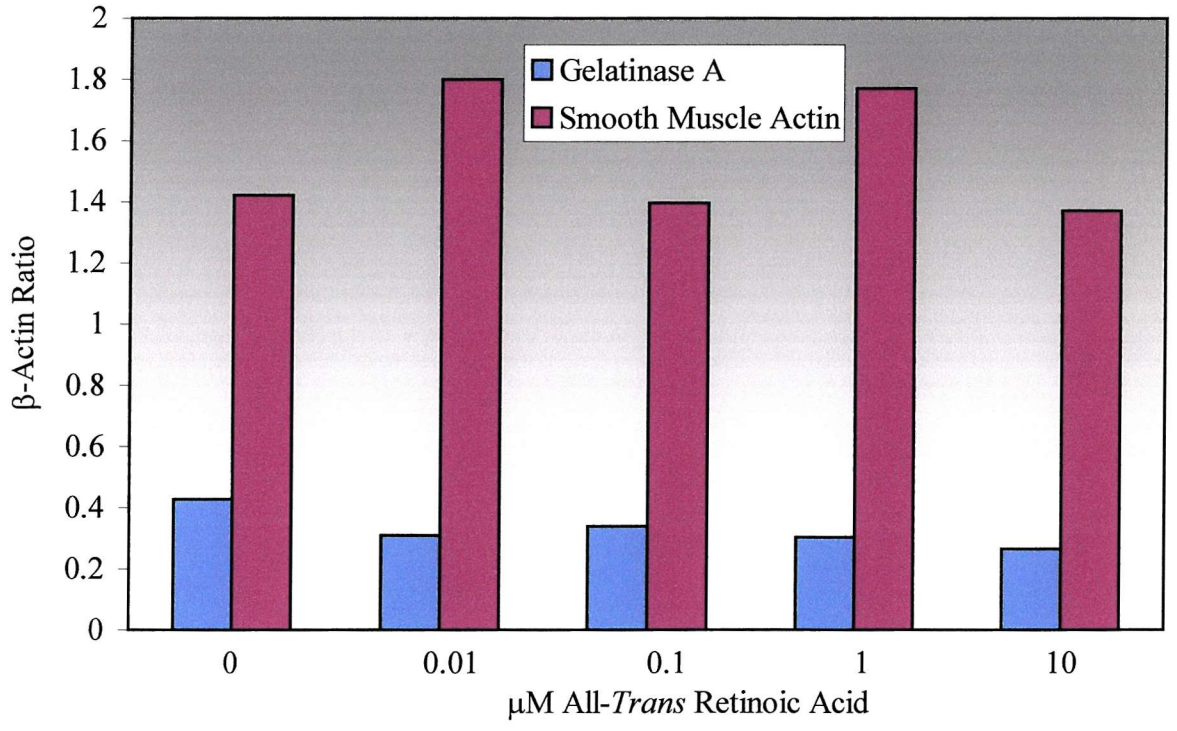
Total RNA from freshly isolated rat HSC (day 0) and rat HSC cultured on plastic in the continuous presence or absence of exogenous all-trans retinoic acid (10 μM), were analysed for expression of gelatinase A mRNA using northern hybridisation (panel A). The band intensity for gelatinase A mRNA was expressed as a ratio to that for β-actin mRNA (panel B).

Figure 3.14: Effect of continuous incubation of all-*trans* retinoic acid on mRNAs in hepatic stellate cells cultured for 6 days

A:



B:

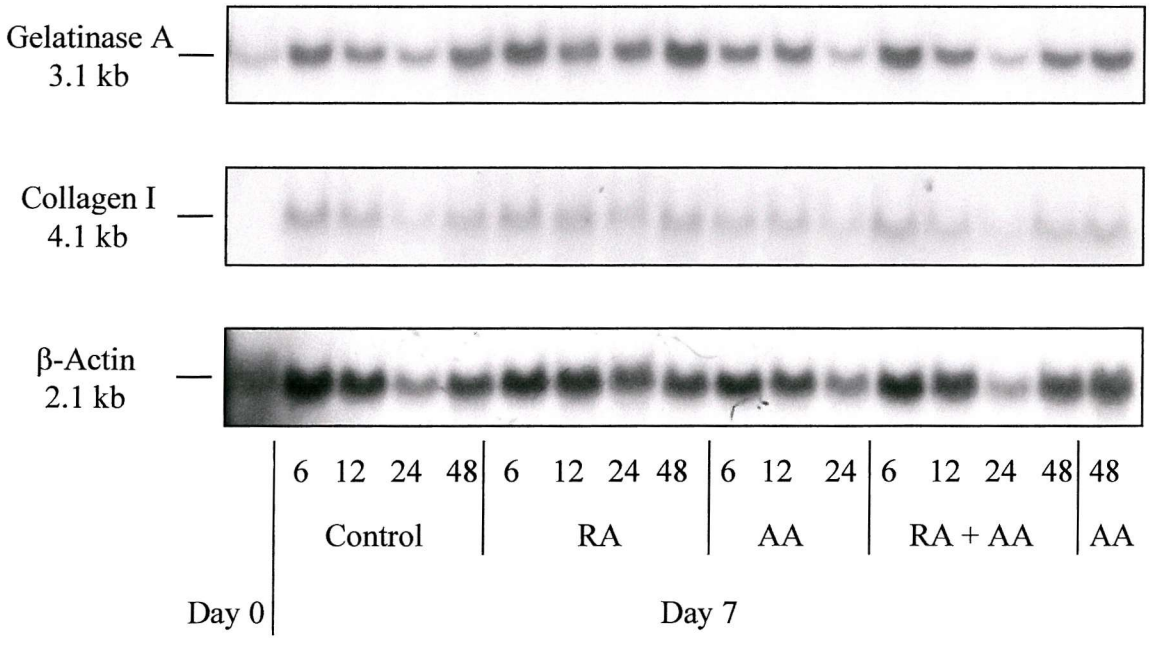


Total RNA from rat HSC cultured on plastic for 6 days in the continuous presence of exogenous all-trans retinoic acid, were analysed for expression of gelatinase A and α-smooth muscle actin mRNA using northern hybridisation (panel A). The band intensity for each of these transcripts was expressed as a ratio to that for β-actin mRNA (panel B).

A study published by Weiner *et al.* (1992) examined the effect of all-*trans* retinoic acid on HSC RARβ mRNA expression. These authors treated primary rat HSC cultured on plastic for 7 days with all-*trans* retinoic acid (1 μM) in the presence of the antioxidant, ascorbic acid (50 μg/ml) for 48 hours. Consequently, the effect of all-*trans* retinoic acid (10 μM) together with ascorbic acid (50 μg/ml) on HSC mRNAs in the present study was also examined (Figure 3.15.). When all-*trans* retinoic acid was applied in combination with ascorbic acid, there was no appreciable concentration-related change in the level of gelatinase A or collagen type I mRNA compared to the level in untreated HSC cultures. This was true for all the time points that were studied (6, 12, 24 and 48 hour treated samples).

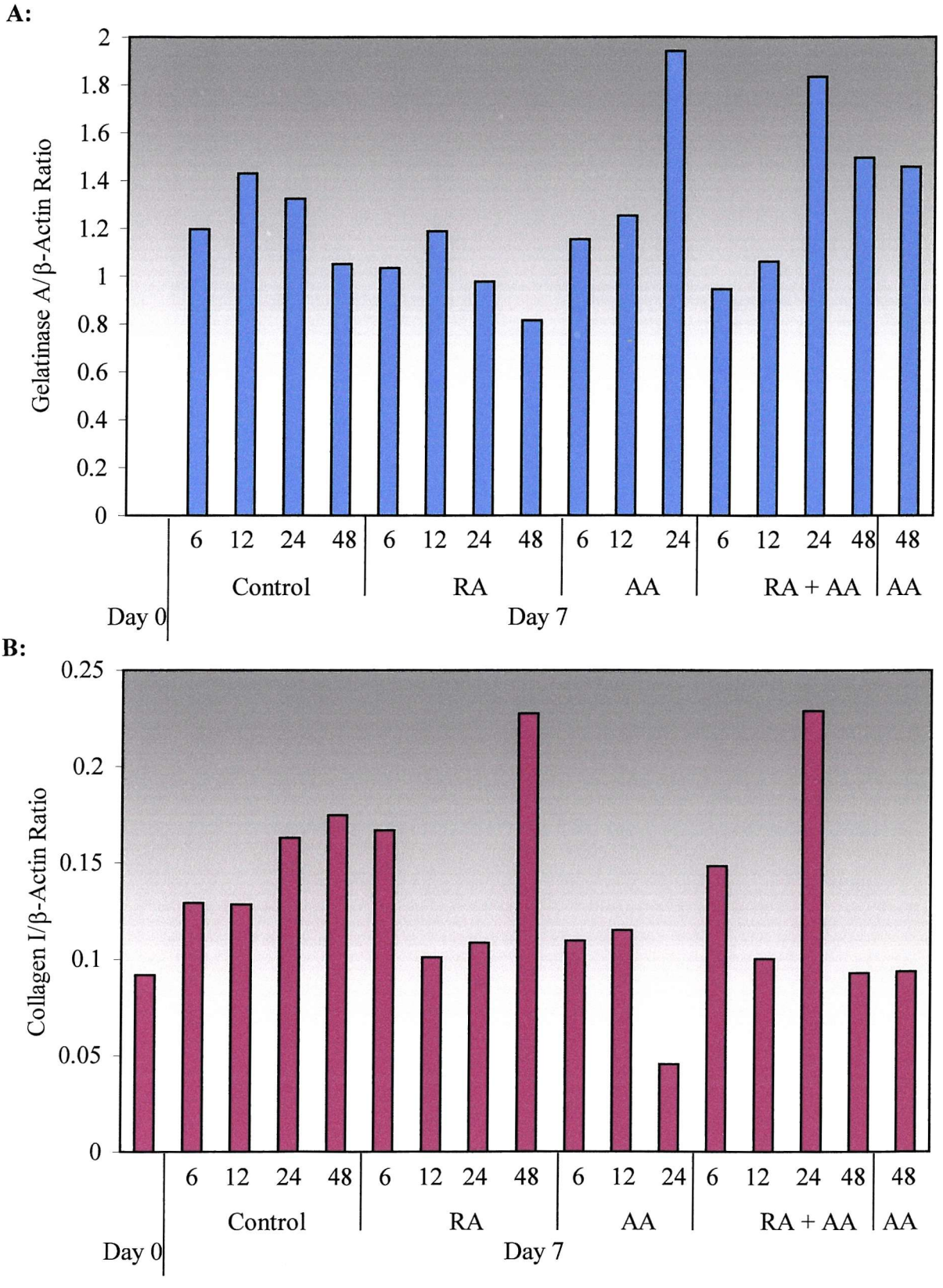
In summary, the data accumulated indicate that in HSC all-*trans* retinoic acid does not have any apparent effect on the expression levels of gelatinase A, collagen I, TIMP-1, TIMP-2, β_1-integrin and α-smooth muscle actin mRNA expression, despite alteration of a number of experimental parameters including the presence or absence of serum, the duration of treatment, or the use of activated versus quiescent HSC.

Figure 3.15.a: Effect of incubation of all-*trans* retinoic acid together with ascorbic acid for 6, 12, 24 and 48 hours on mRNAs in hepatic stellate cells cultured for 7 days



Total RNA was extracted from freshly isolated rat HSC (day 0) and rat HSC cultured on plastic for 7 days without treatment (control) or after treatment with 10 μM all-trans retinoic acid (RA), 50 μg/ml ascorbic acid (AA) or both RA and AA after 6, 12, 24 and 48 hours. The total RNA was analysed for expression of gelatinase A, collagen I and β-actin mRNA using northern hybridisation.

Figure 3.15.b: β-Actin Ratios for mRNAs analysed in Figure 3.15.a following incubation of all-*trans* retinoic acid together with ascorbic acid for 6,12, 24 and 48 hours with hepatic stellate cells cultured for 7 days



The band intensity for each of the mRNAs analysed in Figure 3.15.a was expressed as a ratio to that for β-actin mRNA. Panel A represents the β-actin ratio for gelatinase A mRNA. Panel B represents the β-actin ratio for collagen I mRNA.

3.3.4. Modulation of Protein Synthesis by Retinoids

Although the mRNA levels for gelatinase A and α-smooth muscle actin were unaltered by retinoic acid in HSC cultured on plastic, changes in expression of the gene products may be reflected at the level of protein synthesis.

Little information is available on the effects of retinoic acid metabolites on the synthesis of genes in both HSC and other cell types. Moreover, studies which have examined the anti-fibrotic potential of retinoic acid contain only information concerning the effect of the all-*trans* retinoic acid isomer and most are restricted to examining collagen type I at mRNA and protein levels (Davis *et al.*, 1990; Sato *et al.*, 1995). Therefore, the influence of all-*trans* retinoic acid and two other naturally occurring, metabolically active retinoic acid isomers (9-*cis* retinoic acid and 13-*cis* retinoic acid) on protein levels of gelatinases (MMP-2 and MMP-9) and α-smooth muscle actin in cultured rat HSC were further investigated.

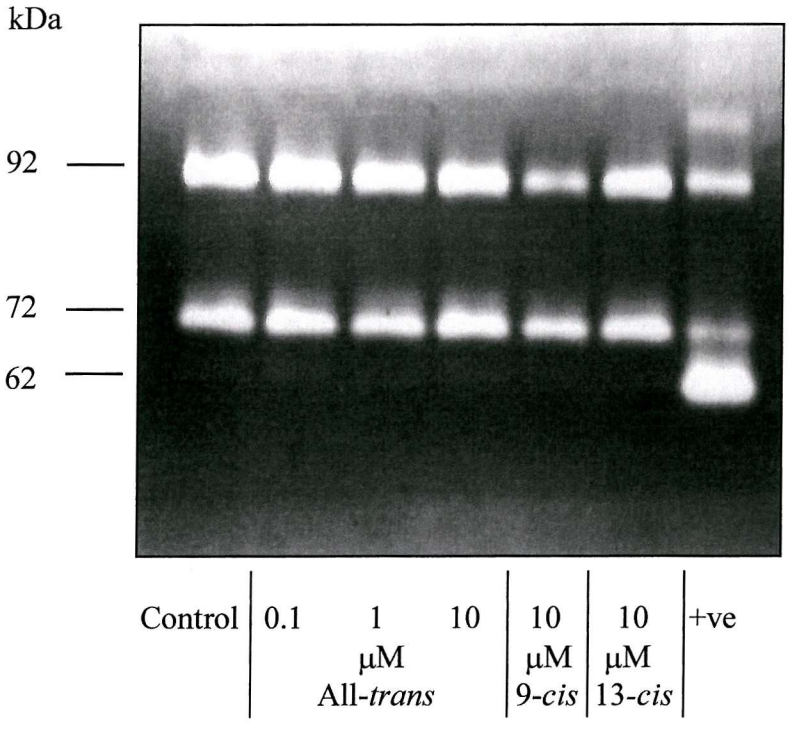
3.3.4.1. Modulation of Gelatinase Protein Synthesis by Retinoids

To determine whether retinoic acid regulates the expression of gelatinases, primary rat HSC cultured for 5 days on plastic were treated with all-*trans* retinoic acid (10, 1, 0.1 μM), 9-*cis* retinoic acid (10 μM) or 13-*cis* retinoic acid (10 μM) in 0.5% serum. After 48 hours, the supernatants were collected and an aliquot (7 μl) of each sample was examined by gelatin zymography (section 2.12.).

Analysis of the media from cultured HSC following treatment with each of the retinoic acid isomers showed gelatinolytic bands at 72 kDa, representing latent gelatinase A (MMP-2; Figure 3.16.). Bands at 62 kDa in the zymogram were barely detectable. This molecular weight represents the fully activated form of gelatinase A (Yu *et al.*, 1996). Gelatinase B (MMP-9; 92 kDa) was also detectable in the supernatants of HSC treated with isomers of retinoic acid. The identity of the HSC derived 92 kDa gelatinase B, the 72 kDa pro-gelatinase A, and the 62 kDa active gelatinase A are supported by the lane containing 5-day old, cultured primary rat HSC treated with concanavalin A (30 μg/ml) for 24 hours. Concanavalin A stimulates gelatinase A activation in HSC (Benyon *et al.*, 1999) and therefore represents a positive control. The gelatinases present in HSC untreated and treated with retinoic acid isomers co-migrated with the gelatinases present in concanavalin A stimulated HSC.

However, both MMP-2 and MMP-9 were uneffected by the retinoic acid isomers when compared to the level of these MMPs present in the media of the untreated HSC cultures.

Figure 3.16: Effect of isomers of retinoic acid on gelatinase A protein expression in hepatic stellate cells



Primary rat HSC, cultured on plastic for 5 days were treated for 48 hours in the absence of serum with different isomers of retinoic acid at the concentrations shown. Rat HSC cultured on plastic for 5 days and treated for 24 hours with concanavalin A (30 μg/ml) were used as a positive control. The cell-free media was harvested and subjected to gelatin zymography.

3.3.4.2. Modulation of α-Smooth Muscle Actin Protein Synthesis by Retinoids

To assess whether α-smooth muscle actin protein expression might be regulated by retinoic acid isomers in HSC, the levels of this protein in HSC that had been treated with retinoic acid isomers were evaluated by western blotting (section 2.10.).

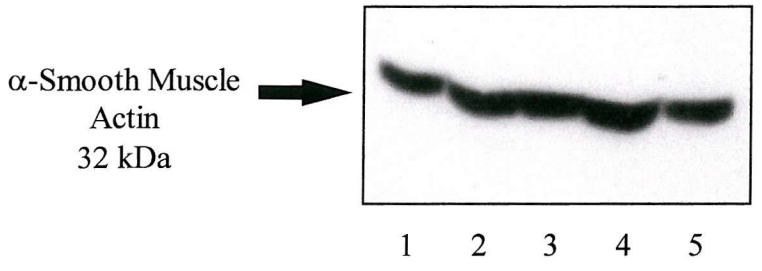
During transformation of HSC from a quiescent to an activated phenotype, overall cellular protein is increased in these cells due to expansion of rough endoplasmic reticulum (Friedman *et al.*, 1989). Therefore to control for any alterations in protein synthesis, instead of loading equal amounts of protein on the SDS-polyacrylamide gel which would underestimate any increase in a specific protein following activation, equal amounts of DNA were loaded.

Hepatic stellate cells cultured on plastic for 4 or 6 days were incubated for 72 or 24 hours respectively with retinoic acid isomers (10 μM). Equal amounts of the HSC lysates (1.5 μg of DNA) were subjected to electrophoresis on a 12.5% denaturing SDS-polyacrylamide gel, prior to western blotting onto PVDF. The blot was then incubated with a monoclonal antibody to α-smooth muscle actin followed by anti-mouse IgG conjugated to horseradish peroxidase. As shown in Figure 3.17., staining of the immunoblots with ECL detected a single band of 32 kDa. The band was specific, since no bands were detected on western blots incubated with the control antibody (mouse IgG) and the molecular weight of 32 kDa is comparable to that of authentic α-smooth muscle actin protein (Skalli *et al.,* 1986).

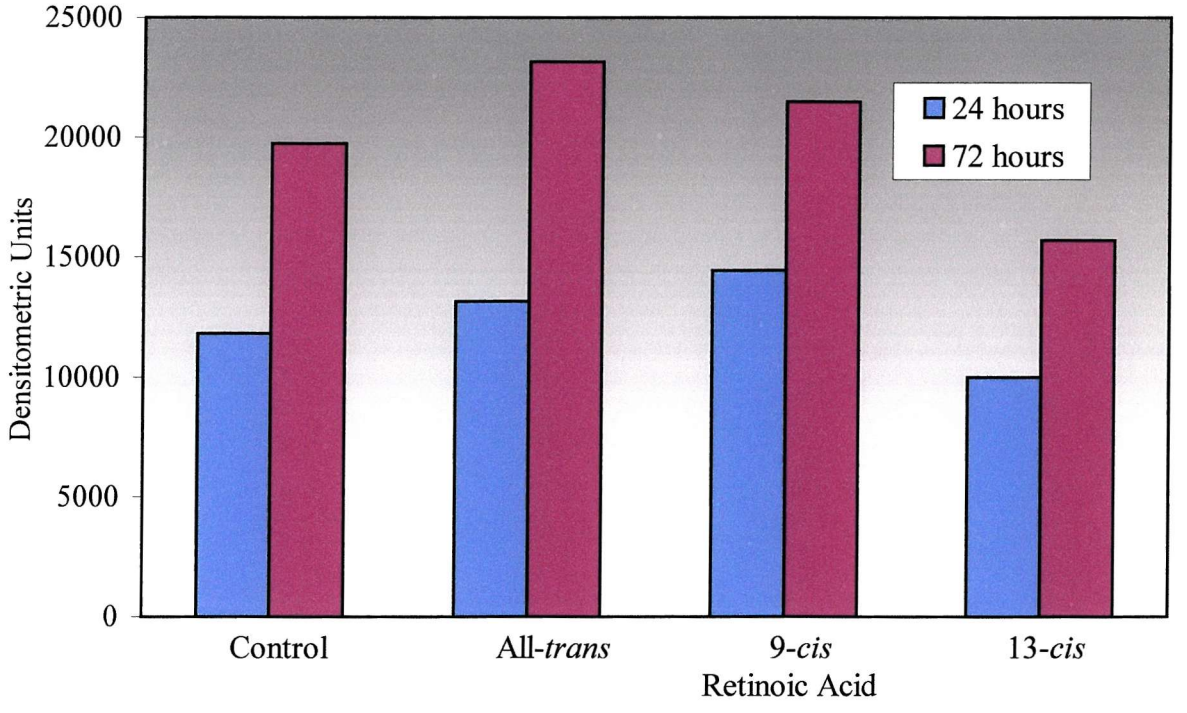
Control cultures synthesised high levels of α-smooth muscle actin protein, demonstrating the activated state of the HSC. However, cultures of HSC which had been treated with retinoic isomers for either 24 or 72 hours were also strongly positive for α-smooth muscle actin protein expression. The results suggest there is no difference between α-smooth muscle actin protein synthesis in HSC treated with retinoic acid isomers for 24 or 72 hours compared with untreated, control cultures.

Figure 3.17: Effect of isomers of retinoic acid on α-smooth muscle actin protein expression in hepatic stellate cells

A:



B:



α-Smooth muscle actin protein levels in rat HSC cultured on plastic for 4 or 6 days were analysed by western blotting following no treatment (lane 1), or treatment with either vehicle (lane 2), or 10 μM of all-trans retinoic acid (lane 3), 9-cis retinoic acid (lane 4) or 13-cis retinoic acid (lane 5) for 72 hours or 24 hours (panel A) respectively. Levels of α-smooth muscle actin were quantified by scanning densitometry (panel B). A further experiment using a separate cell culture gave a similar result.

3.4. DISCUSSION

The liver is a major site for uptake, storage and mobilisation of retinoids. Although hepatocytes have been shown to incorporate and metabolise retinol from chylomicrons, up to 90% of stored retinoids in the liver are found in HSC (Hendriks *et al.,* 1985), mostly as retinyl palmitate under normal nutritional conditions. During chronic inflammation of the liver, HSC proliferate and transform into myofibroblast-like cells (Minato *et al.,* 1983; Mak *et al.,* 1984). The progression to the myofibroblast phenotype during *in vitro* culture and during *in vivo* hepatic fibrogenesis, is characterised by a gradual loss of the intracellular retinoid stores (Friedman *et al.,* 1992a; Leo and Lieber, 1983). Therefore, a direct relationship may exist between retinoid levels and HSC phenotype. Furthermore, any change in HSC vitamin A content has a potential *in vivo* pathological significance. However, the roles of retinoids in the process of liver fibrosis still remain a mystery.

In mammalian species, the main forms of naturally occurring retinoids are retinol and its oxidation products, retinal and retinoic acid together with their *cis/trans* isomers. Therefore, the effects of retinol and isomers of retinoic acid, including all-*trans* retinoic acid, 9-*cis* retinoic acid and 13-*cis* retinoic acid, on the proliferation of HSC have been assayed. The proliferation assays performed with primary cultures of rat HSC show that isomers of retinoic acid and retinol inhibit rather than stimulate the rate of proliferation. Other workers have also demonstrated that retinol and retinoic acid act as growth inhibitors for primary and passaged HSC (Davis and Vucic, 1988; Pinzani *et al.,* 1992b; Davis *et al.,* 1990). Additionally, the results obtained are compatible with the numerous reports that demonstrate the capability of retinoids to regulate cell proliferation and differentiation in a variety of other cell types (Sporn and Roberts, 1984).

The extent of proliferation observed was dependent on the concentration of the retinoid and also the length of time HSC were exposed to the retinoid, suggesting that proliferation is effected by an indirect mechanism. This is in agreement with a report by Hellemans *et al.* (1999) who demonstrated that treatment of passaged HSC with 10^{-7} M 9-*cis* retinoic acid, or treatment of primary HSC with 10^{-7} M all-*trans* retinoic acid required at least 72 hours before inhibition of proliferation could be measured. According to Love and Gudas (1994), at early times after treatment (24 hours or less) of many cell types with retinoic acid, the levels of expression of many retinoic acid responsive genes are altered. At later times after retinoic

acid treatment (48-72 hours) genes encoding proteins that characterise a more differentiated cell are activated, for example the glycoproteins stromelysin and collagenase, involved in the production of extracellular matrix in human fibroblasts (Guerin *et al.,* 1997). Since HSC are responsive to retinoic acid at later times after treatment, it suggests retinoic acid inhibits proliferation by effecting genes encoding proteins that function in cell-matrix adhesion or in the cytoarchitecture.

The *in vivo* implication remains to be determined as most observations of the linkage between retinoids and HSC phenotype have been conducted by examining the effect of exogenous retinoic acid, and the possible roles of endogenously produced retinoic acid (or other retinoids) has not been thoroughly investigated. The findings of one *in vivo* study are at variance with the observations that retinoids can inhibit HSC proliferation. Tanaka *et al.* (1991) claim the capacity of HSC to proliferate in regenerating liver is uneffected by the levels of hepatic vitamin A. The relationship between hepatic vitamin A levels and HSC proliferation was evaluated in rats fed diets containing four different amounts of vitamin A. Despite the striking difference in hepatic vitamin A contents, the proliferative activity of HSC in these livers was uneffected. One possibility for the discrepancy between the results could be that addition of retinol to HSC *in vitro* in media containing FCS is non-physiological since *in vivo* HSC are exposed to retinol complexed to RBP (Blomhoff *et al.,* 1985a). However, Drevon *et al.* (1985) have shown that this *in vitro* culture model, although lacking RBP still permits intracellular retinol uptake and esterification, a key physiological function of HSC *in vivo*.

As with most reports cited (Davis *et al.,* 1990; Pinzani *et al.,* 1992b), retinoic acid was more potent than retinol at inhibiting HSC proliferation. An explanation for this could be that retinol has to be converted intracellularly to other derivatives or isomers, including all-*trans* retinoic acid, for the dominant mode of retinol action (Napoli and Race, 1990; Bhat and Jetten, 1987). In addition, all-*trans* retinoic acid is able to bind directly to the several closely related nuclear retinoid receptors that have been described for retinoic acid; none so far have been described for retinol. Retinoic acid has also been shown to be a more effective anti-proliferative agent than retinol in other cell types (Roberts and Sporn, 1984; Oikarinen *et al.,* 1985). In some instances however, the response observed for retinoic acid is opposite to retinol. For example, in some cells retinoic acid actually augments cell growth whilst retinol is anti-proliferative (Roberts and Sporn, 1984).

The concentration of retinol in the serum of adult rats and healthy humans is 10^{-6} M (Blomhoff *et al.*, 1990). Since about 80% of the vitamin A reserve is stored in the liver (Hendriks *et al.*, 1993), retinoid concentrations are especially high in this organ. Consequently the addition of 10^{-5} M of retinol to a culture system could mimic the physiological conditions in the liver. Since the concentrations used in this study are within the physiological range, endogenous retinoids may potentially have an inhibitory effect on normal HSC proliferation. It is noteworthy that all-*trans* retinoic acid does not occur in serum concentrations at greater than 10^{-8} M (De Leenheer *et al.*, 1982; De Ruyter *et al.*, 1979). Thus it is unlikely that the observed inhibitory action of all-*trans* retinoic acid at concentrations of 10^{-6} M and greater has any physiological significance, although such concentrations have been previously used in studies with HSC (Davis *et al.*, 1990; Weiner *et al.*, 1992). However, 10^{-7} M all-*trans* retinoic acid was still very effective at inhibiting proliferation. Intracellularly, all-*trans* retinoic acid could reach a concentration greater than 10^{-8} M since all-*trans* retinoic acid may represent the intracellular active form for retinol and its derivatives. Furthermore, HSC may receive vitamin A compounds from the neighbouring hepatocytes which could also increase the pericellular vitamin A concentration (Blomhoff *et al.*, 1985b).

The spontaneous differentiation and proliferation of isolated HSC in culture begins to occur only after the loss of the vitamin A stores, which coincides with several days in culture (Davis and Vucic, 1988). In the early stages of liver inflammation, when HSC retain their original vitamin A storing phenotype, cell proliferation could be initiated by growth factors and other mitogens released locally during the tissue repair and inflammatory processes. In later stages, acquisition of the transformed phenotype and loss of intracellular retinoids may be one method by which HSC exert their fibrogenic role independently.

The small, but not significant increase in HSC DNA content between days 4 and 7 in culture, has been noted by others in short-term (day 5 to day 9) HSC cultures under conditions comparable to those used in this study (Friedman and Roll, 1987). In this study, it was observed that retinoid supplementation resulted in a minor (though not statistically significant) reduction in cell number (assessed by DNA measurement), in contrast to its significant and more profound effect on the percentage of cells in the synthesis-phase [S-phase (assessed by [*methyl*-^{3}H]-thymidine incorporation)]. In other cell systems, retinoid induced growth inhibition is cell-cycle specific, with cells accumulating in the Gap$_1$-phase (G_1-phase) of the cycle and more slowly entering the S-phase (Seewaldt *et al.*, 1997; Mummery *et al.*, 1987).

A possible explanation for the observed inhibition of proliferation by HSC, is that retinoids induce cell death. Apoptosis or programmed cell death, is a major physiological mechanism used by multicellular organisms to maintain homeostasis, and recently apoptosis has been shown to be a major factor reducing HSC numbers during the recovery of normal liver histology following liver injury (Iredale *et al.,* 1998). Furthermore, in a variety of cancers, cell types and tissue culture cells, retinoids themselves can cause rapid (less than 24 hours) and extensive apoptosis. Consequently, retinoids may control the rate at which HSC undergo differentiation and apoptosis. To date, there have been no studies investigating the contribution of retinoids to apoptosis of HSC.

This study has demonstrated that exposure of HSC to retinoids does not enhance entry into the apoptotic process, despite mediating growth inhibition. At the retinoid concentrations used in this study, the number of apoptotic nuclei, assessed by acridine orange staining, did not significantly differ in HSC treated with retinoic acid isomers for 72 hours compared with control cultures. This result is not unexpected in view of the fact that HSC DNA content was also not effected following incubation with retinoic acid isomers. Others have also reported that retinoids markedly inhibit cell proliferation in passaged HSC cultures without loss of cell viability (Pinzani *et al.,* 1992b). The concentration (10^{-5} M) used in this study should be of a high enough concentration to induce apoptosis, since in the study performed by Horn *et al.* (1996), 9-*cis* retinoic acid and all-*trans* retinoic acid could induce cell death in P19 embryonal carcinoma cells from a low dose of 10^{-8} M. In the report by Horn *et al.* (1996), 9-*cis* retinoic acid was 5-fold more effective in inducing apoptosis than all-*trans* retinoic acid.

The rapidity of apoptotic events makes measurement of apoptosis difficult. Cells proceed from the onset of apoptosis (characterised by membrane blebbing and cell shrinkage) to fragmented apoptotic bodies in under an hour. In addition, the process of apoptosis is metachronous within a population of cells and therefore at each time point only a snapshot of the apoptotic activity has been determined. This has been demonstrated by Horn *et al.* (1996) who found after treatment with all-*trans* retinoic acid the number of P19 embryonal carcinoma cells undergoing apoptosis was low after 24 hours, peaked at 36-48 hours and thereafter decreased. Consequently, the induction of HSC apoptosis by retinoic acid, may depend on experimental conditions. At earlier time points, for example 48 hours, retinoid treatment may stimulate increased apoptosis compared with control cultures and thus decrease HSC number.

Apoptosis should be examined using a series of complementary techniques. Counting apoptotic bodies in a culture, by for example fluorescence microscopy, only reveals the number of apoptotic cells at any one time. It does not determine the rate of apoptosis in the cell culture. Moreover, it is easy to confuse apoptotic bodies with apoptotic cells, leading to an overestimate of dead cells. Other possible techniques include propidium iodide staining to identify and quantify condensed nuclei, and terminal deoxy-transferase mediated dUTP-biotin nick end labelling (TUNEL) staining to identify and quantify cells with oligosomal DNA fragmentation.

Another possibility for retinoids mediating growth inhibition of HSC, despite not effecting HSC number is that after 72 hours of treatment with retinoids, retinoids may be just beginning to decrease HSC entry into S-phase and the time has been insufficient for this to effect HSC number.

The present study was also undertaken to establish whether retinoic acid could modulate mRNA and protein synthesis in quiescent HSC, and/or myofibroblasts derived from HSC following their spontaneous differentiation in culture. The study conducted a thorough investigation to determine whether genes known to be markers of HSC activation are regulated by isomers of retinoic acid in HSC *in vitro,* as to date published studies have concentrated on HSC proliferation and matrix synthesis.

The present study demonstrates that all-*trans* retinoic acid does not effect the steady state mRNA for gelatinase A, α-smooth muscle actin, TIMP-1, TIMP-2, collagen type I and β_1-integrin in primary HSC cultures. Furthermore, the lack of change is neither effected by the concentration of the retinoid, nor the duration of the treatment.

Many reports have only examined the effect of all-*trans* retinoic acid on collagen expression. As shown in this and other reports, progressive activation of HSC by culture on plastic (demonstrated by an increase in α-smooth muscle actin expression) is associated with an increase in synthesis of collagen type I mRNA. This experiment also indicates that all-*trans* retinoic acid had little, if any, effect on collagen mRNA in these cells. The absence of modification on collagen synthesis in 24-48 hours, is consistent with the results published by Margis *et al.* (1992). However, Davis *et al.* (1987, 1990) and Davis (1988) found that interstitial collagen production is either increased, unchanged or reduced by the exposure of

rat HSC to all-*trans* retinoic acid. This implies differences in experimental conditions (for example different underlying extracellular matrices, *in vivo* or *in vitro* experiments, or whether the HSC cultures are primary or passaged) may cause opposing effects on HSC collagen synthesis. However, the studies in this Chapter eliminate the possibility that differences in when the retinoic acid is added to primary HSC cultures (i.e. during their quiescent state, their transformation or when activated) effect synthesis of mRNAs.

Hepatic stellate cells synthesise many matrix degrading enzymes, MMPs and their specific inhibitors, TIMPs. The mRNA for the MMP gelatinase A was barely detectable in freshly isolated rat HSC but increased dramatically with activation to a myofibroblast phenotype in cell culture (as defined by expression of α-smooth muscle actin). Gelatinase A degrades several basement membrane components including collagen type IV, laminin and fibronectin. Expression of gelatinase A by cultured rat and human HSC has also been reported previously by our group (Benyon *et al.,* 1999) and others (Milani *et al.,* 1994). The study by our group indicates that gelatinase A is a mitogen for HSC and thus its regulation by retinoids is an important issue which has not previously been studied. Low levels of both TIMP-1 and TIMP-2 mRNA were evident in activated HSC. Work from our group and others indicates that TIMPs may have an important role in liver fibrosis and the secretion of TIMPs by activated HSC may prevent degradation of matrix proteins and promote the progression to liver fibrosis, therefore their regulation by retinoids is also an important issue.

Most studies with all-*trans* retinoic acid have been shown to downregulate MMP expression and/or increase levels of TIMPs. Specifically, Clark *et al.* (1987) and Bauer *et al.* (1982) showed all-*trans* retinoic acid to inhibit the expression of interstitial collagenase and increase the expression of TIMP-1 in human fibroblasts. Despite these findings, in this study treatment of HSC with all-*trans* retinoic acid did not have any effect on mRNAs for the MMPs and TIMPs measured.

The mechanisms of cell-matrix interactions involving HSC are likely to be mediated via integrin and non-integrin receptors. Integrins are composed of two non-covalently associated transmembrane heterodimeric proteins termed α and β subunits. Each integrin subunit has a large extracellular domain, a single membrane spanning region and usually a short cytoplasmic domain. The integrins act not only as adhesive molecules but also transduce signals from the extracellular matrix to the cell interior with resultant alterations in cell behaviour, including

gene expression, differentiation and cell growth (Schwartz and Ingber, 1992). This property is largely due to the capability of the intracytoplasmic domain to interact with the cytoskeleton. As prevention of cell adhesion would block cell growth, the effect of all-*trans* retinoic acid on the β_1 mRNA subunit was investigated. β_1-integrin is specialised in cell-matrix adhesion and can bind most of the constituents of the extracellular matrix, including collagens and structural glycoproteins. This study shows rat HSC to express β_1 integrin, in agreement with a previous study which found human HSC to express $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_v\beta_1$ receptors (Carloni *et al.*, 1996). In F9 teratocarcinoma cells, retinoic acid (10^{-7} M) was found to increase the synthesis of β_1-integrin using the technique of immunoprecipitation (Ross *et al.*, 1994). However, in this study retinoic acid treatment did not alter the amount of β_1 integrin mRNA expression during the HSC phenotypic switch to a myofibroblast.

The general lack of response of HSC mRNAs to retinoic acid, could be explained on the basis of retinoid binding to serum proteins (Goodman, 1982). However, even in an experiment where retinoids were added to cultures in a serum-free medium for time periods short enough not to effect the viability of the cells, there was still no effect on the mRNAs. Furthermore, if the retinoids had been sequestered and/or inactivated by binding to the serum proteins, no inhibition of proliferation would have been observed.

Since accumulating evidence suggests that activated HSC are characterised by gelatinase A and α-smooth muscle actin expression, it was investigated whether these two features might be altered at a translational level following treatment with retinoic acid. The results of gelatinase A and α-smooth muscle actin protein expression compares well with the mRNA analysis. The majority of the gelatinase A enzyme released into the media by cultured rat HSC was in pro-enzyme form; only a negligible amount was found in the activated form, which supports studies published recently by our group (Benyon *et al.*, 1999). Like the mRNA expression, both α-smooth muscle actin and gelatinase A proteins were uneffected by any of the isomers of retinoic acid. In numerous other systems, retinoids increase or alter the production of various proteins. However, complementary to this study Davis *et al.* (1991) found by western blotting that passaged HSC treated with 10^{-6} M all-*trans* retinoic acid for 24 hours did not effect α-smooth muscle actin protein expression.

The combination of the experimental studies suggest the inhibition of proliferation induced by the addition of all-*trans* retinoic acid may not represent a global suppression of other cell

functions. In other cell types retinoids have been found to effect the molecules measured in this study at the mRNA and/or the protein level. However, HSC have been shown to contain high concentrations of cytoplasmic RBPs (Blomhoff *et al.,* 1985b). It is possible that differences in the expression of cytoplasmic or nuclear RBPs underlie the observed differences between these and other cell types. The cytoplasmic RBPs can bind retinoic acid and may subsequently sequester the retinoic acid in the cytoplasm or facilitate its catabolism. This would prevent the retinoid from reaching the nucleus and thereby reduce the expression of retinoic acid response genes. To complement this proposal, there is evidence that the higher the level of the CRABP I in the cytoplasm, the less sensitive the cell is to a given external concentration of all-*trans* retinoic acid (Boylan and Gudas, 1991). Consequently, the numerous biological observations made with other cell types may be difficult to extrapolate to HSC.

Although these studies have shown the role of all-*trans* retinoic acid in HSC activation is limited, our group has previously found that 10^{-6} M all-*trans* retinoic acid significantly increases uPA synthesis (Leyland *et al.,* 1996). Urokinase plasminogen activator not only generates plasminogen from plasmin which degrades the extracellular matrix (both directly and by activation of MMP-3) but also activates latent TGFβ which promotes matrix synthesis by HSC. Additionally, although the effect of all-*trans* retinoic acid on mRNAs was thoroughly investigated, the effect of retinoic acid on only two proteins (gelatinase A and α-smooth muscle actin) was examined. It is important to examine the effects of other isomers of retinoic acid on transcription and translation of genes, since Hellemans *et al.* (1999) found all-*trans* retinoic acid and 9-*cis* retinoic acid to exert differential effects on activated rat HSC. Hellemans *et al.* (1999) found all-*trans* retinoic acid to reduce the levels of collagens type I and III, fibronectin and laminin at the mRNA and protein level, whilst 9-*cis* retinoic acid increased the level of collagen type I mRNA (the level of collagen type I protein decreased). 9-*cis* Retinoic acid did not influence the protein levels of collagen type III, fibronectin and laminin. Consequently, the presence of other retinoic acid isomers and/ or the ratio of the various retinoic acid isomers present in quiescent and activated HSC could modulate genes that regulate the fibrogenic and fibrolytic properties of HSC, and this is investigated in the following Chapter (Chapter 4).

CHAPTER 4

Changes in Endogenous Retinoids During Culture Activation of Hepatic Stellate Cells

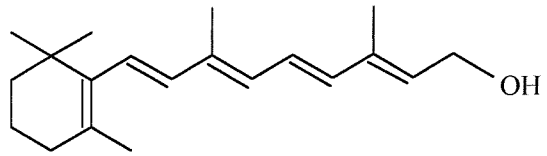
4.1. INTRODUCTION

The liver plays a central role in retinoid homeostasis, by regulating serum retinol levels and by storing the majority of the body's retinoids mainly in HSC. In healthy liver the most characteristic feature of HSC is the presence of large retinoid-rich droplets that are present in the cytoplasm.

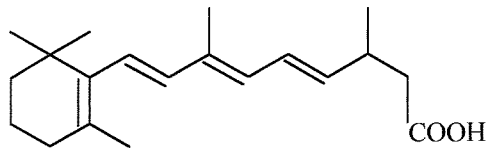
Vitamin A depletion appears to be a common phenomenon during human and experimental liver injury and fibrosis. Following acute and chronic liver injury, the activation and transdifferentiation of HSC correlates with a gradual loss of intracellular vitamin A. The distribution of retinol and its metabolites both in quiescent and activated HSC has not been fully elucidated. As discussed in Chapter 1, retinoids have the capacity to function as autoregulators and reduce cell proliferation and collagen production, the loss of vitamin A may therefore induce HSC to transform with increased collagen synthesis and cell number, thereby contributing to ongoing fibrogenesis. The content and quantity of endogenous retinoids present and released during the activation of HSC therefore warrants further investigation.

The HSC culture model in which HSC are cultured on a substratum of uncoated plastic of type I collagen, mimics many features observed in hepatic fibrosis *in vivo*, including a loss of cellular vitamin A. This *in vitro* model was used to identify and measure changes in intracellular and extracellular levels of several retinoid metabolites during the activation of rat HSC to determine retinoids which may be acting as a potential ligands for the retinoid receptors present in quiescent and activated HSC (Chapter 5). The structures of the retinoid metabolites measured during this study are shown in Figure 4.1.

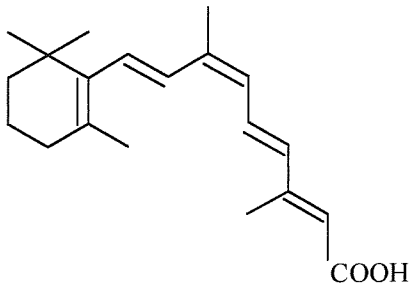
Figure 4.1: Structures of retinol metabolites and internal standards measured by reverse-phase high performance liquid chromatography



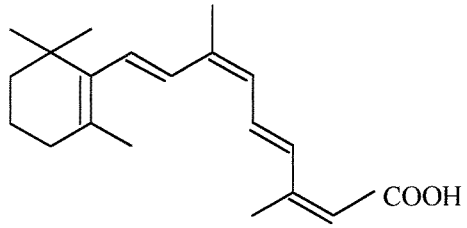
Retinol



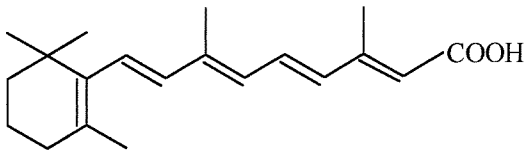
13-*cis* Retinoic Acid



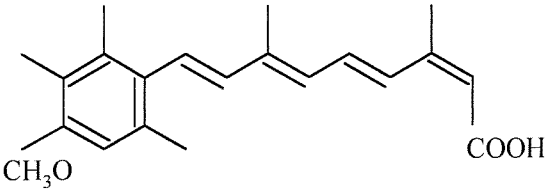
9-*cis* Retinoic Acid



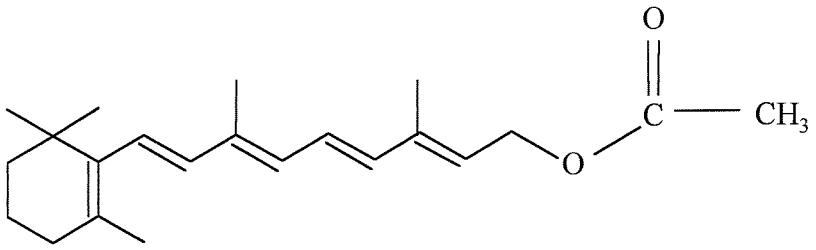
9,13 di-*cis* Retinoic Acid



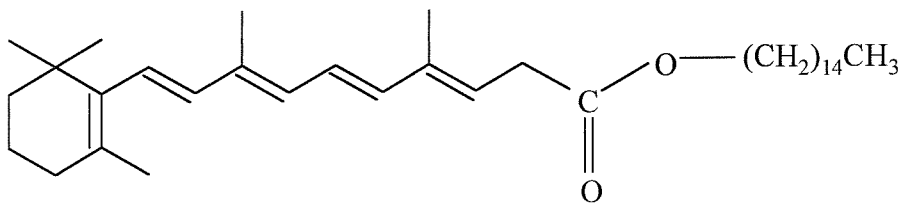
all-*trans* Retinoic Acid



13-*cis* Acitretin (RO 13-7652)



Retinol Acetate

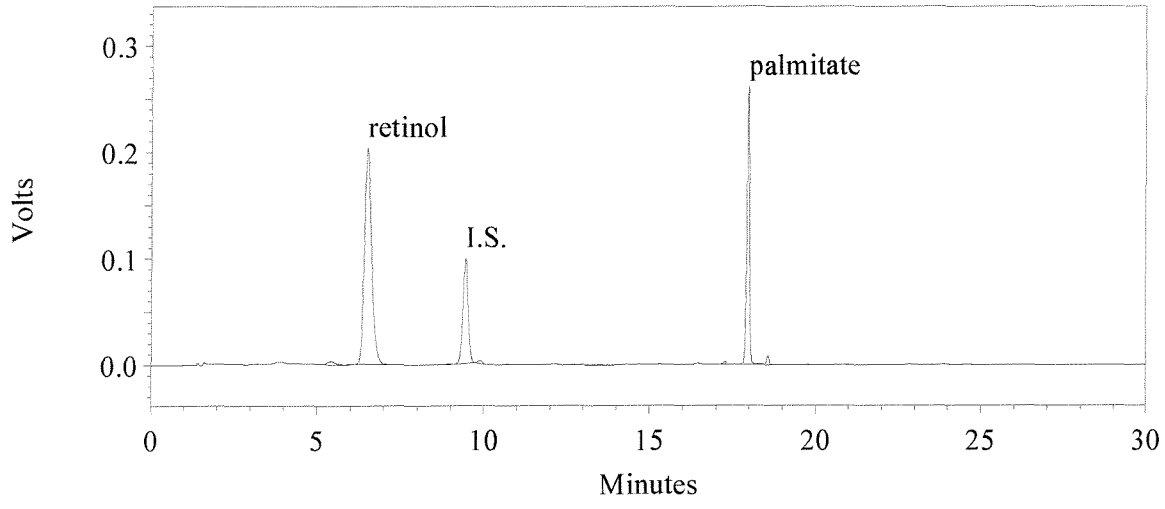


Retinyl Palmitate

4.2. VALIDATION OF THE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHODOLOGY

Before analysing the composition of retinoids in HSC, the HPLC methods (section 2.19.) to be used were first validated. Retention time is normally used to identify a component of a mixture, provided that a known standard is available for comparison. Typical chromatograms following the extraction of the standards retinol, retinyl palmitate, and the retinoic acid isomers from the same biological media (DMEM) to that of the samples are illustrated in Figures 4.2. and 4.3. together with their retention times. The peaks are well separated in both chromatograms. Figure 4.3, panel B also illustrates how for retinoic acids the extraction process, modified as described in section 2.19.3.2.1, selected against the extraction of retinol. Standard samples containing retinoic acids, were additionally spiked with 100 ng of retinol and 100 ng retinyl palmitate to mimic the cell culture samples, as these contain these retinoids at a much higher concentration than retinoic acids. The extraction process decreased the level of retinol appreciably. Retinyl palmitate was not detected on the chromatogram and was probably retained upon the C-18 reverse-phase column due to its higher lipophilicity compared to the other retinoids. Without decreasing the levels of retinol, the measurement of 9-*cis* retinoic acid was hindered but still detectable, even when 9-*cis* retinoic acid was present at a much lower concentration than retinol (Figure 4.4.).

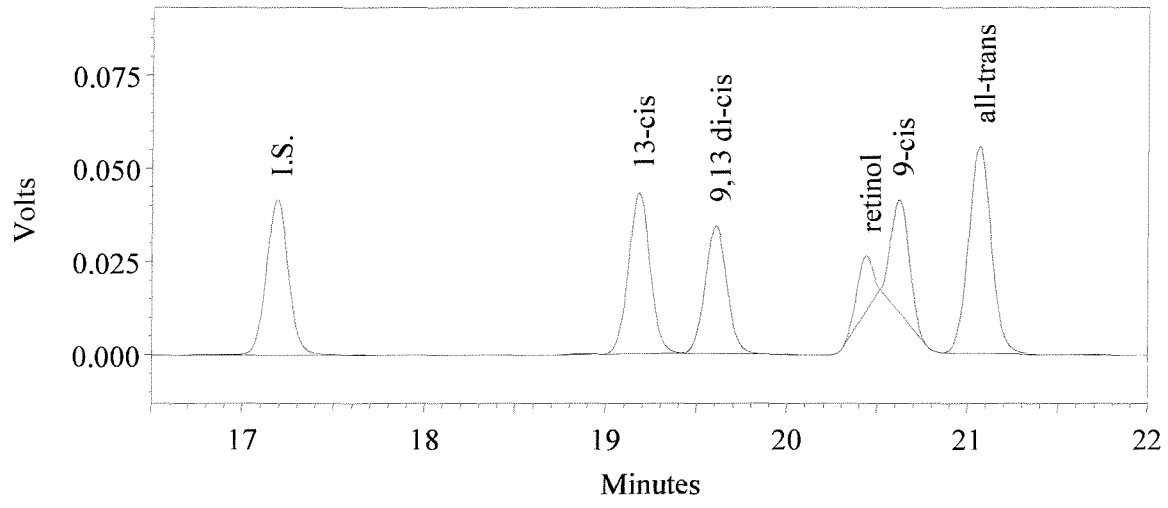
Figure 4.2: Chromatogram of the standards retinol and retinyl palmitate



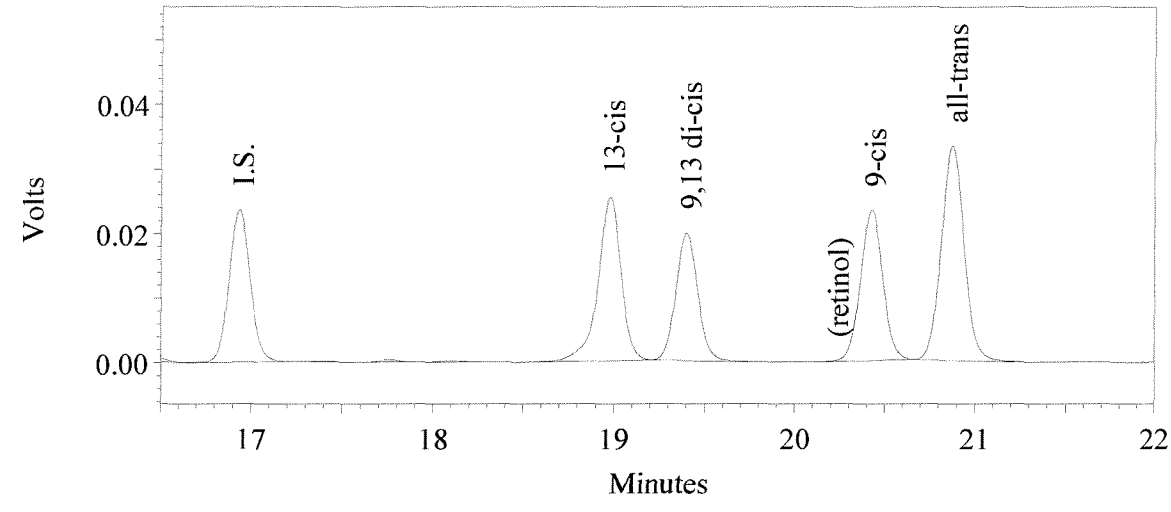
The commercially available retinoids, retinol (1000 ng), retinyl palmitate (1000 ng) and the internal standard (I.S.) retinol acetate (500 ng) were spiked into culture media, extracted and injected onto a reverse-phase HPLC column. Retention times were 6.1 minutes (I.S.), 9.0 minutes (retinol) and 18.0 minutes (retinyl palmitate).

Figure 4.3: Chromatograms of retinoic acid standards

A:

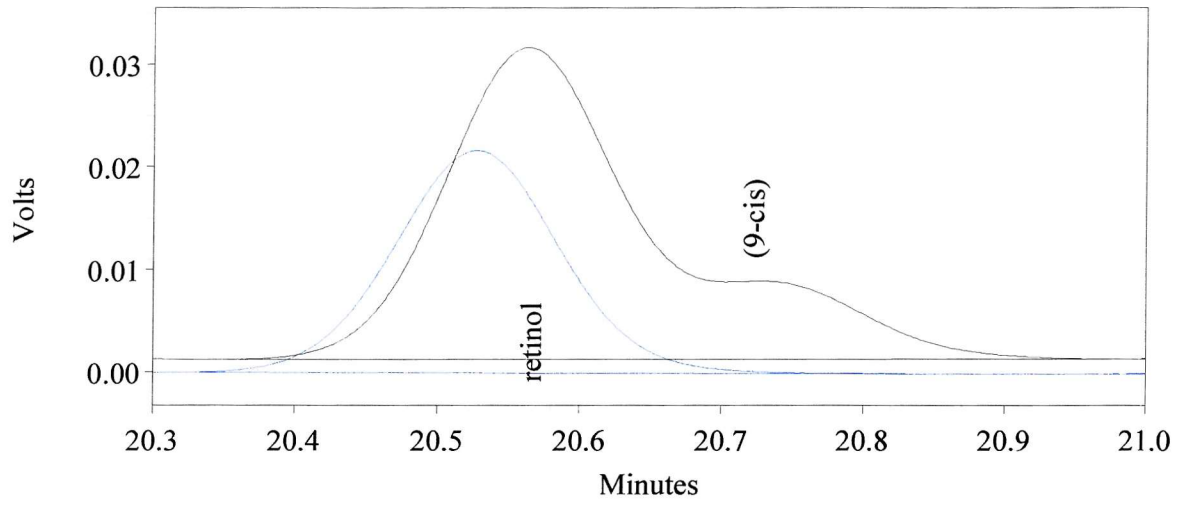


B:



The commercially available isomers of retinoic acid [13-cis retinoic acid (500 ng), 9,13 di-cis retinoic acid (500 ng) 9-cis retinoic acid (500 ng), all-trans retinoic acid (500 ng)], the internal standard (I.S.) 13-cis acitretin (500 ng), retinol (100 ng) and retinyl palmitate (100 ng) were injected directly onto a reverse-phase HPLC column (panel A) or spiked into culture media, extracted and injected onto a reverse-phase HPLC column (panel B). Retention times were 17.0 minutes (I.S.), 19.1 minutes (13-cis retinoic acid), 19.5 minutes (9,13 di-cis retinoic acid), 20.3 minutes (retinol), 20.5 minutes (9-cis retinoic acid) and 21.0 minutes (all-trans retinoic acid).

Figure 4.4: Chromatogram of the standards retinol and 9-*cis* retinoic acid

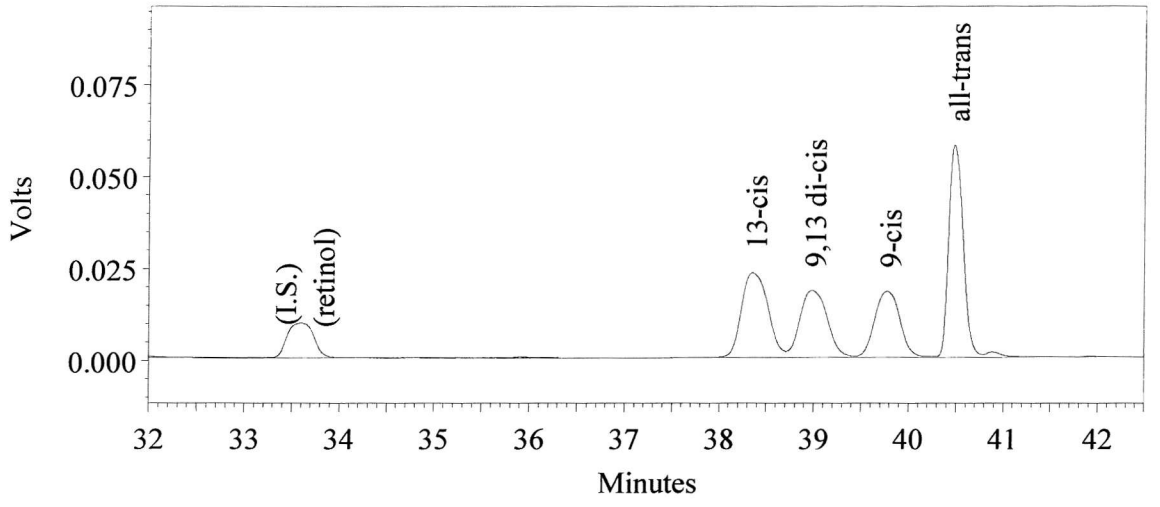


Retinol (100 ng), or retinol (100 ng) and 9-cis retinoic acid (30 ng) was spiked into culture media, extracted and injected onto a reverse-phase HPLC column.

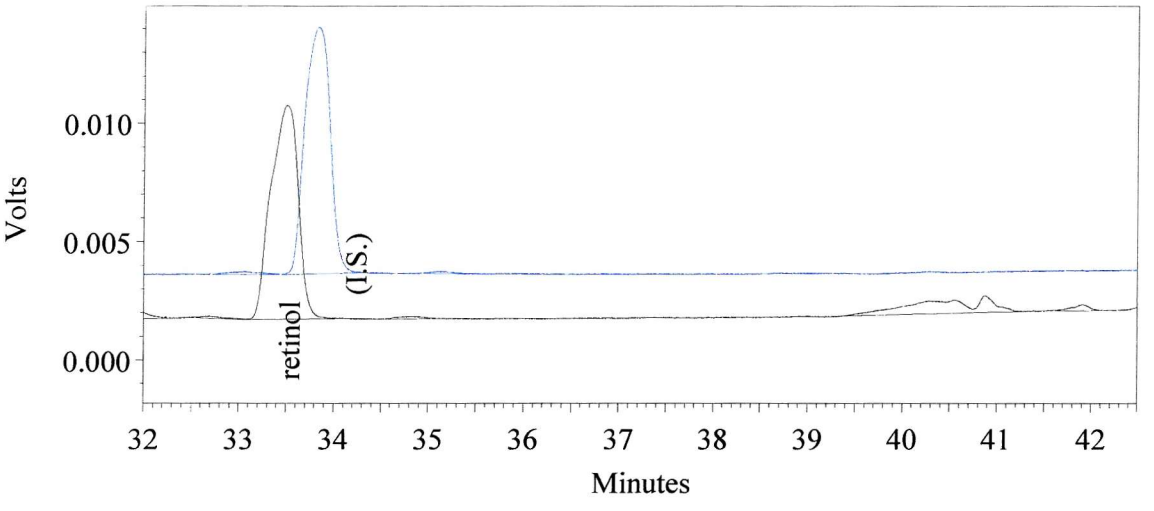
Since retinol and 9-*cis* retinoic acid eluted in close proximity, alternative conditions for resolution of the retinoic acids were investigated. However, to obtain an analytical method for simultaneous quantitation and resolution of four isomers is technically challenging. Conditions stated by Sass and Nau (1994) in which the organic reagent was modified from methanol to acetonitrile, resulted in a greater resolution of retinol from 9-*cis* retinoic acid (Figure 4.5, panel A). This change however, prolonged the retention time of the retinoic acids, contributing to broader peaks. Additionally, the change reversed the elution order of retinol and 9-*cis* retinoic acid. Under these altered conditions, retinol co-eluted with the internal standard, 13-*cis* acitretin (Figure 4.5, panel B). Although two Pfizer compounds, structurally related to retinoic acid were investigated as alternative internal standards, these eluted in close proximity to the retinoic acids. The back extraction technique was finally decided upon to remove retinol, rather than change to the Sass and Nau (1994) method.

Figure 4.5: Chromatogram of retinoic acid standards eluted using the conditions stated by Sass and Nau (1994)

A:



B:

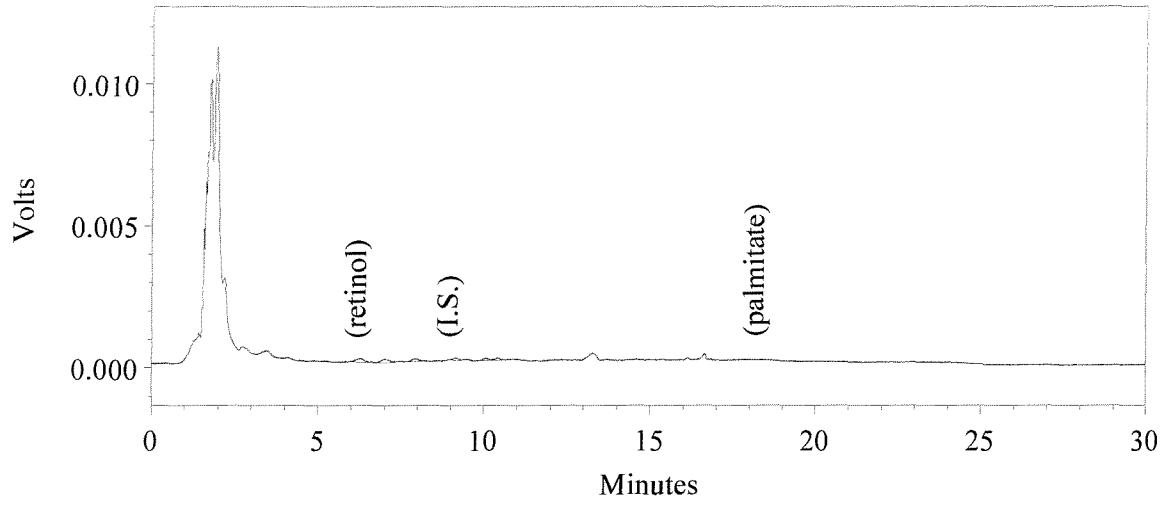


The commercially available isomers of retinoic acid [13-cis retinoic acid (500 ng), 9,13 di-cis retinoic acid (500 ng) 9-cis retinoic acid (500 ng), all-trans retinoic acid (500 ng)], the internal standard (I.S.) 13-cis acitretin (500 ng), retinol (100 ng) and retinyl palmitate (100 ng) were spiked into culture media, extracted, injected onto a reverse-phase HPLC column and eluted under the mobile phase conditions stated by Sass and Nau (1994). Retinol (500ng) and the I.S. (500ng) were also injected separately onto the reverse-phase column and eluted under similar conditions (panel B).

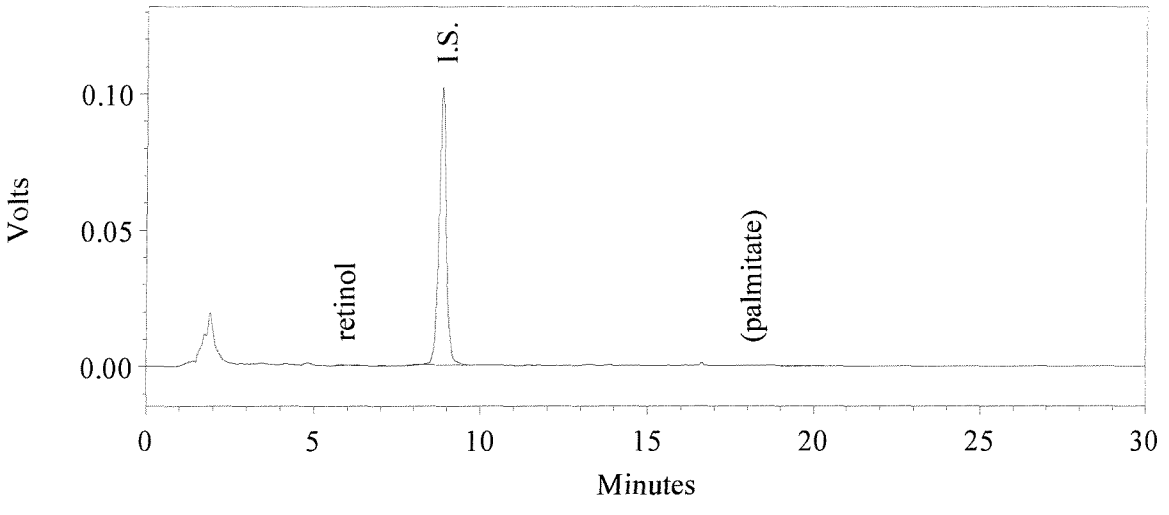
To ensure that blank culture media, or the addition of the internal standards did not produce the peaks observed at the retention times for retinol, retinyl palmitate and the isomers of retinoic acids, extractions were performed on blank media and media spiked with internal standard. The extracts were then analysed using reverse-phase HPLC under similar conditions to the standard samples. For both conditions used to extract and detect retinol and retinyl palmitate, and for those used to detect retinoic acid isomers, typical chromatograms for blank media and for media spiked with internal standard are shown in Figures 4.6, 4.7. and 4.8. Although retinol levels in the control medium in the absence of internal standard could not be detected, a trace (3.4 ng) of retinol was detected when the internal standard, retinol acetate was spiked into the culture medium. Presumably this has arisen from the catabolism of retinol acetate to retinol. However, this small concentration of retinol would not interefere with the assay considering the levels of retinol which are present in HSC. It was therefore concluded that neither DMEM nor the internal standards introduced contaminants which influenced the measurement of retinoids by either HPLC technique.

Figure 4.6: Chromatograms of extracts from culture media and media spiked with internal standard using the conditions applicable for detecting retinol and retinyl palmitate

A:



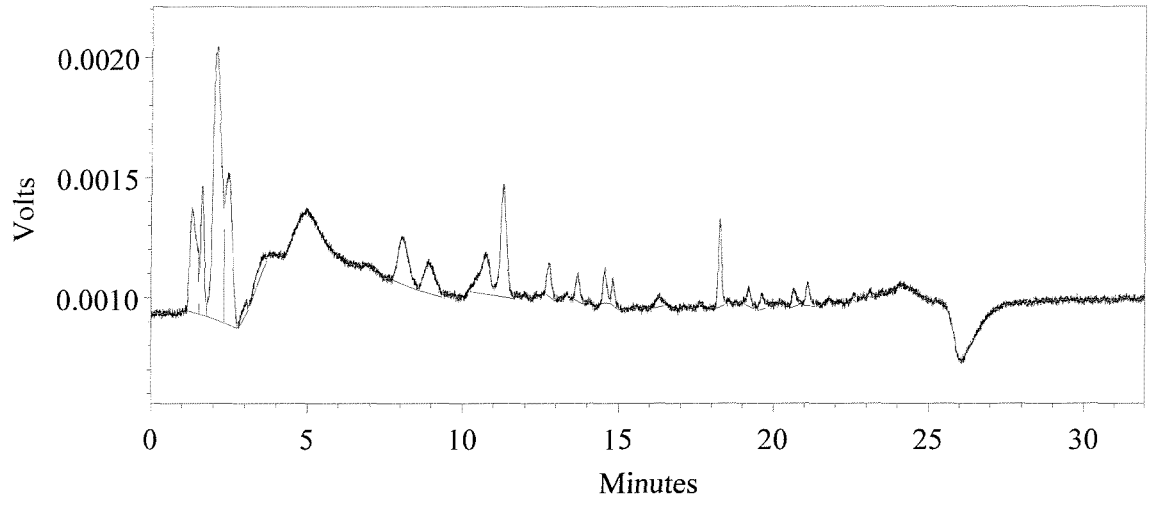
B:



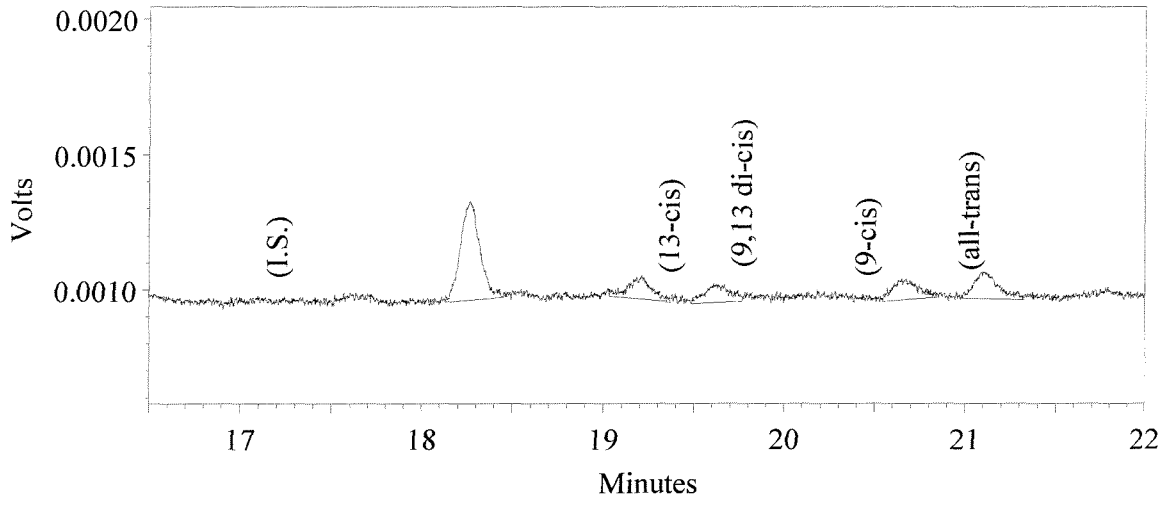
Extracts from culture media (panel A) and media spiked with internal standard [I.S. (panel B)] were injected onto a reverse-phase HPLC column using the conditions applicable to extracting and detecting retinol and retinyl palmitate. The chromatograms are annotated to show where authentic standards of retinol and retinyl palmitate would elute.

Figure 4.7: Chromatograms of extracts from culture media using the conditions applicable for detecting retinoic acid isomers

A:



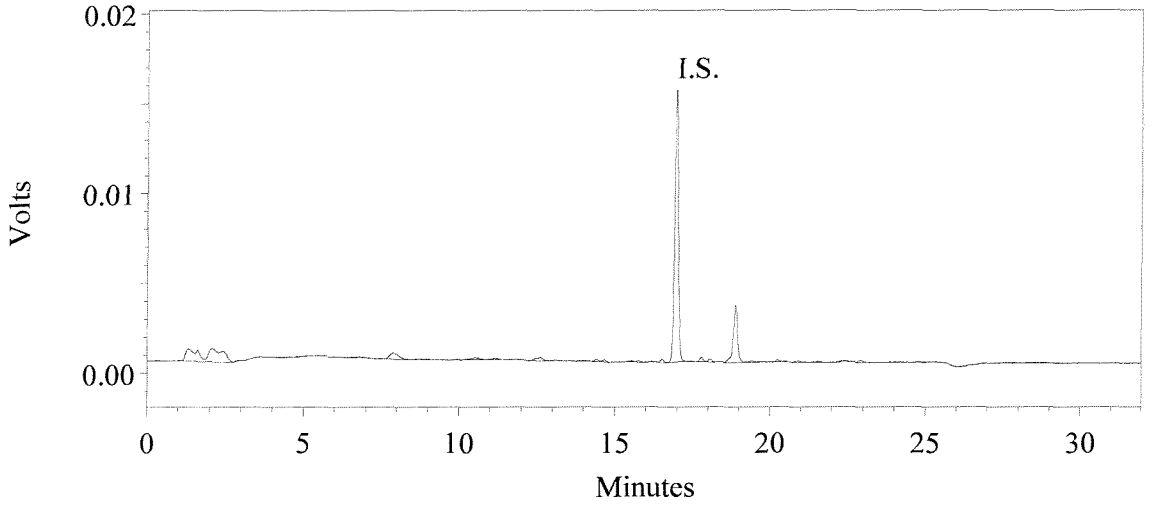
B:



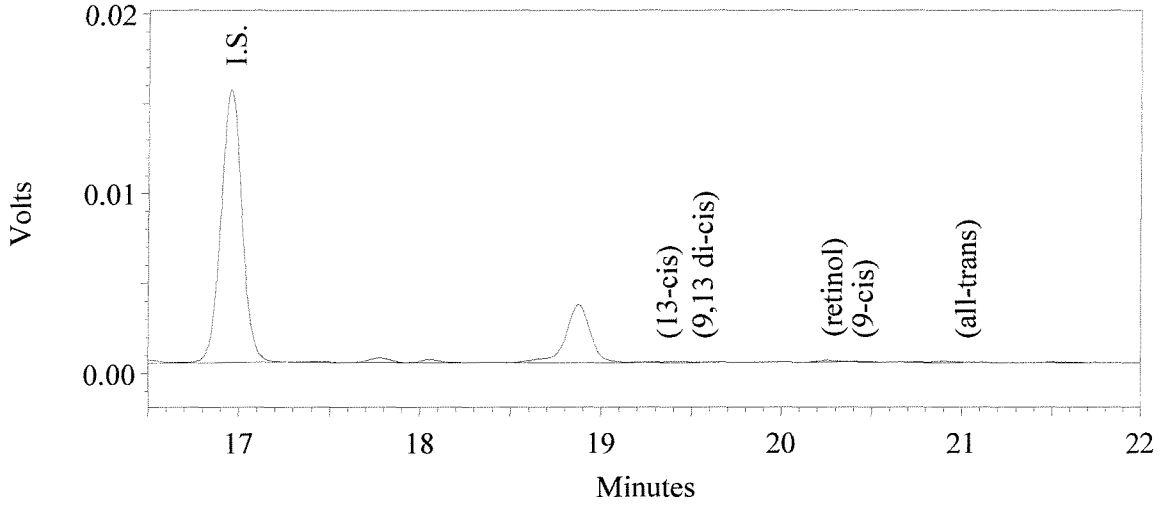
Extracts from culture media were extracted and injected onto a reverse-phase HPLC column using the conditions applicable to extracting and detecting isomers of retinoic acid. Panel B depicts the same chromatogram as in panel A, but with a different X-axis scale to show where the authentic standard isomers of retinoic acid and the internal standard (I.S.) would elute. Note the high sensitivity setting of the Y-axis.

Figure 4.8: Chromatograms of extracts from culture media spiked with internal standard using the conditions applicable to extracting and detecting retinoic acid isomers

A:



B:



Extracts from culture media spiked with internal standard (I.S.) were injected onto a reverse-phase HPLC column using the conditions applicable to extracting and detecting isomers of retinoic acid. Panel B depicts the same chromatogram as in panel A, but with a different X-axis scale to show where the authentic standard isomers of retinoic acid and the internal standard (I.S.) would elute.

To further validate the methods, the precision, reproducibility and linearity of the calibration curves following the extraction procedures were measured. The standard curves covered the entire range of expected concentrations (2-1500 ng/ml). The linear correlation observed between 10 standards of different concentrations of retinol, retinyl palmitate, all-*trans* retinoic acid, 9-*cis* retinoic acid, 13-*cis* retinoic acid and 9,13 di-*cis* retinoic acid were determined by plotting the peak height ratio of the known concentration of authentic retinoid standard to the internal standard, against retinoid concentration (Figures 4.9. - 4.14.). All the calibration curves were shown to have good linearity over the concentration range measured, with correlation coefficients (r^2) greater than 0.996.

Determination of accuracy and precision was accomplished by analysing standards of known concentrations, following their extraction from culture media on 3 separate days. The results for each retinoid are shown in the Tables adjacent to the appropriate calibration curves (Tables 4.1. - 4.6.). The acceptance criteria for precision and accuracy are not more than 15% coefficient of variation for precision and not more than 15% deviation from the actual value for accuracy. At the lower limit of quantitation, 20% is acceptable for both precision and accuracy (Shah *et al.*, 1992). Although most of the values obtained for the standards retinol and retinyl palmitate are within these recommended values, the accuracy values for the retinoic acid isomers at the lower concentrations (less than 10 ng) were found to deviate markedly from these advised limits. This could be corrected by using different mobile phase buffers. However, this could then prevent resolution of the four retinoic acid isomers.

Since internal standards were used in both the extraction procedures for retinol and retinyl palmitate and the isomers of retinoic acid, the percentage recovery following extraction was not determined. Any variation in the injection volume or losses during extraction and/or adsorption on the column will equally effect the internal standard and the test sample.

Table 4.1: Calibration curve reproducibility, accuracy and precision; individual and mean data of retinol from three separate runs

Concentration (ng/ml)						Accuracy[a] (%)	Precision[b] (%)
Prepared	Measured						
	Run 1	Run 2	Run 3	Mean	S.D.		
2	1.4	2	3.2	2.2	0.9	10.0	41.7
5	5.5	6	5.8	5.8	0.3	15.3	4.4
10	10.9	9.5	10.1	10.2	0.7	1.7	6.9
25	27.3	23.6	20.6	23.8	3.4	-4.7	14.1
50	49.8	50.7	48.4	49.6	1.2	-0.7	2.3
100	87.3	101.8	96.3	95.1	7.3	-4.9	7.7
250	242.5	208.6	222.5	224.5	17.0	-10.2	7.6
500	473.9	481.0	475.8	476.9	3.7	-4.6	0.8
1000	1034.2	973.0	970.0	992.4	36.2	-0.8	3.7
1500	1521.3	1512.5	1532.5	1522.1	10.0	1.5	0.7
r^2(1/y)	0.9993	0.9988	0.9986	0.9968	0.0033	-	0.3303
					Mean	0.3	9.0

[a]Accuracy = [(mean measured value-prepared value)(prepared value)] x 100
[b]Precision = (S.D. x 100)/mean

Figure 4.9: A typical standard curve for retinol

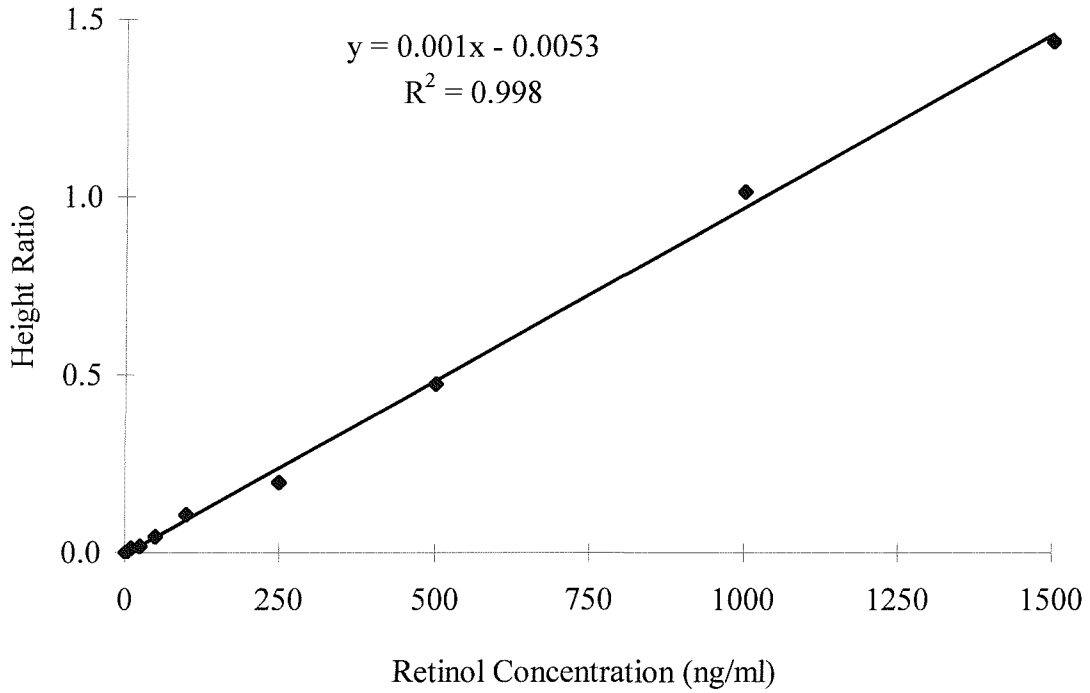


Table 4.2: Calibration curve reproducibility, accuracy and precision; individual and mean data of retinyl palmitate from three separate runs

Concentration (ng/ml)						Accuracy[a] (%)	Precision[b] (%)
Prepared	Measured						
	Run 1	Run 2	Run 3	Mean	S.D.		
2	2.3	1.7	2.2	2.1	0.3	3.3	15.6
5	4.6	4.7	5.7	5.0	0.6	0	12.2
10	9.5	11.1	10.4	10.3	0.8	3.3	7.8
25	20.6	29.2	25.7	25.2	4.3	0.7	17.2
50	48.2	40.6	50.8	46.5	5.3	-6.9	11.4
100	97.7	108.5	98.0	101.4	6.2	1.4	6.1
250	253.0	245.9	245.9	248.3	4.1	-0.7	1.7
500	481.2	535.2	572.0	529.5	45.7	5.9	8.6
1000	1075.1	994.5	1077.2	1048.9	47.2	4.9	4.5
1500	1522.3	1500.7	1525.6	1516.2	13.5	1.1	0.9
r^2(1/y)	0.9985	0.9994	0.9979	0.9986	0.0008	-	0.0756
					Mean	1.3	8.6

[a]Accuracy = [(mean measured value-prepared value)(prepared value)] x 100
[b]Precision = (S.D. x 100)/mean

Figure 4.10: A typical standard curve for retinyl palmitate

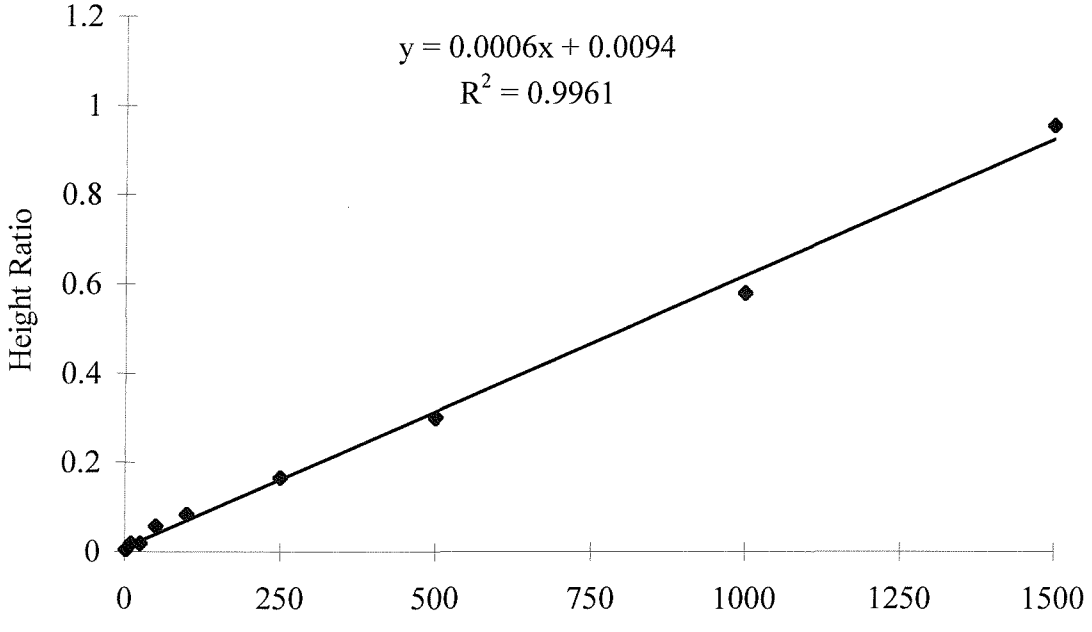


Table 4.3: Calibration curve reproducibility, accuracy and precision; individual and mean data of 13-*cis* retinoic acid from three separate runs

Concentration (ng/ml)						Accuracy[a] (%)	Precision[b] (%)
Prepared	Measured						
	Run 1	Run 2	Run 3	Mean	S.D.		
2	9.2	9.1	9.6	9.3	0.3	365.4	2.8
5	4.3	10.1	11.4	8.6	3.8	71.8	43.8
10	12.2	14.7	15.4	14.1	1.7	40.9	12.1
25	20.1	29.0	28.8	26.0	5.1	3.9	19.6
50	48.4	57.3	56.2	54.0	4.9	7.9	9.0
100	117.0	116.2	112.2	115.1	2.6	15.1	2.2
250	219.1	257.4	219.4	232.0	22.0	-7.2	9.5
500	483.8	512.4	554.3	516.8	34.6	3.4	6.9
1000	1039.3	971.6	1025.7	1012.2	35.8	1.2	3.5
1500	1480.5	1511.1	1464.6	1485.4	23.6	-1.0	1.6
r^2(1/y)	0.9985	0.9993	0.9974	0.9984	0.001	-	0.0955
					Mean	50.1	11.1

[a]Accuracy = [(mean measured value-prepared value)(prepared value)] x 100
[b]Precision = (S.D. x 100)/mean

Figure 4.11: A typical standard curve for 13-*cis* retinoic acid

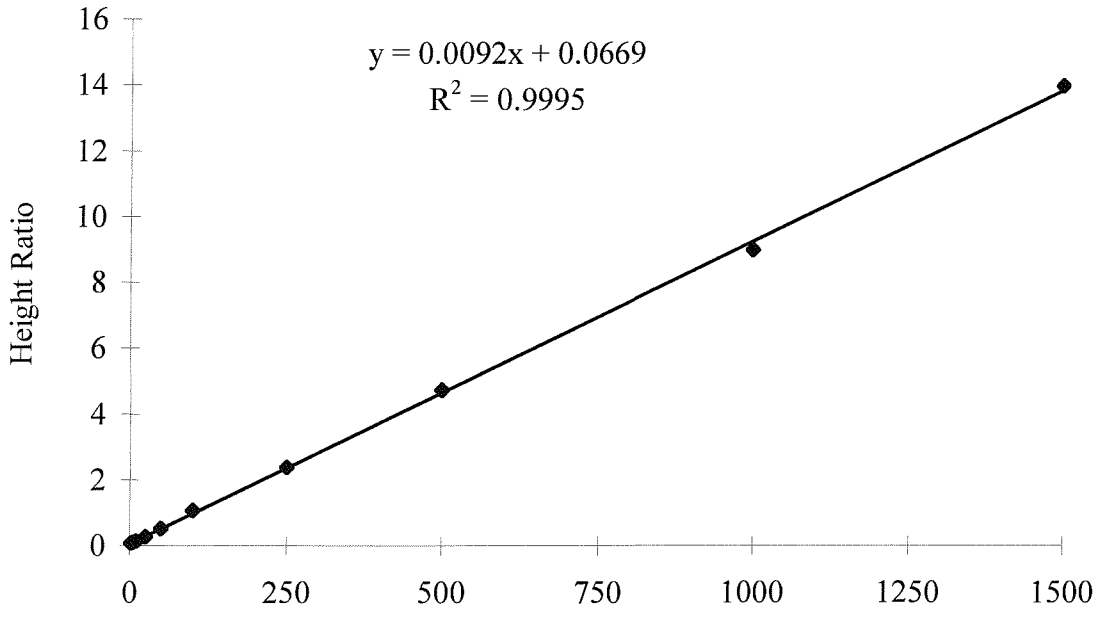


Table 4.4: Calibration curve reproducibility, accuracy and precision; individual and mean data of 9,13 di-*cis* retinoic acid from three separate runs

Concentration (ng/ml)						Accuracy[a] (%)	Precision[b] (%)
Prepared	Measured						
	Run 1	Run 2	Run 3	Mean	S.D.		
2	3.0	2.8	3.2	3.0	0.2	50.9	6.9
5	7.3	6.2	5.7	6.4	0.8	28.4	12.7
10	14.8	13.9	13.3	14.0	0.8	39.8	5.4
25	24.9	27.6	26.4	26.3	1.4	5.2	5.1
50	54.1	56.0	53.4	54.5	1.4	9.0	2.5
100	118.0	113.8	107.9	113.2	5.1	13.2	4.5
250	218.2	259.1	241.7	230.7	24.7	-7.7	10.7
500	479.1	508.2	534.1	507.1	27.5	1.4	5.4
1000	1043.0	972.1	996.7	1003.9	36.0	0.4	3.6
1500	1479.4	1512.3	1494.3	1495.4	16.4	-0.3	1.1
r^2(1/y)	0.9983	0.9994	0.9994	0.9990	0.0006	-	0.6357
					Mean	14.0	5.8

[a]Accuracy = [(mean measured value-prepared value)(prepared value)] x 100
[b]Precision = (S.D. x 100)/mean

Figure 4.12: A typical standard curve for 9,13 di-*cis* retinoic acid

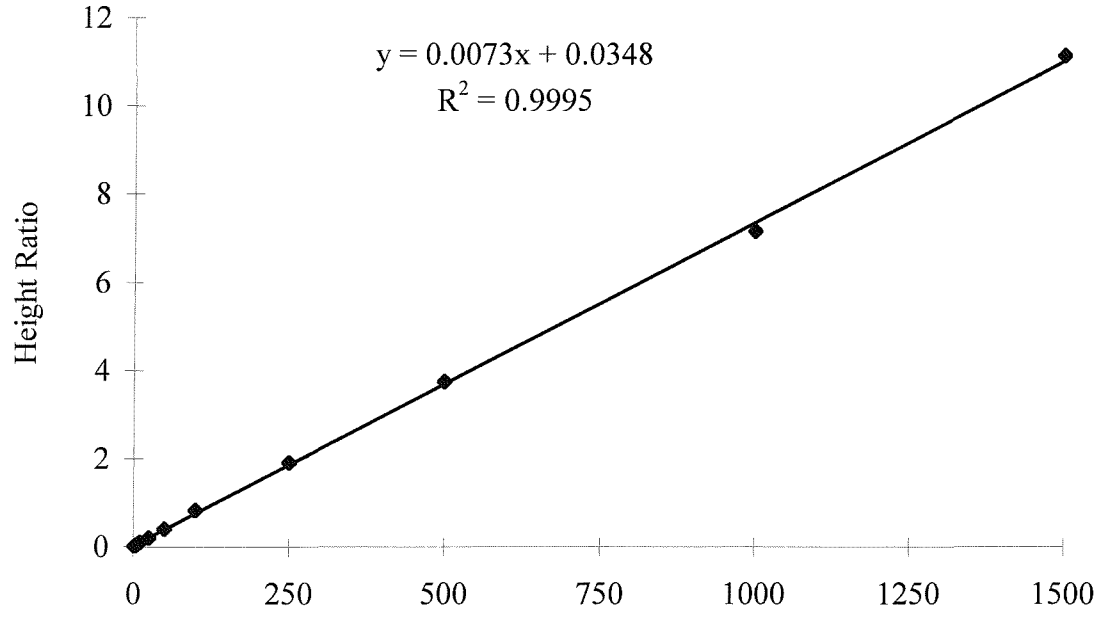


Table 4.5: Calibration curve reproducibility, accuracy and precision; individual and mean data of 9-*cis* retinoic acid from three separate runs

Concentration (ng/ml)						Accuracy[a] (%)	Precision[b] (%)
Prepared	Measured						
	Run 1	Run 2	Run 3	Mean	S.D.		
2	3.0	2.3	3.6	3.0	0.6	48.1	21.5
5	7.7	6.0	5.9	6.5	1.0	30.6	15.2
10	15.6	13.9	13.7	14.4	1.0	44.0	7.2
25	26.0	28.5	26.9	27.1	1.3	8.5	4.8
50	55.8	57.0	54.3	55.7	1.3	11.4	2.4
100	122.1	116.2	109.4	115.9	6.4	15.9	5.5
250	225.0	266.2	217.7	236.3	26.1	-5.5	11.1
500	483.6	506.9	540.9	510.5	28.8	2.1	5.7
1000	1045.2	972.6	992.8	1003.5	37.5	0.4	3.7
1500	1475.0	1510.9	1493.7	1493.2	18.0	-0.5	1.2
r^2(1/y)	0.9983	0.9994	0.9968	0.9982	0.0013	-	0.1308
					Mean	15.5	7.8

[a]Accuracy = [(mean measured value-prepared value)(prepared value)] x 100
[b]Precision = (S.D. x 100)/mean

Figure 4.13: A typical standard curve for 9-*cis* retinoic acid

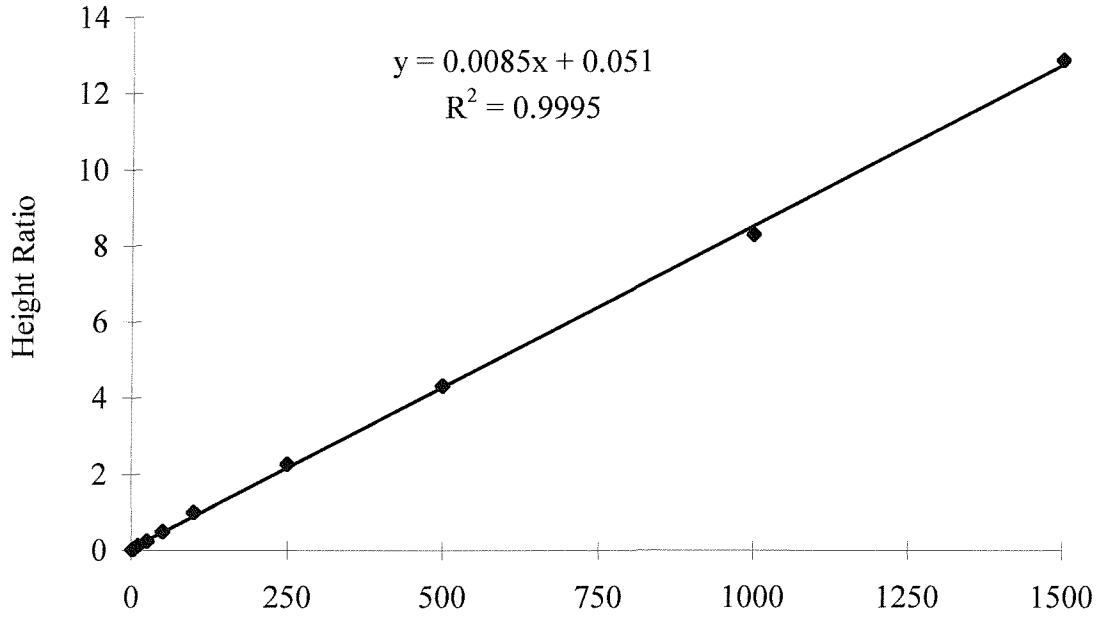
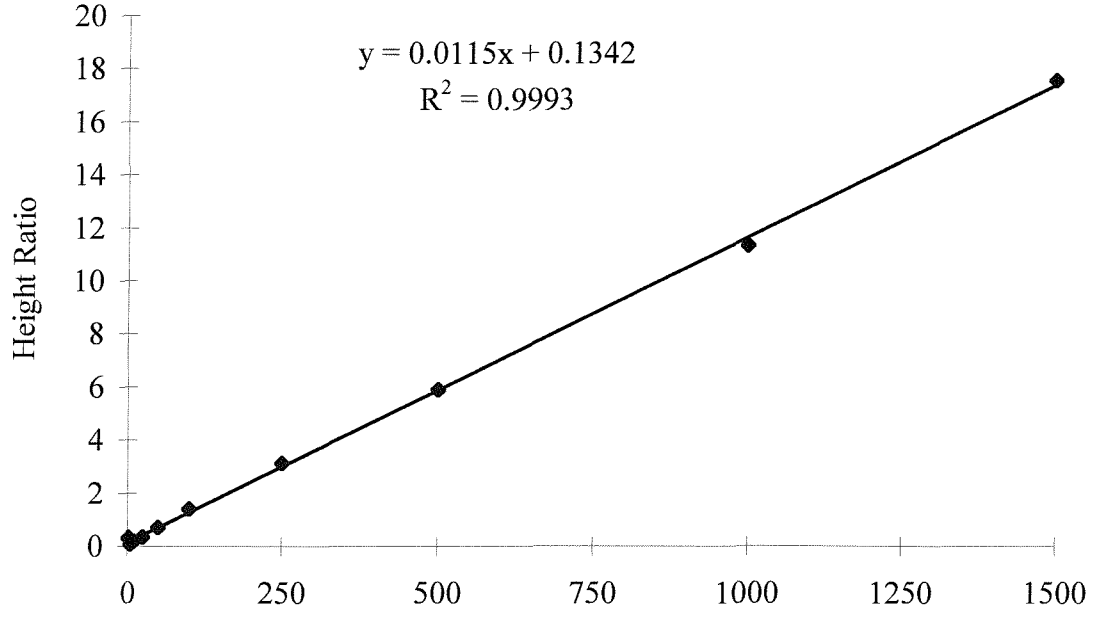


Table 4.6: Calibration curve reproducibility, accuracy and precision; individual and mean data of all-*trans* retinoic acid from three separate runs

Concentration (ng/ml)						Accuracy[a] (%)	Precision[b] (%)
Prepared	Measured						
	Run 1	Run 2	Run 3	Mean	S.D.		
2	4.0	2.8	3.5	3.4	0.6	70.4	18.0
5	8.5	6.3	6.3	7.0	1.3	40.9	18.1
10	17.5	14.7	14.9	15.7	1.6	56.9	10.0
25	28.0	29.3	29.5	28.9	0.8	15.7	2.9
50	58.8	60.0	57.8	58.9	1.1	17.7	1.9
100	128.8	121.1	115.7	121.9	6.6	21.9	5.4
250	234.3	269.6	227.7	243.9	22.5	-2.5	9.2
500	496.8	506.2	553.6	518.9	30.5	3.8	5.9
1000	1039.9	973.2	1010.3	1007.8	33.4	0.8	3.3
1500	1472.3	1509.5	1473.4	1485.1	21.1	-1.0	1.4
r^2(1/y)	0.9985	0.9993	0.9981	0.9986	0.0006	-	0.0612
					Mean	22.5	7.6

[a]Accuracy = [(mean measured value-prepared value)(prepared value)] x 100
[b]Precision = (S.D. x 100)/mean

Figure 4.14: A typical standard curve for all-*trans* retinoic acid



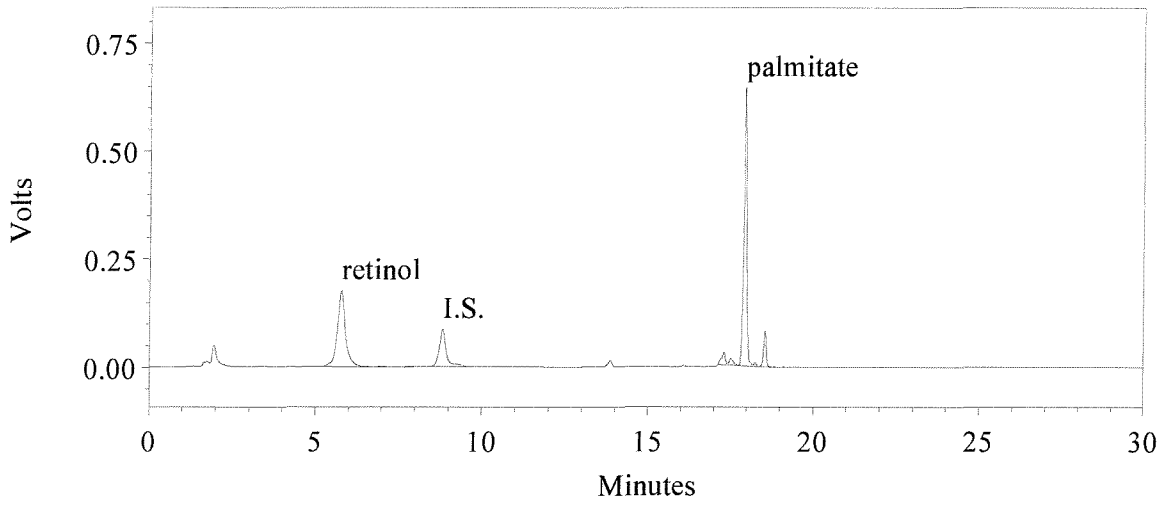
4.3. CHARACTERISATION OF RETINOIDS PRESENT IN QUIESCENT AND ACTIVATED HEPATIC STELLATE CELLS

To survey the changes in metabolites of retinol during the development of HSC activation *in vitro*, rat HSC and their incubation media were collected and their retinoids extracted, desicated and resolubilised in mobile phase buffer (section 2.19.). The concentrations of retinol, retinyl palmitate and isomers of retinoic acid in HSC and their culture media were analysed immediately after isolation (unactivated HSC, day 0) and at days 3, 5, 7 and 9 after culture. As there is a limited number of HSC available from each rat liver, it was unfeasible to measure the quantity of retinoids in HSC on further days in culture. To take into account the change in HSC number during culture, concentrations of retinoids were expressed per microgram of DNA. However, to compensate for the natural variation between different HSC preparations, the quantity of a retinoid present in HSC was also expressed as a percentage of the initial level of that retinoid present at day 0 (unactivated HSC).

4.3.1. Levels of Retinol and Retinyl Palmitate

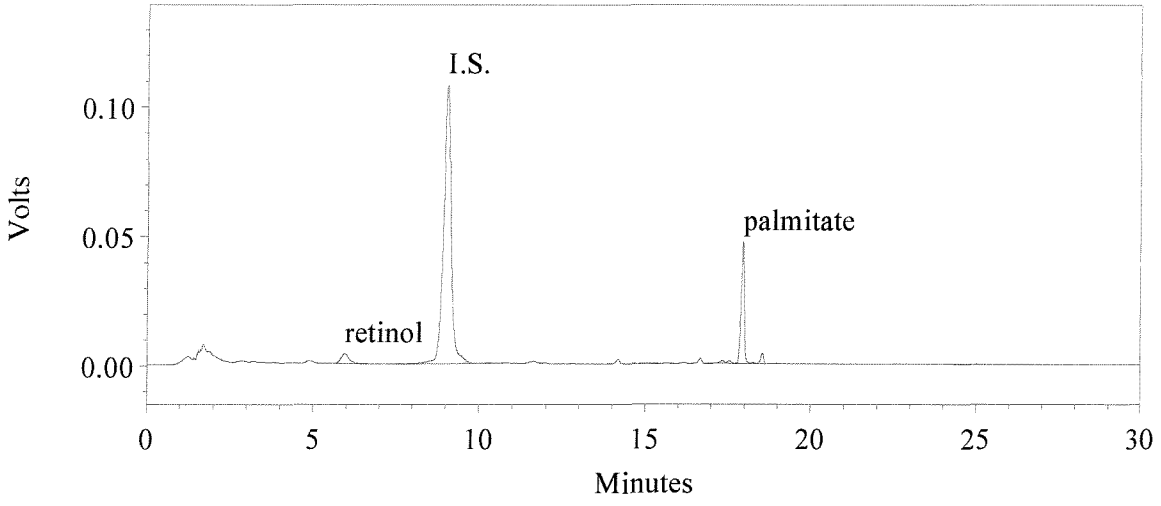
High performance liquid chromatography analysis of the isolated HSC preparations showed that the most abundant intracellular retinoid present was retinyl palmitate, an esterified form of retinol. Retinyl palmitate levels in culture medium alone were undetectable. Small, unidentified peaks with similar retention times to retinyl palmitate were also detected on chromatograms (Figures 4.15. and 4.16.). These may represent other retinyl esters, possibly retinyl stearate, a retinyl ester previously detected and characterised in HSC (Hendriks *et al.*, 1985). However, this is only speculative in the absence of an authentic standard of retinyl stearate.

Figure 4.15: Chromatogram of retinol and retinyl palmitate extracted from hepatic stellate cells



The cell extracts of rat HSC cultured for 7 days on plastic were spiked with internal standard (I.S.). The retinoids were then extracted and injected onto a reverse-phase HPLC column using the conditions applicable to extracting and detecting retinol and retinyl palmitate.

Figure 4.16: Chromatogram of retinol and retinyl palmitate extracted from culture media of hepatic stellate cells



The supernatant from rat HSC cultured for 5 days on plastic was spiked with internal standard (I.S.). The retinoids were then extracted and injected onto a reverse-phase HPLC column using the conditions applicable to extracting and detecting retinol and retinyl palmitate.

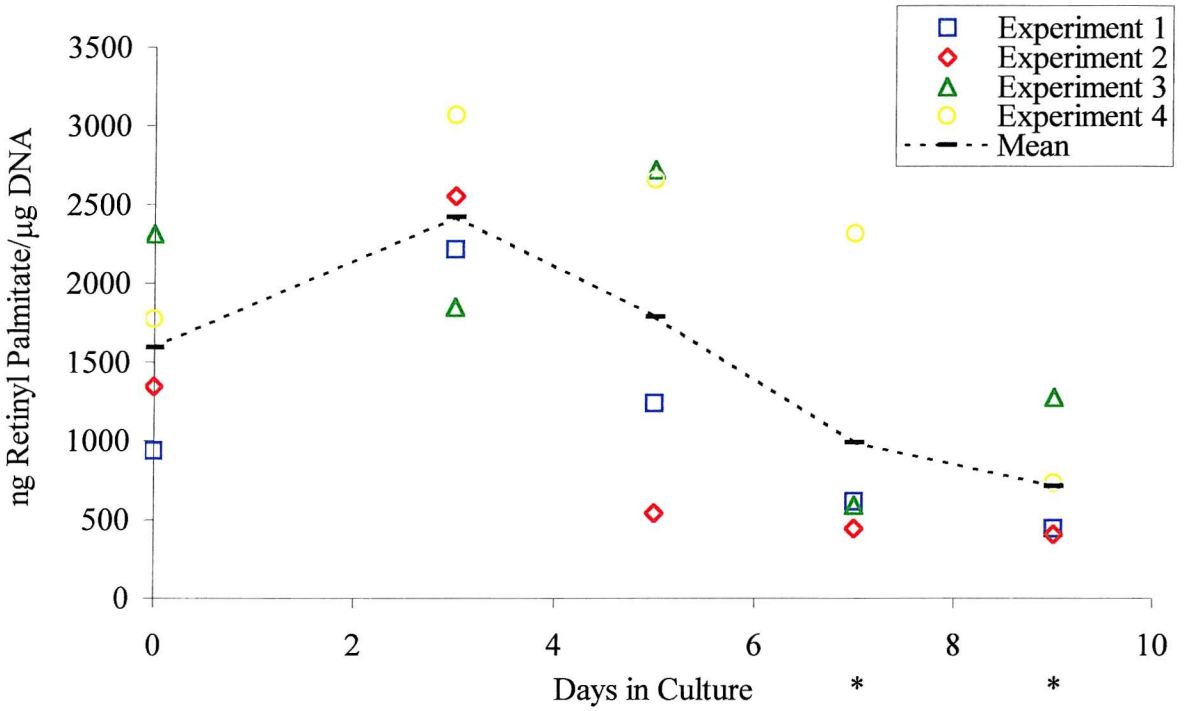
Figure 4.17. shows retinyl palmitate levels were highest in the HSC preparations at day 3 (2419.8 ± 259.8 ng/μg DNA), being over three times the concentration dectected at day 9 (711.6 ± 200.8 ng/μg DNA). It was observed that there was a gradual but significant ($P < 0.05$) decline in the amount of retinyl palmitate present in HSC after 7 and 9 days of culture compared with day 3. The slight elevation of retinyl palmitate between day 0 and day 3 HSC cultures was found not to be significant ($P > 0.05$).

The levels of retinyl palmitate in the culture supernatant from rat HSC showed a decrease from 540.0 ± 297.7 ng/μg DNA to 11.1 ± 3.7 ng/μg DNA following 5 days of culture compared to the initial levels observed at day 3 (Figure 4.18.). Compared to intracellular levels of retinyl palmitate, extracellular levels were much lower (2419.8 ± 259.8 ng/μg DNA intracellularly and 540.0 ± 297.7 ng/μg DNA extracellularly after 3 days of culture). Culture media could not be collected from day 0 HSC cultures, as this represents the time when HSC were isolated from the rat liver.

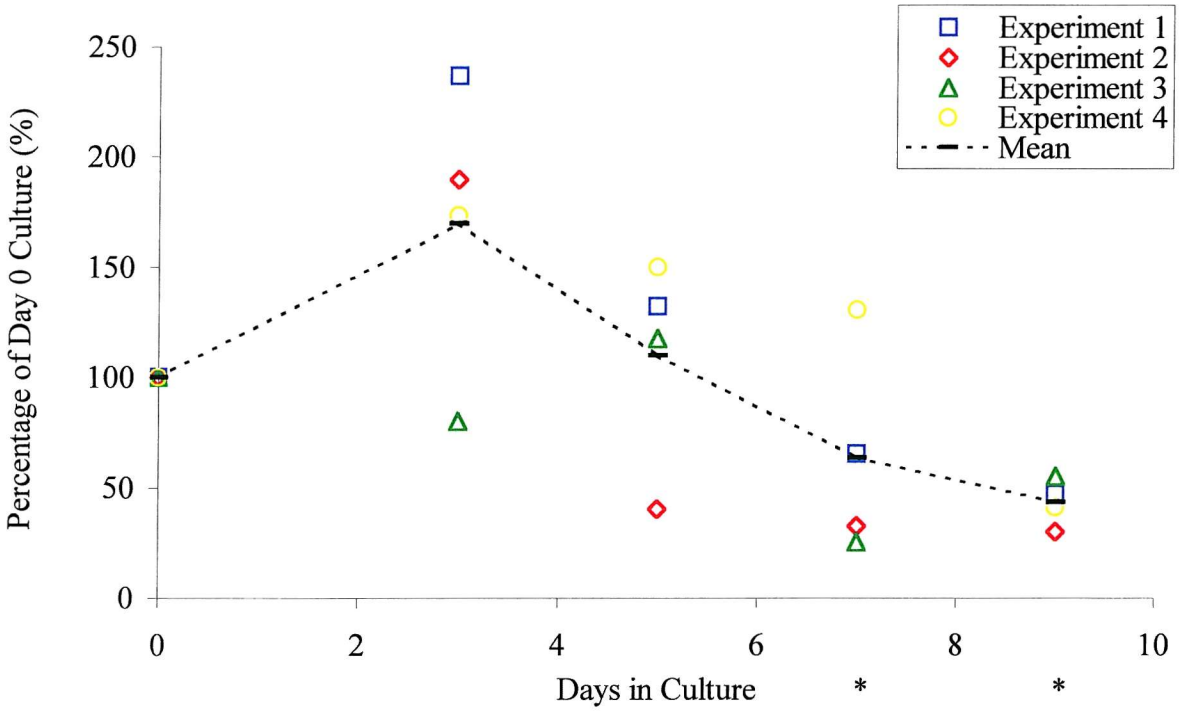
Retinol was the second most abundant retinoid to be detected in the HSC preparations at the time points measured. The levels were at least 10-fold lower than intracellular levels of retinyl palmitate in unactivated HSC (1591.2 ± 294.7 ng retinyl palmitate/μg DNA compared to 75.2 ± 10.7 ng retinol/μg DNA in freshly isolated HSC). In contrast to retinyl palmitate, intracellular levels of retinol were not significantly effected by HSC culture and ranged from 75.2 ± 10.7 ng/μg DNA to 144.3 ± 36.8 ng/μg DNA (Figure 4.20.). However, the extracellular levels of retinol present in the culture media showed a similar trend to the extracellular levels of retinyl palmitate (Figure 4.19.). During the activation of HSC, the extracellular levels of retinol dropped between days 3 and 5 (from 19.6 ± 10.6 ng/μg DNA to 1.3 ± 0.8 ng/μg DNA) and were maintained at this level for the remainder of the culture period. The actual levels of retinol at each time point however were still lower than those measured for retinyl palmitate.

Figure 4.17: Levels of retinyl palmitate in hepatic stellate cells

A:

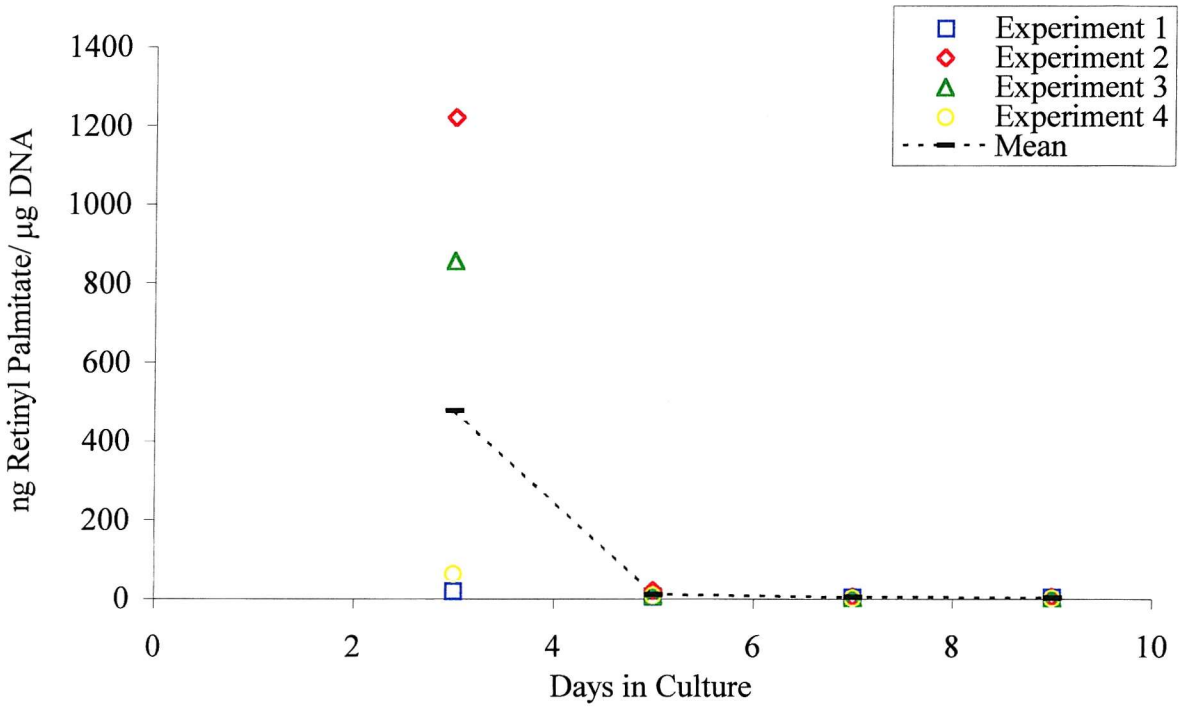


B:



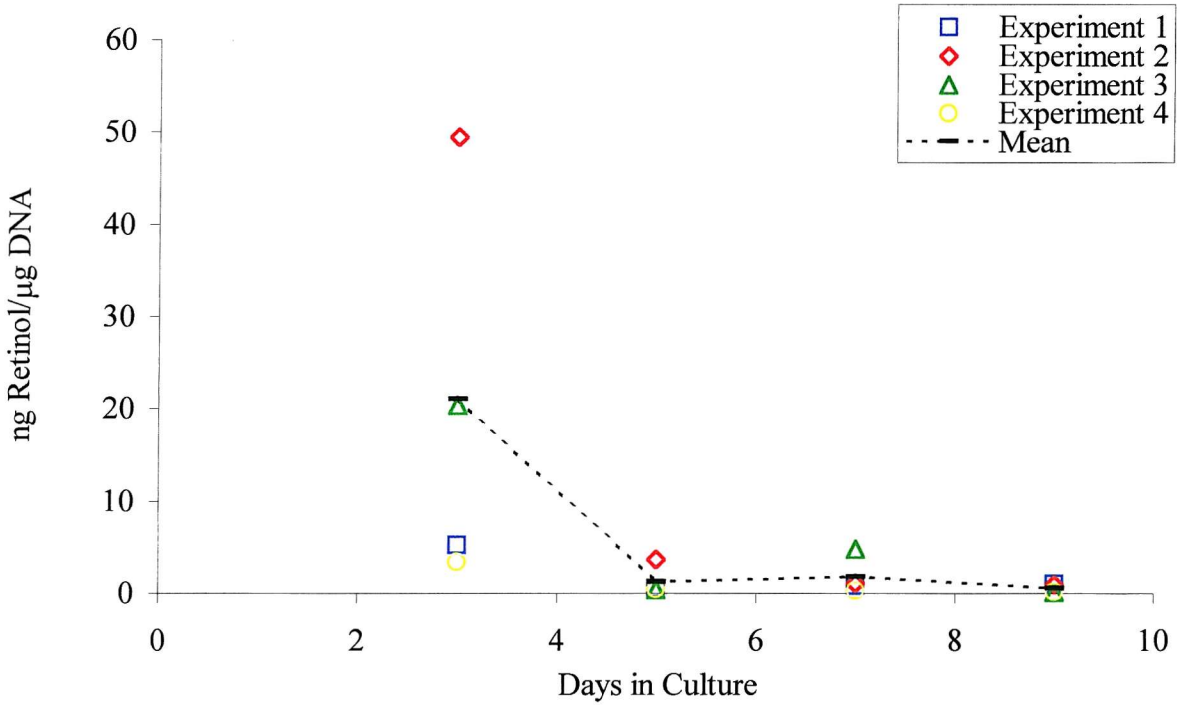
*Levels of retinyl palmitate in extracts from freshly isolated rat HSC and rat HSC cultured on plastic were quantitated by reverse-phase HPLC. Data are expressed per microgram of DNA (panel A) and as percent of the initial level of retinyl palmitate present at day 0 (panel B). Experiments were repeated 4 times with extracts prepared from separate cell cultures. * indicates $P < 0.05$ for difference between these values and HSC cultured for 3 days.*

Figure 4.18: Levels of retinyl palmitate in culture media of hepatic stellate cells



Levels of retinyl palmitate in culture media from rat HSC cultured on plastic were quantitated by reverse-phase HPLC. Experiments were repeated 4 times with extracts prepared from separate cell cultures.

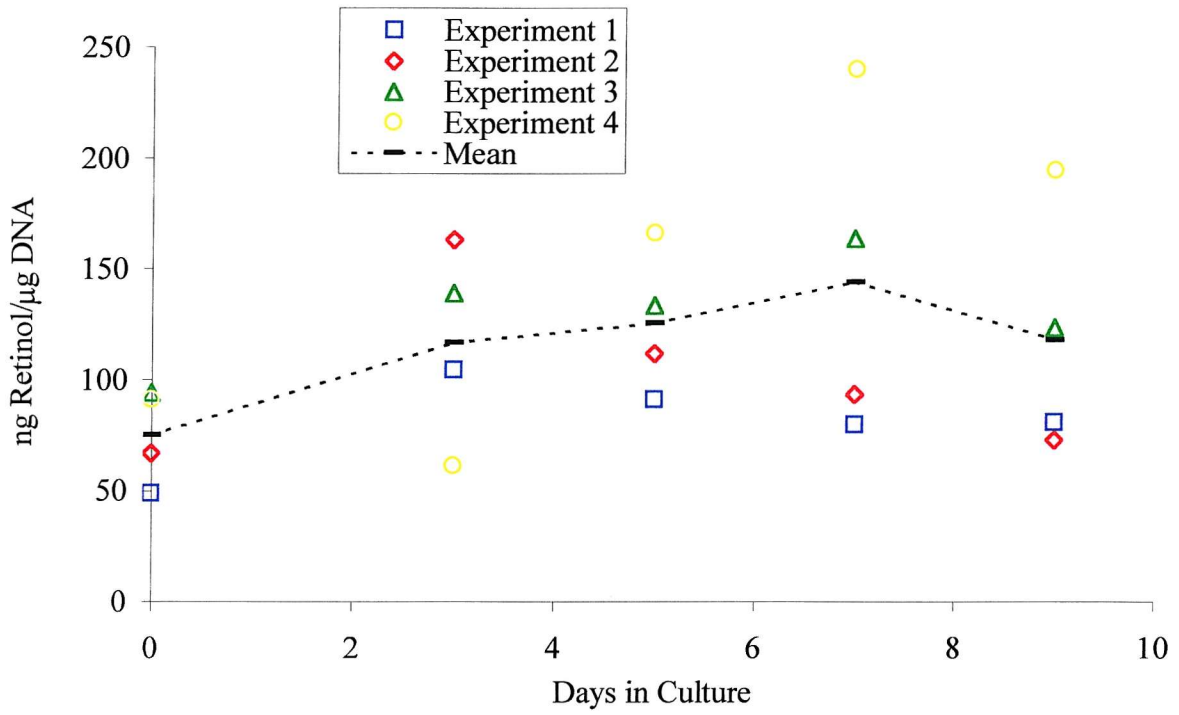
Figure 4.19: Levels of retinol in culture media of hepatic stellate cells



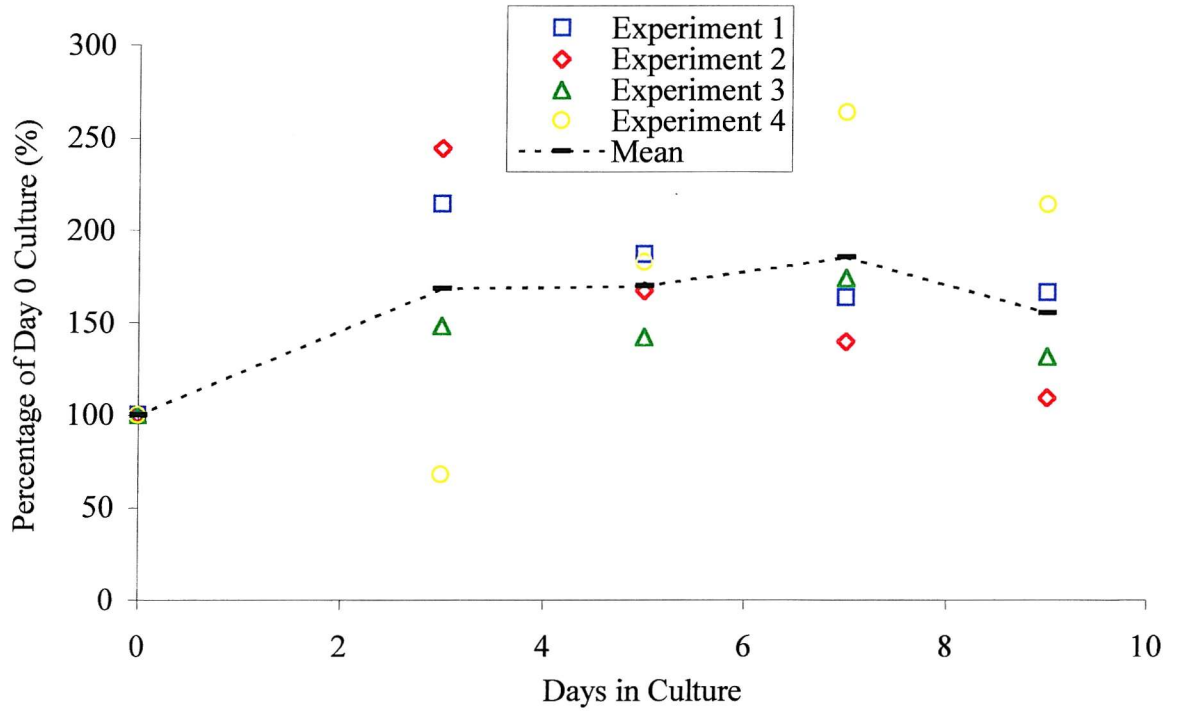
Levels of retinol in culture media from rat HSC cultured on plastic were quantitated by reverse-phase HPLC. Experiments were repeated 4 times with extracts prepared from separate cell cultures.

Figure 4.20: Levels of retinol in hepatic stellate cells

A:



B:



Levels of retinol in extracts from freshly isolated rat HSC and rat HSC cultured on plastic were quantitated by reverse-phase HPLC. Data are expressed per microgram of DNA (panel A) and as percent of the initial level of retinol present at day 0 (panel B). Experiments were repeated 4 times with extracts prepared from separate cell cultures.

4.3.2. Levels of Retinoic Acid Isomers

The reverse-phase HPLC method enabled the simultaneous analysis of 4 isomers of retinoic acid (13-*cis* retinoic acid, 9,13 di-*cis* retinoic acid, 9-*cis* retinoic acid and all-*trans* retinoic acid). Freshly isolated HSC and cultured HSC, together with their culture media were subsequently analysed for these retinoic acid metabolites. Figures 4.21. and 4.22. show representative chromatograms of HSC and culture media collected 5 days after isolation and culture. Coelution of the peaks with authentic standards enabled their identification as retinoic acid isomers.

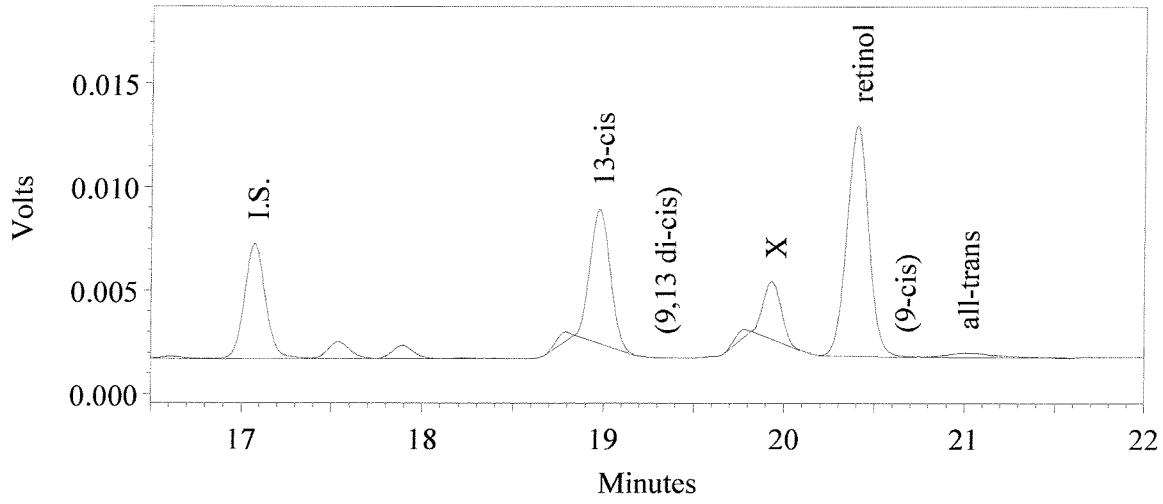
Retinoic acids were detected at lower concentrations than retinol and retinyl palmitate. In a similar way to that found for retinol and retinyl palmitate, the four separate HSC isolations each showed slightly different trends for the isomers of retinoic acid.

Between the isolation and culture of HSC for 3 days, there was a significant ($P < 0.05$) increase in the intracellular level of 13-*cis* retinoic acid (from 1.25 ± 0.14 ng/μg DNA at isolation to 43.7 ± 12.0 ng/μg DNA after 3 days in culture, Figure 4.23.). However, this level was not sustained and by 5 days in culture had decreased back to the level that was observed in freshly isolated HSC. It is noteworthy that individual experiments showed similar trends for levels of 13-*cis* retinoic acid and all-*trans* retinoic acid. For example, levels of both 13-*cis* retinoic acid and all-*trans* retinoic acid were raised in Experiment 1 after 9 days of culture (Figures 4.23. and 4.24.). Extracellular levels of both 13-*cis* retinoic acid and all-*trans* retinoic acid were not significantly effected during culture of HSC from days 3 to 9 (Figures 4.25. and 4.26.). However, there was an observed decrease in both these retinoids in experiments 2 and 3 during days 3 and 5 (Figures 4.25. and 4.26.).

Throughout the culture period monitored, all-*trans* retinoic acid was detected at concentrations of at least 100-fold lower than those found for 13-*cis* retinoic acid (Figure 4.24.). Some levels of all-*trans* retinoic acid were close to the lower limit of quantitation (less than 10 ng), despite performing the extraction on HSC samples containing in excess of 70 μg of DNA. Fewer cells, and hence DNA were obtained from HSC cultured for 3 days. This was caused by washing away unadhered cells following the replacement of culture medium after 48 hours and was also due to the previously reported low rate of HSC proliferation

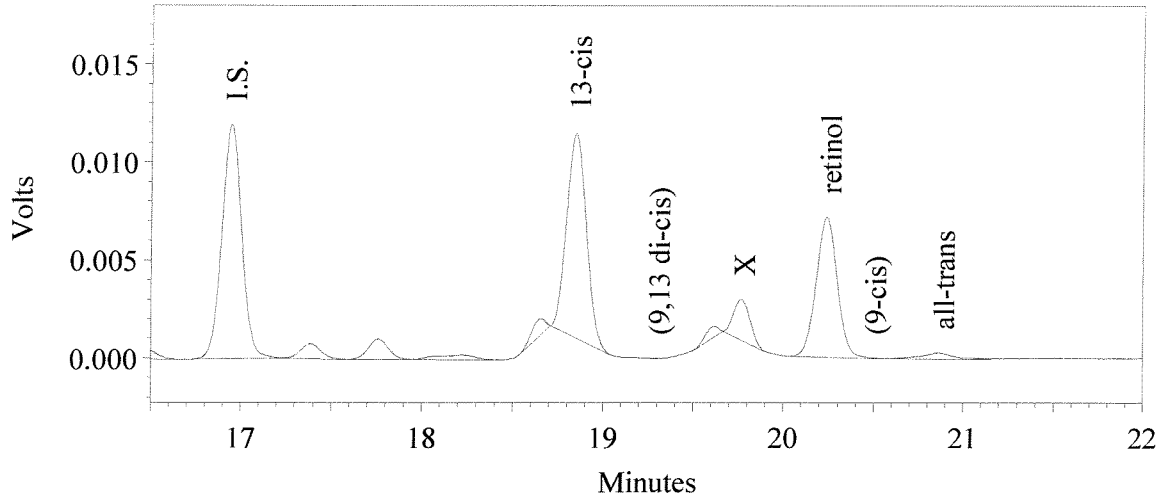
observed during this quiescent phase (Friedman *et al.,* 1992a). As a result, undetectable levels of all-*trans* retinoic acid were found in these samples and this explains the absence of data on the graphs in Figure 4.24. Experiments 2, 3, and 4 exhibited a decrease in the intracellular levels of all-*trans* retinoic acid levels after isolation and remained at this level. However, experiment 1 displayed a sudden increase in all-*trans* retinoic acid in HSC at 9 days of culture.

Figure 4.21: Chromatogram of isomers of retinoic acid extracted from hepatic stellate cells



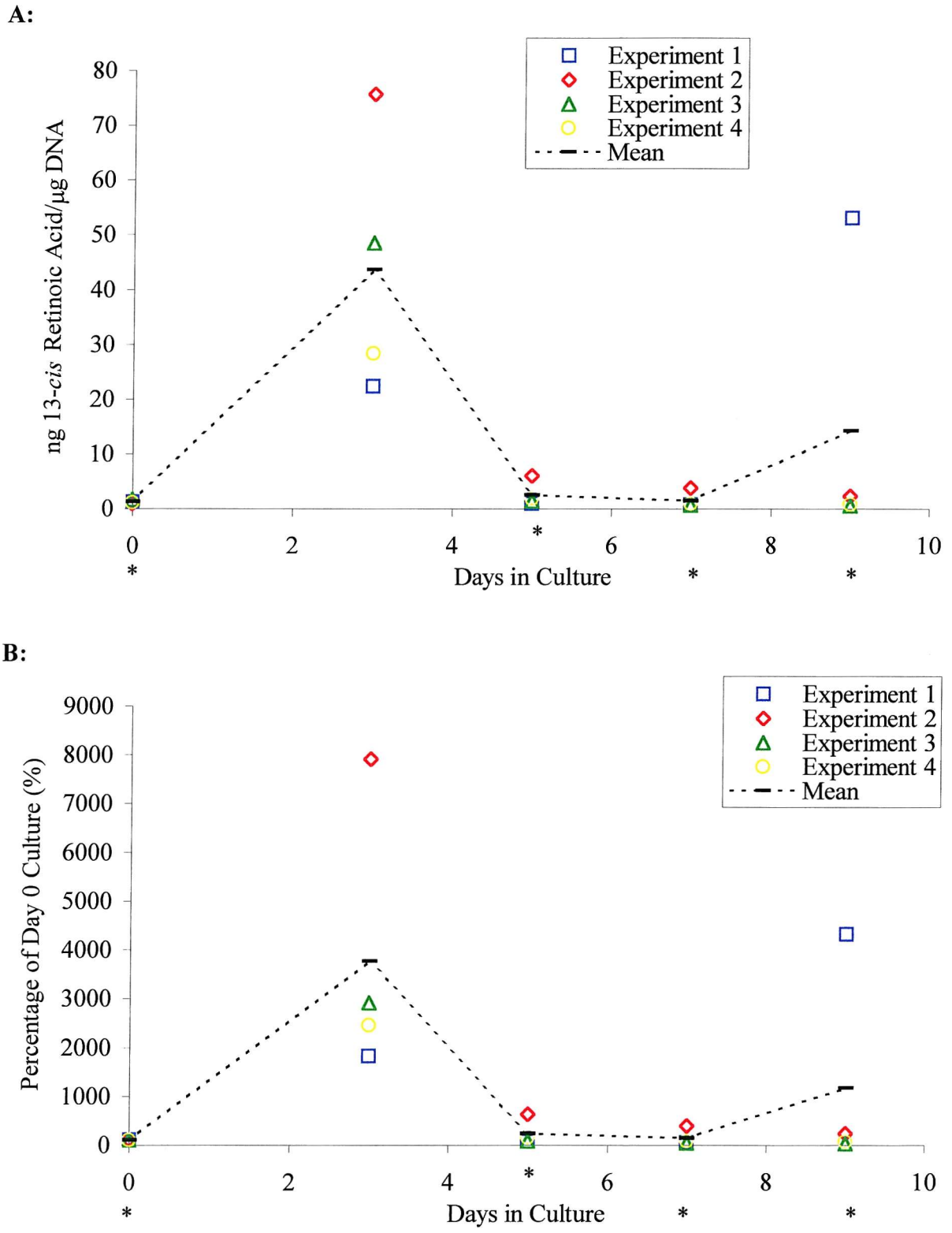
The cell extracts of rat HSC cultured for 5 days on plastic were spiked with internal standard (I.S.), retinol (100 ng) and retinyl palmitate (100 ng). The retinoids were then extracted and injected onto a reverse-phase HPLC column using the conditions applicable to extracting and detecting isomers of retinoic acid.

Figure 4.22: Chromatogram of isomers of retinoic acid extracted from culture media of hepatic stellate cells



The supernatant from rat HSC cultured for 5 days on plastic was spiked with internal standard (I.S.), retinol (100 ng) and retinyl palmitate (100 ng). The retinoids were then extracted and injected onto a reverse-phase HPLC column using the conditions applicable to extracting and detecting isomers of retinoic acid.

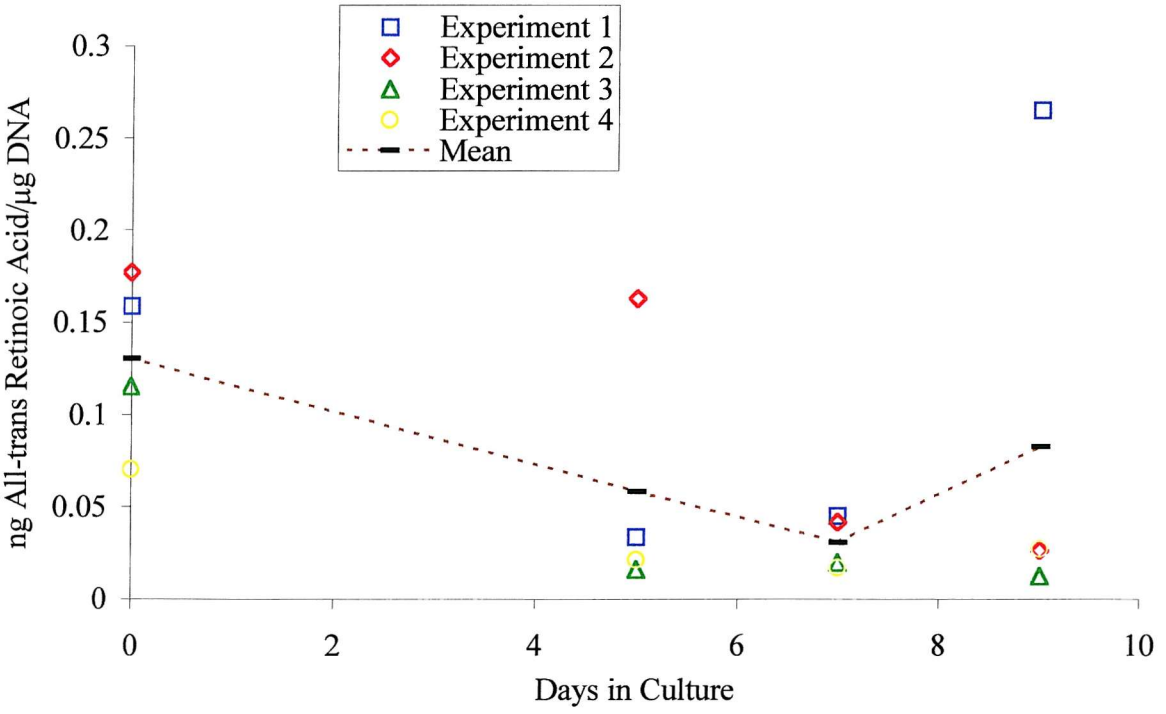
Figure 4.23: Levels of 13-*cis* retinoic acid in hepatic stellate cells



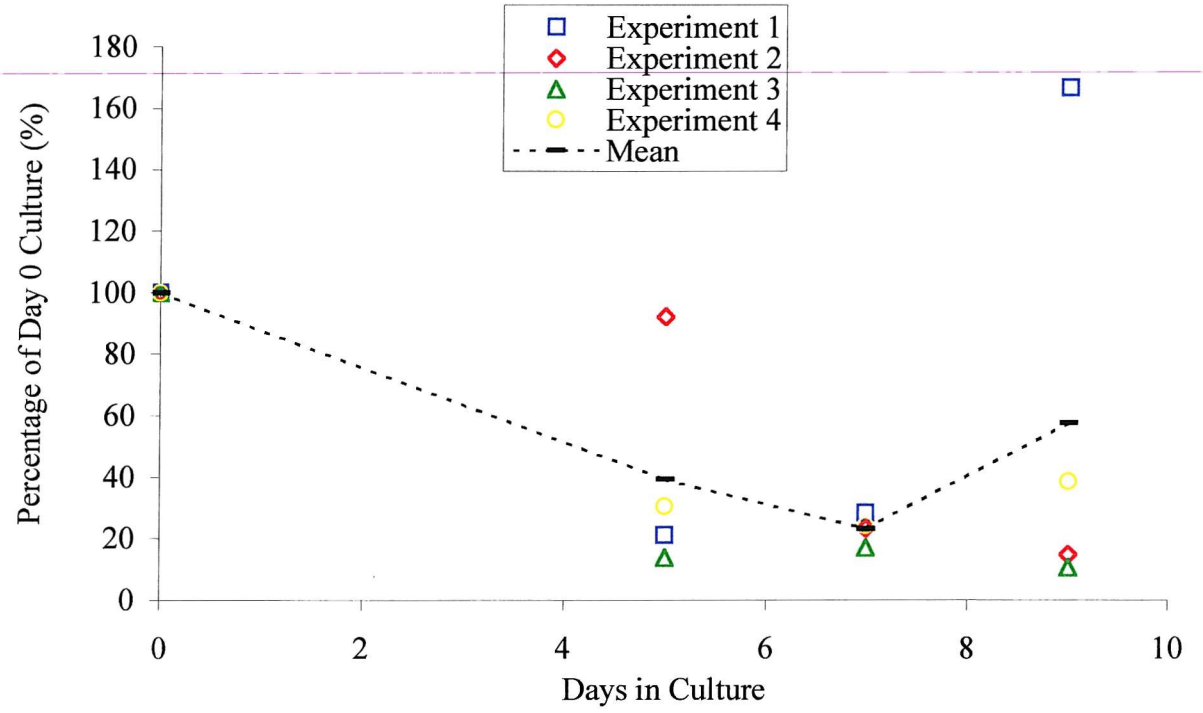
*Levels of 13-cis retinoic acid in extracts from freshly isolated rat HSC and rat HSC cultured on plastic were quantitated by reverse-phase HPLC. Data are expressed per microgram of DNA (panel A) and as percent of the initial level of 13-cis retinoic acid present at day 0 (panel B). Experiments were repeated 4 times with extracts prepared from separate cell cultures. * indicates $P < 0.05$ for difference between these values and HSC cultured for 3 days.*

Figure 4.24: Levels of all-*trans* retinoic acid in hepatic stellate cells

A:

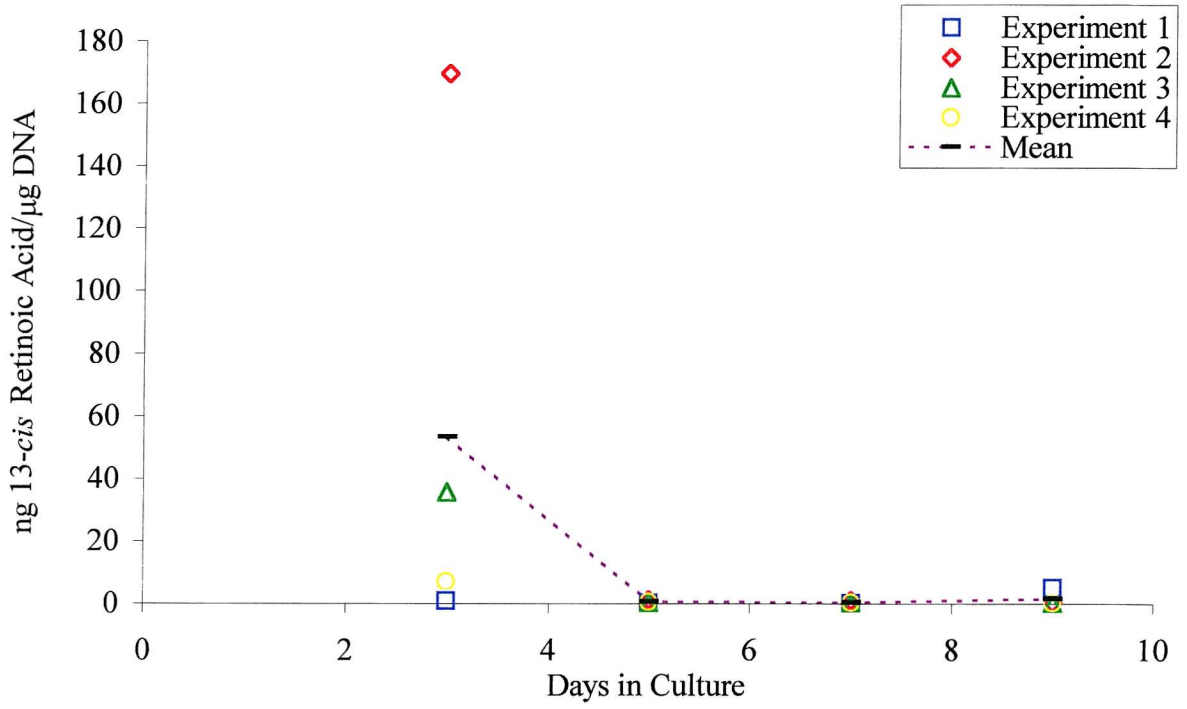


B:



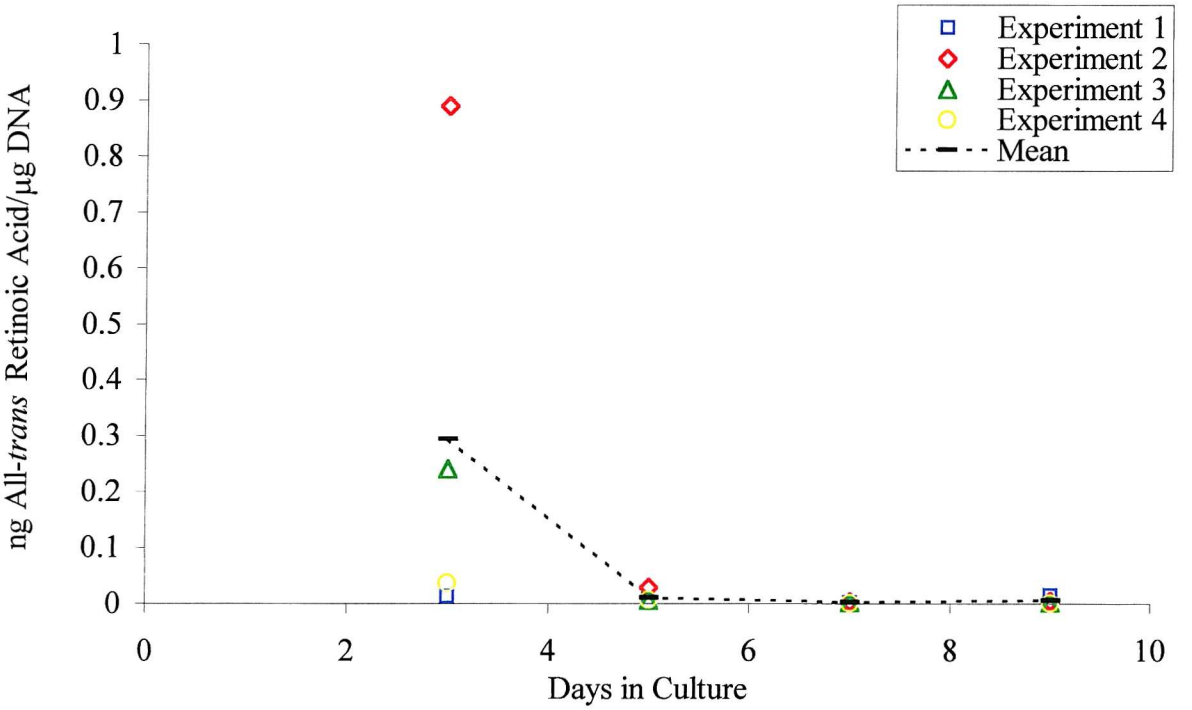
Levels of all-trans retinoic acid in extracts from freshly isolated rat HSC and rat HSC cultured on plastic were quantitated by reverse-phase HPLC. Data are expressed per microgram of DNA (panel A) and as percent of the initial level of all-trans retinoic acid present at day 0 (panel B). Experiments were repeated 4 times with extracts prepared from separate cell cultures.

Figure 4.25: Levels of 13-*cis* retinoic acid in culture media of hepatic stellate cells



Levels of 13-cis retinoic acid in culture media from rat HSC cultured on plastic were quantitated by reverse-phase HPLC. Experiments were repeated 4 times with extracts prepared from separate cell cultures.

Figure 4.26: Levels of all-*trans* retinoic acid in culture media of hepatic stellate cells



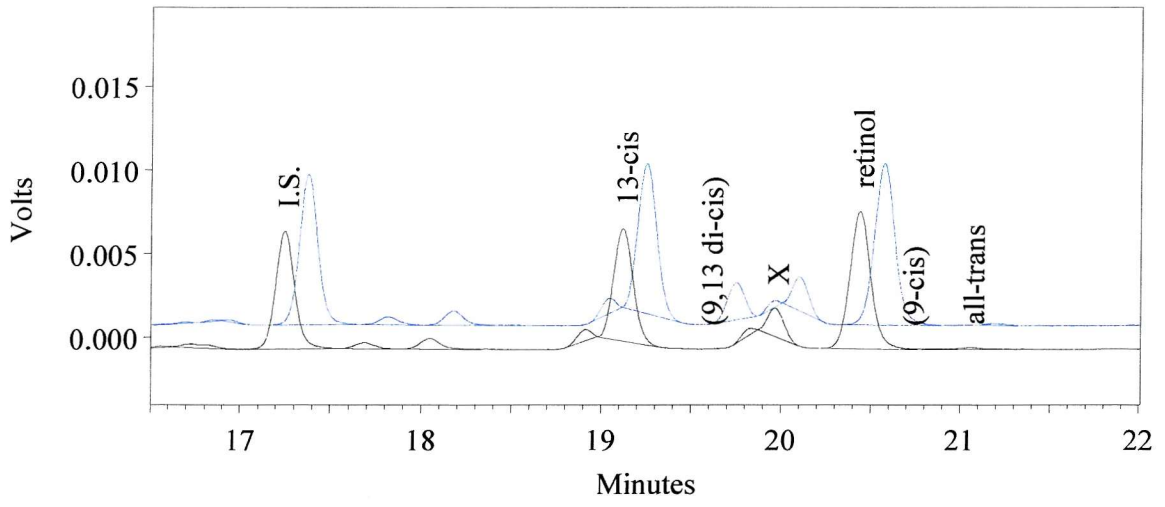
Levels of all-trans retinoic acid in culture media from rat HSC cultured on plastic were quantitated by reverse-phase HPLC. Experiments were repeated 4 times with extracts prepared from separate cell cultures.

No peaks were detected in either HSC or their culture media where the standards 9-*cis* retinoic acid or 9,13 di-*cis* retinoic acid eluted. This suggests either these retinoids were not present in these samples or they existed in too low a concentration (picogram concentration) to be dectected using the current methodology. Separation to base-line level could not be achieved between 9-*cis* retinoic acid and retinol using this methodology, and the extraction procedure was not 100% efficient at selecting against extraction of retinol. It is possible that if 9-*cis* retinoic acid was present at low concentrations it may have been disguised by retinol, although Figure 4.4. demonstrates this is highly unlikely.

In chromatographs of extracts from HSC and their incubation media, a peak (peak X), approximately 5-fold larger than the peak of all-*trans* retinoic acid was found to elute between the metabolites 13-*cis* retinoic acid and all-*trans* retionic acid (Figures 4.21. and 4.22.). This peak represented an unknown species and did not represent 9,13 di-*cis* retinoic acid as the retention time between these two peaks differed (19.5 minutes compared to 19.8 minutes). To strengthen this argument, HSC cultured for 7 days on plastic were spiked or unspiked with authentic 9,13 di-*cis* retinoic acid prior to extraction with organic solvent and was analysed by reverse-phase HPLC. From Figure 4.27, it is evident that 9,13 di-*cis* retinoic acid does not coelute with peak X. Furthermore, none of the available isomers of retinoic acid coeluted at this position either.

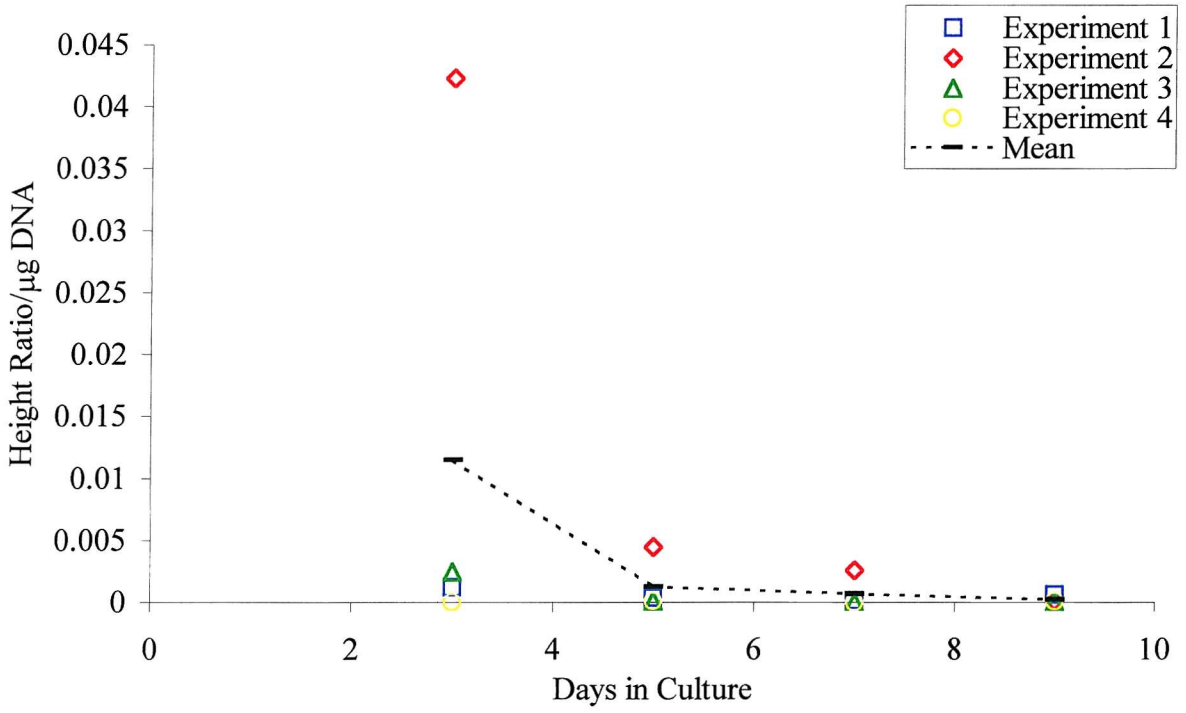
Since peak X elutes in close proximity to other isomers of retinoic acid, peak X may comprise a new form of retinol metabolite, a result of *in vivo* isomerisation of retinol. In the absence of a standard curve for peak X, the change in the height ratio of peak X during culture was measured and standardised to micrograms of DNA. As shown in Figure 4.29, all 4 experiements showed a dramatic and significant ($P < 0.05$) decrement in cellular levels of peak X from 0.04866 ± 0.008064 height ratio/μg DNA to 0.0153065 ± 0.05495 height ratio/μg DNA, following isolation of HSC. This level was maintained over the culture period monitored (day 3 to day 9). There was no significant difference in the extracellular levels of peak X following culture of HSC (Figure 4.28.).

Figure 4.27: Chromatogram of isomers of retinoic acid extracted from hepatic stellate cells spiked with and without 9,13 di-*cis* retinoic acid



The cell extracts of rat HSC cultured for 7 days on plastic were spiked with internal standard (I.S.), and with (blue trace) or without (black trace) 9,13 di-cis retinoic acid. The retinoids were then extracted and injected onto a reverse-phase HPLC column using the condtions applicable to extracting and detecting isomers of retinoic acid.

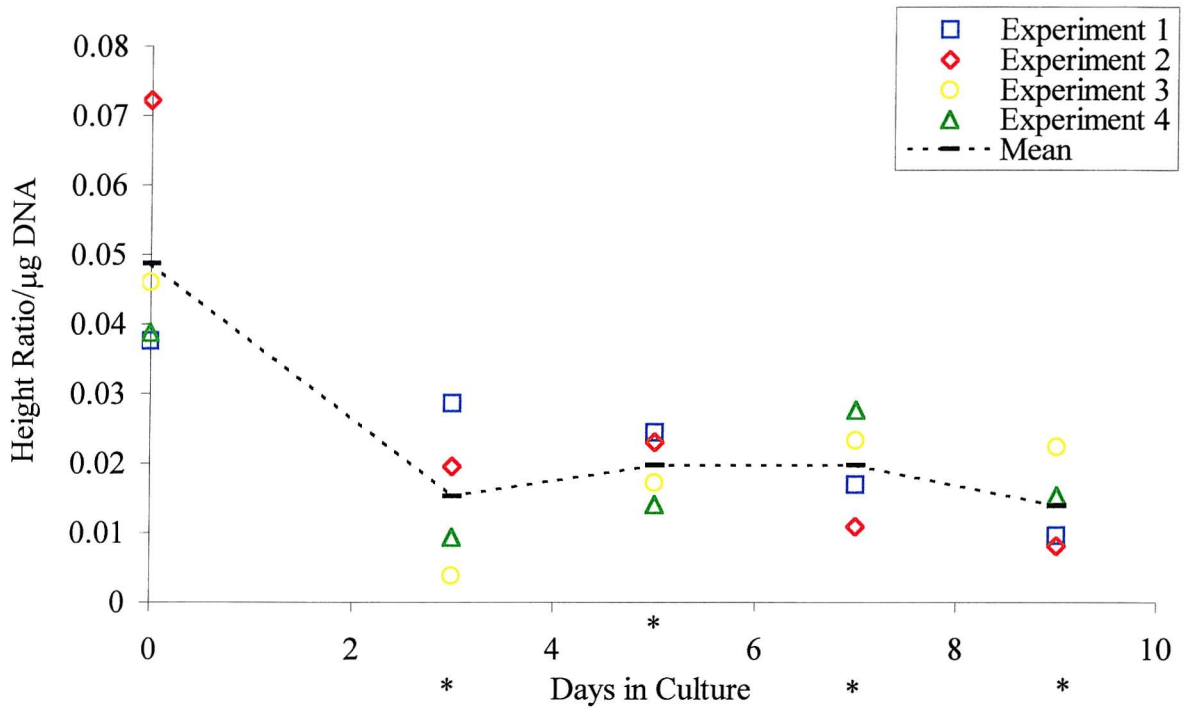
Figure 4.28: Levels of peak X in culture media of hepatic stellate cells



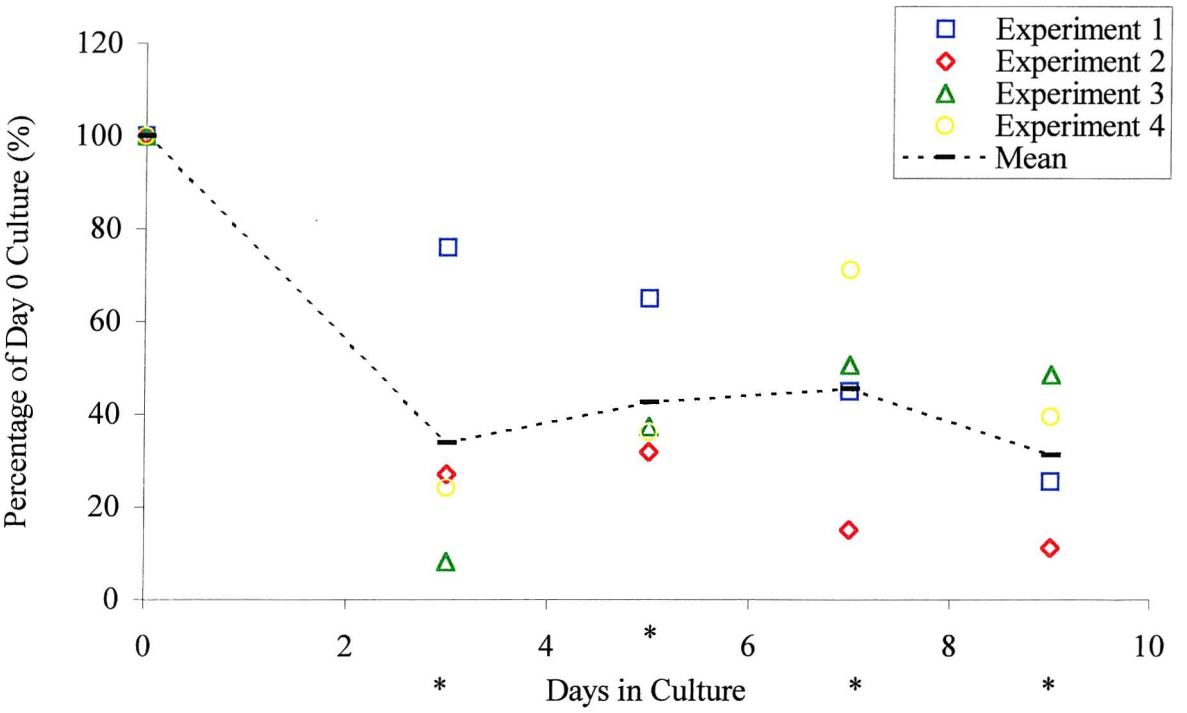
Levels of peak X in culture media from rat HSC cultured on plastic were quantitated by reverse-phase HPLC. Experiments were repeated 4 times with extracts prepared from separate cell cultures.

Figure 4.29: Levels of Peak X in hepatic stellate cells

A:



B:



*The height ratio of peak X in extracts from freshly isolated rat HSC and rat HSC cultured on plastic was measured by reverse-phase HPLC and expressed per microgram of DNA (panel A) and as percent of the initial height ratio at day 0 (panel B). Experiments were repeated 4 times with extracts prepared from separate cell cultures. * indicates $P < 0.05$ for difference between these values and freshly isolated HSC (day 0).*

4.4. DISCUSSION

The aim of this study was to gain information on retinoid metabolism in rat HSC during their progressive activation *in vitro*. These studies complement the studies of the RARs (Chapter 5) by examining generation of their ligands in HSC undergoing activation. These studies are novel in so far as they have examined a wide spectrum of retinoids under standardised conditions and have also taken into account the changes in cell number during culture so that valid comparisons can be made between different culture times.

Between the four different HSC preparations, there was variation among the retinoid content present in the freshly isolated HSC (day 0). This is not unexpected since, as in humans there is a wide variation in liver retinoid content (Hautekeete *et al.*, 1998). Similarly, just as there is overlap between the levels of retinoid present in freshly isolated HSC and in culture activated HSC, there is some overlap between retinoid content of normal patients and in patients with early alcoholic liver disease (Hautekeete *et al.*, 1998). Despite the overlaps, in freshly isolated rat HSC retinoid was stored primarily as the retinyl ester, retinyl palmitate. With activation of the HSC, a clear trend toward a lower percentage of retinyl palmitate compared with initial levels was observed even at early stages of activation (day 5). This succession of events is previously documented (Blaner *et al.*, 1985) and parallel the loss of retinoid droplets consistently observed *in vivo* as HSC transform into myofibroblast-like cells during liver fibrogenesis (Leo and Lieber, 1982; Leo *et al.*, 1983). However, the decrease in the level of intracellular retinyl palmitate in this study is not as dramatic as that reported by Davis and Vucic (1988). These authors detected a decline in the amount of retinyl palmitate from 1.7 ng/μg DNA after 3 days of HSC culture to 0.5-0.8 ng/μg DNA after 10 days of culture, concentrations approximately 1000-fold lower than those detected in this study. The concentration of retinyl palmitate may be lower than that detected in this Chapter as in the method by Davis and Vucic (1988) only a single retinoid extraction was performed on the HSC samples. In addition, the retinoids were monitored via UV absorption at 313 nm, not the maximum absorption for retinyl palmitate (see appendix 3.), and a different reverse-phase HPLC method was used. The results obtained in this Chapter and the results published by Davis and Vucic (1988) suggest that retinyl palmitate could be used as a surrogate marker of hepatic fibrogenesis, although the overlap in retinoid content in normal versus diseased liver mentioned above may make it relatively insensitive.

After 3 days of culture, the highest concentration of retinol was found in culture media. This is unlikely to be a result of leakage of retinol and retinyl esters during isolation of the HSC, since the culture media is replaced 48 hours after isolation. There is a drop in levels of retinol in the culture media observed after 3 days. An explanation could be that during culture, the extracellular retinol may be taken up by HSC to maintain intracellular levels which subsequently are metabolised. This would explain the maintained intracellular levels of retinol and the sustained drop of retinol extracellularly between days 3 and 5. The uptake of retinol from extracellular medium of cultured rat HSC has been previously investigated by Drevon *et al.* (1985) who demonstrated the retinol subsequently inhibited cellular proliferation.

De-esterification of retinyl palmitate is a critical process as it leads to production of the biologically active compound retinol. The intracellular levels of retinol may also be potentially supplemented by hydrolysis of retinyl palmitate as this was found to be significantly decreased during culture. Extracellular levels of retinyl palmitate are probably not involved in maintaining the intracellular levels of retinol since retinyl palmitate cannot be taken up by cells (Blomhoff *et al.*, 1988a). Furthermore, no retinyl ester hydrolase activity has been detected in culture media (Friedman *et al.*, 1993). In contrast, previous studies have shown isolated HSC contain particularly high concentrations of the hydrolase activity (Blaner *et al.*, 1985; Blomhoff *et al.*, 1985b) indicating hydrolysis of retinyl esters to yield retinol can occur intracellularly. Work by Davis and Vucic (1989) and Friedman *et al.* (1993) suggests acute mediators of inflammation, particularly products of Kupffer cells and the surrounding extracellular matrix may alter the de-esterification, or ester hydrolysis process. It is therefore possible that during fibrogenesis *in vivo*, several agents released from other cell types may alter retinyl ester production and hydrolysis, resulting in a decrease in HSC retinol stores.

The sustained intracellular levels of retinol have been demonstrated in other studies. For example, in preliminary studies conducted by Davis and Vucic (1989), it was found that [^{3}H]retinol produced after *in situ* formation of [^{3}H]retinyl ester was released into HSC culture media. As a result, intracellular [^{3}H]retinol formation remained relatively constant. However, there are also contradictory reports which suggest a clear disturbance of intracellular levels of retinol during activation of HSC. Okuno *et al.* (1999) demonstrated that levels of retinol decreased by 66% in fibrotic rat liver. In the same publication they also showed that following 7 days of culture on plastic, levels of retinol in rat HSC decreased by 80% compared to HSC cultured for 1 day. Although Okuno *et al.* (1999) used a similar *in vitro* model to that used

for this study, the HSC were isolated from much lighter Wistar rats (100-120 g compared to 500 g body weight). This may explain the observed differences, since vitamin A content varies according to age and body weight both in humans and in rats (Hendriks *et al.,* 1988; Blomhoff *et al.,* 1988b). In addition, there are few, if any details within their report of the *in vitro* techniques used, including the isolation procedure and how the HSC were cultured. Consequently, a direct comparison cannot be made between these two studies. This is unfortunate, as these authors did not explain how enough HSC were obtained to perform reverse-phase HPLC on HSC cultured for 1 day, considering the low concentrations of retinoids present and without including unadhered, necrotic or apoptotic HSC.

Some authors have suggested there is a sustained release of retinol during transformation of quiescent HSC to myofibroblasts, resulting in a high local concentration of metabolites around the HSC (Friedman *et al.,* 1993; Friedman *et al.,* 1992b). It has been speculated that these metabolites have autocrine effects on HSC and feed back on HSC to regulate their transformation to myofibroblasts (Friedman *et al.,* 1992b). However, the studies in this Chapter found no increase in retinol or isomers of retinoic acid extracellularly following culture of HSC.

Besides retinyl palmitate and retinol, other endogenous metabolites of retinol were also detected, including isomers of retinoic acid. Although they were detected in much lower concentrations than retinol and retinyl palmitate, they are more potent activators of nuclear retinoid receptors. It could be postulated that the higher concentrations of retinol could constitute a reservoir from which a constant supply of short-lived retinoic acid is produced. Rapid turnover would explain why so little retinoic acid was detected. Recent studies in rats, rabbits and monkeys have suggested that 13-*cis* retinoic acid (the most abundant retinoic acid derivative in HSC) could function as a precursor for other active retinoids such as all-*trans* retinoic acid (Collins *et al.,* 1994; Tzimas *et al.,* 1994; Hummler *et al.,* 1994). This suggests HSC hold an abundant source of potential transcriptional activators of the nuclear retinoid receptors. The drop in the retinoic acids (all-*trans* and 13-*cis*) observed in most of the HSC preparations during culture is unlikely to be due to their contributing to maintaining the intracellular levels of retinol since retinoic acids cannot be reduced back to retinol, or stored as retinyl esters (Napoli, 1996).

It has been assumed that most functions of retinoids are mediated at the molecular level by all-*trans* retinoic acid and 9-*cis* retinoic acid since retinoic acid is superior in potency to retinol. However, 9-*cis* retinoic acid could not be detected in the HSC samples. The absence of 9-*cis* retinoic acid was unexpected as endogenous 9-*cis* retinoic acid has previously been detected in mouse liver (Heyman *et al.,* 1992). Under physiological conditions, the low levels of 9-*cis* retinoic acid may be quickly metabolised to related compounds. Heyman *et al.* (1992) report that in mouse liver, the concentration of all-*trans* retinoic acid is approximately 2-3 fold greater than the concentration of 9-*cis* retinoic acid. These authors suggest the mouse liver contains approximately 4 ng of 9-*cis* retinoic acid/g of wet weight. If total liver levels reflect HSC levels, 9-*cis* retinoic acid would be too low to detect with the methodology used in this study since all-*trans* retinoic acid itself was on the limit of detection. However, 9-*cis* retinoic acid has been reported to be 40-fold more potent in activation of RXRα than 13-*cis* retinoic acid and all-*trans* retinoic acid, and therefore undetectable levels might still contribute to activation of RXRα in HSC. Okuno *et al.* (1999) were also unable to detect 9-*cis* retinoic acid, either in fibrotic rat liver or in primary cultured rat HSC.

9,13 Di-*cis* retinoic acid was not detected in HSC in this study. 9,13 Di-*cis* retinoic acid is a novel stereoisomer of all-*trans* retinoic acid, arising from the *in vivo* isomerization of 9-*cis* retinoic acid (Sass *et al.,* 1997). Since 9-*cis* retinoic acid was undetectable in HSC and their culture media, it is therefore not unexpected that 9,13 di-*cis* retinoic acid was also not detectable. However, Okuno *et al.* (1999), who did not detect 9-*cis* retinoic acid, measured an increase of 114% in 9,13 di-*cis* retinoic acid in fibrotic rat livers and an increase of 60% in cultured HSC. These authors argue that the 9,13 di-*cis* retinoic acid is indicative of pre-existing levels of 9-*cis* retinoic acid which has rapidly isomerized. The physiological relevance of the increase in 9,13 di-*cis* retinoic acid is unknown, since this retinoid has little affinity for human RXRα and RXRβ (Horst *et al.,* 1995).

All-*trans* retinoic acid and 9-*cis* retinoic acid are not the only retinol metabolites responsible for the effects attributed to vitamin A. The studies in this Chapter show that HSC produce an as yet uncharacterised metabolite (peak X). The levels of peak X in quiescent and activated HSC cannot be calculated without a standard curve. However, if confirmed to be a retinoid, peak X may comprise a novel retinoid metabolite. Few other substances except retinoids absorb at the wavelength of 330 nm. Perhaps a candidate retinoid for peak X is 11-*cis*

retinoic acid. Heyman *et al.* (1992) measured retinoic acids from mouse liver and showed 11-*cis* retinoic acid to elute between 13-*cis* retinoic acid and 9-*cis* retinoic acid. However, the number of potential stereoisomers, conformers and modifications could potentially generate thousands of alternative molecules. For example, another potential component is 4-oxoretinol, an abundant retinoid in F9 cells (Achkar *et al.,* 1996). 4-Oxoretinol can bind and transcriptionally activate the three RARs, implicating it as a signalling molecule and regulator of cell differentiation. To validate the identity of peak X, the fraction could be collected and analysed using a complementary identification technique such as mass spectrometry. Even if peak X is confirmed to be a retinoid, this metabolite also decreases during activation of HSC, both intracellularly and extracellularly.

Although the results in this study contradict some of the observations reported in the publication by Okuno *et al.* (1999), they support previous finding by Ohata *et al.* (1997). In this paper these workers analysed retinoids in HSC isolated from bile duct ligated rats by HPLC analysis. They demonstrated reduced retinol, 9-*cis* retinoic acid and all-*trans* retinoic acid in HSC from bile duct ligated rats compared to untreated rats. The reason for conflict between the two sets of published results is unclear and this issue has not been addressed by the more recent publication. One possibility is due to the use of different models of fibrosis.

It is important to remember that the response of HSC metabolites in culture may not be the same *in vivo*. Metabolic processes may bring different degrees of isomerization and production of retinoic acids. More isomer exchange may occur between 9-*cis* retinoic acid and all-*trans* retinoic acid in intact rats than would be expected from cultured cells. This is demonstrated by the study by Okuno *et al.* (1999) who found different levels and isomers of retinoic acid to be present during activation of rat HSC *in vitro* than during porcine serum-induced rat hepatic fibrosis *in vivo*. The involvement of Kupffer cells in the mediation of HSC proliferation has been previously reported (Shiratori *et al.,* 1986) and may subsequently influence the pathways of retinoid metabolites. Friedman *et al.* (1993) showed that culture induced activation of HSC by Kupffer cells is associated with a decrease in intracellular retinoids. Furthermore, this was shown to result in a rise in extracellular levels of retinol and retinyl palmitate (Friedman *et al.*, 1993). The metabolism and subsequent levels of the metabolites of retinol may also have been influenced by the exposure of HSC to serum free conditions prior to collection of the cells and their supernatants. *In vivo* HSC are bathed in a constant supply of vitamin A. Nevertheless, using a well-characterised model of HSC

activation which reflects that in liver *in vivo,* retinoid metabolism has been studied specifically in HSC and the levels of retinoids measured during HSC activation from a quiescent to a myofibroblastic phenotype.

In conclusion, the studies in this Chapter have shown that HSC not only contain retinyl palmitate and retinol but also contain retinoic acid derivatives. These retinoic acid derivatives are present during early activation and are not sustained in later activation. Furthermore, these retinoids appear to be effective as RAR activators, as RAR-DNA binding activity can be demonstrated by EMSA, as shown in Chapter 5.

CHAPTER 5

Expression of Retinoic Acid Receptor Beta in Hepatic Stellate Cells

5.1. INTRODUCTION

Although the effect of exogenous retinoids has been examined during transformation of quiescent HSC to myofibroblasts, there are limited data available on the nuclear retinoid receptors responsible for mediating the observed responses. Since different retinoid receptors control different functions, the phenotypic response of HSC to endogenous retinoids may change as they undergo transformation due to different or altered levels of expression of the nuclear retinoid receptors accompanying retinoid release (Weiner *et al.*, 1992; Ohata *et al.*, 1997).

The present study was undertaken to evaluate the expression of RARβ during the progressive activation of HSC from a quiescent to an activated phenotype, mimicked by culturing HSC on plastic. Although several nuclear RARs and RXRs have been described, the expression pattern of RARβ has primarily been examined since it has been shown to mediate the anti-proliferative effects of retinoic acid in other cell types (Seewaldt *et al.*, 1995). Additionally, two studies by independent groups have shown that the expression of RARβ changes during HSC activation (Weiner *et al.*, 1992; Ohata *et al.*, 1997), suggesting that RARβ may regulate HSC activation. If the RARβ receptor decreases in activated HSC as these studies suggested, its loss may contribute to the proliferation observed in activation. To approach this question experimentally, it is important to confirm the published findings and demonstrate that the expression of the RARβ receptor changes during HSC activation. The expression pattern of RARα and RARβ in HSC was therefore examined at the mRNA level and protein level using a range of appropriate techniques.

5.2. DIFFERENTIAL EXPRESSION OF RARα AND RARβ MESSENGER RNA IN HEPATIC STELLATE CELLS

5.2.1. Reverse Transcriptase Polymerase Chain Reaction

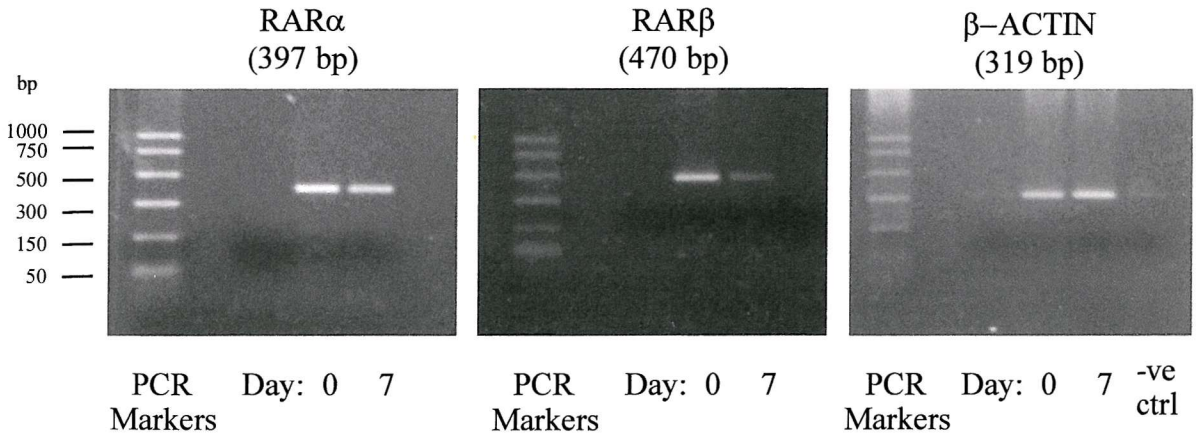
To study the differential expression of the RARα and RARβ genes, initially the RT-PCR technique was performed. Human and mouse RAR genes have been cloned and sequenced (Zelent *et al.*, 1989; Brand *et al.*, 1988; Krust *et al.*, 1989; Benbrook *et al.*, 1988; Giguere *et al.*, 1987; Petkovich *et al.*, 1987). However, the rat RAR genes have never been studied. Since the mouse RARα, RARβ and RARγ cDNA clones can be used to hybridise to rat

RARα, RARβ and RARγ mRNA respectively, it suggests there is a high degree of species homology for each RAR. Subsequently mouse RARα and RARβ sequences were used as the basis to synthesise primers for the RT-PCR.

Total RNA was extracted from freshly isolated rat HSC and primary rat HSC cultured for 7 days. The RNA was reverse transcribed and using sequence specific mouse RARα and mouse RARβ primers the cDNA was amplified using the PCR to determine the expression of RARα and RARβ mRNA (section 2.7.).

Appropriately sized transcripts of 397 bp for RARα, and 470 bp for RARβ were detected in rat HSC upon analysis on an agarose gel (Figure 5.1.). Strong bands were observed for RARα mRNA in freshly isolated rat HSC (day 0) and remained expressed at the same level following activation (day 7). RARβ mRNA however, was present at a considerably higher level in freshly isolated rat HSC (day 0) compared with 7 day cultured rat HSC where it was only present in trace amounts. The rat β-actin cDNA, a house keeping gene (319 bp) and a negative control were also amplified with the RARs to confirm equal loading and equal efficiency of amplification between the different samples.

Figure 5.1: Analysis of RARα, RARβ and β-actin mRNA in hepatic stellate cells by the reverse transcriptase polymerase chain reaction



Electrophoretic analysis of amplified RT-PCR cDNA of RARα, RARβ and β-actin transcripts of freshly isolated rat HSC (day 0) or cultured rat HSC (day 7) on 1.5% agarose/TBE gel. The amount of DNA loaded on each lane of the gel was 5 μl of the amplified product. Similar results were obtained in a further experiment.

5.2.2. Northern Blotting

When mouse cDNA probes for the RARs became available, northern blotting was employed to re-examine expression of RAR mRNA in quiescent and activated HSC (section 2.5.) since this technique is considered to be more quantitative than RT-PCR. Poly $(A)^+$ mRNA and total RNA were extracted from freshly isolated rat HSC and from rat HSC cultured on plastic for various times. Following the separation of RNA molecules (10-20 μg total RNA) by electrophoresis in agarose gels, the RNA was transferred to nylon membranes. The northern blots were subsequently hybridised with [^{32}P]-labelled cDNA probes.

Initially, plasmids containing partial length RARα$_2$ and RARβ$_2$ mouse cDNAs were received from Dr Christopher Redfern, University of Newcastle, Newcastle, UK. The plasmids were amplified by transforming *E.coli* and the cDNA insert retrieved from the plasmid by restriction enzyme digestion. Each insert was purified of contaminants and used as a template for the synthesis of a radioactive cDNA probe. Numerous northern blots were hybridised with these radiolabelled mouse RARα$_2$ and mouse RARβ$_2$ cDNA probes and also with the radiolabelled mouse RARα and mouse RARβ cDNAs purified from the RT-PCR (section 5.2.1.). Using the above cDNA probes, expression of both RARα and RARβ mRNA was undetectable on northern blots containing poly $(A)^+$ mRNA and total RNA extracted from freshly isolated rat HSC and from rat HSC at various times after culture on plastic. However, since these cDNA probes also failed to recognise RARα mRNA in total RNA extracted from the human neuroblastoma cell lines TR14 and SH-SY5Y, cells which are known to express this transcript (Carpentier *et al.,* 1997), the reliability of the cDNA probes was questioned.

Only upon using the full length mouse cDNA sequence for RARα and RARβ$_2$ (a gift from Professor Chambon, IGBMC, Strasbourg, France) as a template to make the cDNA radiolabelled probe, could the appropriate mRNA be detected on northern blots. The representative northern blot in Figure 5.2., panel A, demonstrates that the intensity of the autoradiographic signal representing RARβ mRNA expression rapidly decreases in cultured rat HSC compared with freshly isolated rat HSC (day 0). During transformation of HSC to myofibroblasts, demonstrated by the increased expression of α-smooth muscle actin mRNA, RARβ mRNA became less abundant. To control for differences in loading and to verify good quality RNA, each blot was re-hybridised with a rat β-actin cDNA probe (purified from the

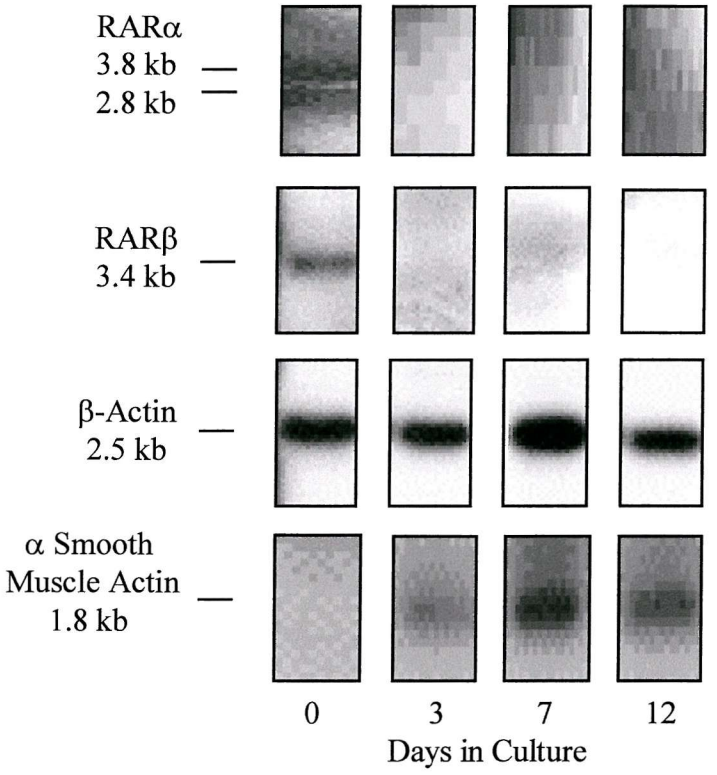
RT-PCR, section 5.2.1.) and the band intensity for RARβ mRNA was expressed as a ratio to that for β-actin mRNA (Figure 5.2., panel B).

Figure 5.2., panel A also shows the result of changes induced in the mRNA level of the RARα gene during progressive activation of HSC in culture. Although present at very low levels, the [^{32}P]-radiolabelled RARα cDNA probe hybridised to two distinct transcripts of 3.8 kb and 2.8 kb with approximately equal intensity in freshly isolated rat HSC. These transcripts are of the expected sizes for RARα mRNA in HSC according to Weiner *et al.* (1992). However, the two mRNAs were undetectable in rat HSC cultured on plastic for 7 days. The suppression of the RARα gene in activated HSC has not previously been investigated using northern hybridisation. Ohata *et al.* (1997) found activated HSC to express RARα mRNA but they detected the transcript by using the highly sensitive RT-PCR technique.

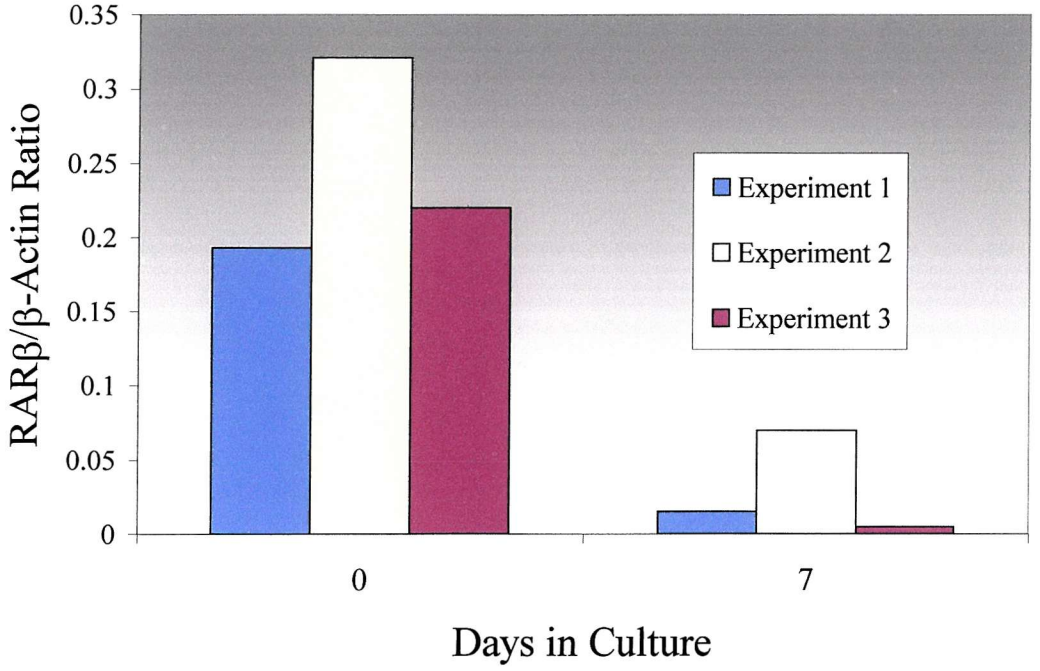
To ensure the DNA used to make the radiolabelled probes represented cDNAs for RARα, RARβ$_2$ and RARγ, restriction enzyme digests were performed on the purified fragments obtained from the pSG5 plasmids that had been cloned either with mouse RARα, RARβ$_2$ or RARγ sequences (Figure 5.3.). The restriction endonucleases chosen were enzymes which recognised specific sites within the cDNAs RARα, RARβ$_2$ and RARγ (see restriction maps, appendix 2.). The purified RARα insert (2261 bp) was digested using the restriction enzymes *Sac* I and *Eco*R V. This generated fragments of sizes 749 bp and 1512 bp, and 1269 bp and 992 bp respectively. Digestion of the RARβ$_2$ insert (1839 bp) generated fragments of the expected sizes; 1428 bp and 411 bp following digestion with *Sac* I, and 1349 bp and 490 bp following digestion with *Bgl* II. The RARγ insert (1907 bp) was digested using the restriction enzyme *Kpn* I and generated fragments of sizes 1373 bp and 534 bp. The restriction enzyme digests verify that each of the purified fragments obtained from the pSG5 plasmids are full length mouse cDNAs for RARα, RARβ$_2$ and RARγ.

Figure 5.2: Analysis of RARβ mRNA expression in hepatic stellate cells by northern blotting

A:

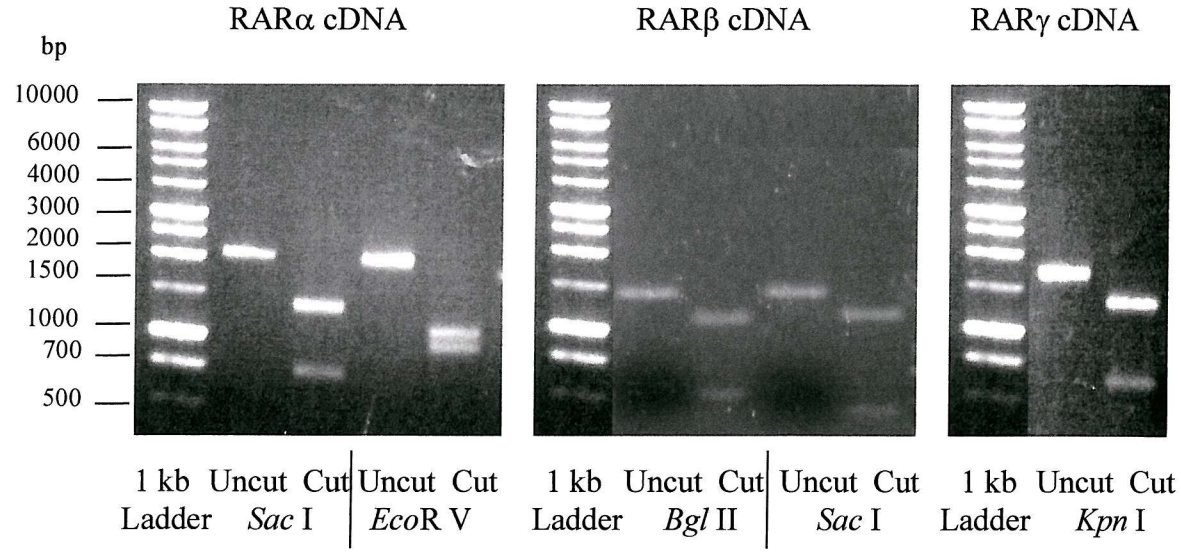


B:



Total RNA from freshly isolated rat HSC (day 0) and rat HSC cultured on plastic were analysed for expression of RARα, RARβ, β-actin and α-smooth muscle actin mRNA using northern hybridisation (panel A). The band intensity for RARβ mRNA was expressed as a ratio to that for β-actin mRNA (panel B). Experiments were repeated at least 3 times with extracts prepared from separate cell cultures and gave similar results.

Figure 5.3: Restriction enzyme digests of the purified fragments obtained from the pSG5 plasmids cloned either with mouse RARα, RARβ$_2$ or RARγ sequences



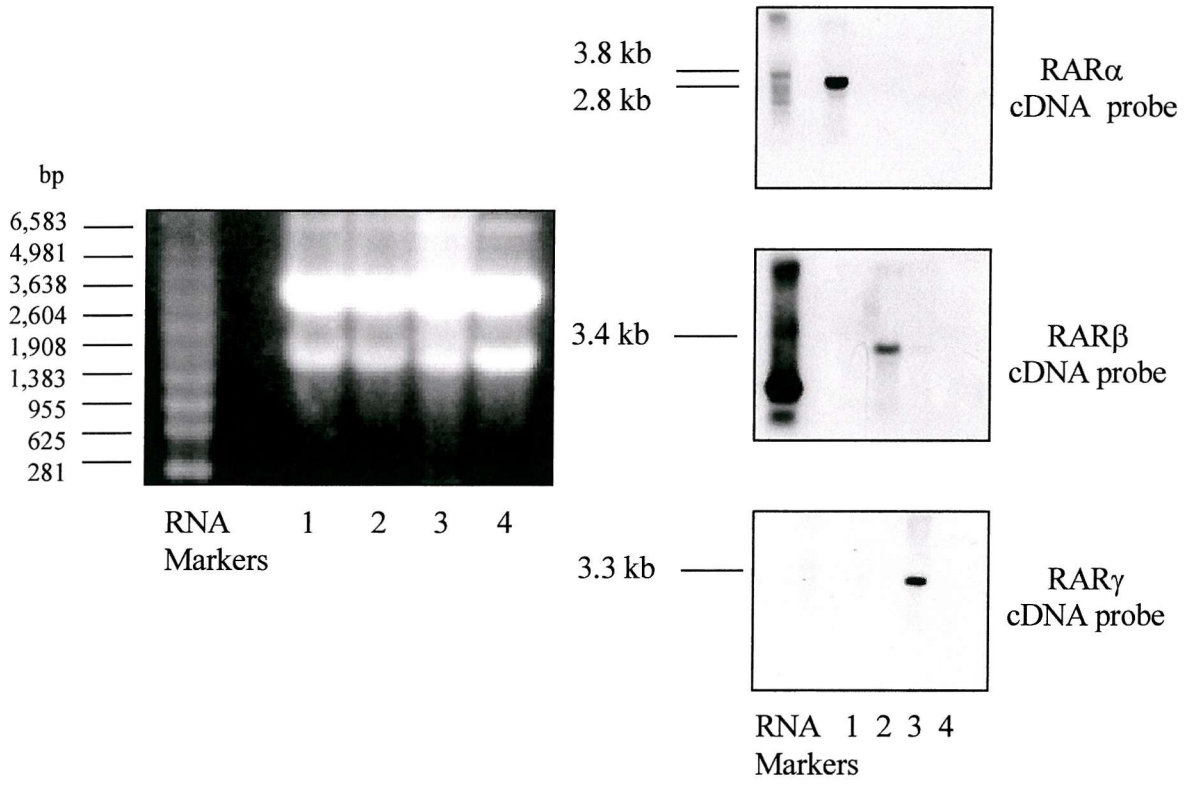
Electrophoretic analysis of DNA restriction digests of the inserts obtained from pSG5 plasmids which had been cloned either with RARα, RARβ$_2$ or RARγ sequences. The digests were analysed on 0.8% agarose/TBE gels together with the uncut insert which underwent a similar reaction, except the restriction enzyme was replaced with distilled water.

Since the three receptor subtypes (RARα, RARβ and RARγ) exhibit a very high amino acid sequence identity in the DNA-binding and ligand-binding domains (Leid *et al.*, 1992), it was important to determine whether the cDNA radiolabelled probes used for hybridisation were selective for the relevant mRNA examined. Applying the calcium phosphate transfection technique (section 2.17.), COS-1 cells were either transfected with pSG5 plasmids containing mouse RARα, RARβ$_2$ or RARγ full length cDNAs or transfected with vector alone (not containing RAR specific cDNAs). Total RNA was extracted, separated by electrophoresis in an agarose gel and transferred to a nylon membrane (section 2.5.). The northern blot was subsequently hybridised with [^{32}P]-labelled full length cDNA probes for mouse RARα, RARβ$_2$ and RARγ.

Hybridisation of the total RNA from RARα, RARβ$_2$ and RARγ transfected COS-1 cells with a [^{32}P]-labelled RARβ$_2$ cDNA probe, revealed a strong signal of approximately 3.4 kb in the RARβ$_2$ transfected COS-1 cell line, the expected size of the transcribed mRNA for RARβ (Figure 5.4.). In contrast, no significant hybridisation signal could be observed in the COS-1 cells transfected with vector alone or in the RARα and RARγ transfected COS-1 cells. Similar results were found when a [^{32}P]-labelled RARα or RARγ cDNA probe was used i.e.

the cDNA probes hybridised to a correctly sized mRNA only in the RNA from COS-1 cells which had been transfected with the same RAR isoform. This demonstrates selectivity of the mouse RAR cDNA probes for each of the isoforms.

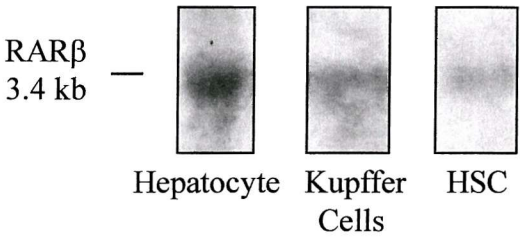
Figure 5.4: Analysis of cross-reactivity among the retinoic acid receptor subtypes



Northern blot hybridisation analysis of total RNA isolated from COS-1 cells transfected either with plasmids containing mouse RARα (lane 1), mouse RARβ$_2$ (lane 2) or mouse RARγ (lane 3) or transfected with vector alone (lane 4).

Although the isolation of HSC results in a highly purified preparation (> 95%), the cell suspension can be contaminated with small quantities of other hepatic cells, particularly Kupffer cells. Analysis of the cellular sources of hepatic RARβ mRNA using total RNA isolated from pure suspensions of rat liver cells revealed RARβ mRNA is also expressed by hepatocytes and Kupffer cells (Figure 5.5.). A single band of 3.4 kb was detected in both hepatocyte and Kupffer cell RNA following hybridisation of the northern blot with a [^{32}P]-radiolabelled RARβ$_2$ cDNA probe. As a positive control, total RNA from freshly isolated rat HSC was also included on the northern blot. Expression of RARβ mRNA was similar in each cell type. As HSC used in these studies were highly purified, it suggests that the RARβ signal detected on northern blots in Figure 5.2., panel A was due to HSC.

Figure 5.5: Analysis of RARβ mRNA expression in various cells of the liver by northern blotting



Total RNA from freshly isolated rat hepatocytes, Kupffer cells and HSC were analysed for expression of RARβ mRNA using northern hybridisation.

5.3. DIFFERENTIAL EXPRESSION OF RARα AND RARβ PROTEIN IN HEPATIC STELLATE CELLS

Detection of the protein in a cell has greater significance than the presence of the corresponding mRNA, since the protein is the biologically active molecule. Whether the transcript levels of the RARs accurately reflect receptor protein levels is unknown. Consequently, the nuclear presence of the RARβ protein was examined in quiescent and activated HSC.

5.3.1. Western Blotting

During transformation of HSC from a quiescent to an activated phenotype, overall cellular protein is increased in these cells due to expansion of rough endoplasmic reticulum (Friedman *et al.*, 1989). Therefore to control for any alterations in protein synthesis, instead of loading equal amounts of protein on the SDS-polyacrylamide gel which would underestimate any increase in a specific protein following activation, equal amounts of DNA were loaded.

Whole cell lysates were prepared from freshly isolated rat or human HSC (day 0) and from rat or human HSC at various time points after culture on plastic. Since there were no differences in the proteins which were recognised by RAR antibodies between samples in reduced (with β-mercaptoethanol) or non-reduced (without β-mercaptoethanol) sample buffer (data not shown), HSC lysates were prepared in a reduced sample buffer. As a positive control, whole cell lysates of COS-1 cells transfected with a pSG5 mouse $RAR\beta_2$ expression vector were

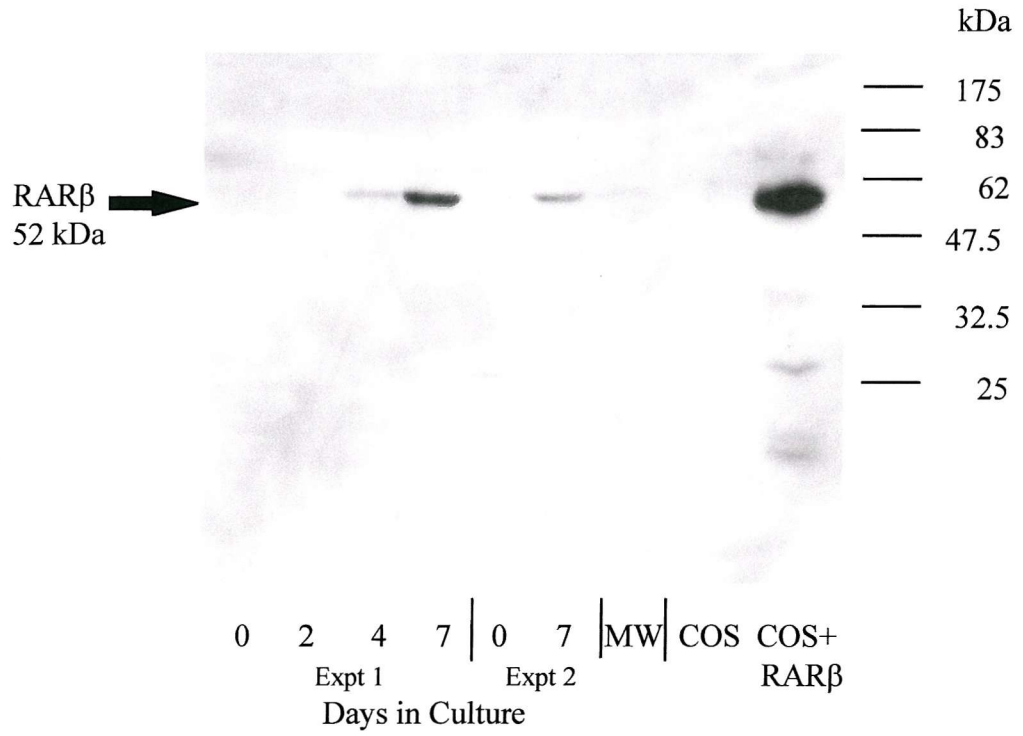
used. Equal amounts (1.5 μg of DNA) of HSC lysates were subjected to electrophoresis on a 12.5% denaturing SDS-polyacrylamide gel (section 2.9.) and western blotted onto PVDF (section 2.10.). Proteins were examined using a double antibody detection method; western blots were incubated with an antibody directed against the target protein or a control antibody (non-immune serum or non-immune IgG), followed by incubation with a second antibody conjugated to horseradish peroxidase. Bands were visualised by ECL.

On western blots incubated with a rabbit polyclonal antibody to human RARβ (Santa Cruz), there were no identifiable protein bands in freshly isolated rat HSC (day 0). However, one single, strong autoradiographic protein band became increasingly expressed during HSC activation (Figure 5.6., panel A). Although the exact molecular weight for each RAR protein is not known for the rat proteins as the molecular weights of the proteins vary depending on the tissue, cell type, and culture conditions, the protein band is within the region reported for RARβ protein (50-60 kDa). More importantly, this protein co-migrated with RARβ protein expressed by COS-1 cells transfected with a $RAR\beta_2$ expression vector. The band detected was specific, since no bands were detected on the western blot incubated with the negative control antibody, rabbit IgG (Figure 5.8., panel B). All three western blots gave similar results (Figure 5.6., panel B). Endogenous RARβ was not detected on western blots of extracts from mock-transfected COS-1 cells, indicating either the absence of, or very low levels of expression of RARβ protein in these cells.

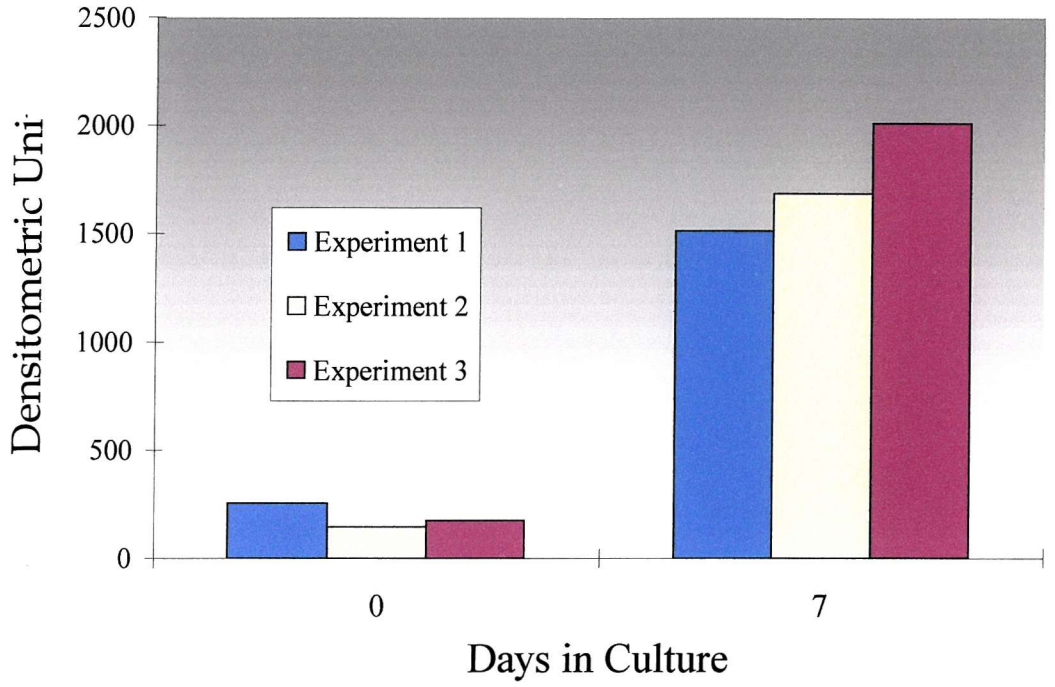
Similar results were found with human cultured HSC. Analysis of human HSC lysates by western blotting using a rabbit polyclonal antibody to human RARβ (Santa Cruz) detected only negligible amounts of RARβ protein in freshly isolated human HSC. However, this protein was found to be strongly expressed in human HSC which had been cultured on plastic for 7 days (Figure 5.7.).

Figure 5.6: Analysis of RARβ protein in hepatic stellate cells by western blotting using a polyclonal antibody to human RARβ (Santa Cruz)

A:

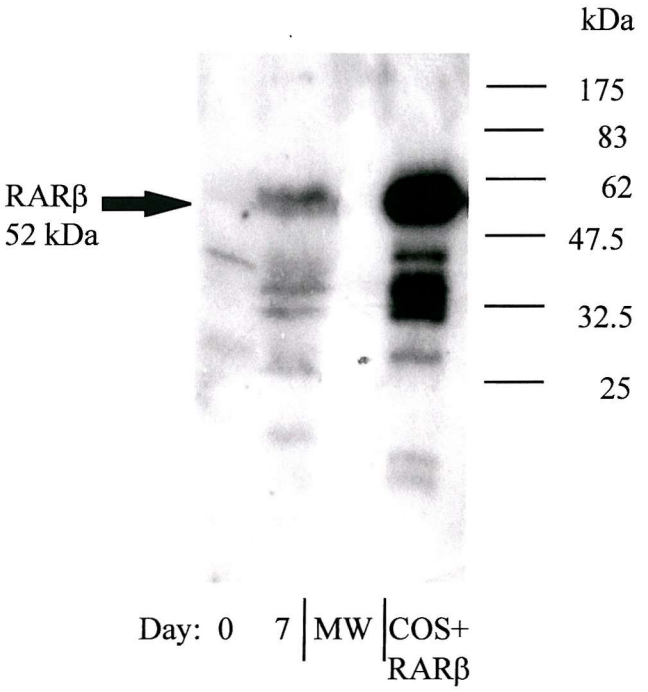


B:



RARβ protein levels in cell lysates from freshly isolated rat HSC (day 0) and rat HSC cultured on plastic were analysed using a rabbit polyclonal antibody to human RARβ (Santa Cruz, panel A). The lysates of COS-1 cells transfected with plasmid containing mouse RARβ$_2$ cDNA were used as positive controls in each experiment. Levels of RARβ protein were quantified by scanning densitometry (panel B). Experiments (Expt) were repeated at least 3 times with extracts prepared from separate cell cultures and gave similar results.

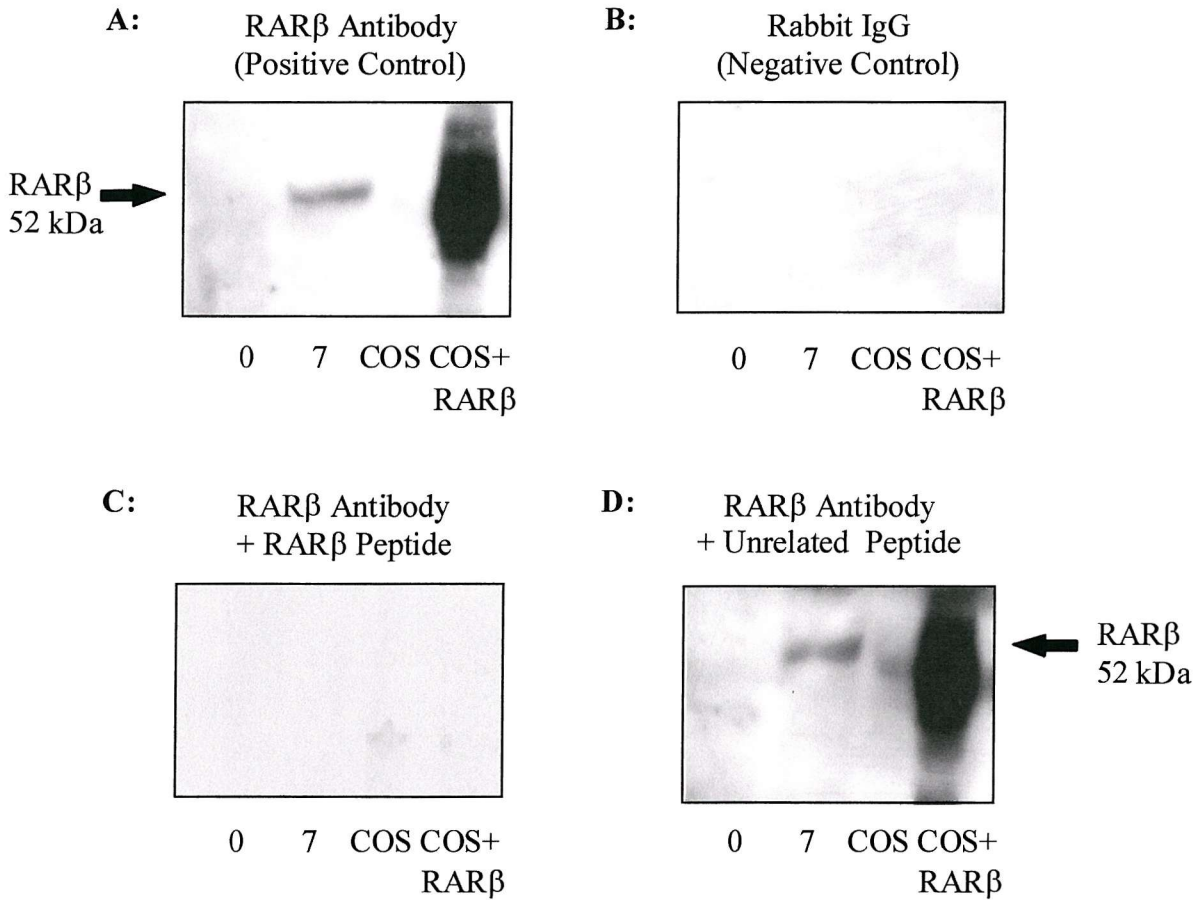
Figure 5.7: Analysis of RARβ protein in human hepatic stellate cells by western blotting using a polyclonal antibody to human RARβ (Santa Cruz)



RARβ protein levels in cell lysates from freshly isolated human HSC (day 0) and human HSC cultured on plastic for 7 days, were analysed using a rabbit polyclonal antibody to human RARβ (Santa Cruz). The lysate of COS-1 cells transfected with plasmid containing mouse RARβ$_2$ cDNA was used as a positive control.

To verify the specificity of the Santa Cruz RARβ antibody used in the western blotting studies, the effect of adding a competing peptide was examined (section 2.10.2.). Preincubation of the Santa Cruz RARβ antibody with a 10-fold excess of a specific RARβ peptide containing the same sequence (SISPSSVENSGVSQSPLVQ) that Santa Cruz used to generate this antibody, effectively blocked detection of the 52 kDa band upon analysis by western blotting (Figure 5.8., panel C). The peptide caused complete disappearance of both the strong signal observed in COS-1 cells transfected with a mouse RARβ$_2$ expression vector, and also the weaker signal observed in cultured rat HSC (day 7). In contrast, a similar competition experiment with a non-immunogenic peptide did not reduce the intensity of the 52 kDa band (Figure 5.8., panel D). This experiment further demonstrates the specificity of the 52 kDa protein band to RARβ protein.

Figure 5.8: Analysis of RARβ protein in hepatic stellate cells using a competing peptide specific to the polyclonal antibody for human RARβ (Santa Cruz)

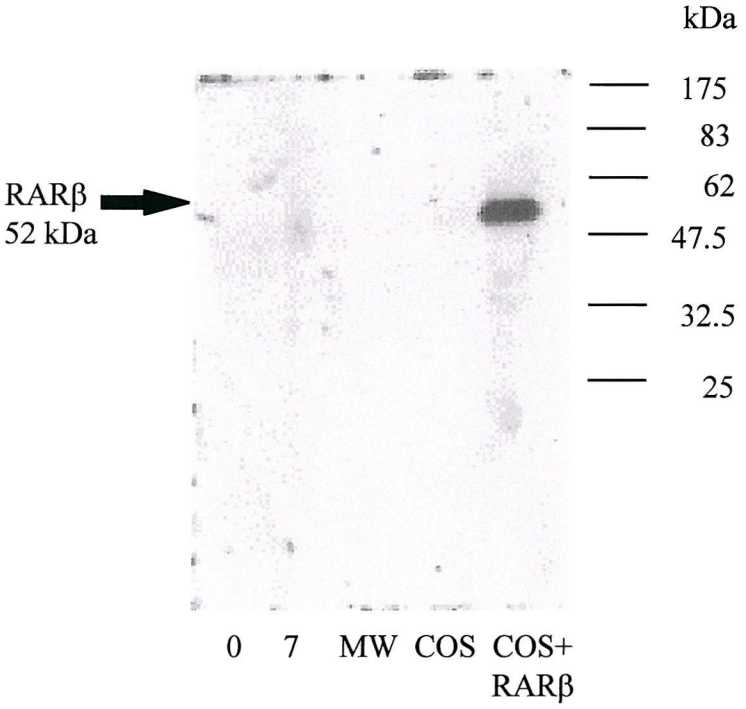


Western blot analysis of whole cell lysates of freshly isolated rat HSC (day 0), HSC cultured for 7 days on plastic and COS-1 cells either mock transfected or transfected with mouse $RAR\beta_2$ expression vector. Blots in panels A and C were incubated with RARβ antibody (Santa Cruz) without or with previous immunoadsorption with a specific RARβ peptide respectively. Panel D shows a blot incubated with RARβ antibody previously immunoadsorped with a non-immunogenic peptide. The blot in panel B was incubated with non-immune rabbit IgG.

To ensure the RARβ antibody obtained from Santa Cruz recognised intact rat RARβ protein, a different RARβ antibody was employed in western blotting studies, with the aim that detection of the same 52 kDa protein by two different antibodies would be conclusive evidence that RARβ protein expression increases with activation. The only alternative RARβ antibody commercially available to date was from Affinity Bioreagents. Although reported to only cross-react with mouse and human RARβ, it was hoped that this monoclonal antibody to mouse RARβ would also recognise rat RARβ protein since interspecies conservation of each member of the RAR family is very high (Krust *et al.*, 1989). However, as shown in Figure 5.9., this RARβ antibody failed to detect rat RARβ protein. The only band identified on the

western blot was detected in COS-1 cells transiently expressed with a mouse RARβ$_2$ vector. Although this antibody probably recognises RARβ protein expressed in mouse HSC, the yield of HSC isolated from a mouse liver is too low to perform substantial primary culture experiments.

Figure 5.9: Analysis of RARβ protein in hepatic stellate cells using a monoclonal antibody to mouse RARβ (Affinity Bioreagents)

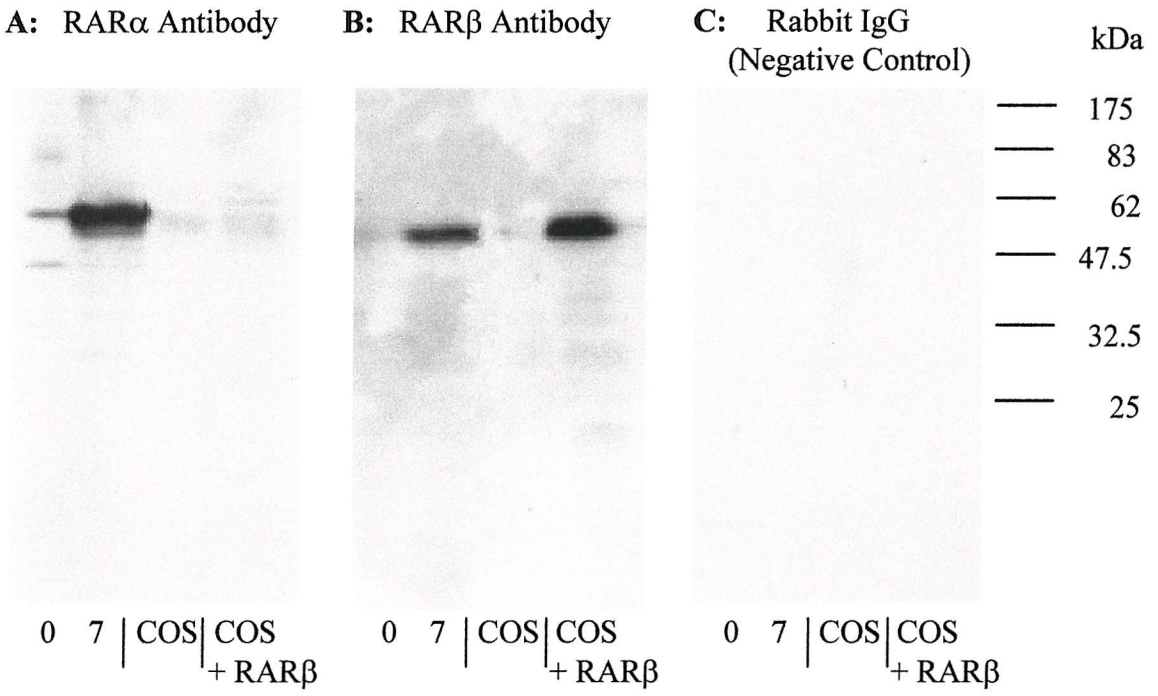


RARβ protein levels in cell lysates from freshly isolated rat HSC (day 0) and rat HSC cultured on plastic for 7 days were analysed using a monoclonal antibody to mouse RARβ (Affinity Bioreagents). The lysates of COS-1 cells, transfected with plasmid containing mouse RARβ$_2$ cDNA were used as a positive control.

By western analysis using a polyclonal antibody to human RARα (Santa Cruz), a protein of approximately 51 kDa, the expected molecular weight for RARα, was detectable in freshly isolated rat HSC (Figure 5.10., panel A). However, HSC cultured for 7 days on plastic showed 3 distinct but closely spaced protein bands of similar molecular weights (50-60 kDa). All signals were specific since bands were not detected on the immunoblot which was incubated with rabbit IgG (Figure 5.10., panel C). Multiple species of RARα have been detected by western analysis in cell lines and shown to result from phosphorylation (Ali *et al.*, 1992). The broad appearance of the RARα protein bands in cultured HSC (day 7) is compatible with the possibility of such post-translational phosphorylation. To support the fact

fact that the RARα antibody was specific only to RARα protein, the western blot shown in Figure 5.10., panel A was stripped of antibody and re-probed using a polyclonal antibody to human RARβ (Santa Cruz). The RARβ antibody reacted specifically to reveal a single protein with an apparent molecular weight of 52 kDa in HSC cultured for 7 days (Figure 5.10., panel B). The RARβ antibody also recognised the COS-1 cells transfected with mouse $RAR\beta_2$ expression vector.

Figure 5.10: Analysis of RARα and RARβ protein in hepatic stellate cells by western blotting using a polyclonal antibody to human RARα and human RARβ respectively (Santa Cruz)



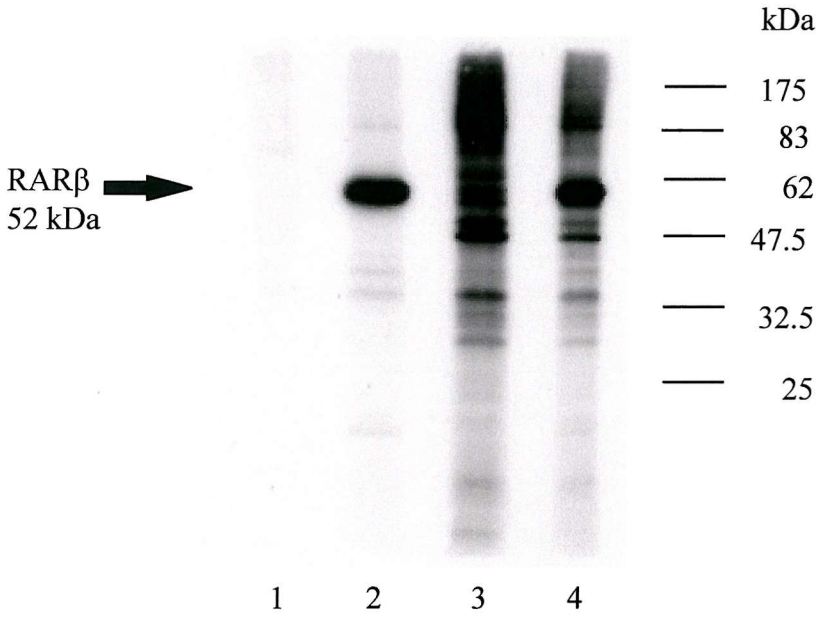
Western blot analysis of cell lysates of freshly isolated rat HSC (day 0), rat HSC cultured for 7 days on plastic and COS-1 cells either mock transfected or transfected with mouse $RAR\beta_2$ cDNA. The western blot in panel A was incubated with a rabbit polyclonal antibody to human RARα (Santa Cruz), subsequently stripped and then re-probed with a rabbit polyclonal antibody to human RARβ (Santa Cruz; panel B). The blot in panel C was incubated with non-immune rabbit IgG.

5.3.2. Immunoprecipitation

Immunoprecipitation, an alternative technique to western blotting was also used to detect RARβ protein expression in HSC. However, prior to performing immunoprecipitation on HSC, conditions for the immunoprecipitation had to be established which vary depending on the rate of synthesis of the protein of interest, the half-life of the protein and its amino acid

composition. To optimise conditions, immunoprecipitations were initally performed on cells that were known to overexpress RARβ, COS-1 cells transfected with a $RAR\beta_2$ expression vector. Figure 5.11. shows the signal obtained after immunoprecipitation was performed on these cells using either a single lysis buffer or a triple lysis buffer to solubilise the target antigen. In this experiment, transfected COS-1 cells were incubated for 4 hours with [^{35}S]-labelled methionine to incorporate [^{35}S]-labelled methionine and cysteine into newly synthesised proteins (section 2.16.). The RARβ protein was then immunoprecipitated with a polyclonal antibody to human RARβ (Santa Cruz). The level of RARβ protein in the immunoprecipitate was analysed by SDS-PAGE and visualised by autoradiography. The result suggests a more efficient and selective extraction of the RARβ protein is achieved using the single lysis buffer. Subsequently, single lysis buffer was used to immunoprecipitate RARβ protein from HSC.

Figure 5.11: Immunoprecipitation of RARβ protein using different lysis buffers



Immunoprecipitation of RARβ protein was performed on COS-1 cells transfected with plasmid containing a $RAR\beta_2$ expression vector using either a single lysis buffer (lanes 1 and 2) or a triple lysis buffer (lanes 3 and 4) to solubilise the target protein. Lanes 1 and 3 represent immunoprecipitations where the RARβ antibody was replaced with non-immune rabbit IgG (negative control).

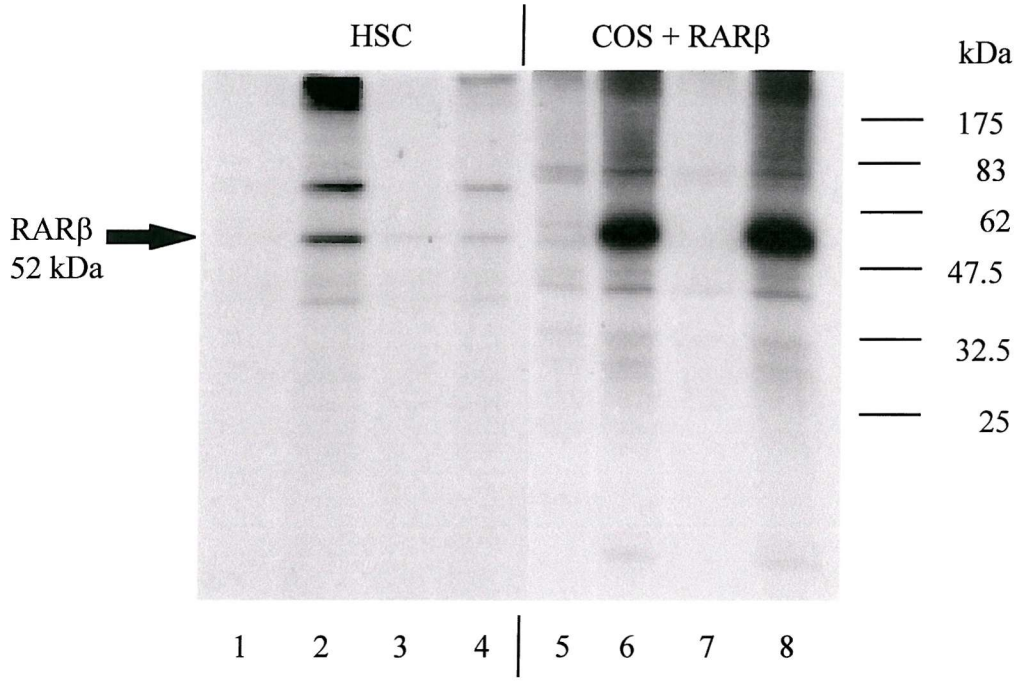
After altering other parameters, including the incubation time with radiolabelled [^{35}S]-methionine and the concentration of the protein antibody, the conditions for immunoprecipitation of radiolabelled RARβ protein from HSC were established. Figure 5.12. demonstrates the result of an immunoprecipitation of RARβ protein from primary rat HSC

cultured for 7 days on plastic. Control immunoprecipitation assays were also performed on COS-1 cells transfected with a mouse RARβ$_2$ expression vector. The COS-1 immunoprecipitates were fractionated on the same SDS-polyacrylamide gel as the HSC immunoprecipitate. A protein at an apparent molecular weight of 52 kDa, the correct molecular weight for RARβ, was detected both in COS-1 cells transfected with RARβ$_2$ expression vector and also in HSC. It is well documented that RARs bind to RAREs as heterodimers, the other two proteins immunoprecipitated from HSC may result from proteins which were previously bound to RARβ before separation on the SDS-polyacrylamide gel.

The signal obtained by immunoprecipitation was confirmed to be specific since previous immunoadsorption of the RARβ antibody with a specific RARβ peptide (sequence: SISPSSVENSGVSQSPLVQ) blocked the signal (Figure 5.12.). However, previous immunoadsorption of the RARβ antibody with a non-immunogenic peptide did not effect the intensity of the signal to as great an extent. Furthermore, the signal was not detected when immunoprecipitations were perfomed using non-immune rabbit IgG instead of the RARβ antibody.

The immunoprecipitation reinforces the findings by western blotting (section 5.3.1.), that RARβ protein is expressed in activated HSC.

Figure 5.12: Analysis of RARβ protein in cultured rat hepatic stellate cells using immunoprecipitation

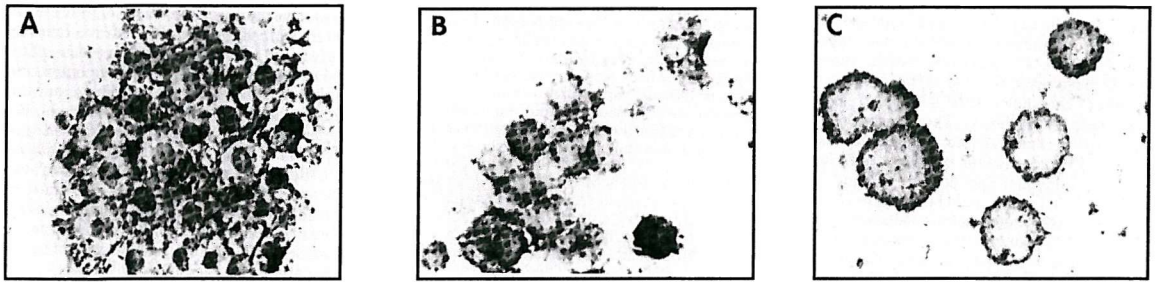


Immunoprecipitated cell lysates of rat HSC cultured for 7 days (lanes 1, 2, 3, 4) and COS-1 cells transfected with plasmid containing a RARβ$_2$ expression vector (lanes 5, 6, 7, 8). Immunoprecipitations were performed without (lanes 2 and 6) or with (lanes 3 and 7) previous immunoadsorption with a specific RARβ peptide. Lanes 4 and 8 represent immunoprecipitations in which the RARβ antibody was previously immunoadsorbed with a non-immunogenic peptide. Lanes 1 and 5 show immunoprecipitations in which the RARβ antibody was replaced with non-immune rabbit IgG (negative control).

5.3.3. Immunocytochemistry

Using several approaches it has been shown that RARβ protein expression increases with activation of HSC. These results apparently contradict the findings by Weiner *et al.* (1992) who, using northern hybridisation and immunocytochemistry, demonstrated that freshly isolated rat HSC not only express RARβ mRNA but also contain nuclear RARβ protein. More importantly, they showed that HSC cultured for 7 days on uncoated plastic had no detectable mRNA or nuclear staining for RARβ. Figure 5.13. shows the immunocytochemistry pictures reproduced from the report by Weiner *et al.* (1992).

Figure 5.13: Immunocytochemical analysis of freshly isolated rat HSC and cultured rat HSC with a polyclonal antibody to human RARβ, SP172
Pictures reproduced from Weiner et al. (1992).



Freshly isolated HSC stained with RARβ antibody

Freshly isolated HSC stained with non-immune rabbit IgG

Day 7 cultured HSC stained with RARβ antibody

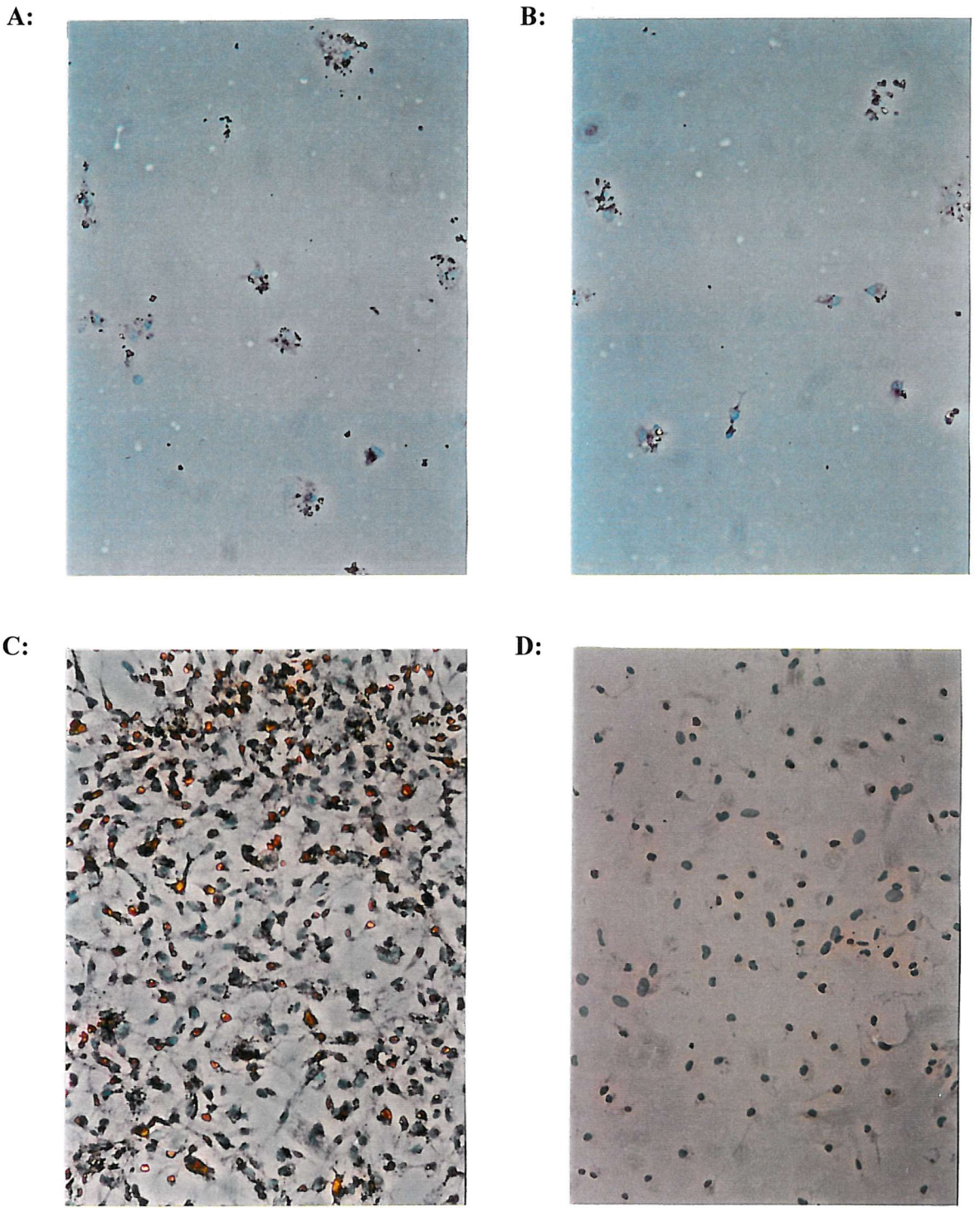
Cytospins of freshly isolated rat HSC and trypsinised rat HSC after 7 days of culture. Cells were stained with a polyclonal antibody to human RARβ, SP172 or with non-immune rabbit IgG. Cells were photographed at 1,000x magnification on a Nikon HFX-II microscope for A and B and at 400x magnification for C.

Attempts were made to verify the findings by Weiner *et al.* (1992) and to determine whether similar results are obtained with the RARβ antibody purchased from Santa Cruz. Therefore, the same antibody used in the study by Weiner *et al.* (SP172, a rabbit polyclonal antibody raised against the F region of human RARβ) was obtained from Professor Chambon (IGBMC, Strasbourg, France) and applied to immunocytochemical staining of HSC (section 2.13.). Primary rat HSC were cultured on glass slides for either 2 or 7 days and fixed using acetone before incubation with either the antibody SP172, the antibody purchased from Santa Cruz, or the appropriate negative control [rabbit serum or non-immune rabbit IgG respectively (section 2.13.)]. Antibodies retained by the fixed cells after extensive washing were detected using a biotinylated secondary antibody and a horse-radish peroxidase labelled streptavidin-biotin complex. The enzyme label was then detected using the substrate chromogen DAB.

Clear, strong nuclear staining was observed in HSC cultured for 7 days on uncoated glass with the antibody purchased from Santa Cruz (Figure 5.14.). Almost all the nuclei of the target cells were strongly stained. No staining was observed when HSC were treated with non-immune rabbit IgG. The staining was found to be attenuated in HSC cultured on glass slides for 2 days. In these slides no cytoplasmic or nuclear staining was apparent in the quiescent HSC, either in the presence of the RARβ antibody or the non-immune rabbit IgG. These immunocytochemistry results are therefore consistent with the results obtained following incubation of western blots with the same antibody (section 5.3.1.) and additionally show that

the Santa Cruz antibody binds to protein with a nuclear localisation as expected.

Figure 5.14: Immunocytochemistry of hepatic stellate cells using a polyclonal antibody to human RARβ (Santa Cruz)

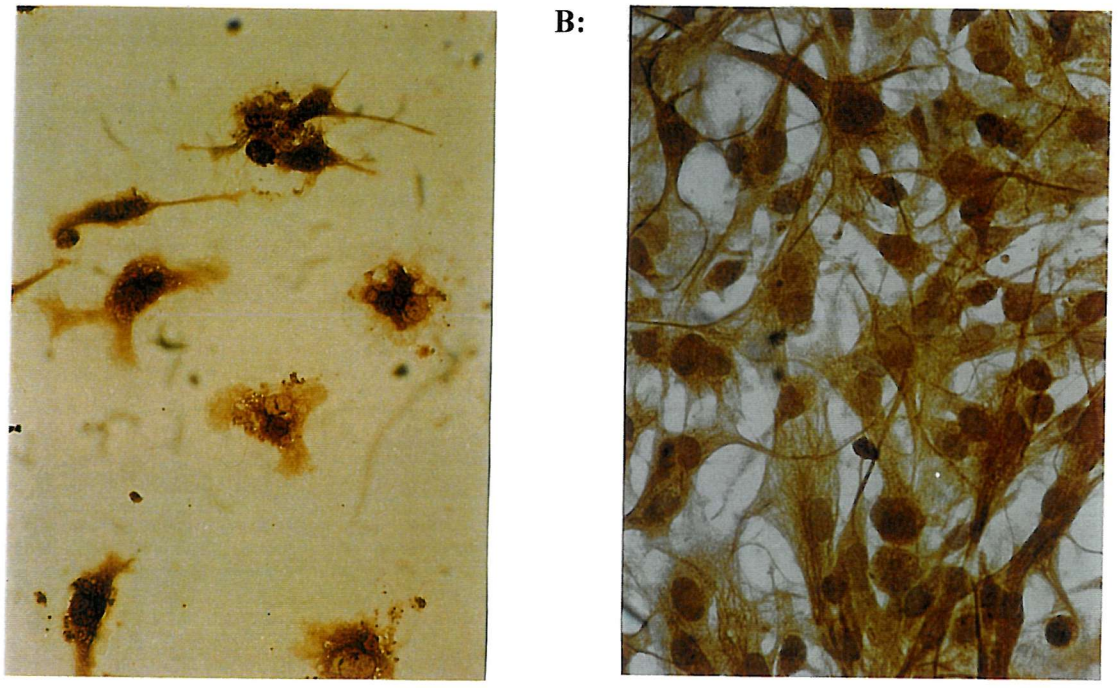


Rat HSC cultured on glass slides for 2 days (panel A and panel B) or 7 days (panel C and D) were immunostained with either a rabbit polyclonal antibody to human RARβ purchased from Santa Cruz [panel A and C (200x magnification)] or with non-immume rabbit IgG [panel B and D (200x magnification)].

To ensure the specificity of the antibodies, the cultured HSC were also incubated with a polyclonal antibody to chicken gizzard desmin (Figure 5.15.). Desmin is expressed by quiescent and activated rat HSC, thus serving as a positive control in these experiments.

Figure 5.15: Immunocytochemistry of hepatic stellate cells using a polyclonal antibody to desmin

A: **B:**

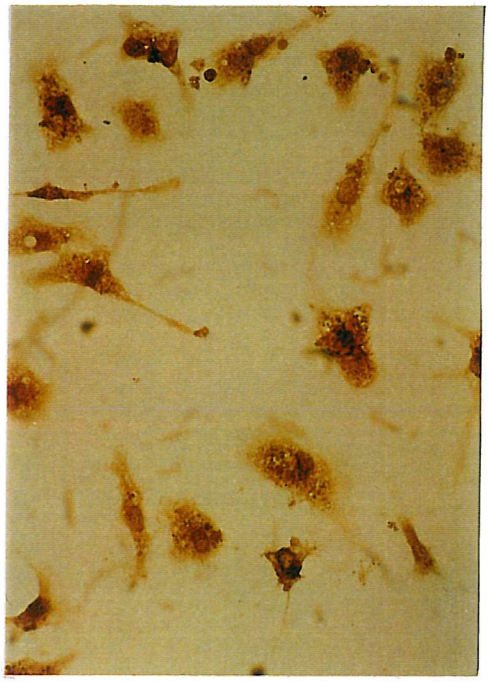


Rat HSC cultured on glass slides for 2 days (panel A) or 7 days (panel B) were immunostained with a rabbit polyclonal antibody to chicken gizzard demsin (400x magnification).

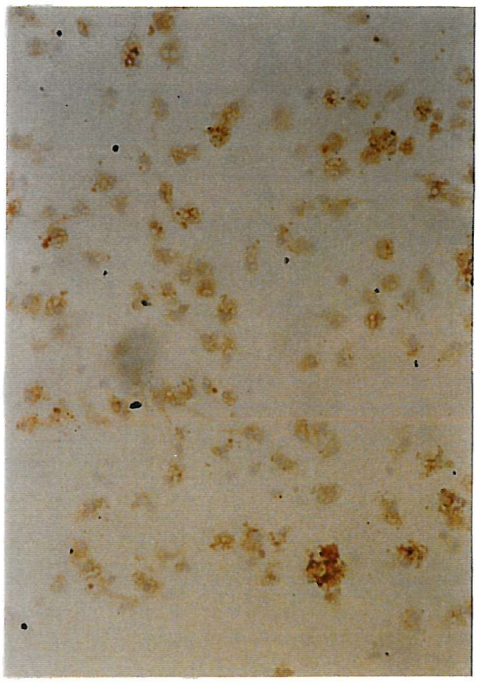
A positive immunoreaction was also revealed in rat HSC incubated with the antibody SP172 (Figure 5.16.). There was a strong nuclear staining in HSC cultured on glass slides for 2 days. However these HSC also had positive staining in the cytoplasm. The corresponding control staining was negative. Less intense staining was detected by SP172 in HSC cultured on slides for 7 days, and the staining was primarily restricted to the nuclei. However, the nuclear staining in HSC cultured for 7 days was not specific, since staining was also detected in the cells stained with rabbit serum. This immunocytochemistry data is in partial agreement with the results published by Weiner *et al.* (1992). However, the data in this paper cannot be compared directly since Weiner *et al.* (1992) performed control immunostainings with non-immune rabbit IgG and not the appropriate control, rabbit serum. Furthermore, a negative control for HSC cultured on plastic for 7 days is absent from this published report.

Figure 5.16: Immunocytochemistry of hepatic stellate cells using a polyclonal antibody to human RARβ, SP172

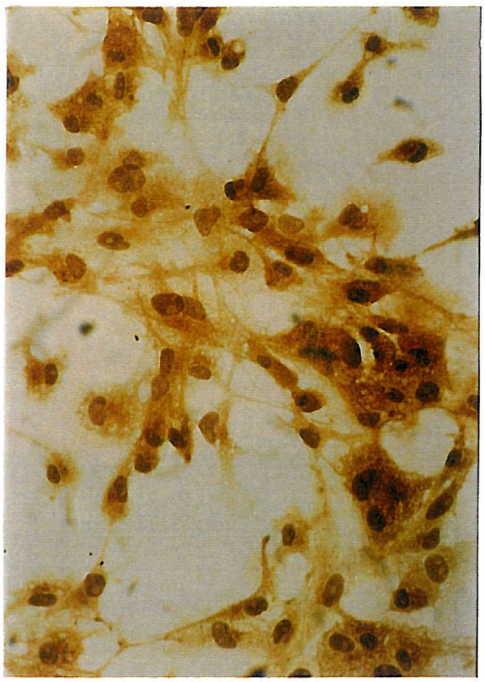
A:



B:



C:

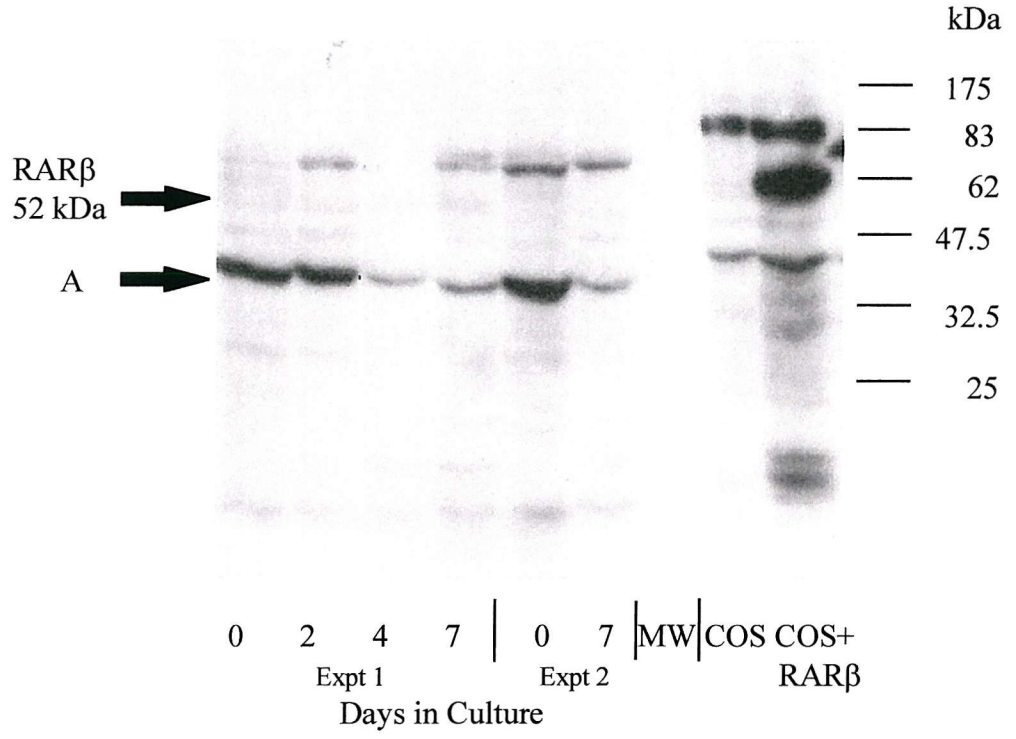


D:

Rat HSC cultured on glass slides for 2 days (panel A and panel B) or 7 days (panel C and D) were immunostained with a rabbit polyclonal antibody to human RARβ, SP172 [panel A and C (400x magnification)] or with rabbit serum [panel B (200x magnification) and panel D (400x magnification)].

To establish a possible reason why the results obtained by Weiner *et al.* (1992) differed from the results using the RARβ antibody obtained from Santa Cruz, western blots were incubated with the rabbit polyclonal RARβ antibody, SP172. This antibody did not detect any protein in rat HSC at the correct molecular weight (52 kDa) for RARβ (Figure 5.17.). Only in the positive control, extracts from COS-1 cells transfected with mouse $RAR\beta_2$ expression vector, was RARβ of correct molecular weight recognised by the antibody. It is noteworthy however, that antibody SP172 detected a lower molecular weight protein of approximately 35 kDa in rat HSC (band A, Figure 5.17.) and the intensity of this band decreased with activation of HSC. The detection of this artefactual band could possibly explain the decrease in nuclear staining observed in activated rat HSC compared with freshly isolated rat HSC when SP172 was used in immunocytochemistry by Weiner *et al.* (1992).

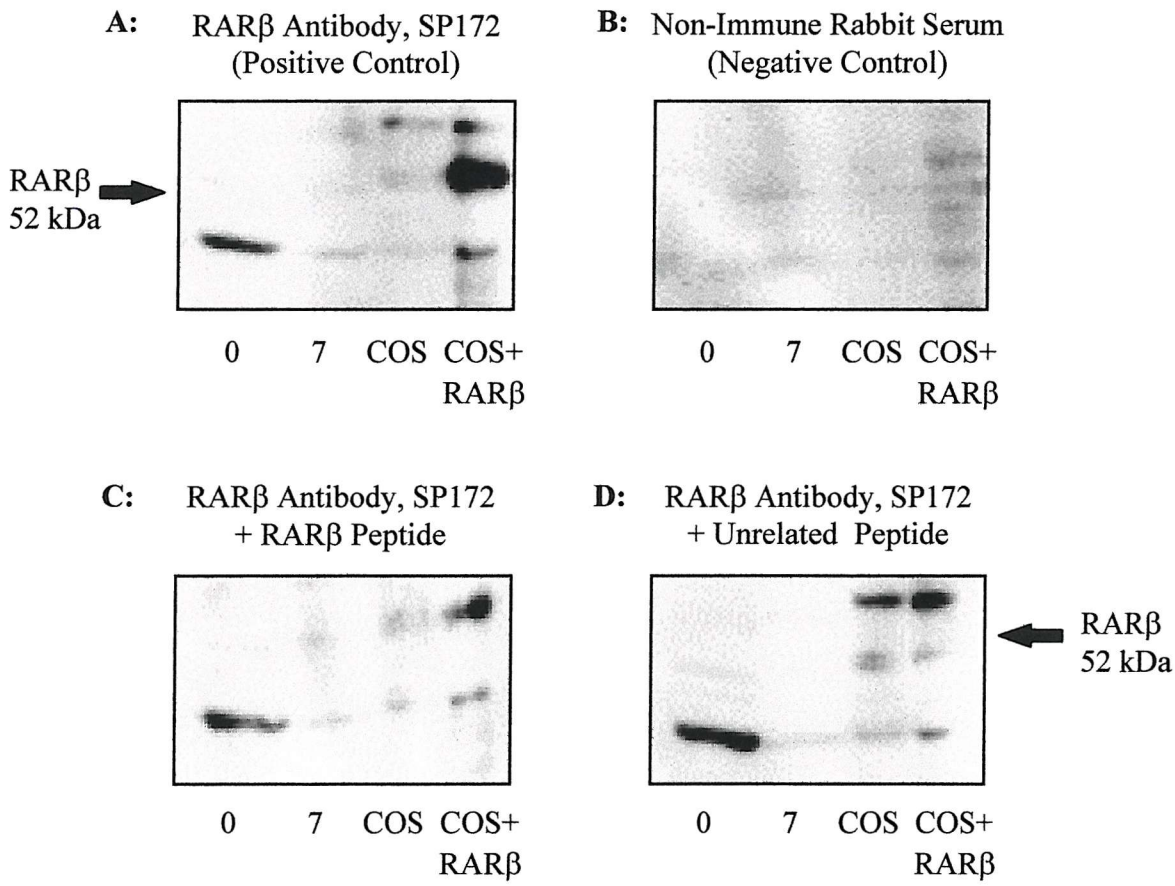
Figure 5.17: Analysis of RARβ protein in hepatic stellate cells by western blotting using a polyclonal antibody to human RARβ, SP172



The same western blot shown in Figure 5.6. was stripped of antibody and re-probed using a polyclonal antibody to human RARβ (SP172) to analyse RARβ protein levels in cell lysates from freshly isolated rat HSC (day 0) and rat HSC cultured on plastic. The lysates of COS-1 cells, transfected with plasmid containing mouse $RAR\beta_2$ cDNA were used as a positive control. Similar results were found in 3 further experiments (Expt). Band A indicates the major protein recognised by SP172 in rat HSC.

To determine whether the RARβ antibody, SP172 specifically reacts with RARβ protein, competition assays were performed (Figure 5.18.). Immunoadsorption of the SP172 RARβ antibody with a 10-fold excess of a RARβ peptide which contained the same sequence (PSISPSSVENSGVSQSPLVQ) used by Professor Chambon to generate SP172, did not effect the intensity of the 35 kDa signal detected in freshly isolated rat HSC. However, the 52 kDa signal detected in COS-1 cells transfected with mouse $RAR\beta_2$ expression vector was no longer seen when the antisera was immunoadsorbed with the corresponding peptide. A similar competition experiment with an unrelated peptide did not effect the intensity of this signal, indicating that SP172 specifically recognises mouse but not rat RARβ protein.

Figure 5.18: Analysis of RARβ protein in hepatic stellate cells using a competing peptide specific to the polyclonal antibody for human RARβ, SP172



Western blot analysis of whole cell lysates of freshly isolated rat HSC (day 0), HSC cultured for 7 days on plastic and COS-1 cells either mock transfected or transfected with mouse $RAR\beta_2$ expression vector. Blots in panels A and C were incubated with RARβ antibody (SP172) without or with previous immunoadsorption with RARβ peptide (specific to SP172) respectively. Panel D shows a blot incubated with RARβ antibody previously immunoadsorped with a non-immunogenic peptide. The blot in panel B was incubated with non-immune rabbit serum.

5.4. ANALYSIS OF RARE BINDING ACTIVITIES IN HEPATIC STELLATE CELLS BY GEL MOBILITY SHIFT ASSAYS

Although the presence of RARβ protein has been demonstrated, it is important to determine whether the receptor is functional. Therefore experiments were performed to examine the DNA binding activity of RARβ in HSC.

To analyse the functional activity of RARβ, EMSA were performed using [^{32}P]-labelled oligonucleotides (RARE) recognised by RAR proteins (section 2.15.). The RARE binding activity was examined in rat nuclear protein HSC extracts (10 μg) during culture activation (Figure 5.19.). After incubation, the reaction mixture was resolved on a non-denaturing polyacrylamide gel and the gel subjected to autoradiography. The EMSA revealed a single band, representing total RARE binding activity in HSC nuclear protein extracts (it is not possible to discriminate between RAR subtypes using EMSA as they all bind to the oligonucleotide).

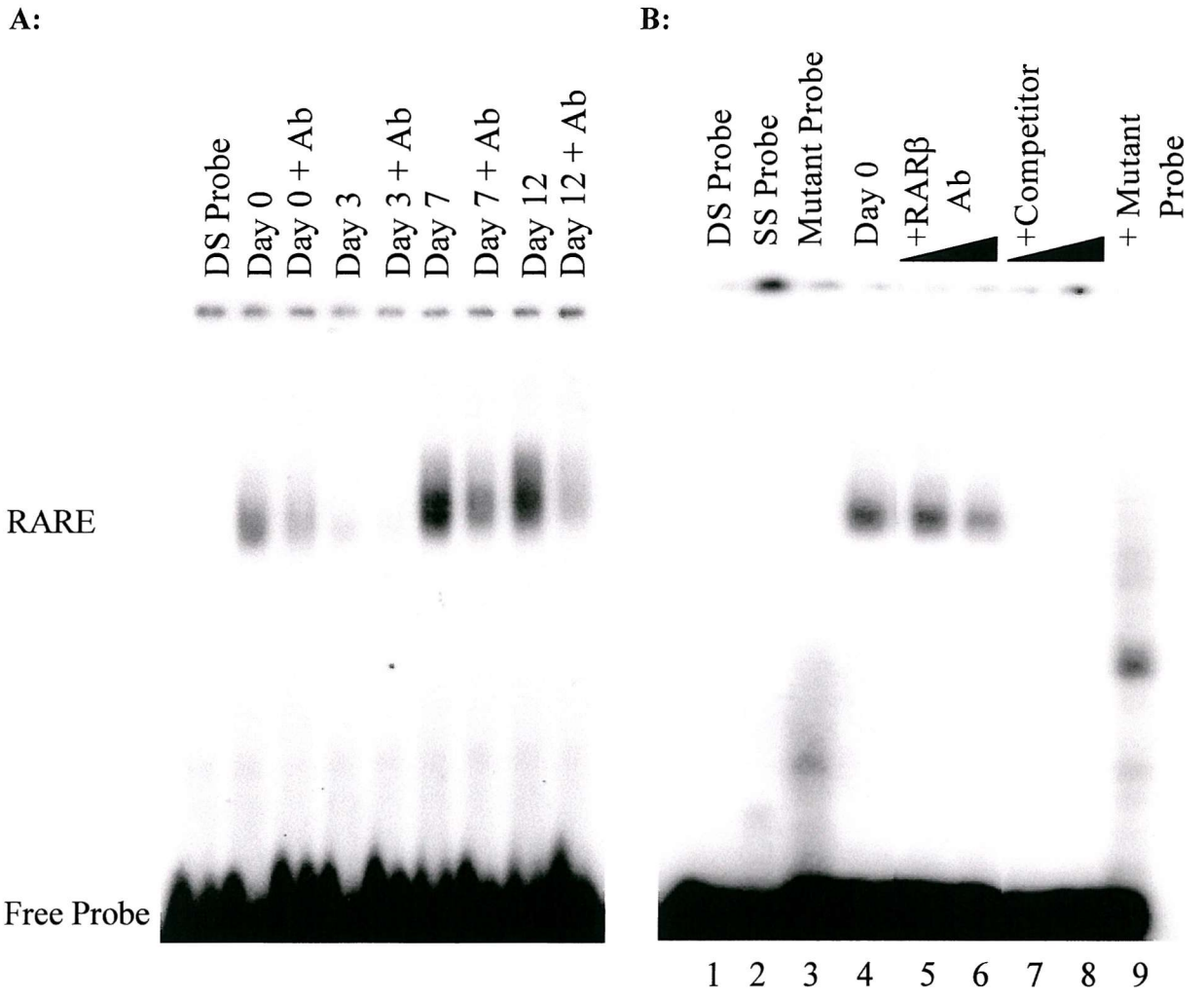
The EMSA broadly agreed with the western blotting analysis shown in Figure 5.6., demonstrating that although there was a transient reduction at day 3, abundance of total RAR protein increased in activated (day 7 and day 12) HSC (Figure 5.19., panel A). Studies using blocking antibody to RARβ (supershift assays) showed that this protein was a major contributor to overall RARE binding activity, particularly in activated HSC since the band diminished in intensity in the presence of the antibody.

Diminution of RARE binding activity by anti-RARβ was confirmed in a further experiment (Figure 5.19., panel B). The specificity of RARE binding activity was tested by competition experiments using an unlabelled consensus oligonucleotide in 10-fold or 100-fold excess of the [^{32}P]-labelled consensus oligonucleotide. The single band was completely eliminated. Additionally, when the [^{32}P]-labelled RARE probe was replaced with a [^{32}P]-labelled mutant RARE probe, the band intensity of the previously shifted complexes was decreased. In both experiments this provides evidence of binding specificity.

The use of neutralising RARβ antibodies in the EMSA confirms the RARβ protein receptor contributes to overall RARE binding in activated HSC and further supports the observation that although RARβ mRNA decreases with HSC activation, expression of the cognate ligand

increases.

Figure 5.19: Electromobility shift assay for RARE binding in hepatic stellate cells



Nuclear protein extracts from freshly isolated rat HSC (day 0) or cultured rat HSC were analysed for RARE binding activities by EMSA (panel A) in the presence or absence of an antibody (Ab) against RARβ (Santa Cruz). The specificity of RARE binding activity was tested using an unlabelled probe in 10-fold (lane 7) or 100-fold (lane 8) excess of [^{32}P]-labelled probe (panel B) and also by replacing [^{32}P]-labelled RARE probe with a [^{32}P]-labelled mutant RARE probe (lane 9). Lanes labelled as DS (double stranded) probe, SS (single stranded) probe and mutant probe, were run with labelled probe without nuclear extracts. Experiments were repeated at least 3 times with extracts prepared from separate cell cultures and gave similar results.

5.5. DISCUSSION

The mechanism by which retinoids regulate the growth and differentiation of normal and fibrotic HSC has not been clearly elucidated but it is thought that these effects require binding to nuclear retinoid receptors. There are 2 families of nuclear retinoid receptors, RARs and RXRs and each receptor family consists of three genes, α, β and γ that generate multiple RAR and RXR isoforms using two promoters (P1 and P2) and alternative splicing [for review see Chambon (1996)]. Both RARs and RXRs are ligand-inducible transcription factors that modify the expression of particular genes by binding to specific DNA sequences, RAREs. To determine the role of retinoids in HSC activation and fibrogenesis requires knowledge of the expression of the RARs and RXRs as HSC become activated. The retinoid receptors are a diverse family of proteins and cannot be feasibly examined in their totality. Therefore, it was decided to concentrate on the RAR family, especially RARβ as at the time this work was initiated, there was evidence that expression of this receptor altered dramatically as HSC became activated *in vitro*.

Concerning the expression of RARβ mRNA, the results from the semi-quantitative RT-PCR agreed with the findings from northern hybridisation; RARβ mRNA was reduced in cultured rat HSC compared with freshly isolated HSC. This suggests a down regulation of the expression of RARβ mRNA during the transformation of the HSC to a myofibroblastic phenotype. This is the first known report that has used RT-PCR to detect a change in the level of RARβ mRNA expression in cultured rat HSC isolated from a normal rat.

Two previous studies show results that are compatible with the mRNA studies. Weiner *et al.* (1992) considered the possibility that reduced or altered expression of one or more of the nuclear retinoid receptors may accompany retinoid release by HSC and result in enhanced fibrotic development. They suggested that as the receptors confer retinoid responsiveness to cells, differences in their relative expression might alter responsiveness. Weiner *et al.* (1992) analysed RARβ expression by northern blot analysis in freshly isolated HSC from normal rats, HSC isolated from CCl_4-treated rats, and primary rat HSC which had been cultured on uncoated plastic for 7 days. The second study by Ohata *et al.* [1997 (published whilst the studies in this Chapter were in progress)], used RT-PCR to assess RARβ mRNA expression in HSC isolated from rats with cholestatic liver fibrosis. In both studies the mRNA for RARβ was diminished in activated HSC. Contrary to the above investigators and to the results

obtained in this study, Friedman *et al.* (1993) detected RARβ mRNA by northern hybridisation in primary HSC which had been activated by culture for 4 days on uncoated plastic.

In addition to HSC, the liver also expresses several other cell types, including parenchymal cells (hepatocytes) and Kupffer cells (macrophages). The parenchymal cells account for 85-90% of the total liver mass and the Kupffer cells comprise approximately 17% of the number of non-parenchymal cells. Measurement by northern blotting demonstrated expression of RARβ mRNA in both these liver cells. According to other reports, RARβ is the predominant RAR in parenchymal cells and HSC (Ulven *et al.,* 1998). In Kupffer cells, all RARs are equally expressed (Ulven *et al.,* 1998; Ohata *et al.,* 2000). Since HSC only represent approximately 8% of the cells in the liver, it is feasible that hepatocytes are the major source of RARβ mRNA in the liver. Weiner *et al.* (1992) who also studied the expression of RAR subtypes using northern blot analysis, observed no detectable mRNA for RARβ in rat liver parenchymal cells or Kupffer cells. Since very similar protocols were used in this study and the one performed by Weiner *et al.* (1992) the disagreement is difficult to explain.

Whilst measurement of mRNA provides information about expression of RARs, it is ultimately expression and regulation of the RAR proteins which is important. Although there are several *in situ* hybridisation and northern blot studies on the distribution of RAR transcripts in the liver, there are very few studies on the distribution of the RAR proteins. Therefore, it is uncertain if transcript levels accurately reflect receptor protein levels. Hence to examine thoroughly the role of the RARs, it was necessary to determine whether the RAR mRNA levels in quiescent and activated HSC could be extrapolated to expression of the endogenous RARα and RARβ protein levels. This was performed using specific polyclonal antibodies in combination with a variety of biochemical methods.

By western blotting, the RARα protein in cultured cells was detected as a protein triplet. This suggests either the possible occurrence of post-translational modifications, such as phosphorylation which is known to alter the electrophoretic mobility of proteins, or the presence of different isoforms of the RARα protein. In western blots containing extracts from both the mouse F9 teratocarcinoma cell line and the human neuroblastoma SH-SY5Y cell line, the RARα protein has also been detected as more than one protein band (Ali *et al.,* 1992).

The major, unexpected finding of this study is that although RARβ mRNA decreases with activation of HSC, expression of the cognate RARβ protein (detected by western blotting, immunoprecipitation and immunocytochemistry) appears to be up-regulated in both human and rat HSC. Use of neutralising RARβ antibodies in EMSA studies confirmed that this receptor made an appreciable contribution to overall RARE binding activity in nuclear extracts from activated HSC. This is also the most direct measurement of RARβ transcriptional regulatory activity.

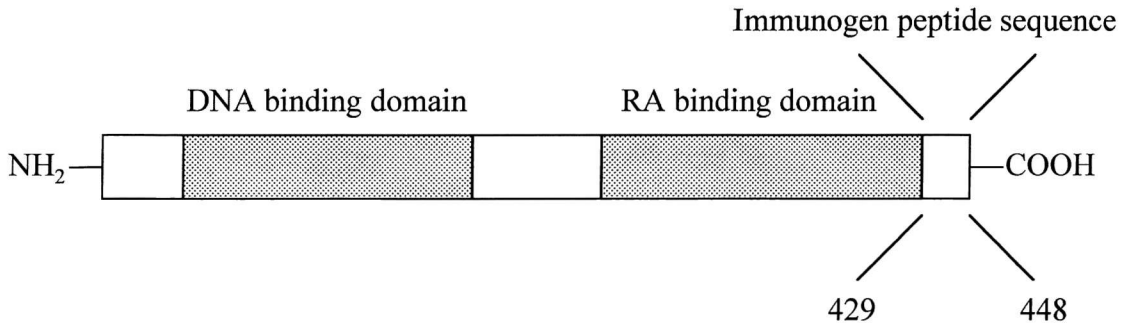
The RARβ protein data contradict the further observations by Ohata *et al.* (1997) who found RARE binding activity was reduced in HSC from cholestatic liver compared with those from normal liver. Currently, the reason for this difference is unclear, but may arise from the use of different models of HSC activation making comparison difficult. Weiner *et al.* (1992) for example, found CRBP mRNA levels to increase in cultured rat HSC yet remain unchanged in HSC isolated from CCl_4 rats. There is further evidence that HSC activation *in vitro* is different from *in vivo*. De Bleser *et al.* (1997) have shown that HSC activated *in vitro* make different TGFβ isoforms compared to those *in vivo*.

The finding that RARβ protein is increased in activated HSC also contradicts the immunocytochemistry observations by Weiner *et al.* (1992) shown in Figure 5.13., apparently showing decreased RARβ protein in culture activated HSC. However, my studies have found that the antibody (SP172) used in the immunocytochemical studies conducted by Weiner *et al.* (1992) to detect RARβ protein in rat HSC, does not recognise rat RARβ protein. A lower molecular weight protein (35 kDa) was consistently detected by SP172, which decreased in expression following HSC activation. However, this species is not derived from RARβ since immunoadsorption of SP172 with an excess of peptide specific to SP172 did not effect the intensity of this band. As immunocytochemistry cannot discriminate between different antibody binding proteins, my western blotting studies therefore provide an explanation for the results obtained by Weiner *et al.* (1992).

It is noteworthy that the amino acid sequence of the RARβ peptide that was used to generate the SP172 antibody showed only a very minor variation to that used to produce the Santa Cruz antibody (Figure 5.20.). Both RARβ antibodies were raised against amino acids at the C-terminus, a region common among RARβ isoforms. However, the minor differences

between the peptides, seems to determine whether the antibody will recognise RARβ protein in the rat or the mouse, as SP172 was able to readily detect mouse RARβ which had been expressed in COS cells (Figure 5.17.)

Figure 5.20: Functional domain structure of the RARβ receptor



Numbers flanking the peptide sequences correspond to the position of the amino acid residues in the sequences of the RARβ peptides.

Antibodies	Amino acid sequence (single letter code) of the synthetic peptides
Polyclonal antibody to human RARβ (Santa Cruz)	-SISPSSVENSGVSQSPLVQ 430 448
Polyclonal antibody to human RARβ (SP172) Antibody used by Weiner *et al.* (1992)	PSISPSSVENSGVSQSPLVQ 429 448
Polyclonal antibody to mouse RARβ (Affinity Bioreagents)	PSVSPSSVENSGVSQSPLLQ 429 448

The finding that RARβ mRNA decreases with activation of HSC but expression of the protein increases, implies that RARβ protein expression in activated HSC is dissociated from steady state mRNA, suggesting that post-transcriptional mechanisms may play a major role in regulating this protein in these cells. In quiescent HSC, detection of RARβ mRNA may arise from the requirement that mRNA needs to be translated in order for its degradation to occur (Sachs, 1993).

An enhanced RARβ protein expression in the absence of a corresponding change in mRNA may be due to increased efficiency of mRNA translation or prolongation of the half-life of the protein. Of relevance to the latter mechanism, it has been demonstrated that intracellular proteases play an important role in regulating the half-life of RXR protein in HuH7 cells (Matsushima-Nishiwaki *et al.*, 1996a and 1996b). Whether this represents an important post-

transcriptional mechanism regulating retinoid receptors in other cell types, including HSC should be investigated (see Chapter 7). It is possible that the 52 kDa protein observed by western blotting represents post-translationally modified RARβ_2 which resists degradation. Other investigators for example have described phosphorylation of the mouse RARβ_2 during *in vitro* translation in rabbit reticulocyte lysates (Rochette-Egly *et al.*, 1992).

Although a discrepancy between the up-regulation of protein levels in the absence of a corresponding change in mRNA is unusual, such a control mechanism does exist for some genes. For example, in M2 melanoma cells the levels of the integrin subunit α_1 protein were found to be higher in cells cultured on an EHS matrix than on plastic but the reverse was not true for the amounts of mRNA (Delcommenne and Streuli, 1995). More recently, and of more relevance, Sommer *et al.* (1999) published evidence that RARβ_2 and RARβ_4 are post-transcriptionally regulated in breast epithelial cells. In an analogy to this study, these authors could only detect RARβ_2 and RARβ_4 mRNA expression by RT-PCR and not by northern analysis in breast tumour epithelial cells. However, the RARβ_2 and RARβ_4 proteins in these cells were at levels comparable to those cells with relatively high levels of RARβ_2 and RARβ_4 mRNAs. Additionally, since normal human mammary epithelial cells express RARβ_4 mRNA but not RARβ_4 protein, the authors suggest there is differential protein synthesis or stability for RARβ_4 between normal epithelial cells and breast cancer cells.

The mechanism for this type of control is not clear but Sommer *et al.* (1999) suggest upstream reading frames may provide a level of regulation for these genes. To initiate translation, the 40-S of eukaryotic ribosomes binds to the capped 5' end of mRNA and initiates translation by scanning for the first AUG sequence. Translation then proceeds until an in-frame stop codon is reached. Most eukaryotic mRNAs have a short 5' untranslated region and no AUG sequences upstream of the translational start site; this feature ensures efficient translation. However, approximately 5-10% of all eukaryotic mRNAs, particularly those encoding regulatory proteins (i.e. tumour suppressors and cell cycle control genes) have complex leader sequences that contain upstream open reading frames. The RARβ_2 mRNA has a long 5' untranslated region that contains 5 partially overlapping upstream open reading frames that precede the major open reading frame. Zimmer *et al.* (1994) showed that introducing mutations in the start/stop codons of the upstream open reading frames effected expression of the downstream RARβ_2 open reading frames. This suggests the upstream open reading

frames act at the level of translation and the presence of AUG sites in the 5' leader sequences is inhibitory to translation at downstream coding regions.

Based on the study by Zimmer *et al.* (1994), Sommer *et al.* (1999) hypothesised that for RARβ$_4$ translation to occur, initiation of upstream open reading frames must be bypassed. Two upstream open reading frames in human RARβ$_4$ begin with AUGs within a strong context for translation initiation but are out-of frame for the RARβ translation start site; translation of either of these upstream open reading frames would consequently proceed past this translation start site. For RARβ$_4$ translation to occur, initiation at these upstream open reading frames must therefore be bypassed. The authors hypothesise that the lack of RARβ$_4$ protein in normal human mammary epithelial cells results from tight inhibition of translation through these upstream open reading frames. Conversely the presence of RARβ$_4$ protein in breast tumour epithelial cells with low transcript levels may indicate that these cells have mechanisms of escaping this translation control.

Applying their hypothesis to the results obtained in this Chapter, the lack of RARβ protein in the presence of readily detectable mRNA in quiescent HSC may result from tight inhibition of translation as a consequence of upstream open reading frames in the RARβ gene. Conversely, the presence of RARβ protein with low transcript levels in activated HSC could result from activated HSC having evolved mechanisms to prevent this translational control. In support of this hypothesis, it has been shown that RARβ$_2$ upstream open reading frames translationally regulate the mouse RARβ$_2$ gene (Zimmer *et al.*, 1994). An alternative possibility is that RARβ mRNA may be segregated into separate cytoplasmic pools that are either translatable or non-translatable, as occurs for some cytoskeletal proteins (Fulton and L'Ecuyer, 1993). As HSC become activated, there may be an increase in the mRNA directed to the translatable cytoplasmic pool.

The detection of RARβ in activated human HSC by western blotting has further demonstrated that these findings can be extrapolated to humans. However, the *in vivo* implications of the findings in this study with respect to the overall hepatic injury response remain to be determined. Animal models are indispensable for the study of the cell biology and pathobiochemistry of liver fibrosis, since studies in cell culture are unlikely to reflect the influences of complex intercellular interactions *in vivo*. Furthermore, fibrogenesis is a tissue

phenomenon which does not depend solely on changes in a single cell type. However, by various parameters, the *in vitro* model of HSC attached to uncoated plastic provides an accurate recapitulation of the process of HSC activation *in vivo* which is a common ultimate pathway leading to fibrosis, regardless of the initiating factor for liver injury.

In order to bind to DNA and regulate gene transcription, an RAR needs to form a heterodimeric complex with an RXR. To understand the relevance of a change in the level of RARβ protein during activation of HSC *in vivo*, it is therefore important to analyse RXRs. However, it is attractive to speculate that the rise in RARβ expression during HSC activation has a physiological relevance that may have a feedback role in preventing or contributing to the development of liver fibrosis and this is supported by studies in Chapter 6.

CHAPTER 6

Functional Studies of Retinoic Acid Receptor Beta in Hepatic Stellate Cells

6.1. INTRODUCTION

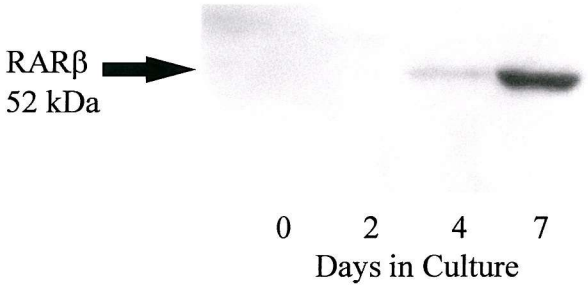
Various RAR and RXR subtypes and isoforms are expressed in distinct patterns throughout development and in the mature organism (Dolle *et al.,* 1989). Furthermore, genes encoding these receptors show greater conservation between species than between each other (Chambon, 1996). These observations suggest that each of the RAR and RXR subtypes, together with their respective isoforms mediate unique, non-overlapping biological functions.

Although my studies have shown that RARβ protein is upregulated in activated HSC (Chapter 5), they provide no information on the possible relevance of this protein to HSC activation. The escalating expression of RARβ protein during HSC activation follows a similar time course to HSC proliferation and α-smooth muscle actin protein expression (Figure 6.1.), both well documented markers of HSC activation (section 1.7.3.). This raises the possibility that RARβ may be involved in regulating specific genes which mediate the activation of HSC and could thereby play an important role in either the progression or suppression of fibrogenesis. Consequently, the observation that RARβ protein is increased during activation of HSC has been exploited and the role of the elevated levels of RARβ protein upon growth and differentiation of HSC has been examined using a variety of molecular biology techniques.

Figure 6.1: Time course of RARβ protein expression in comparison with markers of hepatic stellate cell activation

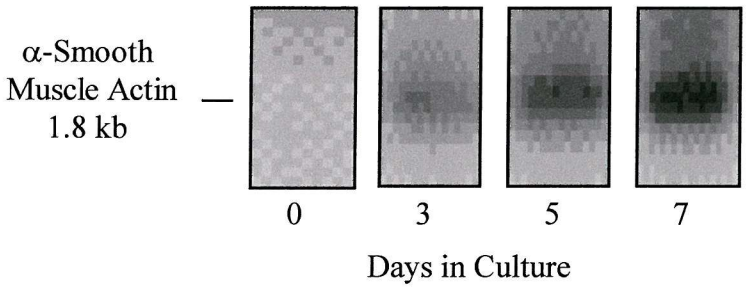
A:

RARβ protein expression (western blot)



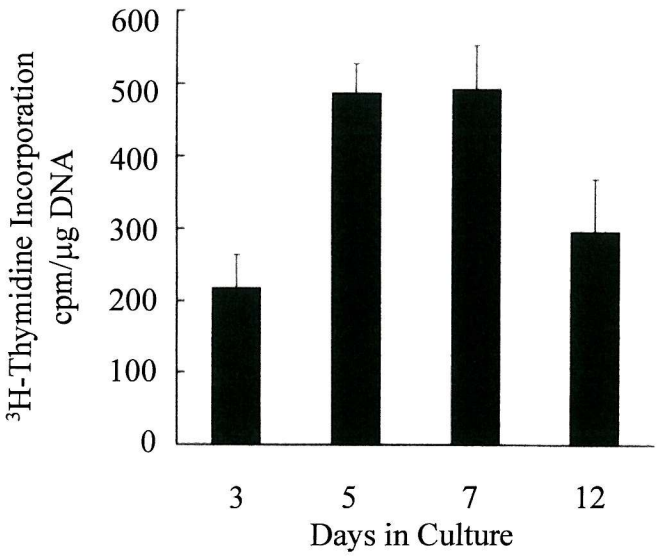
B:

α-Smooth muscle actin mRNA expression (northern blot)



C:

Hepatic stellate cell proliferation



RARβ protein levels in cell lysates from freshly isolated rat HSC (day 0) and rat HSC cultured on plastic were analysed by western blotting using an antibody to RARβ [panel A (section 5.3.1.)]. Total RNA from freshly isolated rat HSC (day 0) and rat HSC cultured on plastic were analysed for expression of α-smooth muscle actin mRNA using northern hybridisation [panel B (section 5.2.2.)]. The rate of rat HSC proliferation at various times in culture was assessed by thymidine incorporation [Data presented are mean ± S.E.M. from 3 separate experiments (panel C)].

6.2. TRANSFECTION OF HEPATIC STELLATE CELLS WITH A RARβ EXPRESSION VECTOR

To study the possible role of RARβ in HSC activation, attempts were made to overexpress this protein in HSC by transfecting a expression vector bearing the RARβ cDNA under the control of a simian virus 40 (SV40) promoter into non-expressing quiescent rat HSC. The consequences upon proliferation and collagen synthesis could then be assessed.

However, before determining the effects of overexpressing RARβ in rat HSC, one obstacle to overcome is the ability to transfect HSC. To date, very few groups have successfully transfected (transiently or stably) HSC with a high transfection efficiency. Since various parameters will be measured in HSC transfected with RARβ, it is important to have a high transfection efficiency to represent a homogenous population. A positive control plasmid, pHook^{TM}-2*lacZ* (Invitrogen) was used to optimize and assess transfection efficiencies (section 2.17.). This plasmid contains the *E.coli* gene encoding β-galactosidase. Successful transfections will result in β-galactosidase expression which can be monitored using a colorimetric assay. Cells expressing β-galactosidase will turn blue in the presence of X-gal. Comparison of the number of blue cells (transfected) versus non-blue cells (untransfected) will allow determination of the transfection efficiency.

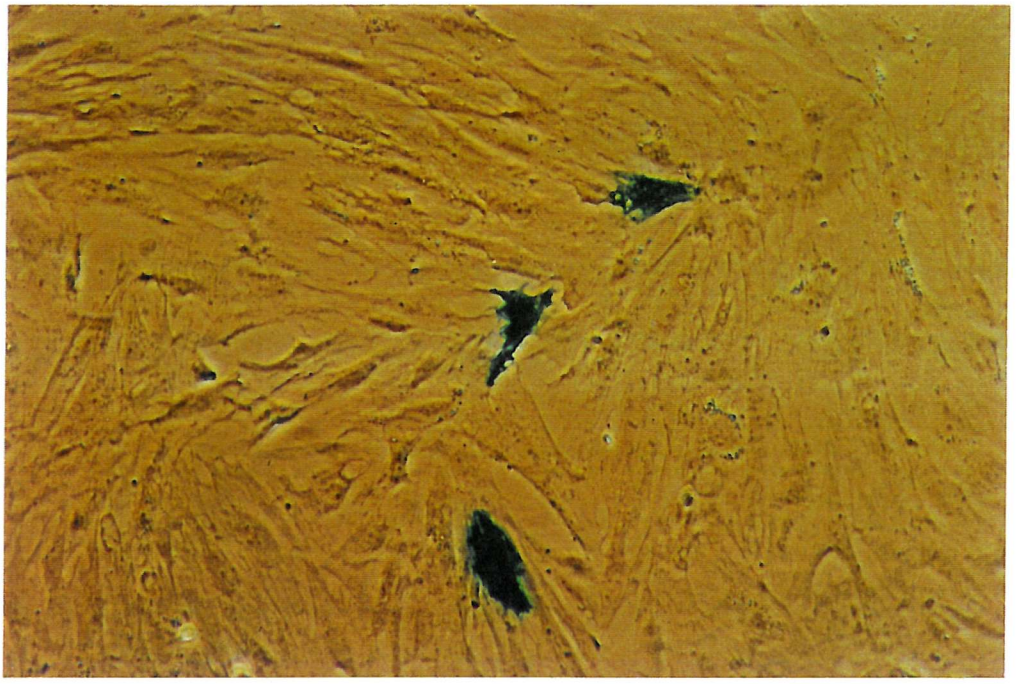
The transfection of primary HSC proved to be very difficult despite applying various protocols, using different transfection reagents and conditions, and attempting to transfect the primary HSC at various stages during culture (2, 4, 7 and 12 days). Only when passaged HSC were used in transfection experiments were any blue cells detectable upon staining with X-gal (Figure 6.2.). The efficiency of transfection obtained with passaged HSC however, is still too low to warrant any further studies using whole cell populations. In addition, as these cells express RARβ, a different approach of transfecting with antisense RARβ would be more rational.

Despite the lack of success in obtaining a high transfection efficiency, the role of RARβ in regulating HSC proliferation and activation can be determined by using a number of synthetic retinoids. Several retinoid analogues have been designed to specifically bind and transcriptionally activate individual isoforms of RARs and RXRs. In addition, selective RAR and RXR antagonists have been synthesised which can be employed to investigate the effect of

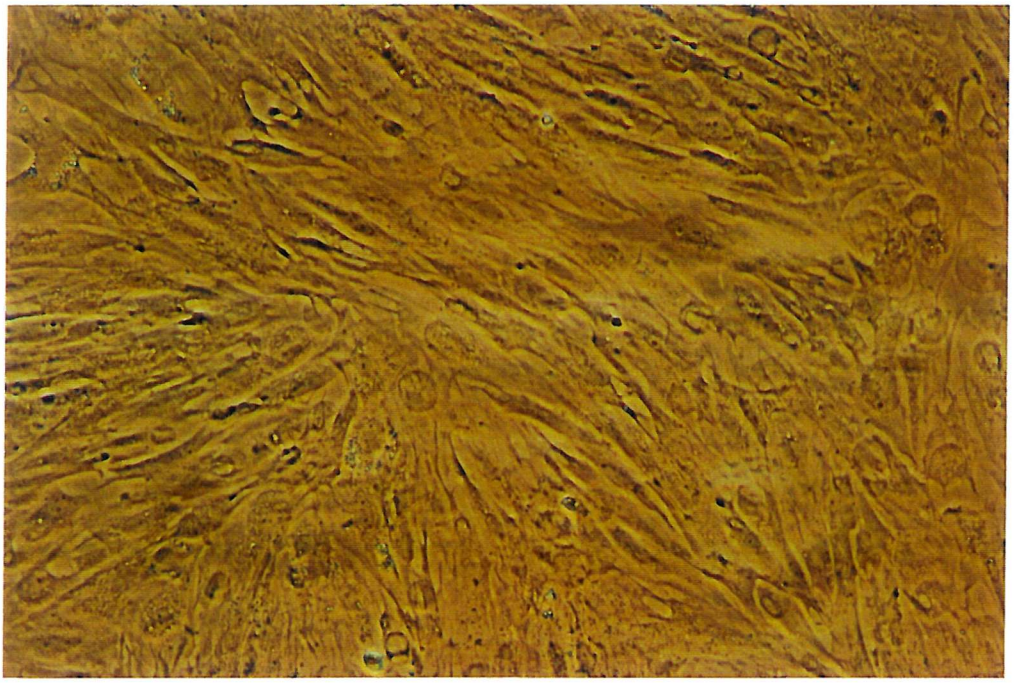
blocking the functional activity of retinoid receptors.

Figure 6.2: A light micrograph of transfected hepatic stellate cells

A:



B:



Passaged rat HSC cultured on uncoated plastic were either transfected with a β-galactosidase expression vector (Panel A) or were mock transfected (Panel B). The cells were subsequently stained with X-gal and photographed (400x magnification).

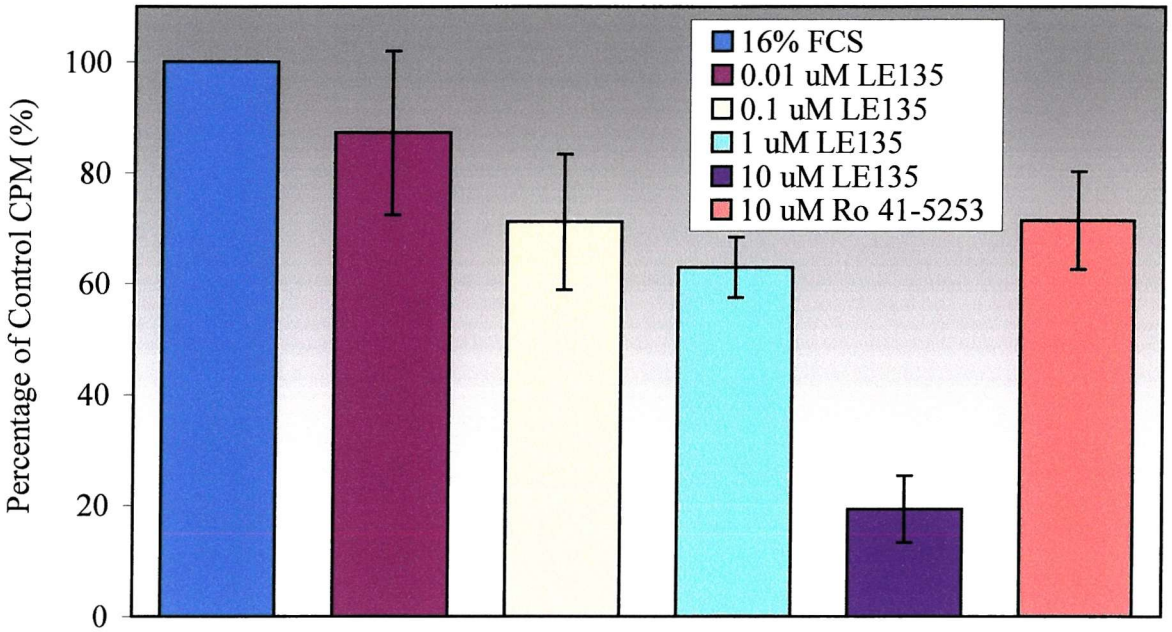
6.3. THE ROLE OF RARβ IN HEPATIC STELLATE CELL PROLIFERATION

6.3.1. [*methyl*-^{3}H]-Thymidine Incorporation

To establish the involvement of RARβ in HSC proliferation, an antagonist with a high selectivity for RARβ [LE135 (Umemiya *et al.*, 1997; Imai *et al.*, 1997)] was employed. Primary rat HSC cultured for 6 days on plastic were incubated either in the presence of the RARβ antagonist or 0.2% ethanol (control cultures) for 24 hours. The rate of proliferation was assessed by incorporation of [*methyl*-^{3}H]-thymidine into nuclear DNA (section 2.3.). Due to different degrees of absolute incorporation between separate experiments, data were expressed as a percentage of control cells.

This assay demonstrated effective and statistically significant inhibition ($P < 0.05$) of HSC proliferation in the presence of 10 μM LE135 (inhibition to 19.4% ± 6.1) and 1 μM LE135 (inhibition to 62.9% ± 5.4) compared to untreated control HSC (Figure 6.3.). Furthermore, the effect was concentration related. To investigate whether the observed inhibition of proliferation in HSC was RAR subtype specific, the experiment was also performed with a selective antagonist directed against RARα [Ro 41-5253 (Imai *et al.*, 1997; Brembeck *et al.*, 1998)]. The exposure of HSC to the RARα antagonist (10 μM) routinely caused inhibition of proliferation (inhibition to 71.4% ± 8.8) but the effect was significantly less ($P < 0.05$) than that detected with LE135 (inhibition to 19.4% ± 6.1 with 10 μM LE135, compared to inhibition to 71.4% ± 8.8 with 10 μM Ro 41-5253). This indicates that RARβ is specifically responsible for the observed decrease in the rate of HSC proliferation.

Figure 6.3: Effect of incubation of an RARβ selective antagonist on hepatic stellate cell proliferation



Rat HSC cultured for 6 days, were incubated for 24 hours with either different concentrations of an RARβ selective antagonist (LE135) or an RARα selective antagonist (Ro 41-5253) and the rate of proliferation assessed by thymidine incorporation. Data are expressed as percent of control, 16% FCS. Data presented are mean ± S.E.M. from 3 separate experiments. $P < 0.05$ for difference between 10 μM and 1 μM LE135 and control.

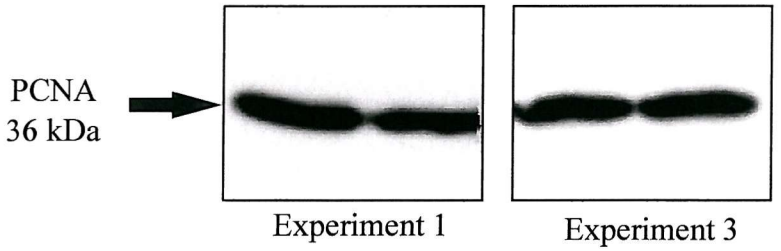
6.3.2. Proliferating Cell Nuclear Antigen

Since the protein PCNA, also known as cyclin is synthesized in early G_1 and S-phases of the cell cycle (Woods *et al.*, 1991), the inhibition of HSC proliferation caused by the RARβ antagonist (section 6.3.1.) may also be reflected by a reduction in the level of expression of this protein. Primary rat HSC were cultured on plastic for 5 or 6 days before incubation with the RARβ antagonist LE135 (10 μM) for 48 hours or 24 hours respectively. Equal amounts (1.5 μg of DNA) of whole cell lysates were subjected to electrophoresis on a 12.5% denaturing SDS-polyacrylamide gel (section 2.9.) and western blotted onto PVDF (section 2.10.). The PCNA protein was examined using a mouse monoclonal antibody directed against PCNA, followed by incubation with anti-mouse IgG conjugated to horse-radish peroxidase. The proteins were visualised by ECL.

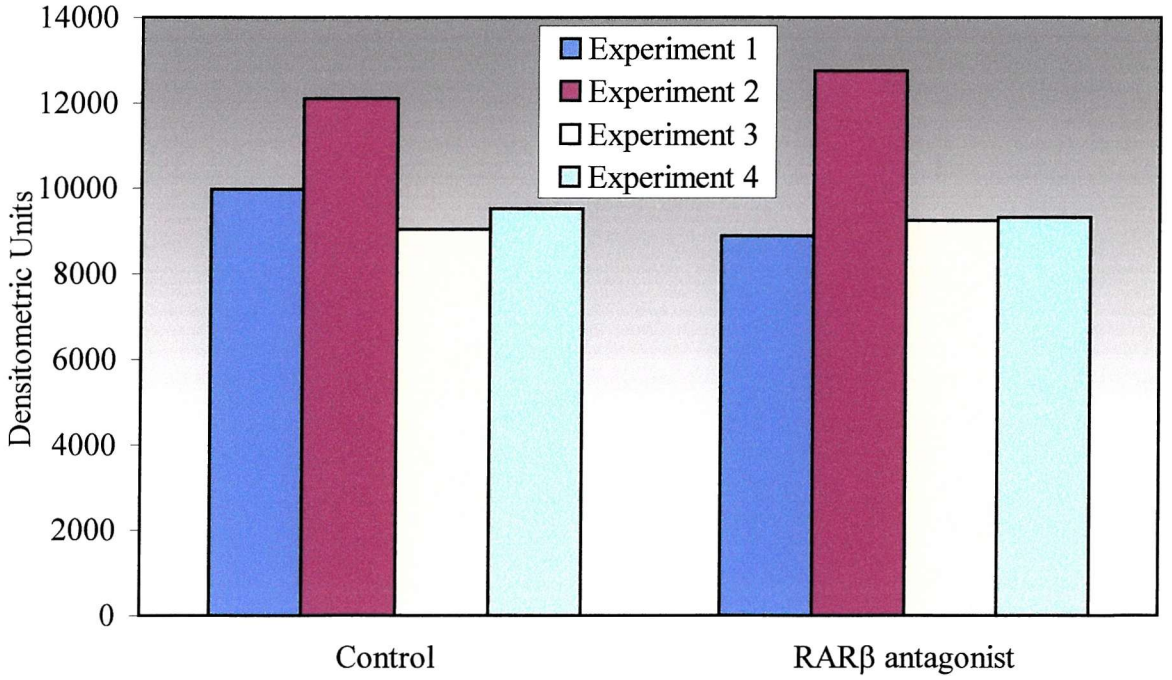
Despite LE135 reducing the proliferation rate of HSC, the PCNA protein was not effected by this compound, either after exposing HSC to LE135 for 24 hours or 48 hours (Figure 6.4.).

Figure 6.4: Effect of an RARβ selective antagonist on proliferating cell nuclear antigen protein expression in hepatic stellate cells

A:



B:

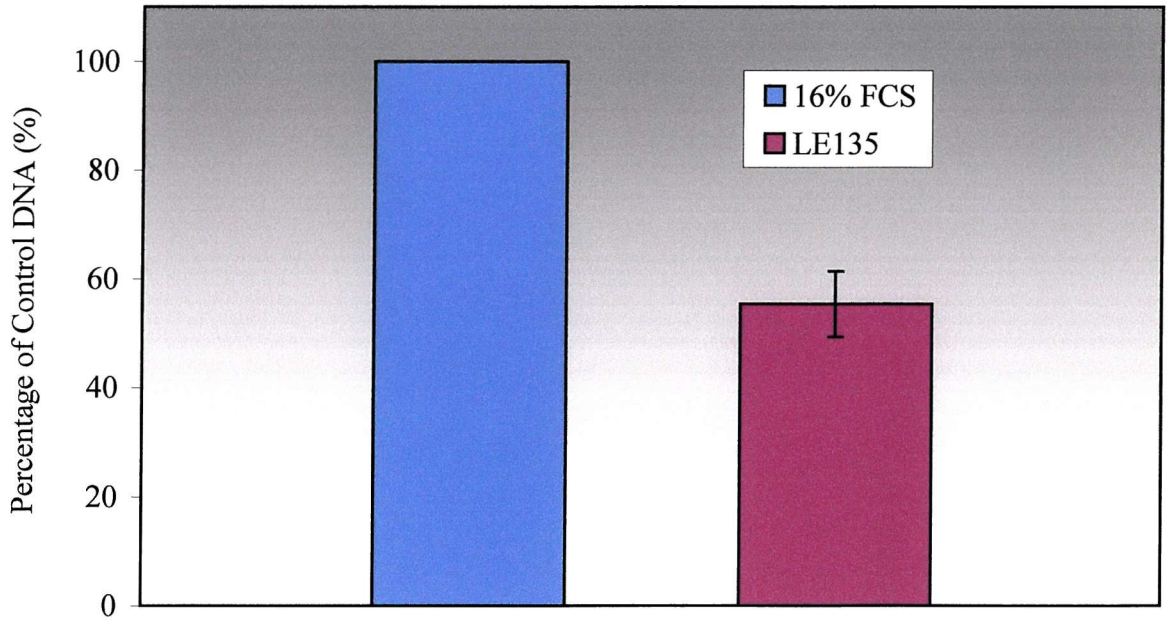


Proliferating cell nuclear antigen (PCNA) protein levels in cell lysates from rat HSC cultured on plastic for 5 or 6 days were analysed by western blotting following treatment with either vehicle or 10 μM of an RARβ selective antagonist (LE135) for 48 hours (Experiments 1 and 2) or 24 hours (Experiments 3 and 4) respectively (panel A). Levels of PCNA were quantified by scanning densitometry (panel B).

6.3.3. Measurement of Changes in DNA

The effect of the selective RARβ antagonist (LE135) on HSC proliferation was also assessed by measuring changes in DNA. Following plating of equal numbers of primary rat HSC on tissue culture plastic, HSC were harvested after 6 days of culture, and also after 7 days of culture following 24 hours treatment with either LE135 (10 μM) or vehicle (the equivalent concentration of ethanol), and the DNA content assayed (section 2.11.1.). The DNA in HSC did not change significantly ($P > 0.05$) between days 6 and 7 (an increase of 0.5% ± 2.3). However, mean DNA content was significantly lower ($P < 0.05$) in cultures treated for 24 hours with LE135 (Reduction to 55.4% ± 6.0) compared with control cultures (Figure 6.5.). This result supports the data obtained following the assessment of HSC proliferation in the presence of LE135 by [*methyl*-^{3}H]-thymidine incorporation (section 6.3.1.).

Figure 6.5: Effect of an RARβ selective antagonist on hepatic stellate cell DNA content



Rat HSC cultured for 6 days, were incubated for 24 hours with 10 μM of an RARβ selective antagonist (LE135) and the changes in the levels of DNA measured on Day 7. Data are expressed as percent of control, 16% FCS. Data presented are mean ± S.E.M. from 3 separate experiments. $P < 0.05$ for difference between LE135 and control.

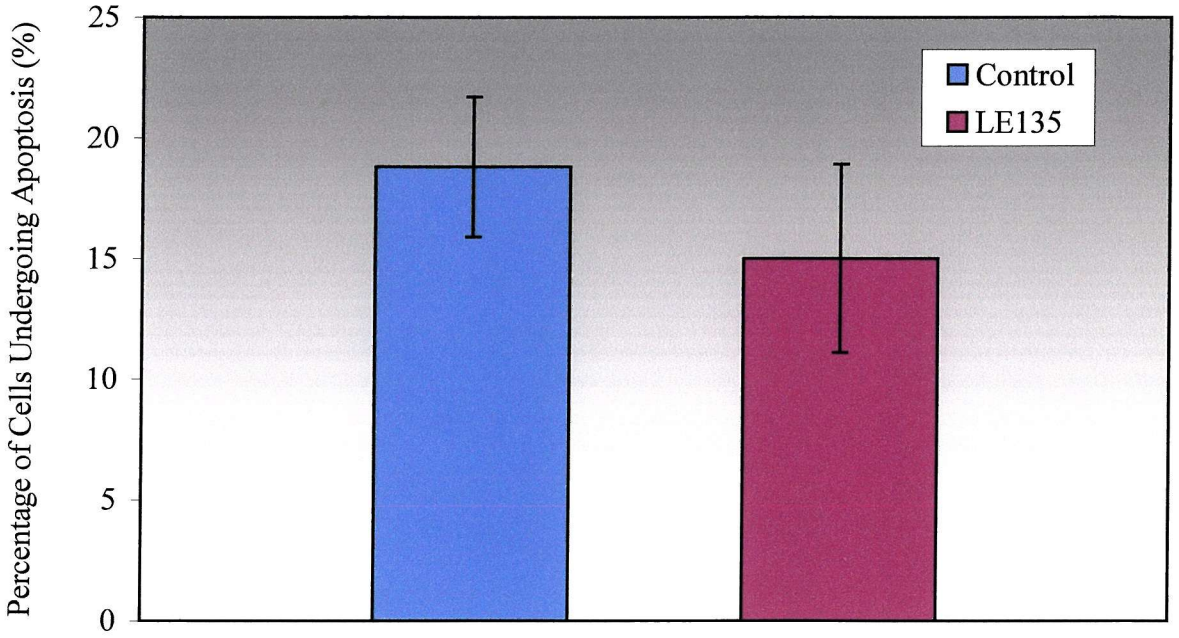
6.4. THE ROLE OF RARβ IN APOPTOSIS OF HEPATIC STELLATE CELLS

Apoptosis, or programmed cell death, is a major physiological mechanism in normal

development and tissue homeostasis, and functions as an autonomous suicide pathway that mediates a reduction in cell number in a variety of contexts. It is well documented that retinoids can cause rapid and extensive apoptosis in a variety of cancers, cell types and tissue culture cells (Bollag *et al.,* 1994). Previous findings have demonstrated that induction of RARβ by retinoic acid correlates with the growth inhibitory effect of retinoids and that RARβ mediates retinoic acid induced growth inhibition by promoting apoptosis (Horn *et al.,* 1996; Monczak *et al.,* 1997; Liu *et al.,* 1996). Since RARβ protein expression increases in cultured HSC (as shown in Chapter 5), RARβ may promote apoptosis in HSC, a process which is known to be increased in activated HSC (Saile *et al.,* 1997).

Figure 6.6. shows the results from 3 separate experiments examining the influence of the selective RARβ antagonist, LE135 on HSC survival. In these experiments, primary rat HSC were plated on 24 well culture plates, 5 days before 10 μM of the RARβ antagonist, LE135 or 0.2% ethanol (control cultures) were added to the medium. After a further 48 hours, acridine orange was added to the wells and normal and apoptotic cells were counted using an inverted fluorescent microscope (section 2.18.). As shown graphically in Figure 6.6., the number of apoptotic nuclei which appeared in HSC treated with the RARβ antagonist was not significantly different ($P > 0.05$) compared with the number of apoptotic cells observed in the control cultures (15% ± 3.9 apoptosis with LE135 compared with 18.8% ± 2.9 in control cultures).

Figure 6.6: Effect of an RARβ antagonist on spontaneous apoptosis of hepatic stellate cells



Rat HSC cultured for 5 days were incubated for 48 hours with 10 μM of an RARβ selective antagonist (LE135) or 0.2% ethanol (control cultures) prior to adding acridine orange and determining the number of apoptotic cells on day 7. Data presented are mean ± S.E.M. from 3 separate experiments.

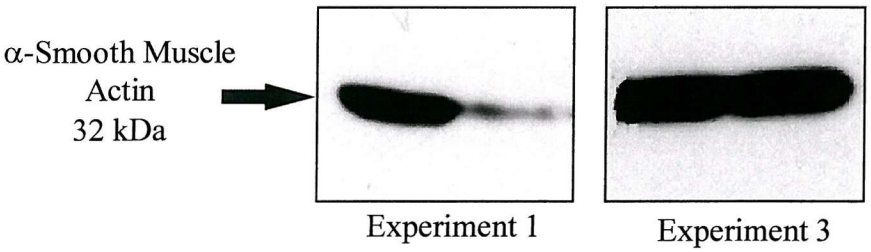
6.5. THE EFFECT OF RARβ ON EXPRESSION OF α-SMOOTH MUSCLE ACTIN PROTEIN IN HEPATIC STELLATE CELLS

Activated but not quiescent rat HSC have been demonstrated to contain high levels of α-smooth muscle actin (Rockey *et al.*, 1992a). The effect of RARβ on this well documented marker of HSC activation was examined using the technique of western blotting. Cell extracts from the same experiments used to determine the effect of RARβ on PCNA protein expression (section 6.3.2.) were analysed for expression of α-smooth muscle actin. Primary rat HSC cultured on plastic for 5 or 6 days were incubated with the RARβ antagonist, LE135 (10 μM) for 48 hours or 24 hours respectively. Equal amounts (1.5 μg of DNA) of whole cell lysates were subjected to electrophoresis on a 12.5% denaturing SDS-polyacrylamide gel (section 2.9.) and western blotted onto PVDF (section 2.10.). The α-smooth muscle actin protein was examined using a mouse monoclonal antibody directed against α-smooth muscle actin, followed by incubation with anti-mouse IgG conjugated to horse-radish peroxidase.

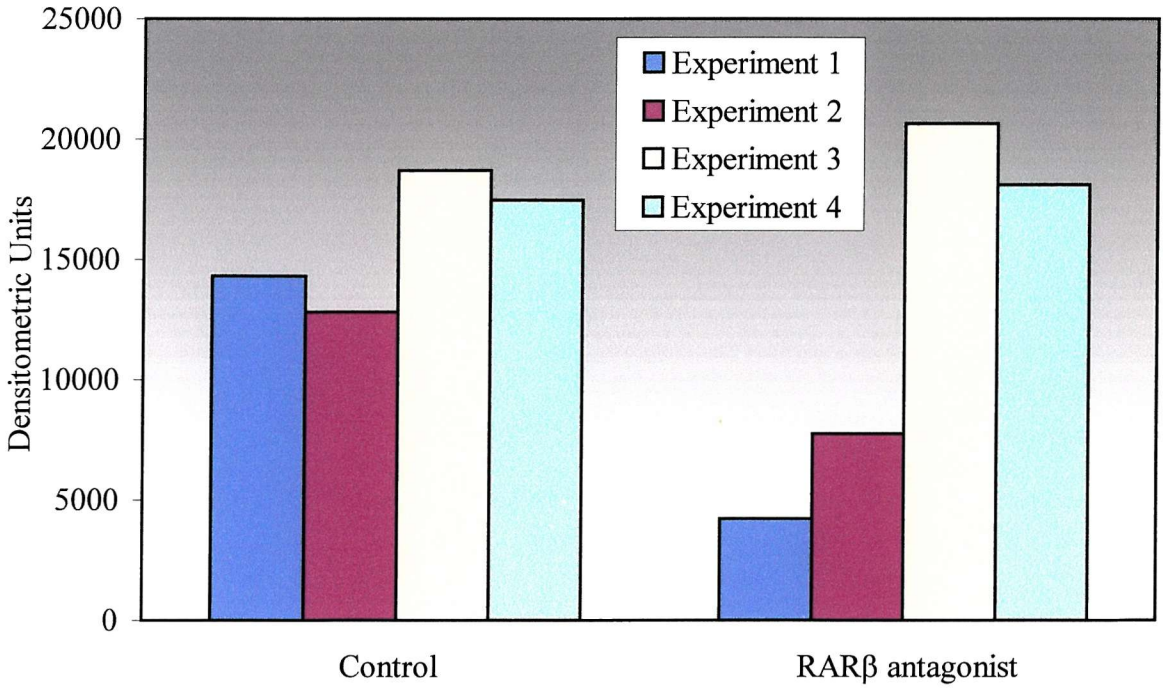
The proteins were visualised by ECL. As shown in Figure 6.7., α-smooth muscle actin was readily detectable in HSC cultured for 7 days on plastic. This is demonstrated by the band observed at the correct molecular weight of 32 kDa (Skalli *et al.,* 1986). Whereas HSC incubated with the antagonist for 24 hours expressed α-smooth muscle actin at the same level as the control cultures, the HSC incubated with the antagonist for 48 hours expressed only faint levels of α-smooth muscle actin protein. The protein bands at 32 kDa were not observed in the immunoblots incubated with mouse IgG (negative control), indicating the binding was specific.

Figure 6.7: Effect of an RARβ selective antagonist on α-smooth muscle actin protein expression in hepatic stellate cells

A:



B:



α-Smooth muscle actin protein levels in cell lysates from rat HSC cultured on plastic for 5 or 6 days were analysed by western blotting following treatment with either vehicle or 10 μM of an RARβ selective antagonist (LE135) for 48 hours (Experiments 1 and 2) or 24 hours (Experiments 3 and 4) respectively (panel A). Levels of α-smooth muscle actin were quantified by scanning densitometry (panel B).

6.6. DISCUSSION

The determination of the regulatory activity *in vivo* for the natural retinoids 9-*cis* retinoic acid and all-*trans* retinoic acid is difficult due to the potential for these retinoids to undergo isomerisation (Urbach and Rando, 1994; Sass *et al.,* 1994a) and/or metabolism to more polar compounds (Sass *et al.,* 1994a; Sass *et al.,* 1994b). However, the recent development of synthetic retinoids that act selectively with RARs or RXRs, or as RAR or RXR antagonists, has made it possible to independently assess the contribution of each RAR and RXR to the various retinoid actions. These analogues of natural retinoids are conformationally restricted, preventing their isomerisation (Hashimoto *et al.,* 1990).

The studies of progressive HSC activation shown in Figure 6.1., indicated that RARβ protein was becoming expressed in HSC coincident with both the peak of proliferation and α-smooth muscle actin expression. Using an antagonist with a high selectivity for RARβ, this study identified RARβ to be involved in the proliferation of HSC and induction of α-smooth muscle actin expression in HSC, suggesting RARβ potentiates the activation response. The rather slow onset in the reduction of α-smooth muscle actin expression (48 hours) compared to the rate at which proliferation was reduced (24 hours) by the antagonist suggests that the role of RARβ in regulating α-smooth muscle actin expression is an indirect one. A second transcription factor, possibly an RXR, may be required by RARβ that is not present, or is present at too low levels under basal conditions. The RARβ receptor may mediate the induction of the second transcription factor which subsequently mediates induction of α-smooth muscle actin. Alternatively, α-smooth muscle actin mRNA may have a long half-life and take time to disappear.

The PCNA is a protein required for cell cycle progression from G_1-phase to S-phase. It is first expressed in mid-G_1 and can be used as a marker of cell cycle entry into the G_1-phase (Celis *et al.,* 1987). Since PCNA expression in HSC was not effected by the RARβ antagonist, the data indicate that RARβ does not regulate the G_1 point of the cell cycle and cells are therefore able to progress from G_0/G_1 to the S, G_2 and mitosis (M-phase) phases of the cell cycle. In HSC, the RARβ protein is possibly required for progression through these latter phases (S, G_2 and M phases), since thymidine incorporation was inhibited and the DNA content in cells was reduced following incubation of the HSC with the RARβ antagonist.

Retinoic acid receptors are involved in regulating numerous genes. In addition to α-smooth muscle actin, RARβ may also be involved in regulating other genes whose expressions are altered during the transformation of quiescent HSC to an activated phenotype. The effects of only an RARα and RARβ antagonist have been studied due to time constraints and reagent availability. Other synthetic retinoids with selectivity in receptor binding and gene transactivation may also prove useful for further studies, for example the selective RARβ analogue (Ro 28-2249), the RARα selective analogue (Am580), and the RARγ selective analogue [CD437 (Shimada *et al.*, 1997)].

The observation that the RARβ antagonist did not effect apoptosis was unexpected considering published data positively associate RARβ expression with programmed cell death. The RARβ gene has been shown to have a suppressive effect in epidermoid lung cancer cells (Houle *et al.*, 1993), it inhibits anchorage-independent growth (Li *et al.*, 1995) and induces apoptosis in breast cancer cells (Seewaldt *et al.*, 1995), and also confers sensitivity to retinoic acid mediated growth inhibition (Houle *et al.*, 1993; Li *et al.*, 1995). These studies have mainly used tumour cell lines which have lost their normal regulation of proliferation. Events that occur in other cell types cannot necessarily be extrapolated to liver cells. Many tumour cells loose their sensitivity to retinoic acid following their transformation. Loss of RARβ gene expression is thought to be responsible for this loss of retinoic acid sensitivity in these cells (Liu *et al.*, 1996). In contrast, HSC which have transformed from a quiescent to an activated phenotype can still respond to retinoic acid (section 3.3.1.) and express increasing concentrations of RARβ protein (section 5.3.). This suggests a different signalling system may exist in HSC compared with these other cell types. To study the involvement of RARβ in growth-inhibition, some of the published studies have been conducted with selective RAR antagonists in the presence of exogenous retinoic acid (Horn *et al.*, 1996; Liu *et al.*, 1996), questioning the physiological relevance of these results. Additionally, some published studies have transfected cells with a RARβ expression vector (Liu *et al.*, 1996). Transfection experiments do not necessarily reflect the regulatory mechanisms under physiological conditions since transfected cells will express concentrations of RARβ at non-physiological high levels. It is possible that HSC may express different RXR isoforms to tumour cells and the resulting RARβ/RXR dimer have proliferative and not apoptotic actions. An alternative concept, is that HSC may generate their own retinoids which activate different RXRs than tumour cells. When these RXRs heterodimerize with RARβ an overall effect of proliferation

and not apoptosis may be observed. In summary, the strength of the studies in this Chapter is that endogenous RARβ has been inhibited in HSC, and hence the activities of relevant RAR/RXR dimers has been diminished.

The discovery that the RARβ antagonist did not stimulate or prevent apoptosis does not indicate that RARβ is not involved in this process. The rapidity of apoptotic events makes measurement of apoptosis difficult. Cells go from the onset of apoptosis (characterised by membrane blebbing and cell shrinkage) to fragmented apoptotic bodies in under an hour. In addition, the process of apoptosis is metachronous within a population of cells and therefore at each time point only a snapshot of the apoptotic activity has been determined. At earlier time points, for example after 24 hours treatment, the RARβ antagonist may stimulate increased apoptosis compared with control cultures. Alternatively, RARβ may induce necrosis of HSC rather than their apoptosis.

Apoptosis should be examined using a series of complementary techniques. Counting apoptotic bodies in a culture, by for example fluorescence microscopy, only reveals the number of apoptotic cells at any one time. It does not determine the rate of apoptosis in the cell culture. Moreover, it is easy to confuse apoptotic bodies with apoptotic cells, leading to an overestimate of dead cells. Other possible techniques include propidium iodide staining to identify and quantify condensed nuclei, and TUNEL staining to identify and quantify cells with oligosomal DNA fragmentation.

Retinoic acid receptors are regulated by several genes. Furthermore, RARs (α_2, β_2 and γ_2) contain RAREs in their promoter regions and respond to their own activation, thus forming an autoregulatory loop (Hoffmann *et al.,* 1990; de Thé *et al.,* 1990). The effects of retinoids may therefore be amplified through the upregulation of RARs in this autoregulatory loop, so that small increases in retinoids may result in disproportionately greater biological changes. In this study the extent to which the antagonist blocks the functional activity of RARβ was not investigated. Such studies would require co-transfection of HSC with an RARβ responsive reporter gene which is technically not achievable given the resistance of these cells to transfection (Figure 6.2.). If the RARβ antagonist is not in excess, only a fraction of the functional activity of RARβ will be inhibited.

Manshouri *et al.* (1997) found expression of antisense RARα in transgenic mice led to a 30-80% reduction in the level of RARα protein in various tissues and also showed complementary increases in RARβ and RARγ proteins. The authors suggest that the autoregulatory function of the RARs is contributing to the compensatory increase of the RARβ and RARγ proteins. Applying this concept, it is possible that in the presence of the RARβ antagonist, remaining RARs in the HSC may bind with other factors to RAREs in the RAR promoters and lead to compensatory increases in RARα, RARβ and RARγ. The increases may conceal the contribution made by RARβ. In support of this, it has been found that a certain degree of redundancy exists in the retinoid signalling pathway. For example, knock-out mice for all RARα isoforms showed a phenotype that is relatively discrete considering the ubiquitous expression of RARα (Lufkin *et al.,* 1993). Furthermore, knock-out mice either for all isoforms of RARβ (Luo *et al.,* 1995), or specific RAR isoforms [$RAR\alpha_1$ (Li *et al.,* 1993), $RAR\beta_2$ (Mendelsohn *et al.,* 1994), $RAR\gamma_2$ (Lohnes *et al.,* 1993)] have no apparent alterations in phenotype. Similarly, some of the natural promoters that have been tested in cotransfection experiments could be activated albeit to different extents by more than one RAR or RXR isoform (Vivanco-Ruiz *et al.,* 1991).

A growing amount of information has demonstrated the important role of heterodimerization of RARs and RXRs in transactivation of gene expression. It is possible that the unnaturally low levels of RARβ in activated HSC in the presence of the antagonist, may result in an imbalance in the relative concentrations of heterodimers formed between members of the RARs and RXRs, and the heterodimers formed between RXRs and other hormone receptors. This suggests that more than one technique should be performed inorder to establish the role of RARβ.

The data from this study suggest RARβ may be one of the major factors responsible for the progression of HSC activation, which subsequently has important pathogenic consequences for the development of hepatic fibrosis. These initial findings therefore warrant further investigation both *in vitro* and *in vivo* since they indicate a potentially novel, HSC directed therapeutic approach to liver fibrosis.

CHAPTER 7

General Discussion

and

Future Studies

7.1. KEY FINDINGS OF THIS STUDY

The purpose of this thesis was to examine the retinoids which are present in quiescent and activated HSC and to critically evaluate the role of these retinoids in activation of HSC. The thesis also aimed to examine the expression and role of the nuclear retinoid receptor RARβ.

Chapter 3 demonstrated that although exogenous retinoic acid metabolites inhibited HSC proliferation, retinoic acid metabolites, in particular all-*trans* retinoic acid failed to modify expression of a variety of genes (gelatinase A, pro-collagen I, TIMP-1, TIMP-2, β_1-integrin and α-smooth muscle actin) whose transcripts are considered to be markers of HSC activation. Chapter 4 analysed the changes in the content of several endogenous retinoid metabolites during activation of rat HSC by reverse-phase HPLC. The vitamin A metabolites present in the HSC varied depending on the status of the HSC. In a quiescent state the main retinoids present in HSC were shown to be retinyl esters. With the development of fibrosis the retinyl ester content of the HSC decreased. A fraction of this may be converted to retinol and retinoic acid metabolites since it was demonstrated that intracellular retinol levels remained relatively constant and retinoic acid metabolites (all-*trans* retinoic acid and 13-*cis* retinoic acid) were endogenously produced in quiescent HSC and during the activation process.

Chapter 5 demonstrated that HSC do not only contain retinol and metabolites of retinol but also contain nuclear retinoid receptors necessary for retinoids to exert their effects. In particular, this Chapter demonstrated that RARβ protein is dissociated from RARβ mRNA expression and increases with activation of HSC. Furthermore, it was demonstrated by EMSA that the RARβ protein was functionally active and the studies in Chapter 6 provided novel evidence that the RARβ is potentially important to HSC activation as it is a mitogen and activation factor for HSC; increasing HSC proliferation and α-smooth muscle actin expression.

7.2. CONTRIBUTION OF THE DATA TO THE AVAILABLE LITERATURE

Current research in retinoid metabolism in the liver has been directed towards the specific roles of HSC in this process. The HSC is considered to play an important role in the hepatic storage and metabolism of retinol. Although there is some evidence for the involvement of

parenchymal cells (Blaner *et al.,* 1985; Blomhoff *et al.,* 1985b) and Kupffer cells (Earnest *et al.,* 1986; Hendriks *et al.,* 1987a) in retinoid metabolism, their possible contribution to this process is unclear. Many issues about the roles of retinol and its derivatives in liver fibrosis are still unresolved and researchers have principally examined the mechanisms responsible for HSC retinoid loss and the potential role this loss may have in facilitating cellular activation and hepatic fibrosis. The experiments conducted in this thesis have contributed novel information to this field.

The complex effects of all-*trans* retinoic acid and 9-*cis* retinoic acid are mediated via the numerous isoforms of nuclear retinoid receptors which are produced through alternative splicing and promoter usage [for review see Chambon (1996)]. These ligand dependent transcription factors are thought to confer retinoid responsiveness to cells, thus differences in their relative expression might indicate different levels of retinoid responsiveness. The possibility that during HSC activation changes in nuclear retinoid receptors accompany retinoid release was therefore considered in this thesis.

Whilst measurement of mRNA provides information about expression of RARs, it is ultimately expression and regulation of the RAR proteins which is important. Although there are several northern blot studies on the distribution of RAR transcripts in the liver (Ulven *et al.,* 1998; Ohata *et al.,* 2000; Weiner *et al.,* 1992; Friedman *et al.,* 1993), there are very few studies on the distribution of the RAR proteins and these are potentially flawed. This thesis has demonstrated that RARβ transcript levels do not reflect expression of the RARβ protein levels in quiescent and activated HSC.

Although the finding that RARβ protein is increased in activated HSC contradicts the immunocytochemistry observations by Weiner *et al.* (1992) (Figure 5.13., apparently showing decreased RARβ protein in culture activated HSC), my studies found that the antibody (SP172) used in these immunocytochemical studies does not recognise rat RARβ protein. A lower molecular weight protein (35 kDa) was consistently detected by SP172, which decreased in expression following HSC activation. However, this species is not derived from RARβ since immunoadsorption of SP172 with an excess of peptide (specific to SP172) did not effect the intensity of this band. As immunocytochemistry cannot discriminate between different antibody binding proteins, my western blotting studies therefore provide an explanation for the results obtained by Weiner *et al.* (1992).

The implication of this novel finding that RARβ protein is increased during activation of HSC, warrants further research since this thesis signifies a potential pro-fibrogenic role for RARβ. From the research performed in carcinoma cells RARβ is implicated as a tumour suppressor (Liu *et al.*, 1996; Houle *et al.*, 1993). In tumour cell lines, the RARβ gene is not deleted but instead the $RAR\beta_2$ promoter is less responsive to retinoic acid (Bartsch *et al.*, 1992). This thesis has demonstrated that the promoter of RARβ is responsive in HSC since RARβ protein is upregulated during activation of HSC. As retinoic acid is still present in activated HSC (Chapter 4), synthesis of this receptor may be induced by autoregulation resulting in amplification of the RARβ protein level. Studies of HSC in prolonged culture would confirm whether a saturation level for RARβ is reached.

Since retinoids can exert their effects via RARβ and previous studies have shown RARβ to be anti-proliferative (Liu *et al.*, 1996; Houle *et al.*, 1993), it was unusual to find the retinoids exerted an anti-proliferative effect on HSC yet a RARβ antagonist also decreased HSC proliferation. However, since HSC have developed as the major storage site of retinoids in the body (Blomhoff *et al.*, 1991; Wake, 1971) and have very high retinoid content, different retinoids and retinoid receptors may exist within HSC. Subsequently, events that occur in other cell types cannot necessarily be extrapolated to HSC.

This thesis has also examined how retinoid metabolites change during the activation of HSC from a quiescent to an activated phenotype *in vitro*. Despite Okuno *et al.* (1999) publishing their work whilst these studies were performed, my studies are the first to demonstrate, in a detailed analysis of primary cultures of HSC, that changes observed in this culture model parallel the loss of retinoid droplets observed *in vivo* during liver injury (Hautekeete *et al.*, 1998). These observations therefore support the validity of the culture model of HSC activation. Furthermore, the decrements in levels of cellular and extracellular retinoic acids observed during the activation of HSC (Chapter 4) suggests the retinoids are metabolised to further, perhaps novel, isomers of retinoic acid. The retinoids produced during activation of HSC may play a central role in increasing hepatic fibrosis since the vitamin A status of the liver has been shown to play an important role in the development of liver fibrosis (Shiratori *et al.*, 1987; Senoo and Wake, 1985; Seifert *et al.*, 1994). This may be caused not only by an autocrine effect on HSC but also by a paracrine effect; retinoids have been shown to activate Kupffer cells and lower the threshold for endotoxin liver injury (Earnest *et al.*, 1986; Hendriks *et al.*, 1987a).

In hepatic fibrosis *in vivo,* several agents may act in concert to alter the retinyl ester production and hydrolysis, and subsequently deplete the hepatic retinyl ester levels. This may involve growth factors from Kupffer cells (Friedman *et al.,* 1993), soluble cytokine factors as well as the surrounding extracellular matrix (Davis and Vucic, 1989).

Chapter 3 demonstrates that although exogenous retinoic acid metabolites inhibit HSC proliferation, retinoic acid metabolites, in particular all-*trans* retinoic acid fail to modify expression of gelatinase A, pro-collagen I, TIMP-1, TIMP-2, β_1-integrin and α-smooth muscle actin. In other cell types, the induction of cellular differentiation by retinoic acid involves the ability of this signalling molecule to alter the expression of a wide variety of genes (Love and Gudas, 1994). It is possible that in HSC there are differences in the interactions of the receptor subtypes with the endogenous retinoic acids. The response to 9-*cis* or all-*trans* retinoic acid may differ depending on the population of receptor subtypes present in the cell. Allenby *et al.* (1994) showed for example that transiently transfected RARs (RARα, RARβ, and RARγ) in COS-1 cells bound with differential affinities to labelled 9-*cis* and all-*trans* retinoic acid. Overall, the physiological response of a target cell for retinoid action and transcription of a particular gene is dependent on a complex interplay of several factors, including the isoforms and levels of RXRs and RARs, the efficiency with which different heterodimers are formed, competition with homodimers and the ratio of the various types of active retinoids. These factors may explain the observed fibrogenic effects of retinoids under some conditions (Okuno *et al.,* 1997) and their anti-fibrogenic actions under others (Davis *et al.,* 1990).

7.2.1. A Model of Intracellular Retinoid Actions in Quiescent and Activated Hepatic Stellate Cells

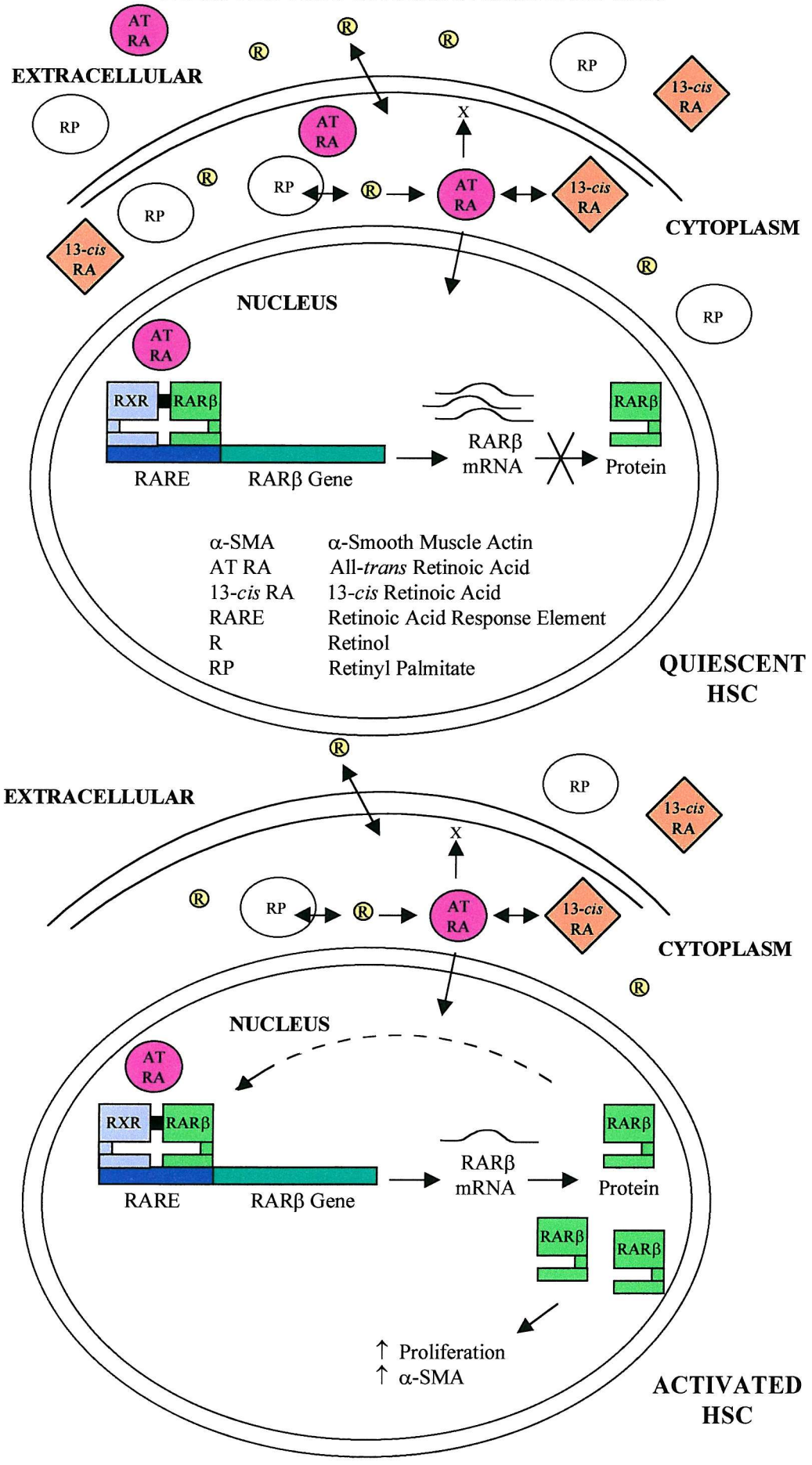
Based on the results reported in this thesis, a model reflecting the intracellular retinoid actions in quiescent and activated HSC has been developed as shown in Figure 7.1.

The vitamin A metabolites present in the HSC vary depending on the status of the HSC. In quiescent HSC the major morphological characteristic is the presence of large retinoid-rich droplets that are present in the cytoplasm. The retinoid is primarily stored as the retinyl ester, retinyl palmitate, the most abundant intracellular retinoid present. With activation of the HSC, and coincident with the loss of retinoid droplets, retinyl palmitate is metabolised. Retinyl

palmitate is presumably metabolised to retinol whose levels are maintained intracellularly and represents the second most abundant retinoid detected. The levels of retinol may also be supplemented by extracellular levels of retinol since retinol can be taken up by cells and the extracellular levels of retinol decrease with HSC activation. Although much lower concentrations of retinoic acid isomers are found in HSC compared with the concentrations of retinol and retinyl palmitate, the retinol may constitute a reservoir from which a supply of retinoic acids are produced. The retinol may be converted to all-*trans* retinoic acid and subsequently to other metabolites including, 13-*cis* retinoic acid, detected at even higher (x100) concentrations than all-*trans* retinoic acid. In contrast to studies by Okuno *et al.* (1999), neither 9-*cis* retinoic acid nor 9,13 di-*cis* retinoic acid was detected, and hence has been omitted from Figure 7.1. Since the retinoic acid metabolites decrease with activation they have possibly been converted to other retinoid metabolites.

Retinoic acid receptors are activated by both all-*trans* and 9-*cis* retinoic acid. Retinoid X receptors are activated by 9-*cis* retinoic acid only. Within the nucleus, all-*trans* retinoic acid binds to an RAR heterodimerised to an RXR. Once bound, the complex acts as a ligand inducible transcription factor activating expression of target genes by binding to RAREs located in the promoter region of the target gene. The expression of $RAR\beta_2$ is itself modulated by retinoic acid through an RARE present in its promoter. In quiescent HSC the RARβ mRNA is detectable but there is a down regulation of the expression of RARβ mRNA during the transformation of the HSC to a myofibroblastic phenotype. However, more importantly expression of RARβ protein is upregulated. This could be due to increased efficiency of mRNA translation or prolongation of the half-life of the protein. The increased RARβ protein in activated HSC then potentiates fibrosis by contributing to α-smooth muscle actin expression and proliferation of the HSC.

Figure 7.1: A model showing intracellular retinoid actions in quiescent and activated hepatic stellate cells based on results obtained in this thesis



7.3. A CRITICAL APPRAISAL OF STUDIES PERFORMED IN THIS THESIS

The studies in Chapter 3 contain mainly information concerning the effect of the all-*trans* retinoic acid isomer on the anti-fibrotic potential of retinoic acid. Since RARs are activated by both all-*trans* and 9-*cis* retinoic acid and RXRs are activated by 9-*cis* retinoic acid, most of the experiments in Chapter 3 have therefore examined the response of RARs. However, 9-*cis* retinoic acid is undetectable in HSC (Chapter 4) and the relevance of examining the response of HSC to this retinoid could therefore be queried.

Due to time constraints and availability of equipment, the fractions which eluted from the HPLC column in Chapter 4 could not be collected and analysed using the complementary identification technique, mass spectrometry. This technique would have reinforced the findings and possibly identified the compounds which eluted in close proximity to all-*trans* retinoic acid and 13-*cis* retinoic acid. It is important to determine whether particular retinoic acid isomers are causally related to fibrogenesis. In particular, peak X may represent 14-HRR or anhydroretinol. These retro-retinoids are a novel class of retinol metabolites which have agonist/antagonist properties in B cells and T cells (Buck *et al.*, 1993). Furthermore, a previous study has detected 14-HRR in activated HSC in CCl_4 and bile duct ligated models of liver injury, as well as during culture activation (Friedman *et al.*, 1992b). Since 14-HRR appears early in HSC activation (Friedman *et al.*, 1992b), retro-retinoids may have regulatory roles in the development of HSC activation and hepatic fibrosis. Although authentic 14-HRR and anhydroretinol compounds were available from Dr Derguini, Sloan-Kettering Institute, New York, America, due to time constraints the effect of these compounds on HSC activation was not examined in detail. Another limitation of the reverse-phase HPLC studies was that some levels of retinoic acid isomers were close to the lower limit of detection and some retinoids, including 9-*cis* retinoic acid may even have been in too low a concentration to detect. However, to date reverse-phase HPLC is the only appropriate method available to measure the retinoid metabolites.

To ensure the RARβ antibody obtained from Santa Cruz recognised intact rat RARβ protein, a different RARβ antibody was employed in western blotting studies with the aim that detection of the same protein by two different antibodies would be conclusive evidence that RARβ protein expression increases with activation. The only alternative RARβ antibody commercially available to date was from Affinity Bioreagents and this failed to detect rat

RARβ protein. Limitations on the availability of reagents was also experienced when detecting the RAR transcripts in HSC using the technique of northern blotting. Although the human and mouse RAR genes have been cloned and sequenced (Zelent *et al.,* 1989; Brand *et al.,* 1988; Krust *et al.,* 1989; Benbrook *et al.,* 1988; Giguere *et al.,* 1987; Petkovich *et al.,* 1987), the rat RAR genes have not been studied. Consequently, the mouse RAR cDNAs were used to hybridise to rat mRNAs in a similar way to other published studies (Weiner *et al.,* 1992; Kato *et al.,* 1992).

To study the role of RARβ in HSC, the effect of an RARα and RARβ antagonist was studied. Other synthetic retinoids with selectivity in receptor binding and gene transactivation, for example the selective RARβ analogue (CD2019), the RARα selective analogue (Am580), and the RARγ selective analogue [CD437 (Shimada *et al.,* 1997)] are available. However, in addition to time constraints, further studies using these compounds was prevented because of commercial funding. The selective RARβ retinoid (CD2019) and the RARγ selective retinoid (CD2019) are not available commercially but can be obtained from CIRD/GALDERMA, Sophia Antipolis Cedex Valbonne, France, provided this company have ownership of any results obtained. This thesis was, in part, sponsored by Pfizer Limited, Sandwich, Kent, preventing the collaboration with another company. To date, the RARβ antagonist LE135 is the only selective RARβ antagonist available, preventing confirmation of the results with a different compound.

7.4. POTENTIAL CONTRIBUTION OF MY STUDIES TO DEVELOPING THERAPIES FOR LIVER FIBROSIS

Conventional treatment of chronic liver disease (e.g. abstinence from alcohol, corticosteroid treatment of autoimmune hepatitis) is relatively ineffective in preventing the progression of liver fibrosis. If RARβ is expressed at low levels in other cell types, then the utility of a selective RARβ antagonist as a potential HSC directed therapeutic agent for liver fibrosis is feasible. Alternatively, it may be possible to focus on key target genes for RARβ or other retinoid receptors for the development of a liver fibrotic agent in the future. Since my studies have found RARβ to increase the rate of HSC proliferation but in other cell types RARβ has been shown to act as a tumour suppressor (Liu *et al.,* 1996; Houle *et al.,* 1993), this property could be utilised as a focus for therapeutic development. As RARβ knockout mice appear

healthy (Luo *et al.*, 1995; Mendelsohn *et al.*, 1994), antagonising RARβ long term also indicates a potential area for treatment of fibrosis. However, this could lead to the development of tumours in other cell types since, as mentioned previously, in other cell types RARβ acts as a tumour suppressor (Liu *et al.*, 1996; Houle *et al.*, 1993). Alternatively following liver injury, the generation of retinoids which are ligands for RARβ by HSC could be suppressed by targeting retinyl ester hydrolase, required for mobilising retinol from the abundant vitamin A granules in HSC (Azais-Braesco *et al.*, 1995).

7.5. FUTURE STUDIES

The reason for the increase in RARβ protein levels following HSC activation should be examined. Since the conditions for immunoprecipitation of RARβ protein from HSC have been established (Chapter 5), pulse-chase experiments could be performed on quiescent and activated HSC to establish whether the half-life of RARβ changes following activation. Experiments could also be conducted to determine whether intracellular proteases play a role in regulating the half-life of RARβ in HSC, as has been detected for the regulation of RXR in HuH7 cells (Matsushima-Nishiwaki *et al.*, 1996a). The RARβ protein may resist degradation by increased phosphorylation of the receptor as previously shown for RARα and RXRα (Yen *et al.*, 1998; Solomon *et al.*, 1999; Lefebvre *et al.*, 1995).

Retinoic acid receptors need to form heterodimeric complexes with RXRs in order to bind to DNA and regulate gene transcription. To understand the relevance of a change in the level of RARβ protein during activation of HSC *in vivo*, it is therefore also important to analyse the expression of RXRs. Recent studies for example have found RXRα mRNA levels to be decreased following activation of HSC (Ohata *et al.*, 1997). Serial immunoprecipitations could identify the RXRs which bind to RARβ at different stages of HSC activation as binding is not disrupted using this technique.

To further address the role of RARβ in retinoic acid mediated gene regulation, the ability to transfect HSC with a high transfection efficiency needs to be conquered. A dominant negative RARβ construct could then be designed that lacked the DNA binding domain but retained the ability to dimerize, resulting in the formation of non-functional dimers. Following transfection in HSC the effect of the construct on HSC activation could then be examined. The

contribution of RARβ to apoptosis of HSC also warrants further investigation since apoptosis should be measured by more than one technique. Other possible techniques include propidium iodide staining to identify and quantify condensed nuclei, and TUNEL staining to identify and quantify cells with oligosomal DNA fragmentation. Clearly, the physiological role of RARβ cannot be established solely from molecular and transfection studies in isolated cultured tissues or cells.

Although it has been shown that HSC are responsible for retinoic acid generation, the possibility cannot be excluded that *in vivo* retinol might be transferred from HSC to surrounding hepatic cells and there converted to retinoic acid. It is important to clarify the results which have been obtained in this thesis by determining whether similar responses occur in animal models of liver fibrosis. Retinoid metabolism and subsequent biological changes may differ between different models of hepatic fibrosis. Animal models are indispensable for the study of the cell biology and pathobiochemistry of liver fibrosis, since studies in cell culture are unlikely to reflect the influences of complex intercellular interactions *in vivo.* Furthermore, fibrogenesis is a tissue phenomenon which does not depend solely on changes in a single cell type. However, by various parameters the *in vitro* model of HSC attached to uncoated plastic provides an accurate recapitulation of the process of HSC activation *in vivo,* which is a common ultimate pathway leading to fibrosis regardless of the initiating factor for liver injury. Furthermore, established cell lines and primary cell cultures have provided simpler, retinoid-dependent model systems that are more amenable to experimental study and manipulation than *in vivo* models.

To further examine the loss or conversion of retinoid metabolites during the transition of HSC from a quiescent to an activated phenotype, and to examine whether this change has a significant effect upon HSC behaviour, reverse-phase HPLC studies could be performed on activated HSC re-plated on a substratum which resembles the subendothelial matrix of normal liver, for example EHS. It has previously been found that when activated HSC are re-plated onto EHS they adopt a quiescent phenotype and regain intracellular lipid droplets (Gaça *et al.,* 2000). However, these droplets have not been analysed to determine whether the lipid droplets contain retinoids and, if present the composition of these retinoids.

Another *in vitro* model which could be used to analyse changes in retinoids and their receptors in HSC and in whole liver is the rat CCl_4 recovery model (Iredale *et al.,* 1998). With this

model, liver fibrosis or cirrhosis can be induced in 4-6 weeks and the pattern of pathologic evolution resembles in some respects that seen in alcoholic liver injury in man and in animal models. For example, in both cases regeneration of hepatocytes occurs after necrosis and fibrotic infiltration is almost irreversible in the advanced stages of cirrhosis (Tamayo, 1983). Retinoid content and retinoid receptor expression could be examined in HSC during the recovery phase.

7.6. CONCLUSION

In conclusion, not only has this thesis shown that retinoid metabolites change during HSC activation but also that the nuclear retinoid receptor, RARβ protein is dissociated from its mRNA and increases with activation. Furthermore, this thesis has found that RARβ is a mitogen and activation factor for HSC.

The thesis has attempted to build on the information currently available in the literature with emphasis on the physiological role of retinoids and their receptors at the cellular level in relation to their transcriptional and post-transcriptional activities. Evidence has been presented for the appearance of specific retinoids in HSC as they undergo activation. However, like other important discoveries many more questions have been raised. Most importantly definitive proof that retinoid receptor subtypes have specific functions in HSC is awaited because evidence to support this is still largely circumstantial. The continuing study of retinoids and their receptors will provide us with significant new information about the regulatory control of retinoids in quiescent and activated HSC. Moreover, these studies will lead to discoveries relevant to the clinical use of retinoids, not only in the area of liver fibrosis but also perhaps in other areas, including cancer prevention and treatment, and dermatology.

APPENDICES

APPENDIX 1.

SOLUTIONS AND REAGENTS

Cell Culture Reagents

PSG:	32 μg/ml Gentomycin 10 milliUnits/ml Penicillin 10 μg/ml Streptomycin (in sterile water)

DNA Assay

TE buffer (1 x):	1 mM EDTA 10 mM Tris-HCl, pH 7.5

DNA Electrophoresis Reagents

DNA loading buffer (6 x):	0.25% Bromophenol blue 30% Glycerol (in water)
TAE buffer (10 x):	10 mM EDTA 0.4 M Tris-acetate Adjust to pH 8.0
TBE buffer (10 x):	0.9 M Boric acid 20 mM EDTA 0.9 M Tris Adjust to pH 8.0

Electromobility Shift Assay Reagents

Buffer C:	See nuclear extract reagents
Forward exchange buffer (10 x):	15 mM DTT 100 mM Magnesium chloride 1 mM Spermidine 500 mM Tris-HCl, pH 7.5
Gel loading buffer (6 x):	1.7% Bromophenol blue 0.5 M EDTA, pH 8.0 3.75 mM Ficoll 400 1.7% Orange G 1.7% Xylene cyanol FF
TBE buffer (10 x):	See DNA electrophoresis reagents
TE buffer (1 x):	See DNA assay reagents

DNA Purification Reagents

Wizard™ Minipreps and Maxipreps Reagents	
Cell lysis solution:	1% SDS 0.2 M Sodium hydroxide
Cell resuspension solution:	10 mM EDTA 100 µg/ml RNase A 50 mM Tris-HCl, pH 7.5
Column wash solution:	5 mM EDTA 200 mM Sodium chloride 20 mM Tris-HCl, pH 7.5
Neutralisation solution:	Potassium acetate, pH 4.8
TE buffer:	See DNA assay reagents

Gelatin Zymography

Coomassie blue stain:	0.5% (v/v) Coomassie brilliant blue R250 10% Glacial acetic acid 45% Methanol
Destain:	10% Glacial acetic acid 30% Methanol
Incubation buffer:	5 mM Calcium chloride 0.05 M Tris Adjust to pH 8.0
Sample buffer (2 x):	0.5% Bromophenol blue 20% Glycerol 10% β-Mercaptoethanol 4% SDS 125 mM Tris-HCl, pH 6.8 (Omit β-mercaptoethanol for non-reducing conditions)

Immunocytochemistry

Harris haematoxylin:	100 g Aluminium potassium sulphate 950 ml Distilled water 50 ml Ethanol 5 g Haematoxylin 5 g Mercuric oxide

TBS:	0.14 M Sodium chloride 5 mM Tris Adjust to pH 7.6

Immunoprecipitation Reagents

NET-gel buffer:	1 mM EDTA 0.25% Gelatin 0.1% Nonidet P-40 (NP-40) 0.02% Sodium azide 150 mM Sodium chloride 50 mM Tris-HCl, pH 7.5
Sample buffer (2 x):	See gelatin zymography reagents
Single lysis buffer:	1 μg/ml Aprotinin 100 μg/ml Phenylmethylsulphonyl fluoride (PMSF) 0.02% Sodium azide 150 mM Sodium chloride 50 mM Tris-HCl, pH 8.0 1% Triton-X 100
Triple lysis buffer:	As single lysis buffer plus: 1% NP-40 0.1% SDS 0.5% Sodium deoxycholate
Wash buffer:	10 mM Tris-HCl, pH 7.5 0.1% NP-40

Northern Blotting and Hybridisation Reagents

DEPC treated water:	1 ml DEPC 1 litre Distilled water Leave overnight then autoclave for 1 hour to break down DEPC to ethanol and CO_2
Hybridisation buffer:	0.5% Blocking reagent (Roche Diagnostics, Lewes, UK) 2 x Denhardts reagent 50% Formamide 20 μg/ml Herring sperm DNA 1% SDS 1 x Sodium orthophosphate EDTA 5 x SSC Make up with DEPC treated water

SSC (20 x):	3 M Sodium chloride 300 mM Sodium citrate Make up with DEPC treated water, adjust to pH 7 and autoclave.

Nuclear Extract Reagents

Buffer A:	0.5 mM DTT 10 mM HEPES (pH 7.9 at 4 °C) 1.5 mM Magnesium chloride 0.2% NP-40 10 mM Potassium chloride
Buffer C:	2 mM DTT 0.2 mM EDTA 0.2% Glycerol 20 mM HEPES (pH 7.6 at 4 °C) 100 mM Potassium chloride

Poly (A^+) Messenger RNA Extraction

Micro-Fast Track™ Kit Reagents

Binding buffer:	500 mM Sodium chloride 10 mM Tris, pH 7.5 (in DEPC treated water)
Elution buffer:	10 mM Tris, pH 7.5 (in DEPC treated water)
Glycogen carrier:	2 mg/ml in DEPC treated water
Low salt wash buffer:	250 mM Sodium chloride 10 mM Tris, pH 7.5 (in DEPC treated water)
Stock buffer:	1.5 mM Magnesium chloride 2% SDS 200 mM Sodium chloride 200 mM Tris, pH 7.5 (in DEPC treated water)

Protein Assay

Buffer C:	See nuclear extract reagents

RNA Electrophoresis Reagents

DEPC treated water:	See northern blotting reagents

MOPS (10 x):	10 mM EDTA 0.2 M MOPS 50 mM Sodium acetate Make up with DEPC treated water, adjust to pH 7 and autoclave. Cover bottle with foil to prevent light oxidation.
RNA loading buffer:	0.5% Bromophenol blue 2 M Formaldehyde 0.75 M Formamide 7% Glycerol 1 x MOPS (Make up with DEPC treated water)

RNA Isolation Reagents

DEPC treated water:	See northern blotting reagents
4 M GIT:	4 M GIT 26 mM Sodium acetate Adjust to pH 7.0

SDS-PAGE Reagents

Running buffer (5 x):	0.2 M Glycine 1 mM SDS 25 mM Tris

Transfection Reagents

HBS (10 x): (to prepare 2 x HBS, dilute the 10 x stock with water and adjust the pH to 7.12)	0.2% (w/v) Di-sodium hydrogen orthophosphate 5.94% (w/v) HEPES 8.18% (w/v) Sodium chloride
PSG:	See cell culture reagents
X-gal solution:	2 mM Magnesium chloride hexahydrate in PBS 4 mM Potassium ferricyanide 4 mM Potassium ferrocyanide 1 mg/ml X-gal in N'-N'-dimethylformamide

Transformation of *E.Coli*

Terrific broth:	Terrific broth consists of medium containing 3% phosphate buffer
Medium (900 ml):	4 g Glycerol 12 g Tryptone 24 g Yeast extract
Phosphate buffer:	0.72 M Di-potassium hydrogen orthophosphate 0.17 M Mono-potassium hydrogen orthophosphate

Western Blotting Reagents

Blocking solution (5%/10%):	5 g/10 g 'Marvel' milk powder 100 ml TTBS
Coomassie blue stain:	See gelatin zymography reagents
Destain:	See gelatin zymography reagents
Runnning buffer:	1.9 M Glycine 1% SDS 0.25 M Tris
Transfer buffer:	27 mM Glycine 20% Methanol 50 mM Tris
TTBS:	0.5 M Sodium chloride 50 mM Tris 0.05% Tween 20 Adjust to pH 7.4

Western Blotting Stripping Reagents

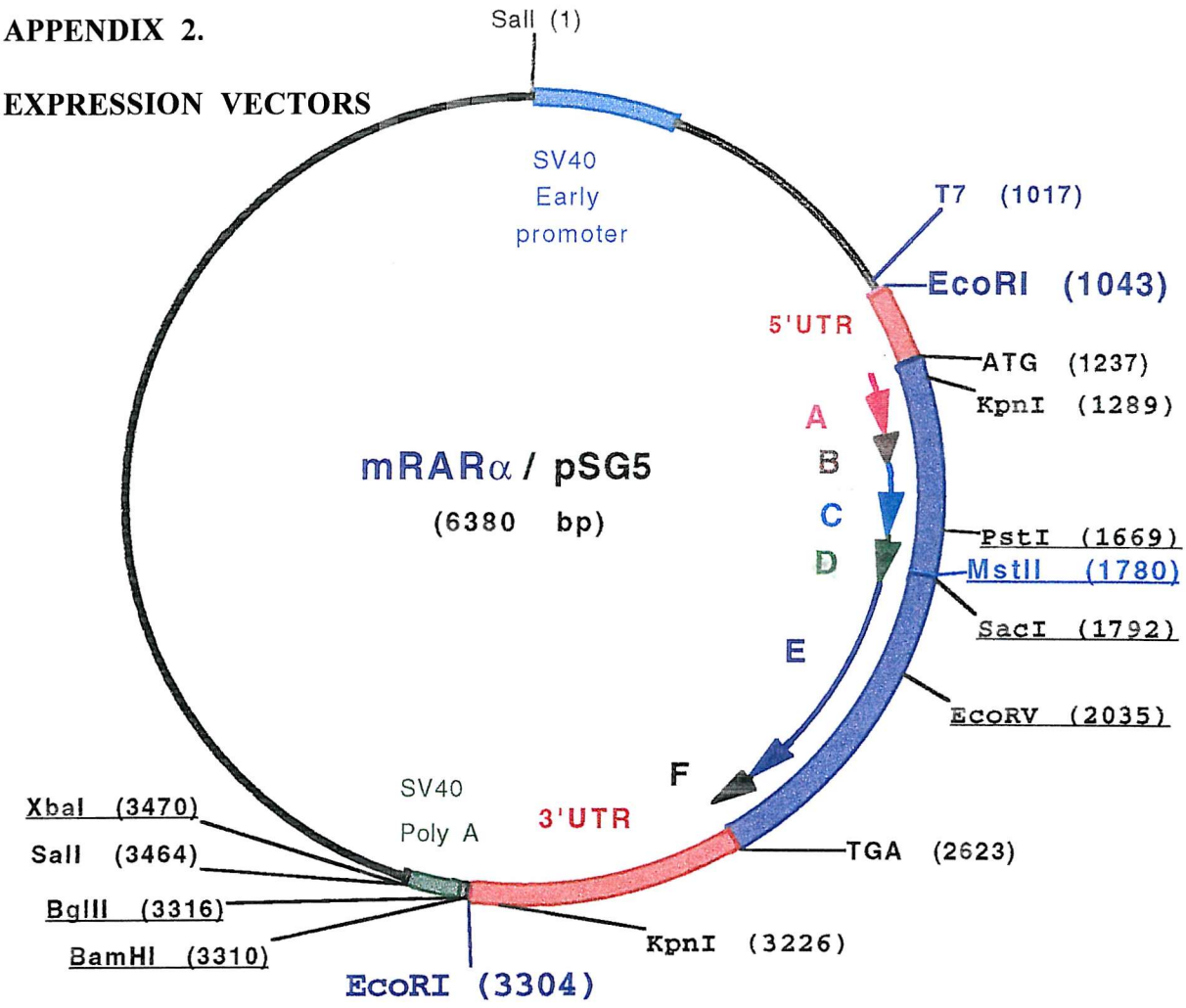
Stripping buffer:	100 mM β-Mercaptoethanol 2% SDS 62.5 mM Tris-HCl, pH 6.7
TTBS:	See western blotting reagents

Whole Cell Lysate Reagents

Sample buffer (2 x):	See gelatin zymography reagents

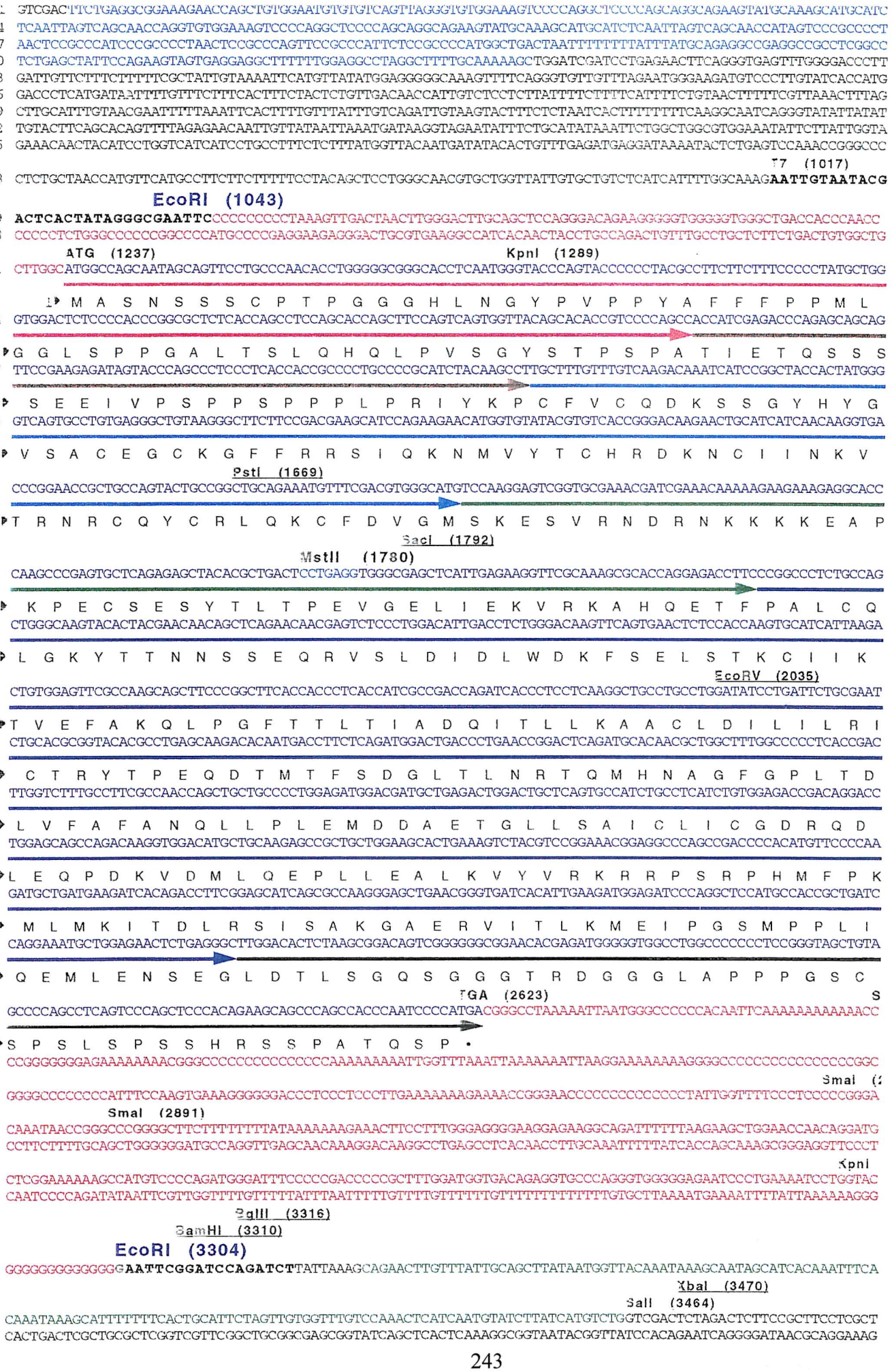
APPENDIX 2.

EXPRESSION VECTORS



mRAR α COMMENT

DESCRIPTION:	Two overlapping clones reconstituted at MstII site and put into EcoRI of pSG5		
INSERTED INTO:	EcoRI	**RESISTANCE:**	Ampicilin
EXCISE WITH:	EcoRI	**SIZE:**	Environ~2Kb
REFERENCES:	Zelent et al. Nature 339:714-717,89		
CONSTRUCTED BY:	A.KRUST	23/01/89	


SalI (1)
T7 (1017)
EcoRI (1043)
ATG (1237)
KpnI (1289)
PstI (1669)
SacI (1792)
MstII (1780)
EcoRV (2035)
TGA (2623)
SmaI (2
SmaI (2891)
KpnI
BglII (3316)
BamHI (3310)
EcoRI (3304)
XbaI (3470)
SalI (3464)

3597 AACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATC
3700 GACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCT
3803 TACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTG
3906 GGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAG
4009 CAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGG
4112 TATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAG
4215 CAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGG
4318 TCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTA
4421 CCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGC
4524 TTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAA
4627 GTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCAT
4730 TGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAA
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5039 GGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGT
5142 TCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGG
5245 GAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGA
5348 ATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAA
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5760 TGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGA
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5966 TTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGG
6069 AAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGT
6172 CGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCG
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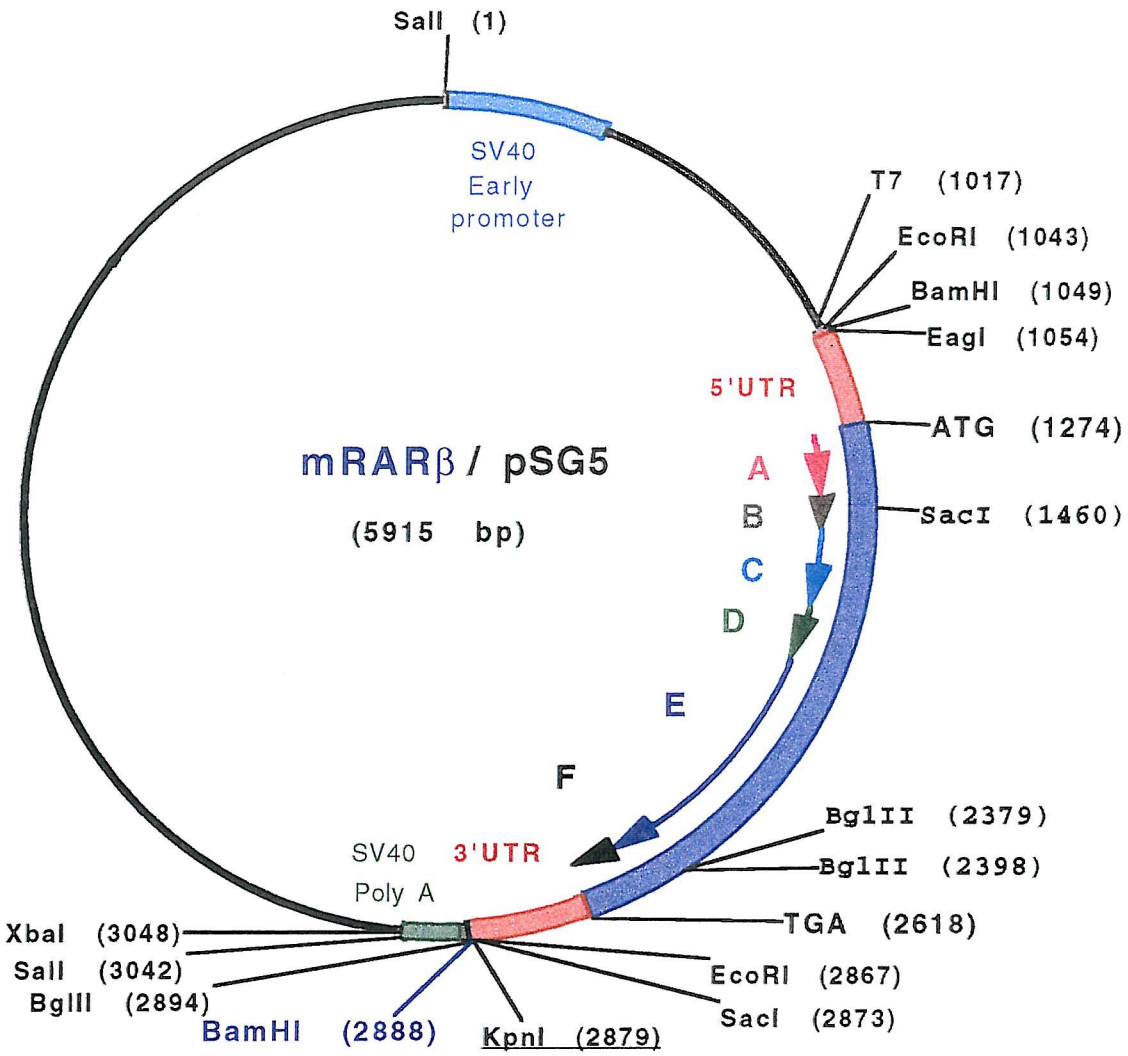


mRAR β 2 COMMENT

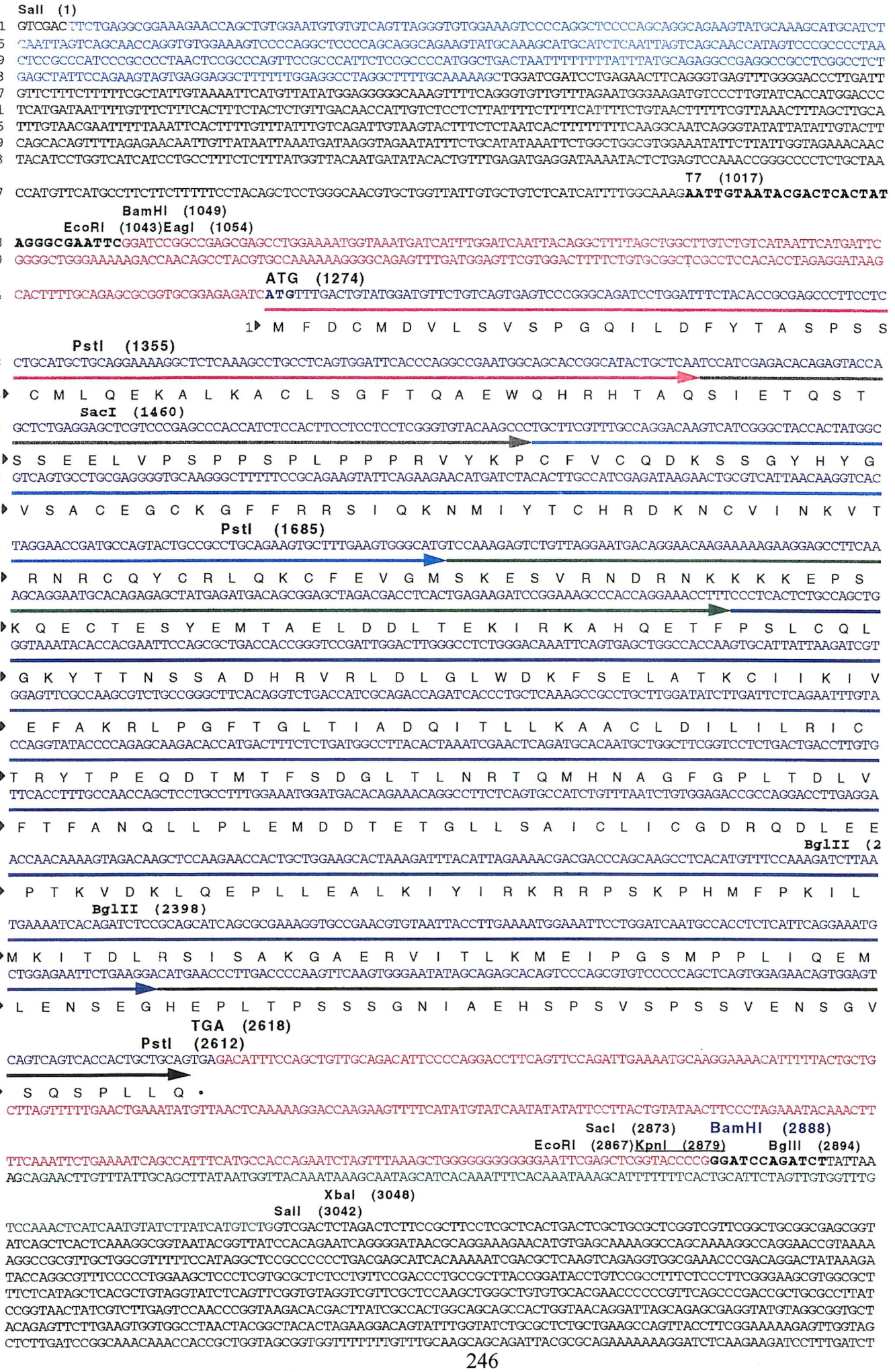
DESCRIPTION: There are two internal EcoRI sites in the cDNA. Part of polylinker from pTZ 19R. All sites between BamHI-EcoRI. Can linearize with KpnI. Constructed from EagI/BamHI of mRAR β digest into BamHI of pSG5.

EXCISE WITH: BamHI

RESISTANCE: Ampicilin **SIZE:** Environ 1,8 Kb

REFERENCES: Zelent et al Nature 339:714-717,89

CONSTRUCTED BY: A.Zelent 23/01/89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T7 (1017)
BamHI (1049)
EcoRI (1043)EagI (1054)
ATG (1274)
PstI (1355)
SacI (1460)
PstI (1685)
BglII (2
BglII (2398)
TGA (2618)
PstI (2612)
SacI (2873) BamHI (2888)
EcoRI (2867)KpnI (2879) BglII (2894)
XbaI (3048)
SalI (3042)

3842 TTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAA
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4154 CTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAA
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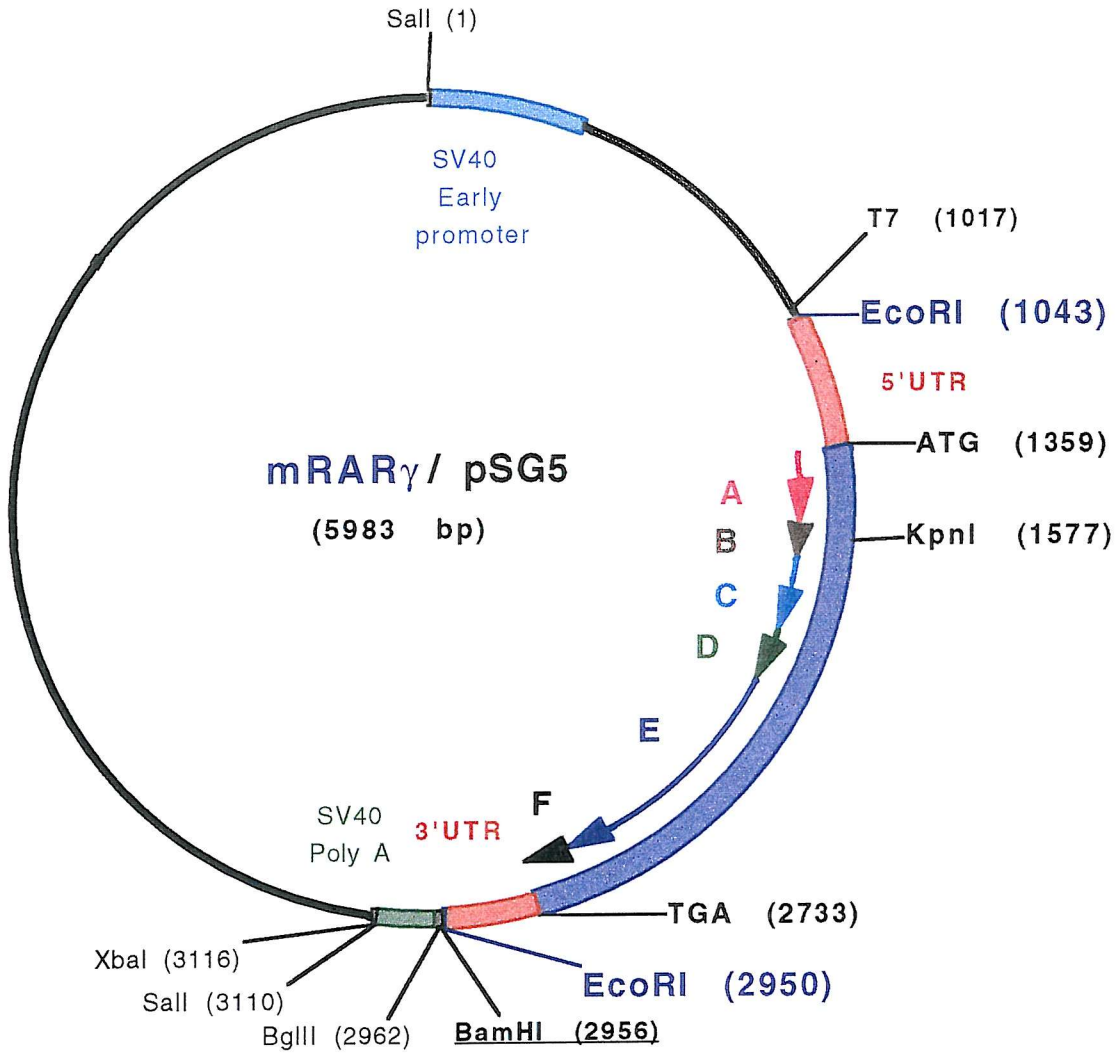


mRAR γ COMMENT

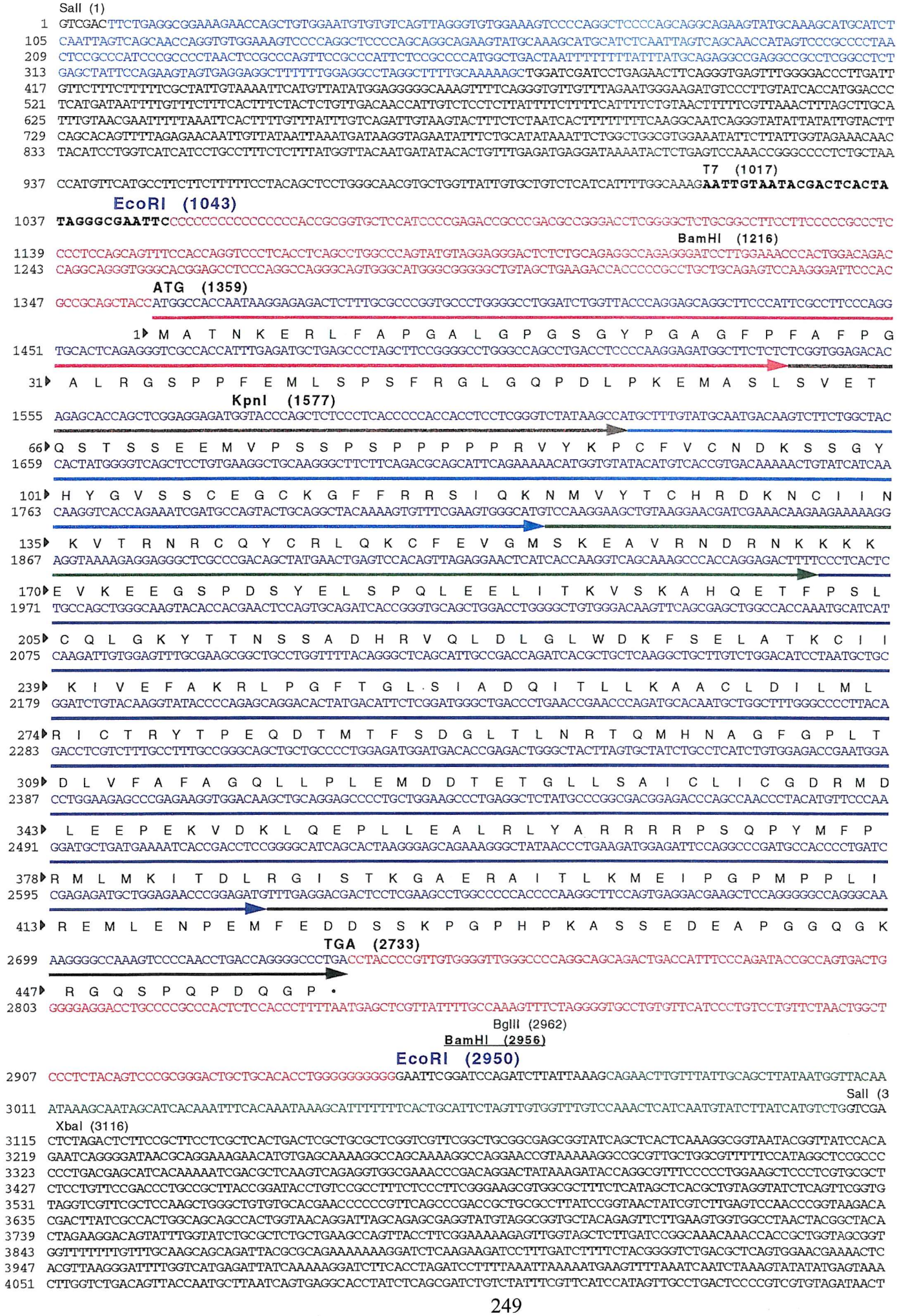
DESCRIPTION: cDNA cloned into EcoRI of pSG5. Can linearize with BamHI.

EXCISE WITH: EcoRI **INSERTED INTO:** EcoRI

RESISTANCE: Ampicilin **SIZE:** Environ 1,9 Kb

REFERENCES: A.Zelent et al Nature 339:714-717,89

CONSTRUCTED BY: A.Zelent 23/01/89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EcoRI (1043)
BamHI (1216)
ATG (1359)
KpnI (1577)
TGA (2733)
BglII (2962)
BamHI (2956)
EcoRI (2950)
SalI (3
XbaI (3116)

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4363 ACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCC
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5403 TTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTG
5507 GAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTT
5611 TTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGG
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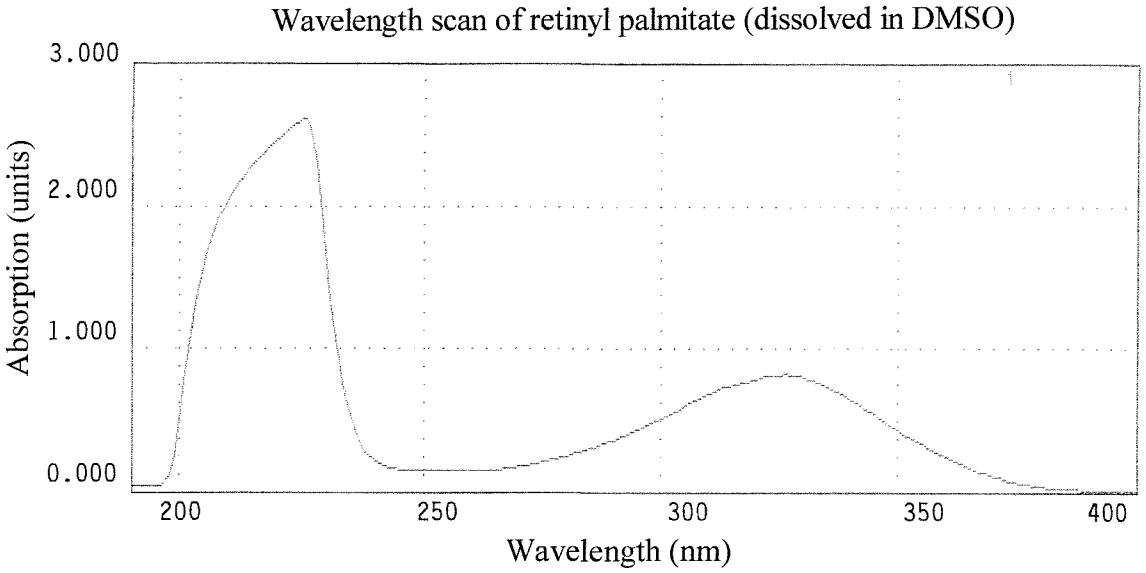
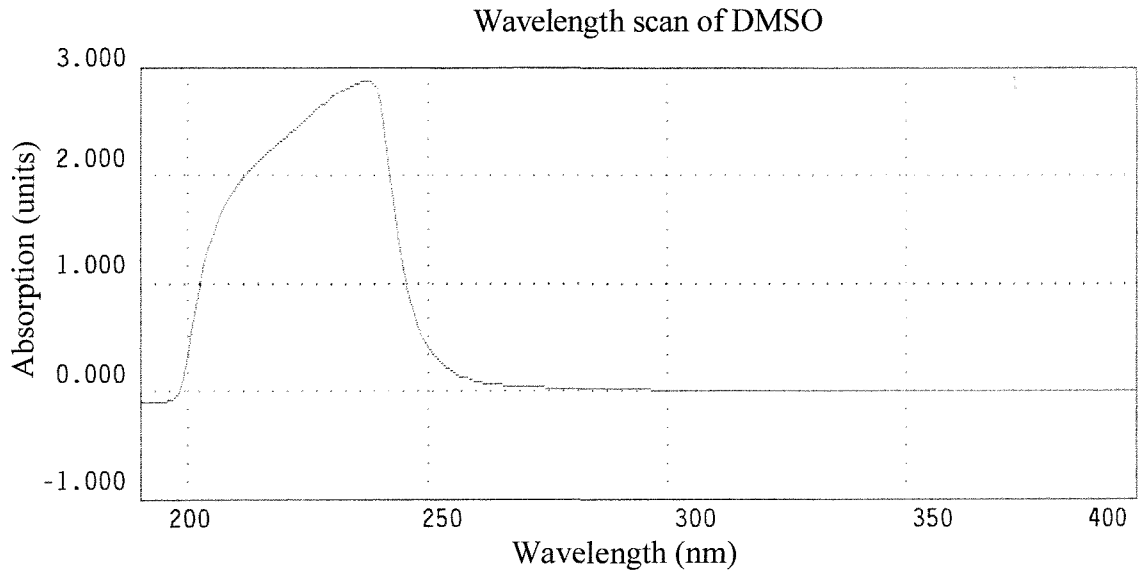
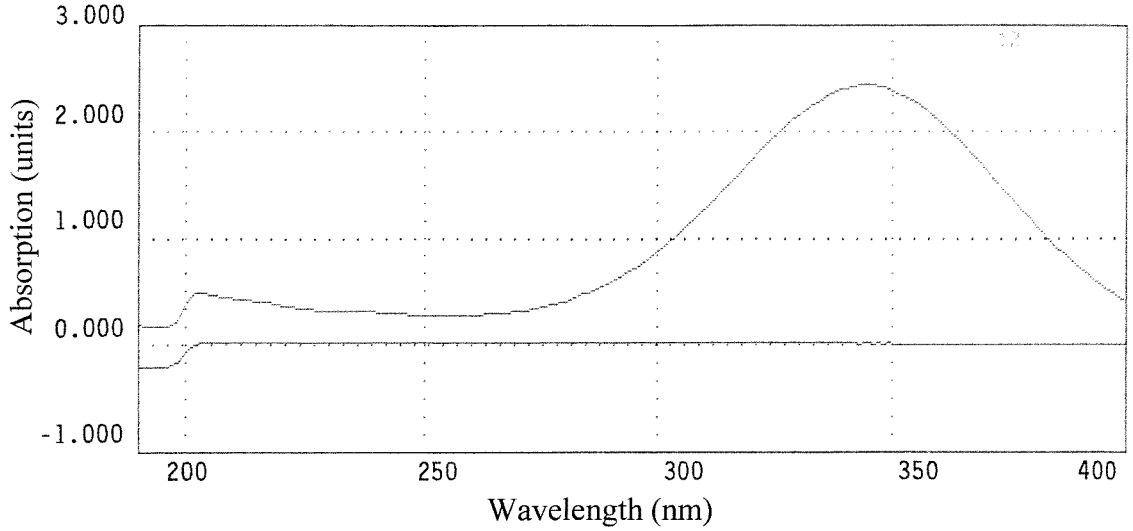
APPENDIX 3.

WAVELENGTH SCANS OF RETINOIDS

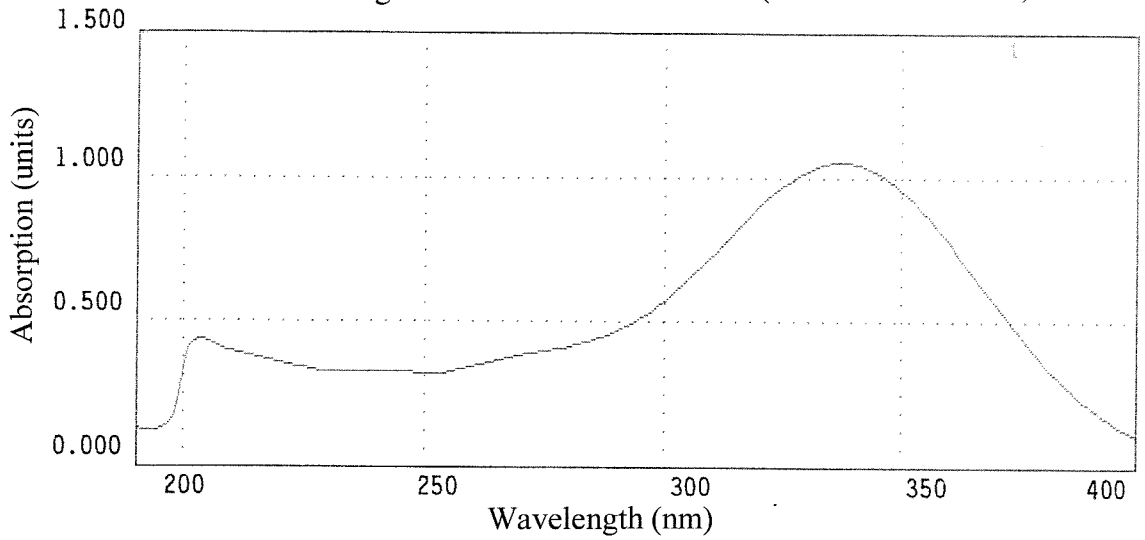




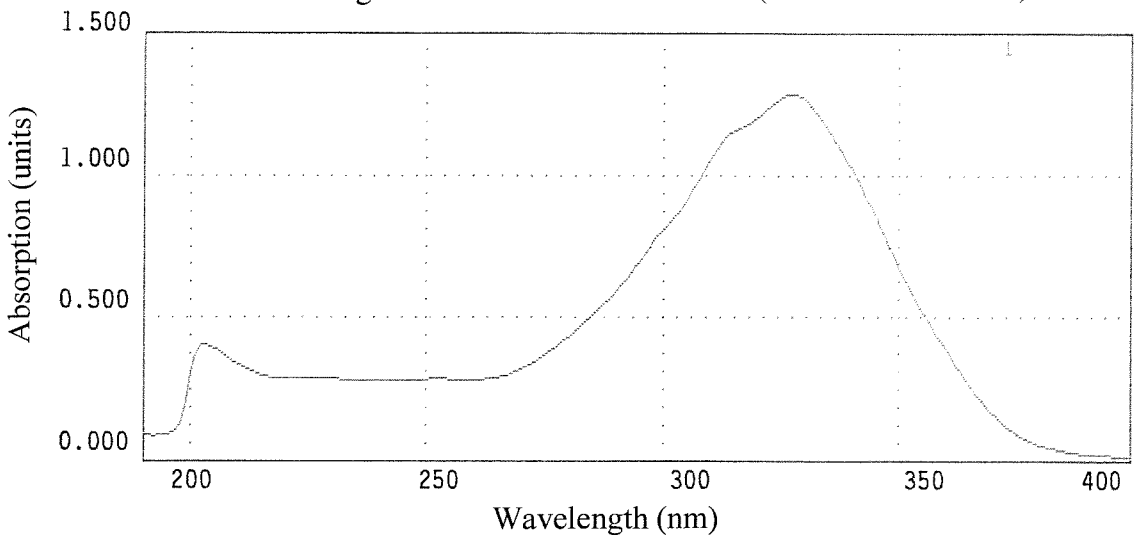
Wavelength scan of all-*trans* retinoic acid dissolved in ethanol (lower trace)

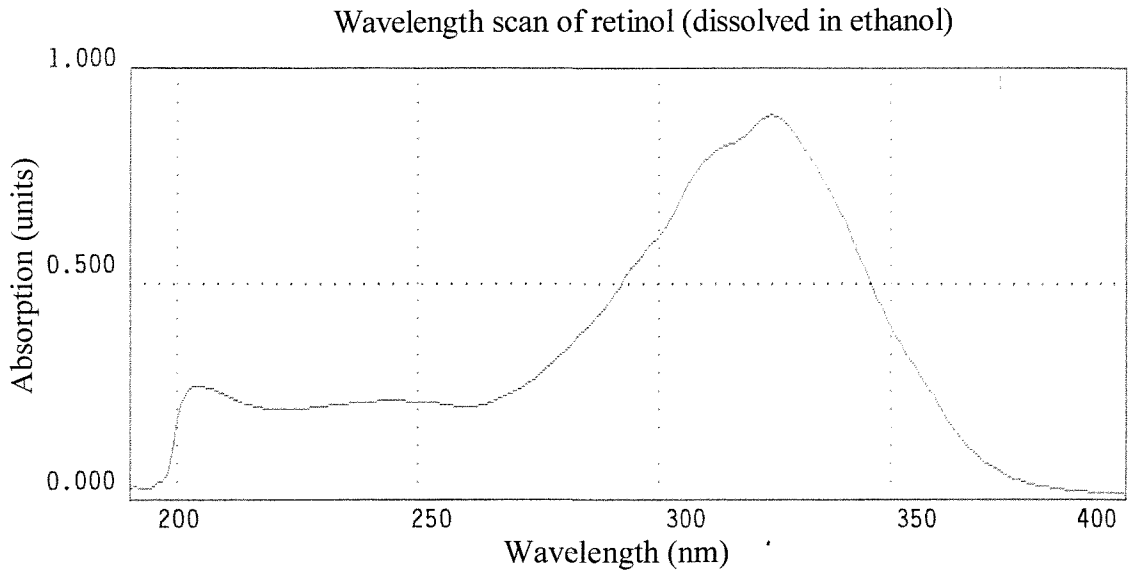


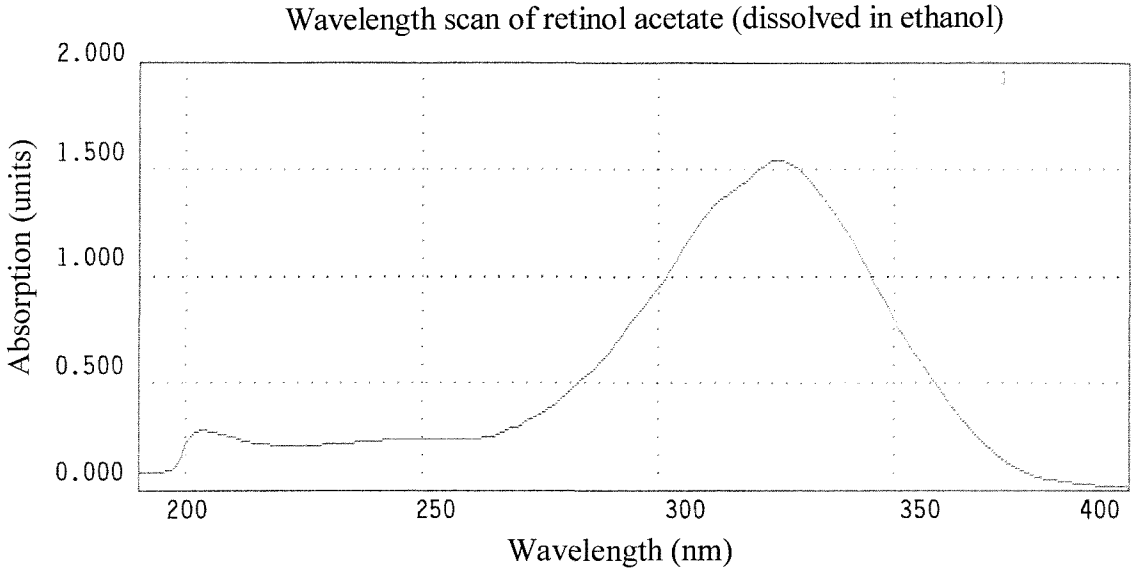
Wavelength scan of 9-*cis* retinoic acid (dissolved in ethanol)



Wavelength scan of 13-*cis* retinoic acid (dissolved in ethanol)




Wavelength scan of retinol (dissolved in ethanol)
1.000
0.500
0.000
Absorption (units)
200
250
300
350
400
Wavelength (nm)


Wavelength scan of retinol acetate (dissolved in ethanol)
2.000
1.500
1.000
0.500
0.000
Absorption (units)
200
250
300
350
400
Wavelength (nm)

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