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Studies on the Expression and Regulation of Transcription Factors in Hepatic Stellate Cells

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

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Doctor of Philosophy

STUDIES ON THE EXPRESSION AND REGULATION OF TRANSCRIPTION FACTORS IN HEPATIC STELLATE CELLS

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A key event in the pathogenesis of liver fibrosis is the activation of hepatic stellate cells (HSCs). However, the transcriptional control of HSC activation is currently poorly understood. This thesis has, therefore, further elucidated the transcriptional mechanisms that may be involved. In the following studies, three major transcription factors (MyoD, Sp1 and AP-1) that may be important regulators of HSC activation have been analysed.

Both *in vitro* and *in vivo* models of HSC activation were associated with changes in E-box DNA binding activities. E-box DNA sequences are found in the regulatory regions of most muscle-specific genes and also many other lineage-specific genes. Freshly isolated HSCs from untreated rats expressed a high mobility complex (C) that was replaced upon activation with two low mobility complexes (A and B). The assembly of complexes A and B was inhibited with an antibody against MyoD. Further studies demonstrated that HSCs did express the transcription factor, MyoD, and its expression may be controlled by a post-transcriptional mechanism. Furthermore, activation of rat HSCs was accompanied by reduced expression of the inhibitory protein, Id1. Investigation of cell-signalling pathways involved in the induction of MyoD expression demonstrated the involvement of both phosphatidylinositol 3-kinase (PI 3-kinase) and the transcription factor, NF-κB.

Both *in vitro* and *in vivo* models of HSC activation revealed induction of a low mobility GC-box (LMGC) binding complex and was subsequently identified as Sp1. Culture activated HSCs showed strong transient induction of Sp1 at 24 hours. Cell-signalling experiments demonstrated that Herbimycin A could inhibit the Sp1 complex, suggesting induction was via a tyrosine kinase dependent pathway. Further culture of HSCs was associated with loss of Sp1 binding and protein expression but was instead replaced by a high mobility GC-box (HMGC) activity that was not reactive with antibodies recognising any of the Sp protein family.

Culture activation of rat HSCs (*in vitro*) indicated transient expression of low mobility AP-1 (LMAP-1) complexes that were maximally induced at 24 hours of culture and then fell to undetectable levels with further culture. Supershift Electromobility Shift Assay (EMSA) identified its components as c-Jun, JunB, JunD, c-Fos and FosB. Further culture of HSCs led to the detection of sustained high mobility AP-1 (HMAP-1) complexes. Supershift EMSA described these proteins as JunD, Fra2 and FosB. Finally, *in vivo* studies revealed a slightly different AP-1 profile where the complexes consisted of JunB, JunD, Fra1 and Fra2.

Further experiments are now required to elucidate the functions of these transcription factors in the HSC. Greater understanding of the molecular events that underlie liver fibrosis may lead to the development of new therapeutic strategies in the future.

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RESEARCH PUBLICATIONS

Modulation of GC-box DNA binding activities and Sp1 expression during *in vitro* and *in vivo* activation of rat hepatic stellate cells. **Vincent K.J.**, Bahr M.J., Arthur M.J.P., Trim J.E., Wright M.C., Smart D.E., Benyon R.C., Mann D.A. Submitted to J Hepatology.

Regulation of E-box DNA binding during *in vivo* and *in vitro* activation of rat and human hepatic stellate cells: Evidence for expression of MyoD. **Vincent K.J.**, Jones E., Arthur M.J.P., Smart D.E., Trim J.E., Wright M.C., Mann D.A. Submitted to Biochem J.

Control of the tissue inhibitor of metalloproteinases-1 promoter in culture-activated rat hepatic stellate cells: Regulation by activator protein-1 DNA binding proteins. Bahr M.J., **Vincent K.J.**, Arthur M.J.P., Fowler A.V., Smart D.E., Wright M.C., Clark I.M., Benyon R.C., Iredale J.P., Mann D.A. Hepatology, 1999, **29** (3), 839-848.

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ABBREVIATIONS

AEBSF	4-(2-Aminoethyl)benzenesulfonyl Fluoride
ANT2	Adenine Nucleotide Translocase 2
AP-1	Activator Protein-1
APS	Ammonium Persulphate
ATP	Adenine Triphosphate
AMV	Avian Myeloblastosis Virus
αSMA	Alpha smooth-muscle actin
BTE	Basic Transcription Element
BTEB	Basic Transcription Element-Binding Protein
bFGF	Basic Fibroblast Growth Factor
bHLH	Basic-Helix-Loop-Helix
BIM	Bisindolylmaleimide 1, Hydrochloride
BSA	Bovine Serum Albumin
bZIP	Basic Region/Leucine Zipper
C2	C2C12 Myoblast
CAT	Chloramphenicol Acetyl Transferase
CBP	CREB-Binding Protein
CCl_4	Carbon Tetrachloride
Cdk	Cyclin-dependent kinase
CK2	Casein Kinase 2
CRE	cAMP-Response Element
CREB	cAMP-Response Element-Binding Protein
CSF-1	Colony Stimulating Factor-1
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
dNTPs	Deoxynucleoside Triphosphates
ECL	Enhanced Chemiluminescent Reagent
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EMSA	Electromobility Shift Assay
ERKs	Extracellular Signal-Regulated Kinases

ET-1	Endothelin-1
F9	F9 Embryonic Carcinoma Cell
FBS	Fetal Bovine Serum
FRA	Fos-Related Antigen
γ-IFN	Gamma-Interferon
HATs	Histone Acetyltransferases
HBS	Hanks Buffered Saline
HCA	Human Cardiac Alpha-Actin
HDACs	Histone Deacetylases
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HMAP-1	High Mobility AP-1
HMGC	High Mobility GC-Box
HOB	Homology Box Motif
HSC	Hepatic Stellate Cell
Id	Inhibitor of DNA Binding; Inhibitor of DNA Differentiation
IGF	Insulin-Like Growth Factors
IκB	Inhibitor of κ Binding
IKK	IκB Kinase
IL	Interleukin
JAB1	Jun Activation Domain Binding Protein 1
JAK	Janus Kinase
JNKs	c-Jun NH_2-Terminal Kinases
LB	Luria Broth
LMAP-1	Low Mobility AP-1
LMGC	Low Mobility GC-Box
MADS	MCM1, Agamous, Deficiens, and Serum Response Factor
MAPKs	Mitogen-Activated Protein Kinasaes
MCK	Muscle Creatinine Kinase
MCP-1	Monocyte Chemotactic Peptide-1
MEF-2	Myocyte-Specific-Enhancer-Binding Factor
MMPs	Matrix Metalloproteinases
MOPS	3-[N-Morpholino]propanesulfonic acid
MT1-MMP	Membrane-Type 1 MMP
MyoR	Myogenic Repressor
NF-κB	Nuclear Factor Kappa B

NIK	NF-κB-Inducing Kinase
NLS	Nuclear Localisation Signal
NP40	Nonidet P-40
NRE	Negative Regulatory Element
PAF	Platelet Activating Factor
PAGE	Polyacrylamide Gel Electrophoresis
PAR-2	Proteinase-Activated Receptor-2
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PDTC	Pyrrolidinedithiocarbamate
PI 3-Kinase	Phosphatidylinositol 3-Kinase
PIC	Pre-initiation Complex
PK	Protein Kinase
PKR	Double-Stranded RNA Activated Kinase
pRB	Retinoblastoma Protein
RHD	Rel homology Domain
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SIE	Sis-Inducible Enhancer
SRE	Serum Response Element
SRF	Serum Response Factor
STAT	Signal Transducer and Activator of Transcription
SV40	Simian Virus 40
TAFs	TBP-Associated Factors
Taq	Thermus Aquatus
TEMED	N,N,N',N'-Tetramethylethylenediamine
TBM	TBP-Binding Motif
TBP	TATA-Binding Protein
TCFs	Ternary Complex Factors
TF	Transcription Factor
TGF	Transforming Growth Factor
TIMPs	Tissue Inhibitors of Metalloproteinases
TLC	Thin Layer Chromatography
TNF-α	Tumour Necrosis Factor-Alpha

TPA	12-O-Tetradecanoylphorbol-13-Acetate
TRE	TPA-Response Element
uPA	Urokinase Plasminogen Activator
UTE-1	Upstream TIMP-1 Element-1

CHAPTER 1

INTRODUCTION

1.1 LIVER FIBROSIS

1.1.1 INTRODUCTION

Liver cirrhosis is a major world-wide health care problem, causing more than six thousand deaths a year in the United Kingdom alone[1]. Cirrhosis is the end result of a protracted time course of progressive liver fibrosis. Hepatic fibrosis is a common response to chronic liver injury caused by various factors (see Table 1.1). The commonest causative factor in the West is alcohol. However, in other countries hepatitis B and C remain the major aetiological factors. Current medical therapy is very unsatisfactory, with 5-year survival rates at only 25%. Regardless of the cause, hepatic fibrosis is characterised by both an increase in the amount of extracellular matrix (ECM) deposited in the liver and also a change in the composition of the ECM[2].

AETIOLOGY OF CIRRHOSIS	
Drugs and toxins	Alcohol, methotrexate, isoniazid, methyldopa.
Infections	Hepatitis B & C, *Schistosoma japonicum*.
Autoimmune/immune-mediated	Primary biliary cirrhosis, autoimmune hepatitis, primary sclerosing cholangitis.
Metabolic	Wilson's disease, haemochromatosis, α_1-antitrypsin deficiency, porphyria (rarely glycogen storage diseases, galactosaemia, abetalipoproteinaemia, etc)
Biliary obstruction (secondary biliary cirrhosis)	Cystic fibrosis, atresia, strictures, gallstones.
Vascular	Chronic right heart failure, Budd-Chiari syndrome, veno-occlusive disease.
Miscellaneous	Sarcoidosis, intestinal bypass operations for obesity.
Cryptogenic	Unknown.

Table 1.1 Table showing various aetiological factors that can lead to liver fibrosis/cirrhosis[3].

Common complications of chronic liver disorders, regardless of their cause, are: portal hypertension (a rise in portal blood pressure above the normal range of 3 to 6mm Hg), variceal bleeding, ascites (a detectable collection of fluid within the peritoneal space), hepatic encephalopathy (neuropsychiatric syndrome associated with neurological and psychiatric disturbances, and occurs as a consequence of acute and chronic liver

failure), disturbed glucose metabolism, and deficiencies of vitamins and trace elements[4].

1.1.2 STRUCTURE OF THE LIVER

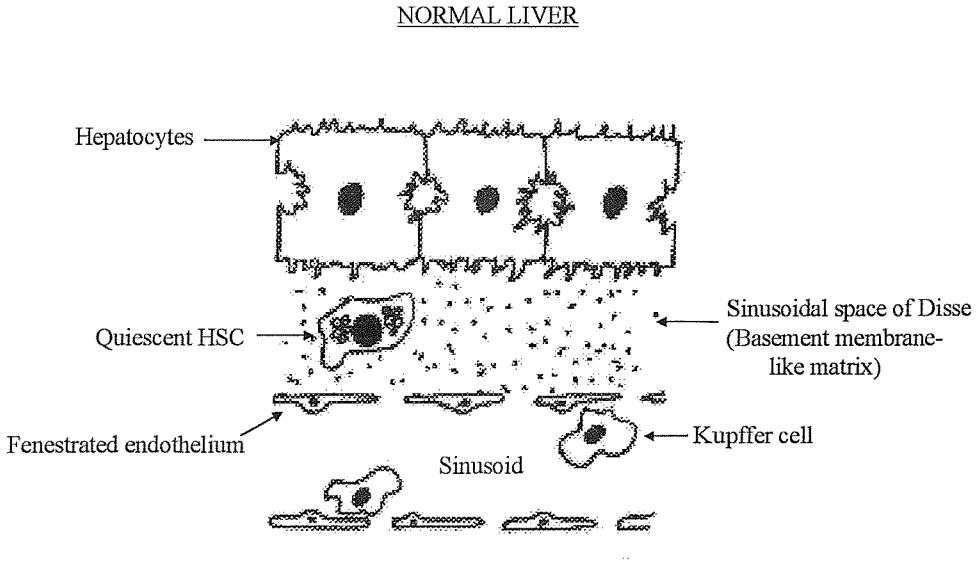
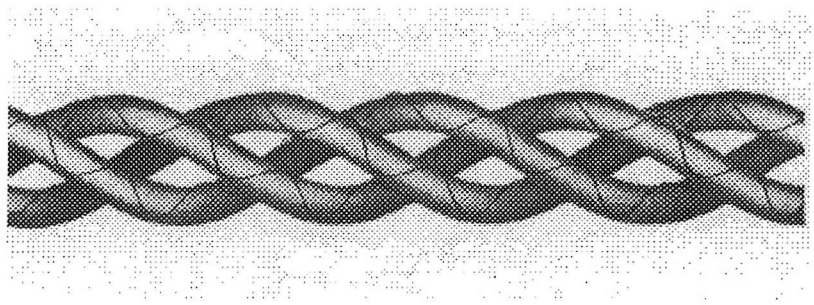


Figure 1.1 Structure of the normal liver. Adapted from Arthur *et al.*, 1994[1].

The functional unit of the liver is the acinus, which receives blood from a single terminal portal venule and hepatic arteriole (termed together the axial vessels) and passes its bile into a single small efferent duct in the same portal tract. The blood supplies feed into the hepatic sinusoids, which are bounded by plates of hepatocytes, and finally drains into the efferent central vein[5]. Hepatocytes make up over 80% of the volume of the human liver and are the site of almost all liver synthetic and degradative functions. Normal hepatocytes are cuboidal in shape and are arranged in palisades along the sinusoid, in close contact with the sinusoidal blood. The vascular sinusoid is lined by endothelial cells and contains, within its lumen, Kuppfer cells and pit cells. Kuppfer cells are the resident tissue macrophages found predominantly in the periportal areas of the sinusoid and are responsible for removing bacterial antigens and endotoxin from the portal blood. Endothelial cells in the liver are highly specialised, possessing many fenestrae which allow free passage of macromolecules from sinusoidal blood into the space of Disse (lies between the sinusoidal endothelium and the brush-bordered sinusoidal surface of the hepatocyte) thus permitting contact with hepatocytes. Pit cells

bear a strong resemblance to the natural killer cell in the spleen which play a role in clearing opsonised particles and tumour cells from the blood[5].

1.1.3 LIVER MATRIX IN NORMAL LIVER



Portion of a collagen molecule

Figure 1.2 Schematic diagram of a collagen molecule.

Collagens have left-handed triple-helical polypeptides of variable length with repeating sequences of (Gly-X-Y)n whereby the repeated glycine at every third position allows regular folding and the formation of the helices. The collagens are broadly classified as fibril-forming (interstitial) which include collagens type I, II, III, V, XI and non-fibril-forming types which include IV, VI, VII, VIII, IX, X and of these, types I, III, IV, V and VI have been identified in the liver. Non-collagenous components of the ECM are collectively termed glycoconjugates and are by weight far more abundant than collagens in normal and fibrotic liver. Glycoconjugates can be further classified as glycoproteins and include laminin, nidogen, fibronectin, tenascin, undulin, elastin and proteoglycans, which are a family of molecules with a protein core and carbohydrate side-chains. These include heparan sulphate, the most common proteoglycan in normal liver[6], heparin, dermatan and chondroitin sulphate. Collagen types I and III account for approximately 90% of total liver collagen, with less of types IV, V and VI[7]. In a normal liver, fibril-forming matrix (collagens I, III and V) is most prominent in the perivascular spaces, around portal tracts and subcapsular areas. Type IV and VI collagens are located in the space of Disse, as well as in vascular and bile duct basement membranes[8,9]. Type IV collagen together with laminin and proteoglycans forms a flexible lattice structure[10] while type VI collagen monomers interact with each other to form chains of molecules within the space of Disse. Periductal and perivascular areas are rich in fibronectin and vitronectin. The basement membrane-like matrix in

the space of Disse contains fibronectin[8] and laminin in the same distribution as type IV collagen. Undulin is found in small amounts in association with the fibril-forming collagens[7]. Heparan sulphate is found predominantly at the portal end of the sinusoid with heparin proteoglycan at the venous end. Dermatan and chondroitin sulphate are found in very small amounts in normal liver[11].

1.1.4 LIVER MATRIX IN FIBROTIC LIVER

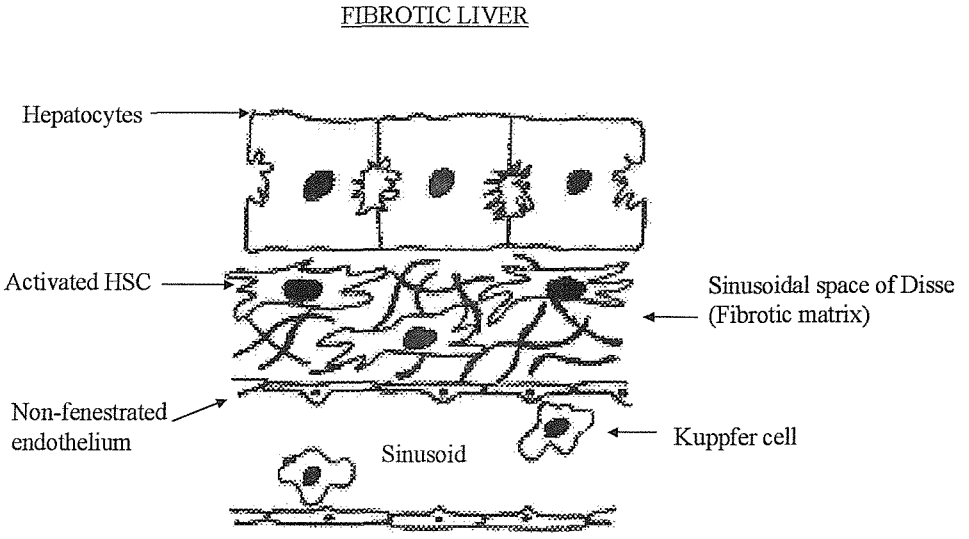


Figure 1.3 Structure of fibrotic liver. Adapted from Arthur *et al.*, 1994[1].

During hepatic fibrosis, as a consequence of hepatic stellate cell (HSC; located in the space of Disse) activation, there is an accumulation of collagens (especially the fibrillar types I and III) as well as other matrix molecules[12]. These matrix proteins are laid down in the space of Disse, initially causing an alteration in the appearance of the sinusoid. With further progression of fibrosis there are changes in the phenotype of the endothelial cells which lose their fenestrae and disturbance of hepatocyte function. If matrix deposition continues there is more gross distortion of the sinusoidal structures with whole septae of connective tissue extending throughout the parenchyma[5]. The final result of this process is the end-stage cirrhotic liver in which the whole anatomy is macroscopically distorted by thick bands of collagen, within which nodules of hepatocytes, often with regenerative foci, are surrounded. Cirrhotic liver contains approximately six times the amount of matrix as normal liver[7]. There is an increase in the amounts of types I, III and IV collagen in the space of Disse but for type I collagen

this increase is disproportionate so that the ratio of type I to types III and IV increases[12]. There is also an increase in laminin within the space of Disse and alterations in both the quantity and the type of fibronectin produced[7]. The spectrum of glycoproteins expressed changes with a relative decrease in heparin and heparan sulphate proteoglycans and an increase in dermatan and chondroitin sulphate proteoglycans. The latter contain the core proteins decorin and biglycan which have a potentially important role in cytokine binding within the extracellular matrix. Hyaluronan, which is only present in small quantities in normal liver, is increased 8-fold in diseased liver and is secreted by HSCs[13].

1.1.5 MATRIX REMODELLING

There is a baseline turnover of this matrix in the normal liver. This is mediated by a family of zinc-dependent proteases, the matrix metalloproteinases (MMPs)[14]. Two major groups of MMPs are believed to be important in matrix degradation during liver fibrosis:

1) Gelatinases and stromelysins

This group have activity against type IV collagen and other components of the normal basement membrane-like matrix[14]. These enzymes may therefore significantly alter HSC activation through degradation of the normal matrix. There is evidence that members of these groups are expressed by activated Kupffer cells (gelatinase B)[15], HSCs (gelatinase A and stromelysin)[16,17]and endothelial cells (stromelysin)[18].

2) Collagenases

Interstitial collagenase is derived from fibroblastic cells including activated myofibroblastic HSCs whereas neutrophil collagenase is released from neutrophil phagosomes in areas of inflammation[14]. Because none of the other MMPs can initiate breakdown of interstitial collagens, interstitial or neutrophil collagenase must be expressed for matrix remodelling to occur in established fibrosis. It has been shown that MMP activity in liver decreases as fibrosis progresses[14].

1.1.6 REGULATION OF METALLOPROTEINASES

The extracellular activity of MMPs are regulated at various stages:

1) Transcriptional activation at the level of the gene[19]. A series of growth factors and cytokines including interleukin-1 (IL-1), tumour necrosis factor-alpha (TNF-α),

platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) also regulate MMPs at the level of the gene[14].

2) Conversion of the pro-MMP to active MMP by cleavage of the propiece. HSCs express urokinase plasminogen activator (uPA) during activation and through expression of this protein can generate active plasmin from plasminogen. The plasmin in turn cleaves the propiece from pro-stromelysin, which can then cleave interstitial collagenase. As activation of HSCs becomes more advanced, expression of uPA decreases and is replaced by the plasminogen activator inhibitor with a resulting decrease in uPA activity[20].

3) Extracellular inhibition by the specific Tissue Inhibitors of Metalloproteinases (TIMPs) or more general protease inhibition such as α_2-macroglobulin.

1.1.7 TISSUE INHIBITORS OF METALLOPROTEINASES

Four TIMPs have been identified to date (TIMP 1, 2, 3, 4) and each one is encoded by a separate gene[21-24]. However, there remains a 40% sequence homology between TIMP-1 and 2. All TIMPs can inhibit the degradative activity of MMP enzymes by binding to their catalytic site, and this is mediated by the N-terminus of TIMPs[1]. In addition, certain TIMPs (via their C-terminus) can stabilise specific pro-MMP species and thus prevent their activation[25-27]. Recently, a novel membrane-bound MMP, membrane-type 1 MMP (MT1-MMP), has been identified as an essential enzyme required for cleaving the pro-peptide sequence of the pro-gelatinase A molecule. It has been suggested that low levels of TIMP-2 are necessary to anchor pro-gelatinase A to MT1-MMP on cell membranes. Another active MT1-MMP molecule, not bound to TIMP-2, could then activate pro-gelatinase A. An excess amount of TIMP-2, however, will bind to most of both MT1-MMP and pro-gelatinase A molecules, resulting in the inhibition of pro-gelatinase A[401]. TIMPs are regulated at the level of transcription by cytokines and growth factors that also govern MMP expression[28]. Expression of mRNA for TIMPs in diseased human liver and animal models of fibrosis indicates that transcripts for both TIMP-1 and 2 are significantly upregulated and, in the case of TIMP-1 are probably HSC derived[29].

Even in comparatively advanced fibrosis, the potential for matrix degradation still exists. However, the process of matrix degradation is either overwhelmed by synthesis,

and/or that matrix degradation is inhibited as a result of TIMP and plasminogen activator inhibitor expression as well as other more general protease inhibitors.

1.1.8 THE HEPATIC STELLATE CELL

The HSC (also known as fat-storing cell, lipocyte or Ito cell) was first identified by von Kupffer in 1876 and comprises approximately a third of the nonparenchymal cells found within the liver[30,31]. The main functions of the HSC appear to be storage and metabolism of retinoids in fat droplets (the liver is the body's main store of vitamin A) and collagen synthesis[32,33]. HSCs in the perisinusoidal space of Disse have been shown to express desmin (a muscle-specific member of the intermediate filaments protein multigene family) and because of their location, it has been suggested that these cells may act as pericytes, regulating blood flow through the sinusoid[31,34].

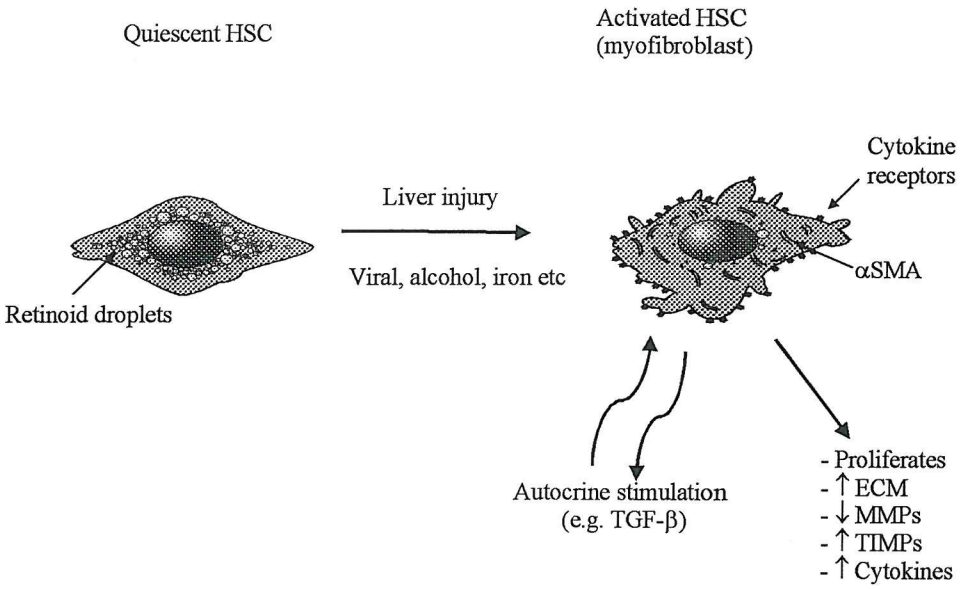


Figure 1.4 Model of HSC activation.

Activation of the HSC is now recognised as one of the most crucial events in the pathogenesis of hepatic fibrosis[2,5]. In response to liver injury the cell undergoes a progressive phenotypic transformation from a quiescent retinoid-storing cell to a proliferating, alpha smooth-muscle actin (αSMA) positive myofibroblast-like cell. Histologically, the activation process is characterised by cell enlargement and

spreading, the development of long cytoplasmic outgrowths, loss of fat droplets, the appearance of one or more nucleoli and hypertrophy of the rough endoplasmic reticulum, along with other signs of high protein synthesis[35]. The cells appear to be activated in two steps; enlarging and becoming more responsive to proliferative and fibrogenic cytokines as the number of receptors for these increases, followed by a second perpetuation stage where the activation seems to become self-sustaining[35]. The process of activation is also associated with the expression of various factors that play a role in the disease process. Activated HSCs express very high levels of collagens I, III and IV, as well as other matrix proteins such as fibronectin, hyaluronic acid and laminin[36,37]. They also synthesise MMPs, such as interstitial collagenase and gelatinases. However the activity of these degradative enzymes is offset by the expression of high levels of TIMPs, resulting in an overall matrix deposition within the liver (see section 1.1.7)[38]. In addition, activated HSCs express cytokines such as IL-6, monocyte chemotactic peptide-1 (MCP-1), endothelin-1 (ET-1), platelet activating factor (PAF), colony stimulating factor-1 (CSF-1), transforming growth factor alpha and beta (TGF-α & -β), as well as the receptors for some of these[39,40]. During HSC transformation, *de novo* expression of αSMA is induced which may contribute to the fact that activated HSCs become contractile and respond to vasoactive agents such as ET-1[31,41].

Several factors are believed to be important in the activation of HSCs. HSCs respond to changes in their substratum by undergoing phenotypic changes, especially if the substratum contains collagens I and III. Conversely activated HSCs can be induced to adopt a quiescent state by culturing them on a basement membrane-like matrix layer[42]. An initial inflammatory change to the matrix in the space of Disse may, therefore, initiate the process of HSC activation. Retinoids have been shown to revert activated HSCs to quiescence *in vitro*, although their role *in vivo* remains uncertain[43]. Similarly, many cytokines that are secreted by various other cells in the liver and by the activated HSC itself have been shown to increase the fibrogenic capacity of these stellate cells[2]. These include TGF-β, PDGF, IL-1, TNF-α, prostaglandins, thromboxanes and bFGF. The best studied among these are TGF-β and PDGF. The former is the most fibrogenic cytokine known and stimulates HSC production of collagen, while repressing its production of interstitial collagenase. The latter is the most potent stimulator of HSC proliferation known[5]. Both are expressed by activated HSCs and, therefore, believed to be responsible for the perpetuation of the activated state by autocrine methods[35].

1.1.9 MODELS OF HEPATIC STELLATE CELL ACTIVATION

The study of HSC activation has been greatly assisted by the development of an *in vitro* model[44]. This involves the isolation of HSCs from human, rat or murine livers, relying on the fact that they are buoyant due to their vitamin A content. Activation is then achieved by culturing freshly isolated HSCs on tissue culture plastic in the presence of serum. The cultured cells develop a phenotype that is very similar to that of activated HSCs *in vivo* and express many of the same factors[45]. This model has greatly advanced understanding of molecular events that underlie hepatic fibrosis. In addition, there are several *in vivo* models that have aided the study of liver fibrosis. For example, the carbon tetrachloride (CCl_4) model is a hepatotoxic *in vivo* model of liver injury and is the oldest and most commonly used toxin for the experimental induction of hepatic injury in rats[46]. It is usually administered by intraperitoneal injection, inhalation or gastric lavage. CCl_4 is a haloalkane that requires bioactivation by mixed function oxidases to yield free radicals that causes lipid peroxidation and an alteration in the gene expression of HSCs. Repetitive dosing results in distinctly identifiable histological states of injury[46]. Immunologically-induced liver fibrosis provides another alternative model, for example, intravenous injections of heterologous serum or albumin or intraperitoneal injection of bacterial cell walls can cause hepatic fibrosis in rats[47]. Furthermore, metabolic-induction (i.e. chronic feeding of rats with an iron-load diet or intramuscular injection of iron dextran), common bile duct ligation and alcohol-consumption can all be used to induce liver fibrosis. Finally, transgenic mice have been created that express certain cytokines, such as TGF-β and gamma interferon (γ-IFN). These models may therefore be used to reveal *in vivo* effects of individual cytokines in mediation of the fibrotic process[47].

1.1.10 THERAPEUTIC STRATEGIES FOR LIVER FIBROSIS/CIRRHOSIS

To date, no antifibrotic treatment (including corticosteroids, prostaglandins and colchicine) has been shown to improve survival in patients with cirrhosis. Further research into the cellular mechanisms underlying liver fibrosis and cirrhosis may lead to development of novel therapeutic approaches that can specifically block the fibrogenic cascade. These may include controlling HSC activation, for example, neutralising antibodies to TGF-β1 have been demonstrated to have a pronounced effect on inhibiting scar formation in experimental skin wounds[48] and have been reported to be effective in preventing experimental liver fibrosis in bile duct ligated rats[49]. However, TGF-β1 gene knockout studies indicate that this cytokine has important

immunomodulatory effects[50], suggesting that this approach may not be applicable to human disease. γ-IFN is another candidate because it inhibits proliferation and activation of HSCs in cell culture and decreases their expression of fibronectin and procollagen I genes[51]. γ-IFN has also shown to have antifibrogenic effects in murine hepatic schistosomiasis[52] but this compound has not yet been used in human liver disease. α-IFN has also been reported to inhibit proliferation of HSCs and their synthesis of collagen[53] as well as decreasing parameters of fibrosis when used for the treatment of chronic hepatitis C[54]. It is not clear whether this is a direct *in vivo* antifibrogenic effect or whether it is mediated via its antiviral and immunomodulatory properties.

Potential targets for interfering with the biosynthesis of fibrillar collagens include formation of the triple-helical procollagen molecules, their cellular secretion and fibril formation in the extracellular space. Following procollagen peptide biosynthesis, there is post-translational modification of proline residues to hydroxyproline by prolyl-4-hydroxylase. This enzyme is important for triple-helix formation and is inhibited by the compound HOE-077, which is given as a pro-drug and then converted to the active form in hepatocytes. However although HOE-077 was effective in reducing development of fibrosis[55] in CCl_4 liver injury and bile duct ligation models, it caused cataracts in dogs, and was therefore withdrawn from trial use in humans. Colchicine has also been used in the treatment of human liver fibrosis and its main mechanism of action is thought to be inhibition of microtubule formation and prevention of pro-collagen secretion. However, it remains of unproven benefit and few hepatologists use this drug[56]. An important step in collagen fibril formation is cross-linking in the extracellular space. This can be affected by lysyl oxidase and thus forms a potentially important therapeutic target as failure of cross-linking may render deposited collagens more susceptible to matrix-degrading proteinases. These inhibitors are under development but none have yet been demonstrated to prevent liver fibrosis[56]. However, excess synthesis and secretion of the other non-collagenous matrix proteins would remain unaltered with the approaches described above, and as these other matrix proteins form a significant component of fibrotic tissue, interfering with collagen biosynthesis alone may not be so useful.

Stimulation of matrix degradation can be advantageous in the treatment of liver fibrosis because many patients often present with relatively advanced disease. Three, largely theoretical, approaches exist:

1. In addition to activation of HSCs, TGF-β1 can also up-regulate TIMP-1 and down-regulate interstitial collagenase expression in most mesenchymal cells. Treatments that inhibit TGF-β1 may thus be beneficial via effects on matrix degradation.

2. The hormone, relaxin (derived from the corpus luteum in pregnancy), has many effects on the cardiovascular system[57] but it is also involved in cervical softening, widening of the pubic rami and uterine involution-effects which are mediated via upregulation of interstitial collagenase and down-regulation of TIMP-1. Similar effects of relaxin have now been described on the regulation of these genes in dermal fibroblasts[58].

3. Data also suggest that polyunsaturated lecithin can decrease liver fibrosis in ethanol-fed baboons. This is probably mediated via increased release of collagenase activity by HSCs, particularly after they have been exposed to acetaldehyde[59].

1.2 TRANSCRIPTIONAL REGULATION

1.2.1 INTRODUCTION

The previous section has described the dramatic activation of HSCs during the pathogenesis of liver fibrosis, a key event in this process. In addition, section 1.1.10 has discussed various therapeutic strategies for treating liver fibrosis. However, an alternative strategy to those already mentioned may be to transcriptionally control genes that are up-regulated during HSC activation and which contribute to the pathogenic effects observed during liver fibrosis. Very little is currently known about the transcriptional regulation of HSC activation although new light is being shed on several potential transcription factors (see section 3.1). Further characterisation of changes in the activity of transcription factors during HSC activation may therefore provide alternative insights into the molecular basis for liver fibrosis and may lead to the development of new therapeutic strategies.

1.2.2 TRANSCRIPTION INITIATION

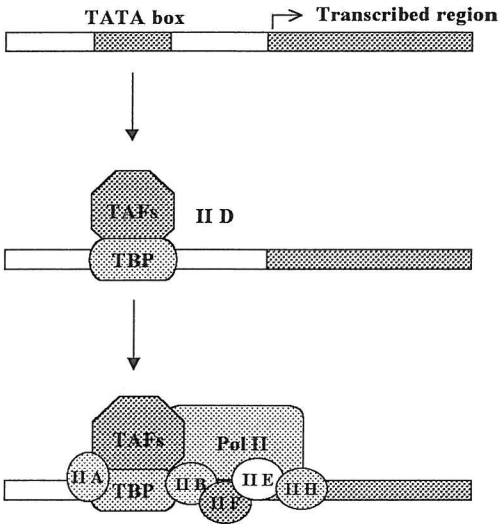


Figure 1.5 Schematic representation of the basal apparatus consisting of RNA polymerase II and general factors.

The association of histones with DNA leads to the formation of nucleosomes, the unit particles of chromatin. If transcription initiation is to occur, chromatin must first be activated. The unravelling of chromatin may be regulated by acetylation or methylation of the free amino groups of lysine residues at the N-terminal end of histone molecules, resulting in a decreased net positive charge and a subsequent lower affinity for the

DNA phosphate backbone[60]. Acetylation of the N-terminal tails of the core histones is a reversible process. Histone acetyltransferases (HATs) catalyse the forward acetylation reaction whereas histone deacetylases (HDACs) catalyse the reverse reaction[402].

General factors are required for both the positioning of RNA polymerase II at its start-point and the subsequent initiation of RNA synthesis. The general factors together with RNA polymerase II constitute the basal transcription apparatus. Transcription factor IID (TFIID) is a multisubunit protein complex comprised of the TATA-binding protein (TBP) and multiple TBP-associated factors (TAFs). Polymerase positioning involves binding of TFIID to the TATA element, which serves as a core for the sequential binding of basal transcription factors IIA, IIB, Pol II/IIF, IIE and IIH[61]. The resulting complex, known as the preinitiation complex (PIC), can initiate transcription *in vitro* in the presence of nucleoside triphosphates. In addition to the TATA box, another important control element is CCAAT[62]. However, some promoters do not contain either of these sequence motifs and instead contain Sp1-binding sites that have been shown to control transcriptional initiation in these genes (see section 1.4.5).

1.2.3 REGULATION OF TRANSCRIPTION INITIATION

Whereas the TBP subunit of TFIID is sufficient for basal transcription, positive or negative regulation of transcription requires the TAFs of TFIID as well as sequence-specific DNA-binding proteins. These eukaryotic transcription factors generally have separable DNA binding and activation/repression domains[63,64].

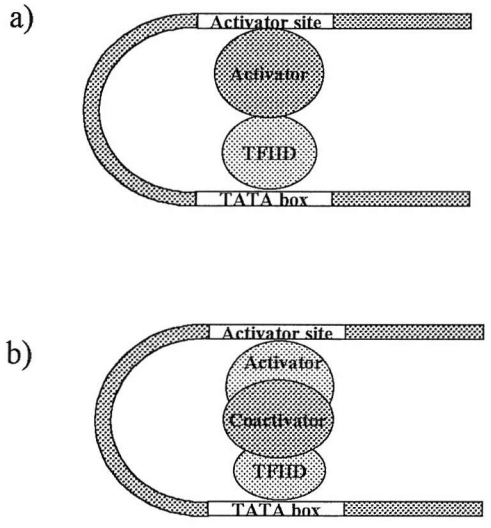


Figure 1.6 Schematic representation of interactions between upstream sequence-specific DNA-binding proteins (e.g. activators) and the basal apparatus.

Transcription factor binding in the vicinity of the promoter modulates the binding and the activity of the basal transcription factors in a way that is only partly understood[65]. Multiple protein-protein interactions have been suggested to explain the activation process. Several activators interact (via their transcription activation region) with TFIID either directly through TBP or through the TAFs (see figure 1.6a)[66,67]. These interactions are thought to facilitate and stabilise TFIID interactions with DNA[68]. TFIIB is also a target for activators[65]. However, some upstream transcription factors do not directly contact the basal apparatus, but instead bind co-activators/-repressors that in turn contact the basal apparatus (see figure 1.6b). Several transcriptional co-activators are HATs (e.g. GCN5, CBP, p300, PCAF, ACTR and $TF_{II}250$), and HDACs are active components of transcriptional co-repressor complexes[402]. Heinzel *et al*, for example, have identified a complex consisting of the co-repressors, N-CoR and mSin3, as well as the HDAC, mRPD3, and show that these components are essential for both nuclear receptor- and Mad-dependent transcriptional repression[403]. For examples of specific co-activators of MyoD, Sp1 and AP-1 transcription factors, see sections 1.3.6, 1.4.5 and 1.5.5, respectively.

Transcription of genes by RNA polymerase II can be inhibited by methylation at CpG dinucleotides. For example, methylation of cytosine residues at CpG sites has been shown to provide a binding site for MeCP2, an abundant mammalian protein capable of transcriptional repression. MeCP1 has also been shown to form a complex with DNA, where methylation exceeds a threshold level, and it seems likely that this complex results in transcriptional repression. Histone H1 and methylated DNA-binding protein H1 have also been suggested as candidates for generalised methyl-CpG binding proteins[60]. Many fully methylated genes can be transcribed at nearly normal rates under conditions where methyl-CpG binding proteins are absent[60]. Tissue specific genes are methylated in most cell types whereas housekeeping genes are constitutively unmethylated and necessary for the activity of these genes (see section 1.4.8).

1.2.4 ROLE OF ENHANCERS

In addition to the promoter (defined operationally by the demand that its sequence components must be located in the general vicinity of the transcriptional start-point), enhancer elements can also regulate transcription. The promoter may contain several short sequence elements (<10bp) that bind transcription factors, dispersed over approximately 200bp upstream of the start-point. In contrast, an enhancer contains a

more closely packed array of elements that bind similar or the same transcription factors and may be located several kb from the start-point, either upstream or downstream of it, and in either orientation. Nucleosome condensing, DNA looping and protein flexibility may be possible mechanisms by which transcription factors at the promoter and at the enhancer can interact to form large protein complexes and increase transcription[69].

1.2.5 ROLE OF SILENCERS

Transcriptional repression in eukaryotes is achieved through silencers, of which there are two main types, silencer elements and negative regulatory elements (NREs). Furthermore, the transcription factors that interact directly with these silencers are referred to as repressors. Silencer elements are position-independent elements that direct an active repression mechanism. For example, on DNA binding of the repressor, a co-repressor may bind which in turn can interfere with the basal apparatus and prevent general transcription factor assembly. In contrast, NREs are position-dependent elements that direct a passive repression mechanism. For example, repressors bound to NREs can overlap neighbouring elements and therefore physically inhibit the interaction of other transcription factors with their specific DNA-binding sites. Many transcription factors have dual functionality i.e. they can act as both repressors and activators of transcription depending on which element they bind[60].

1.2.6 REGULATION OF TRANSCRIPTION FACTORS

Both the concentration and the activity of transcription factors can be regulated. The concentration of transcription factors may be regulated at any of the steps leading from DNA to protein i.e. transcription, RNA processing, mRNA degradation and translation[71]. However, once a transcription factor has been synthesised, its activity can be controlled in a variety of ways. For example, ligand binding is one mode of transcription factor activation and is typical for the large superfamily of nuclear hormone receptors. Heterodimerisation between transcription factors is another important regulatory step. In addition, non-DNA binding factors (e.g. co-activators/-repressors) may mediate a diverse range of functions e.g. acting as a 'bridging factor' between the transcription factor and the basal transcription machinery, stabilising the DNA-binding complex or changing the specificity of the target sequence recognition[71]. Another frequently used mechanism in regulating transcription factor activity is post-translational modification. Although glycosylation of transcription factors can alter

their activity, the most widely used form of modification is phosphorylation. There are many ways in which phosphorylation can alter transcription factor activity[72]:

i) Phosphorylation can either positively or negatively affect the DNA binding properties of a transcription factor.
ii) Phosphorylation can directly regulate the transcriptional activation potential of a transcription factor. For example, it may either positively or negatively influence the ability of a transcription factor to interact with other components of the transcriptional apparatus.
iii) Phosphorylation can also regulate the ability of certain transcription factors to enter the cell nucleus.
iv) Phosphorylation might also affect other properties of transcription factors, such as their intracellular stability.

Many short-lived regulatory proteins are degraded by the ubiquitin system. Initially, multiple molecules of ubiquitin are conjugated to the target protein. Conjugation proceeds in a three-step mechanism. First, the C-terminal Gly of ubiquitin is activated by ATP to a high energy thiol ester intermediate in a reaction catalysed by the ubiquitin-activating enzyme (E1). Ubiquitin is then transferred by the ubquitin-conjugating enzyme (E2) to the target protein that is already bound to the ubiquitin-protein ligase (E3). E3 then catalyses formation of a covalent bond between the activated C-terminal Gly of ubiquitin and an amino group of a Lys residue of the target protein. The target protein is then degraded by a multi-subunit catalytic proteasome and ubiquitin is released[73].

1.3 MYOD TRANSCRIPTION FACTOR

1.3.1 MYOGENIC REGULATORY FACTORS

The myogenic regulator MyoD belongs to the basic-helix-loop-helix (bHLH) family of transcription factors and was first identified in 1987[74]. Subsequent to this, three more related mammalian factors, myogenin[75,76], myf-5[77] and MRF4[78] were isolated. It was then shown that transfection of these genes into a wide variety of normal cells converted them into a myogenic phenotype[79,80]. These factors possess a highly basic domain immediately adjacent to the HLH region.

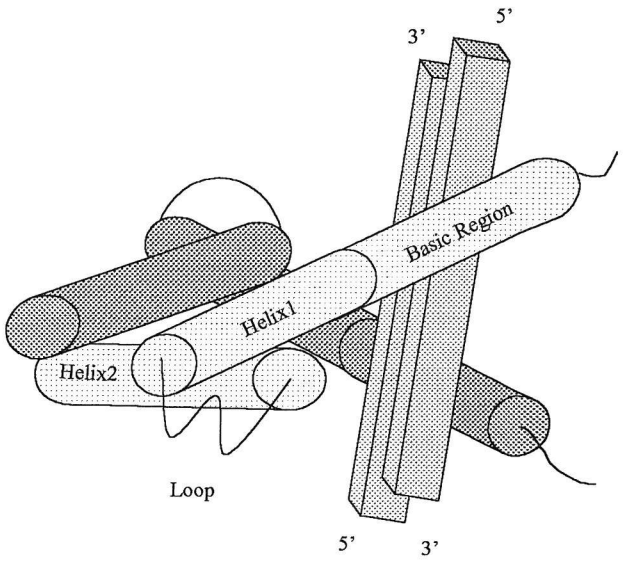


Figure 1.7 Diagrammatic representation of a bHLH motif. Adapted from Lewin, 1998[69].

The HLH comprises two amphipathic alpha helices separated by an intervening loop. Homodimers and, more commonly, heterodimers can be formed by means of interactions between the hydrophobic residues on the corresponding faces of the two helices. The loop is probably important only for allowing the freedom for the two helical regions to interact independently of one another. The basic region is responsible for sequence-specific binding to conserved E-box DNA sequences, CANNTG (where N can be any base)[81] which regulates the transcription of genes essential for terminal cell differentiation in a variety of lineages[82 83]. The myogenic factors show about 80% homology for the bHLH motif. There is also conservation of sequence in the

cysteine/histidine and serine/threonine rich regions. Apart from these regions however, they are structurally unrelated and the genes are dispersed on different chromosomes[77].

1.3.2 ADDITIONAL FUNCTIONS OF THE MYOGENIC FACTORS

In addition to playing a role in cell cycle regulation (see section 1.3.7) and activation of muscle-specific genes associated with myogenesis, each member of the MyoD family can autoregulate its own and cross-activate one anothers' expression to varying degrees. The mechanism of autoactivation is thought to involve an intermediate factor whose expression is induced by the myogenic bHLH factors. When expressed, the intermediate factor acts as a positive regulator of the myogenic bHLH factor. For instance, myogenin activates the expression of the myocyte-specific enhancer-binding factor (MEF2)[84]. There are four *mef2* genes (*mef*2A, *B*, *C*, *D*) identified in vertebrate species[85] and they belong to a superfamily of transcription factors that have a highly conserved region termed the MCM1, Agamous, Deficiens, and Serum Response Factor (MADS) domain. The MEF2 factors are transcriptional activators that recognise specific DNA elements (MEF2 sites) found in regions controlling expression of several muscle-specific genes. The myogenin promoter contains a MEF2 binding site which on binding results in the activation of myogenin expression. This positive regulatory loop is thought to lead to stable expression of large amounts of the myogenic bHLH factors, above a threshold value that is required to induce and maintain the differentiated status of the cells[86].

Gene knockout studies have shown that in the embryo, MyoD and Myf5 play overlapping roles in specification of myoblasts; in the absence of one factor or the other, myogenesis is unaffected, whereas in the absence of both, no myoblasts are formed[87]. In contrast, myogenin is required for muscle differentiation; in its absence, myoblasts are specified but their ability to differentiate is impaired[88,89]. MRF4 and MyoD also have overlapping functions in the differentiation pathway, such that either factor alone is dispensable, whereas in the absence of both factors, myoblasts fail to differentiate[90].

1.3.3 THE MYOGENIC FACTORS BELONG TO A SUPERFAMILY OF BASIC-HELIX-LOOP PROTEINS

Proteins that contain the bHLH motif belong to a superfamily of transcription factors (see Table 1.2). Class A bHLH proteins include several widely expressed gene products

which preferentially dimerise with cell type-specific class B proteins. It has been shown that E47 homodimers and MyoD heterodimers with E12 or E47 bind to E-box target sites avidly whereas E12 homodimerises efficiently but binds poorly. An inhibitory domain N-terminal to the basic region of E12 has been shown to prevent E12 homodimers but not E12/MyoD heterodimers from binding to DNA. In contrast, MyoD homodimerises poorly but, once formed, binds to DNA strongly. The reasons why MyoD homodimerises poorly are unclear[91].

Additionally, the bHLH motif is found in the oncogenic, cell transforming proteins belonging to the Myc family (c-Myc, N-Myc, L-Myc, B-Myc, S-Myc). These proteins, however, do not dimerise with class A or B proteins[92,93]. c-Myc is a nuclear protein that acts as a key regulator of vertebrate proliferation. Its N-terminus acts as a transcriptional activator while its C-terminus contains a HLH and a leucine zipper domain, preceded by a basic region (bHLHZip). c-Myc cellular functions are carried out through heterodimerisation with another bHLHZip protein, Max. c-Myc/Max heterodimers can activate transcription. Max, however, does not contain a functional transactivation domain[95] and when expression levels of Max are high, transcriptionally inactive Max homodimers can inhibit transcription by competing with c-Myc/Max heterodimers for binding at the CACGTG sequence motif[94]. Max may also interact with other bHLHZip proteins, for example Mad1, 3, 4 and Mxi1. The Mad and Mxi1 proteins possess interaction specificities that are very similar to c-Myc; they homodimerise poorly and do not interact with Myc family members but interact strongly with Max. Consequently, Mad proteins compete with c-Myc for Max heterodimer formation. Mad1 does not contain a transactivation domain and is able to suppress transcriptional activation from a promoter containing the CACGTG consensus sequence[96]. Thus, as for Max homodimers, Mad/Max and Mxi1/Max are able to inhibit c-Myc/Max transcriptional activity.

The network of interactions with Max provided by the Myc and Mad families may be fundamental to the choice between cellular proliferation and differentiation. Max is a constitutively expressed protein that is highly stable, possessing a half-life in excess of 24 hours[97]. In contrast, its preferred dimerisation partners, c-Myc and Mad, display very short half-lives, while their expression is highly regulated throughout the cell-cycle [96]. When quiescent cells are stimulated by mitogenic agents, *c-myc* expression is

Table 1.2 EXAMPLES OF bHLH PROTEINS AND THEIR FUNCTIONS [98]			
	Protein	**Source**	**Biological function**
Class A	E12, E47, E2-5	Mammalian	Generated by alternative splicing of the E2A gene. Known collectively as E-proteins, they form homodimers in B lymphocytes and heterodimerise with class B bHLH proteins in other cell types. Required for B cell differentiation.
	E2-2, HEB	Mammalian	Also referred to as E proteins, produced from separate genes.
	DAUGHTERLESS	Drosophila	Functional homologue of mammalian E-proteins
Class B	**Myogenic**		
	MyoD, Myf-5, MRF4, Myogenin	Mammalian	Ectopic overexpression initiates the myogenic programme in many cell types. Their functional heirarchy has been studied by gene targeting. Mice lacking MyoD and Myf-5 are devoid of skeletal myoblasts and muscle, suggesting a role in muscle cell commitment. MRF4 and myogenin are expressed during muscle cell terminal differentiation to regulate myotube formation and fusion.
	Neurogenic		
	Neurogenin, NeuroD1, NeuroD2, NeuroD3	Mammalian	Involved in terminal neuronal differentiation. Ectopic overexpression activates neurone-specific genes and initiates the neural differentiation programme in non-neural cells. Expression of neurogenin precedes that of the NeuroD family.
	NSCL-1, NSCL-2	Mammalian	Exhibit a highly restricted expression pattern confined to the nervous system. Implicated in early neural development.
	Mash-1, Mash-2	Mammalian	Homologues of the Drosophila ACHAETE-SCUTE proteins. Mash-1 and -2 are required for neural and trophoblast development respectively during embryogenesis.
	Hes-1, Hes-2, Hes-3, Hes-5	Mammalian	Homologues of ENHANCER OF SPLIT. Hes-1 and –2 are expressed widely in embryonic and adult tissues where they repress promoters containing both E- and N-box motifs. Hes-3 expression is restricted to the cerebellar Purkinje and Hes-5 to the developing nervous system. Hes-5 binds to E-boxes only, whereas Hes-3 binds to neither E- nor N-boxes but suppresses E-box-dependent transcription by sequestering E47, much like Id proteins.
	ACHAETE-SCUTE	Drosophila	Proneural proteins, involved in the determination of neural precursors.
	ENHANCER OF SPLIT	Drosophila	Protein complex that binds to E- and N-boxes, repressing achaete-scute expression.
	Haemopoietic		
	SCL	Mammalian	Required for haemopoiesis. Involved, by translocation, in T-cell acute lymphoblastic leukaemia. Represses E-box-dependent transcription by E47, much like Id proteins.
	Lyl-1	Mammalian	Closely related to SCL. Involved, by translocation, in T-cell acute lymphoblastic leukaemia.
	Cardiogenic		
	eHand, dHand	Mammalian	Regulate morphogenetic events of asymmetric heart development. eHand expression is controlled by the homeodomain factor Nkx2-5.

induced, leading at the protein level to an equilibrium shift from Max/Max to Myc/Max heterodimer formation. Conversely, withdrawal of mitogenic agents or addition of differentiation agents represses *c-myc* expression, concomitant with the loss of Myc/Max heterodimers. Moreover, Mad1 and Mxi1 expression is induced upon differentiation[96]. Thus, the short half-life of c-Myc, coupled to the stability of Max and the Mad or Mxi1 ratio, provides a very sensitive mechanism for the regulation of c-Myc activity. In this way, cell-cycle exit or entry may be controlled by the formation or disruption of transcriptionally active c-Myc/Max heterodimers.

1.3.4 THE BASIC REGION CONFERS MUSCLE-SPECIFIC GENE TRANSCRIPTION

Studies with MyoD and myogenin show that the basic region plays an important role in mediating cell type-specific transcription. The replacement of the basic regions of MyoD and myogenin with those of E12 or achaete-scute complex results in loss of muscle-specific transcription, although the chimeric proteins retain the ability to bind DNA and dimerise with the E-proteins[99,100]. Two adjacent amino acids, alanine-114 and threonine-115, which are in the centre of the basic region of MyoD and myogenin, comprise a myogenic code which consists of crucial basic region residues required for myogenic conversion and activation of muscle-specific genes[99,100]. Sequence analysis of the basic region has shown that these two amino acid residues are conserved in myogenic bHLH factors but not in other bHLH proteins. Other studies show that when these two amino acids in the basic region of MyoD, plus a single amino acid from the junction that separates the basic region and helix1, were substituted into corresponding positions of the E12 protein, this modified E12 protein acquired the ability to induce myogenesis[101].

1.3.5 INTRAMOLECULAR REGULATION OF MYOD ACTIVATION DOMAIN

Arg-111 of MyoD is also highly conserved throughout the bHLH and bHLH/leucine zipper families. However, Arg-111 adopts a unique conformation in myogenic bHLH protein-DNA cocrystals[102]. Due to the small size of Ala-114, Arg-111 is buried in the major groove and contacts the G of CANNTG. In other bHLH proteins, this arginine residue lies outside of the major groove and position 114 equivalents are occupied by bulkier amino acids, which themselves contact the G of CANNTG. Thus, the exposure of Arg-111 may prevent myogenic function. It has been proposed that DNA binding of

the MyoD basic region leads to recruitment of a recognition factor (see section 1.3.6) that unmasks the transcriptional activation domain in the N-terminus of myogenic factors. It has been shown that an A114 mutant exhibits an altered conformation in the basic region and that this local conformational difference can lead to a more global change affecting the conformation of the activation domain, rendering the molecule incapable of responding to the coactivators. Thus, the activation domain is unmasked only upon DNA binding by the correct basic region. Such a coupled conformational relationship may have evolved to restrict myogenic specificity to a small number of bHLH proteins[103].

1.3.6 MOLECULAR MECHANISMS OF MYOGENIC CO-ACTIVATION

Sartorelli *et al* have searched for factors that might play the role of MyoD co-activator by testing candidate molecules for their ability to augment transcriptional activation or myogenic conversion by MyoD. They have shown that p300 and the related CREB-binding protein (CBP), two molecules with the properties expected of transcriptional adaptors[104], co-activate myogenic bHLH proteins. p300 can interact directly with the amino-terminal activation domain of MyoD. They have also shown that p300 and CBP serve as transcriptional co-activators of MEF2C (see section 1.3.2) by a mechanism involving the interaction of p300 with the MADS domain of MEF2C. These results indicate that p300, besides directly co-activating the N-terminal domain of MyoD, may promote myogenic differentiation by reinforcing the positive autoregulatory loop that operates between the myogenic bHLH proteins and MEF2 transactivators[105].

It has been shown that MyoD can affect transcription during the assembly of the basal transcription factors to form the PIC. MyoD is involved in transcription during two stages. In the first stage, it interacts with TFIID or TBP. This physical interaction results in the stabilisation of MyoD binding to its own DNA binding site without affecting the binding of TFIID or TBP. A study with deletion mutants of MyoD suggests that the interaction with TBP is required for MyoD function in transcription. In the second stage, MyoD recruits TFIIB to the PIC[65] (see section 1.2).

1.3.7 ROLE OF MYOGENIC FACTORS IN CELL CYCLE REGULATION

Cell cycle withdrawal and onset of differentiation are tightly linked processes that depend on growth factor activity. Cell cycle arrest, a prerequisite for differentiation, occurs before S phase during the G_1 phase of the cell cycle[106,107]. Cyclin-dependent

kinase (cdk) inhibitors such as p21 can inactivate G_1/S cdk activity and block cell cycle progression before S phase[108,109]. p21 expression is up-regulated during myogenesis while, in parallel, cdk activities decline[110]. This p21 up-regulation has been associated with permanent cell cycle arrest of muscle cells[111]. One target of the cdk is the retinoblastoma protein (pRb). Inactivation of pRb is required for G_1/S progression and occurs in late G_1 as a result of its phosphorylation by cdks[112]. In its hypo-phosphorylated form, pRb prevents S phase entry by sequestering E2F transcription factors, a family of proteins essential for G_1/S transition[113]. Interestingly, pRb is found hypo-phosphorylated in myotubes and it has been implicated in the maintenance of the permanent cell cycle withdrawal in myotubes[114,115]. During myogenesis, these different cell cycle regulatory pathways can be antagonised or, in contrast, reinforced by the myogenic factors. Over-expression of MyoD directly modulates the cell cycle of normal and transformed cells by blocking G_1/S progression[116]. Different mechanisms may explain the cell cycle inhibitory activity of MyoD. MyoD enhances p21 transcription[110] ultimately leading to cdk down-regulation. Additionally, the interaction between MyoD and the hypo-phosphorylated form of pRb may maintain pRb in its active form[114]. Finally, MyoD has also been shown to down-regulate cyclin B1 expression[117]. Conversely, cyclin D1 is up-regulated in response to growth factors and antagonises the myogenic activity of MyoD[110,118]. Thus, it appears that the decision to differentiate relies on cross-talk between MyoD and cell cycle signalling pathways. In this context, slight variations in MyoD expression and/or activity may change the balance between proliferation and differentiation. In many myogenic cell lines, the capacity of cells to differentiate appears linked to the level of MyoD expression[119]. In addition, the known positive inducers of myogenesis such as insulin-like growth factors (IGF), thyroid hormones and retinoic acid enhance both MyoD expression and muscle differentiation[120,121] implying that a minimal threshold of MyoD protein must be reached before differentiation can take place.

1.3.8 REGULATION OF MYOGENIC DIFFERENTIATION

Although myogenic bHLH proteins are expressed in proliferating, undifferentiated myoblasts, they do not activate muscle differentiation genes until myoblasts exit the cell cycle. The activity of myogenic factors is highly controlled in particular by growth factors, oncogenes and negative HLH proteins such as Id (see section 1.3.9). Treatment of myoblasts with fetal bovine serum (FBS), bFGF-2 or TGF-β1 is known to inhibit differentiation of both primary myoblasts and established myoblast cell lines[122]. The

events responsible for the inhibition of differentiation by these agents are complex and occur via different molecular mechanisms. FBS suppresses myogenesis by increasing intracellular levels of Id[123,124]. TGF-β1, on the other hand, suppresses the activity of myogenin by a mechanism that is independent of Id and that does not disrupt the DNA binding properties of the protein[124]. It is thought that TGF-β may inhibit the activity or expression of a coregulator that recognises the basic region and is essential for activation of the myogenic program[124,125]. Inhibition of DNA binding has been implicated as the mechanism through which FGF-2 represses myogenin activity, since FGF-2 induced phosphorylation of the conserved threonine residue in the basic domain inhibits myogenin from binding to DNA. The phosphorylation is probably mediated by protein kinase C[126]. Interestingly, this repression mechanism is not universal for all myogenic factors, since FGF-2 fails to induce phosphorylation of the conserved threonine residue in MRF4[127], and MyoD mutants lacking the conserved threonine residue remain repressed by FGF-2[128]. These conflicting results suggest that FGF-2 functions to inhibit myogenesis by targeting myogenin directly and by repressing MRF4 and MyoD activities indirectly. In addition, both FGF and TGF-β have been shown to repress the transcription of MyoD[122]. However, in contrast to the findings with FGF-2 and TGF-β, other studies have shown that the growth factors, IGF-I and IGF-II, can stimulate myogenic differentiation[129].

Myogenesis can also be negatively regulated by many oncogenes, such as *c-myc*, *c-jun*, *c-fos*, *H-ras*, and *E1a*, depending on their expression levels[130]. For many of these oncogenes, their expression in myoblasts leads to cell transformation and the repression of the myogenic factors at the transcriptional level[131,132]. Oncoproteins can also attenuate the formation of active myogenic-E heterodimers by increasing levels of Id expression[130]. Lastly, certain oncoproteins inhibit myogenesis by directly silencing the transcriptional activity of the myogenic factors. In the case of Fos and Jun, for example, a direct interaction between these oncoproteins and MyoD or myogenin blocks muscle-specific gene activation[133,134]. The mechanism for repression by c-Myc is not currently understood. c-Myc does not appear to interact directly with the myogenic regulatory factors or with E-proteins[132]. However, Myc is known to bind to pRb[135]. Whether Myc inhibits myogenesis through the pRb arm of the heterodimer/E-box binding complex is not known but remains a possibility.

Myogenesis can also be positively regulated by some oncogenes. For example, the *c-mos* proto-oncogene product, Mos, is a serine/threonine kinase that can activate myogenic differentiation by specific phosphorylation (mainly in the C-terminal portion) of MyoD which favours heterodimerisation of MyoD and E12 proteins and inhibits binding of MyoD homodimers. Lenormand *et al* show that MyoD protein phosphorylated by Mos behaves differently from the unphosphorylated protein or the MyoD protein phosphorylated by the serine/threonine kinases such as protein kinase C (PKC) and protein kinase A (PKA) that inhibit myogenesis[136]. In addition, the oncogene *p3k*, coding for a constitutively active form of phosphatidylinositol 3-kinase (PI 3-kinase) can enhance myogenic differentiation in cultures of chicken-embryo myoblasts. It increases the size of the myotubes and induces elevated levels of the muscle-specific proteins MyoD, myosin heavy chain, creatine kinase and desmin. Inhibition of PI 3-kinase activity with LY294002 or with dominant-negative mutants of PI 3-kinase interferes with myogenic differentiation and with the induction of muscle-specific genes[137].

In addition, recent studies by Lu *et al* have described a bHLH protein, called myogenic repressor (MyoR), that is expressed at high levels in proliferating myoblasts in culture and is down-regulated during myogenesis. MyoR forms heterodimers with E proteins and can bind the same DNA sequences as myogenic bHLH factors, but it acts as a transcriptional repressor and inhibitor of myogenesis[138]. These results suggest a role for MyoR as a lineage-restricted transcriptional repressor of the muscle differentiation program.

1.3.9 THE ID PROTEIN

The Id (inhibitor of DNA binding, inhibitor of DNA differentiation) family of HLH proteins consists of four members in mammals (Id1 to Id4)[83]. The Id proteins lack a basic region and as a consequence are able to antagonise the DNA binding and transcriptional activation functions of bHLH proteins through heterodimerisation[83,123]. Although the Id proteins bind with high affinity to the class A proteins both *in vitro* and *in vivo*, interactions between the class B bHLH proteins and Id proteins have also been reported[91,123,139]. In most cell types, however, Id proteins are thought to modulate the function of class B proteins through sequestration of their class A partners. The Id proteins are all of similar size (13-20kDa). Outside of the highly conserved HLH domain, their sequences are largely divergent and are encoded by individual genes.

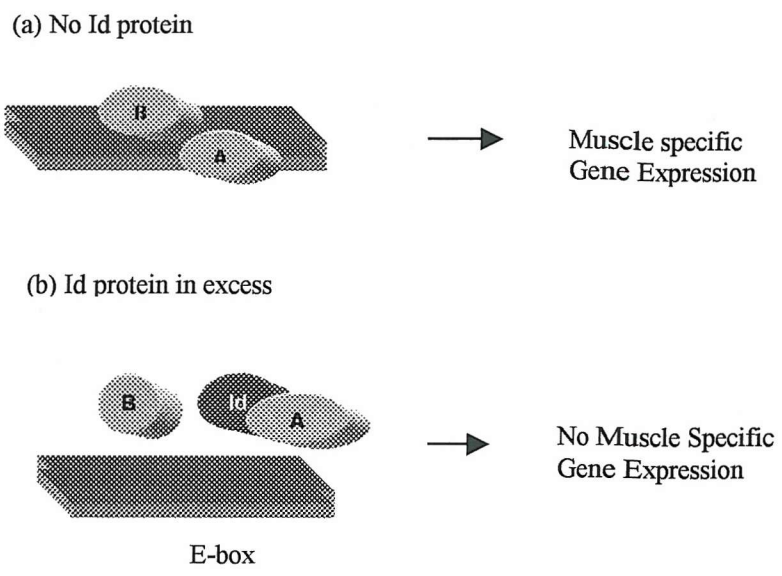


Figure 1.8 Id proteins as dominant-negative antagonists of bHLH transcriptional regulators. (A)=class A bHLH; (B)=class B bHLH. Adapted from Norton *et al.*, 1998[98].

Generally, in cell lines, Id expression is highest in proliferating cells and low or absent in quiescent and/or terminally differentiated cells. There are exceptions however, for example, Id2 is up-regulated during monocyte-macrophage differentiation[140,141]and is constitutively expressed in resting T lymphocytes but down-regulated in response to mitogenic stimulation[142]. In addition, Id1 is abundantly expressed in serum-deprived, quiescent glomerular mesangial cells but transiently down-regulated by addition of serum mitogens[143]. However in most other cell types, Id gene expression is strongly induced in response to mitogen/growth factor stimulation, with a peak of expression occurring after 1-3hrs, similar to other early-response genes[144-147]. During traverse of the cell cycle, this immediate-early peak of Id expression declines and followed by a second peak of expression prior to the onset of S phase[146]. Also, in common with most other proteins encoded by early-response genes, Id proteins have a relatively short half-life (~1.5h) but are stabilised through heterodimerisation with bHLH targets, a process that also leads to a destabilisation of the bHLH protein dimer partner[148].

Expression of various Id genes is down-regulated on exit from the cell cycle and on terminal differentiation in a number of *in vitro* mammalian cell line models representing diverse lineages. Induced differentiation in these cell-line models is impaired with enforced expression of Id and prevents both bHLH protein binding *in vitro* and transactivation of E-box-dependent gene expression *in vivo* in transfected cells[98]. In addition, studies using a tethered dimer of MyoD and E47 proteins created by

incorporating a flexible polypeptide linker between each monomer[149] showed resistance to Id1 and, moreover, acted as a dominant-positive myogenic factor capable of initiating myogenic determination even in the presence of cellular Id protein[149]. *In vivo* gene-targeting studies show that mice with targeted disruption of individual Id genes are essentially normal. However, double knockouts are embryo lethal[150]. Thus in mammals, at least, Id functions are indispensable for survival and their loss cannot be compensated for by other classes of dominant-negative bHLH antagonists.

Recent studies on how Id protein functions are integrated with the cell-cycle regulatory machinery have now implicated mechanisms that do not directly involve bHLH protein antagonism. For example, Id2 binds to pRb and can reverse the growth arrest and cell cycle block elicited by over-expression of this suppressor[151]. Id2 can also antagonise the growth-suppressive activities of the cdk inhibitors p16 and p21[152]. Id1 and Id3, however, do not display either of these functions. Recent data have shown that E2A (E12/E47)-dependent transactivation of the $p21^{Cip1/Waf1}$ gene is inhibited by Id1 and that the enhanced cell growth in response to ectopic overexpression of Id1 correlates with inhibition of p21 expression[153]. In addition, cdk2 phosphorylation of Ser5 in mammalian Id2 and Id3 proteins has recently shown to occur during late G_1-to-S phase transition of the cell cycle, and this neutralises the function of Id2 and Id3 in preventing E-box-dependent bHLH homo- or heterodimer complex formation in *vitro*. Cdk2-dependent phosphorylation therefore provides a switch during late G_1 to S phase that both nullifies an early G_1 cell cycle regulatory function of Id2 and Id3 and modulates their target bHLH specificity. A potential cdk2 site is present at the same position in Id4 but, interestingly, not in Id1[154,155].

1.3.10 REGULATION BY NF-κB

Nuclear Factor Kappa B (NF-κB) was first identified in 1986 by Sen and Baltimore as a regulator of the kappa light-chain enhancer in B-cells[156]. Since then, it has been found in a wide range of different cells and has been shown to be one of the most crucial transcription factors in immune and inflammatory processes[157]. Five different members of the mammalian NF-κB/Rel family have been so far identified; p105/p50 (NF-κB1), p100/p52 (NF-κB2), p65 (RelA), RelB and c-Rel, with the viral oncogene v-rel being related. They are all characterised by the presence of the Rel homology domain (RHD), an N-terminal stretch of about 300 amino acids. This RHD functions in DNA binding, dimerisation and interaction with a group of specific inhibitory proteins, known as the

Inhibitors of κ Binding (IκB)[157]. All the NF-κB proteins bind to DNA as dimers and all possible combinations of homo and heterodimers have been shown to exist.

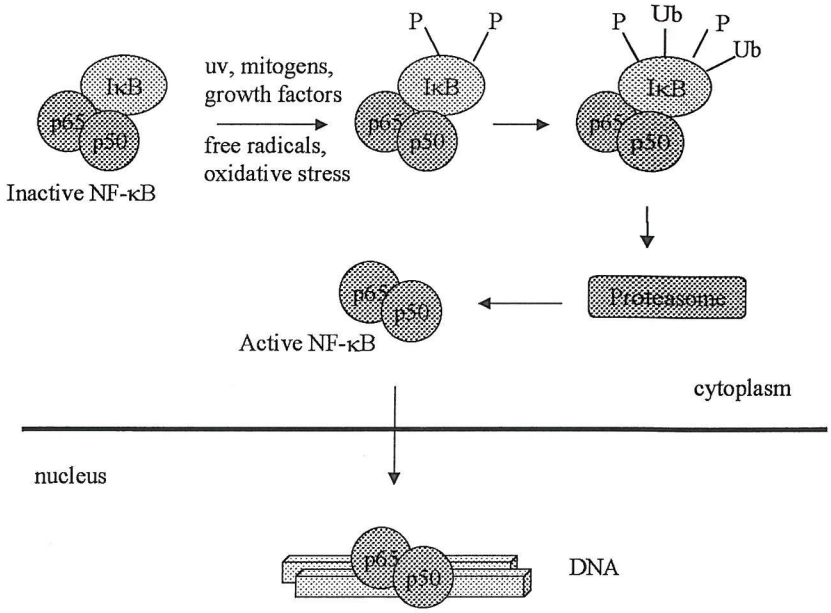


Figure 1.9 Schematic diagram showing the mechanism of activation of NF-κB.

The NF-κB proteins are found in most cell types sequestered in the cytoplasm by the IκB inhibitory proteins. These proteins form a structurally and functionally related family of which there are currently seven members-Iκ-α, IκB-β, IκB-γ, IκB-ε, Bcl-3 and the C-terminal tails of p100 and p105[158]. The interaction of the IκBs with the various NF-κB dimers is mediated through the RHD and masks the nuclear localisation signals (NLS) of the various monomers, preventing nuclear translocation. Induction of NF-κB activity classically involves signals that cause the dissociation and subsequent degradation of IκB proteins, allowing NF-κB dimers to enter the nucleus and induce gene expression[159]. The mechanism of activation is similar regardless of the stimulus. This involves stimulus-induced activation of IκB kinase (IKK) which consists of IKK-α and IKK-β, along with the NF-κB-inducing kinase (NIK) and other as yet uncharacterised proteins[160]. The result of this is phosphorylation of two conserved serines on IκB (ser 32 and 36 in IκB-α). This targets the molecule for phosphorylation-

dependent ubiquitination on two conserved lysines[161]. The ubiquitinated protein is then degraded by the multisubunit catalytic proteasome, releasing the NF-κB dimer which is then translocated to the nucleus using its NLS and can activate gene transcription.

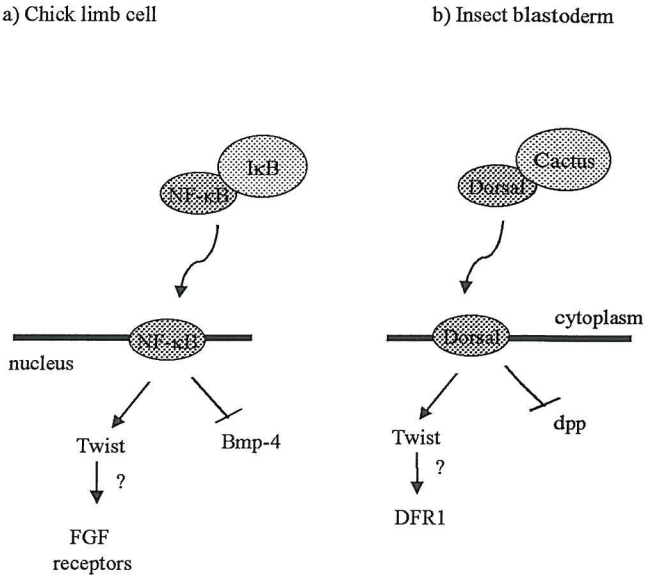


Figure 1.10 NF-κB signalling in chick limb cells and insect blastoderm.

Various studies with HSCs have reported the induction of NF-κB DNA binding in response to growth factors or mitogens[162-166]. For example, Lee *et al* have shown that activation of HSCs by TGFα is accompanied by NF-κB induction. Kanegae *et al* and Bushdid *et al* have shown that signalling through NF-κB in chick limb cells occurs by its release from IκB and translocation to the nucleus where it regulates target genes (activation of the bHLH protein, Twist, and repression of Bmp-4). When NF-κB signalling is blocked, Twist expression is reduced. Genes(s) encoding receptors for FGF could lie downstream of Twist. In Drosophila blastoderm, they show that signalling through Dorsal, a homologue of NF-κB, occurs by its release from cactus and translocation to the nucleus, where it activates Twist and represses expression of decapentaplegic (dpp, of which Bmp-2 and Bmp-4 are vertebrate homologues). A Drosophila homologue of vertebrate FGF receptors (DFR1) appears to lie downstream of Twist, at least in the imaginal discs. A human connection comes from the finding

that mutations in the Twist gene cause Saethre-Chotzen syndrome, which is characterised by limb anomalies[167,168].

1.4 SP1 TRANSCRIPTION FACTOR

1.4.1 INTRODUCTION

Sp1 was one of the first eukaryotic transcription factors to be identified and cloned, by virtue of its ability to selectively increase transcription *in vitro* from the Simian virus 40 (SV40) early promoter at GC-rich sites[169]. In normal cells, Sp1 exists as an approximately equimolar mixture of two polypeptide species, with apparent molecular masses of approximately 95 and 105kDa. *In vivo* labelling studies have revealed that the two Sp1 species arise through differential phosphorylation; the 105kDa form being a phosphorylated derivative of the 95kDa form[170]. Sp1 is an abundant nuclear protein found in many cell types in higher eukaryotes. It is involved in the regulation of a wide variety of different genes, including the early promoter of SV40[171], genes involved in proliferative response[172,173], extracellular matrix protein genes[174,175], housekeeping genes[176,177] and a number of growth factor genes[173,178]. A decanucleotide consensus sequence, known as the 'GC-box', that is characteristic of Sp1 binding sites has been defined: 5'-GGGGCGGGGC-3'[179]. Sp1 binding sites are functional in either orientation. Recent studies indicate that the Sp1 family of transcription factors can also bind to GT-rich sequences: 5'-GGGGTGTGG-3'[180,181].

1.4.2 FAMILY OF SP PROTEINS

Several proteins with homology to Sp1 have been identified in a number of different studies. A rat protein, basic transcription element-binding protein (BTEB), was cloned by virtue of binding the basic transcription element (BTE) box, a GC-box sequence in the promoter of the rat P4501A1 gene[182]. Both Sp1 and BTEB affect expression of P4501A1 through the BTE sequence. Using the rat BTEB cDNA as a probe, a related cDNA designated BTEB2 was isolated from human placenta library[183]. This gene was expressed specifically in testes and placenta, but no known function has yet been described. Analysis of a T-cell antigen receptor promoter revealed a GT-box motif that could bind to Sp1 as well as two other proteins, Sp2 (~80kDa) and Sp3 (~100kDa), coded by two separate genes[181]. Studies with the uteroglobin promoter GT-box led to the identification of two Sp1-like proteins, Sp3 and Sp4 (~82kDa)[180]. Sp1, Sp2 and Sp3 are widely expressed at a high level in many different cell types. However, Sp4 is highly expressed only in the brain, suggesting a tissue-specific role for this factor[180]. In addition, recent studies by Ratziu *et al* have led to the identification of another GC-box binding protein, Zf9. Zf9 is induced during the *in vitro* and *in vivo* activation of rat

HSCs[184]. Further studies have shown that Zf9 can interact with GC-boxes in the promoters of TGF-β1 and TGF-β receptors (types I and II), and thus transactivate these genes. The regulation of these genes by Zf9 may involve co-operation with Sp1[185].

1.4.3 DNA-BINDING DOMAINS AND HOMOLOGY OF SP-LIKE PROTEINS

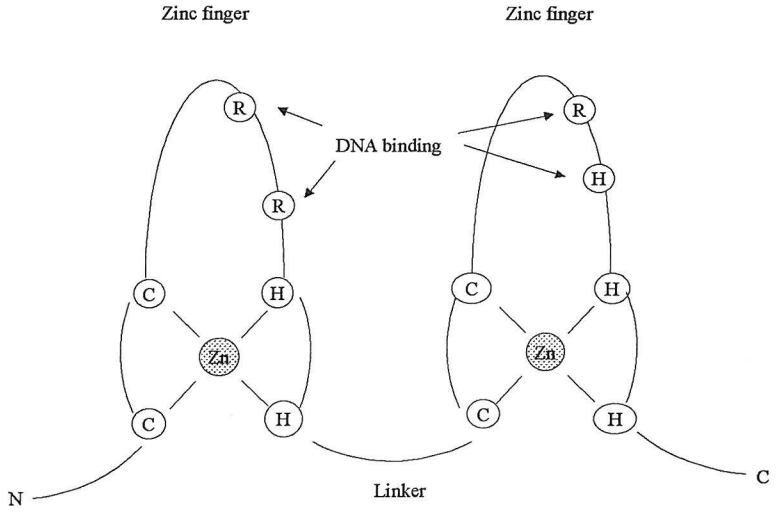


Figure 1.11 Diagrammatic representation of zinc finger motifs. Adapted from Turner *et al.*, 1997[186].

The Sp-family have highly conserved DNA binding domains located near the C-terminus that consist of three zinc fingers. Each finger has a loop of 12 amino acids anchored by two cysteine and two histidine residues that tetrahedrally co-ordinate a zinc ion[187]. This motif folds into a compact structure where the N-terminal part of each finger forms a β-sheet and the C-terminal part forms an α-helix, the latter binding in the major groove of DNA. The α-helical region contains conserved basic amino acids which are responsible for interacting with the DNA[188,189]. Homology to Sp1 at the zinc finger domain varies from 59% for BTEB2 to 90% for Sp3 and Sp4. Analysis of the DNA-binding activities reveal that Sp3, Sp4, BTEB, BTEB2, and Zf9 proteins recognise GC and/or GT-box motifs with specificity and affinity very similar to that of Sp1[180,185,181,190]. Contrary to these factors, Sp2 binds with lower affinity to a GT-box motif and not at all to the GC-box motif. The predicted Sp3 and Sp4 protein sequences have extensive homology to Sp1 throughout the entire open reading frame, whereas

homology outside the zinc finger domain of Sp2 is much more limited. Both BTEB and BTEB2 do not show any homology to Sp1 outside the DNA-binding domain[187].

1.4.4 TRANSACTIVATION DOMAINS OF SP-LIKE PROTEINS

Sp1, Sp3 and Sp4 have very similar structural features. In addition to the highly conserved DNA binding domain close to the C terminus, all three proteins contain two glutamine- and serine/threonine-rich amino acid stretches in the N-terminal part of the molecule. For Sp1, the glutamine-rich domains have been identified as transactivation domains (A and B). Two additional domains of Sp1 (C and D) located adjacent to the zinc finger region also influence the transcriptional activation function, one being weakly basic (C) and the other (D) showing no significant homology to known activation domains. On the other hand, Sp2 has a serine/threonine-rich region followed by a glutamine-rich domain (B), a charged domain (C) and a DNA binding domain located at the C terminus[191].

1.4.5 SYNERGISTIC TRANSACTIVATION

There are several examples of promoters that contain one or more independent Sp1 sites. However, there has been considerable interest in recent times in the ability of Sp1 to interact with other factors to modulate transcription in various ways. Many transcription factors are known to multimerise to form an active species. Although DNA binding by Sp1 does not require dimerisation, it is now evident that two adjacent Sp1 sites can act synergistically to activate transcription nearly 100-fold[192]. The regions of the Sp1 molecule which are necessary for synergistic activation, have been mapped extensively. Sp1 requires three of its domains to activate transcription synergistically from two adjacent sites in a proximal promoter construct and for long-range synergistic activation between a proximal site and a distal (enhancer) site. These domains include the glutamine-rich activation domains A and B and the most C-terminal region of Sp1, domain D. Domains A and B are also required for activation at a single site, unlike domain D[191].

Synergistic Interactions with Cell-Specific and Growth-Regulatory Transcription Factors

Interactions between a ubiquitous transcription factor like Sp1 and a cell-specific transcription factor can lead to high levels of cell-specific transcription. Sp1 has been shown to synergistically interact with cell-specific transcription factors such as GATA-

1, the major erythroid transcription factor[193], HNF3α in lung epithelial cells[194] and the myeloid-specific factor PU.1[195]. In addition, several muscle-specific genes are positively regulated by a combination of Sp1 and myogenic bHLH proteins, including the regulatory regions of human cardiac α-actin (HCA)[196], troponin I[197], α and δ subunits of the acetylcholine receptor[198], muscle sarcoplasmic reticulum Ca^{2+}-ATPase gene[199], and muscle phosphofructokinase P2[200]. Sp1 has also been shown to interact synergistically with growth-regulatory transcription factors, such as the E2F family of proteins. There is considerable evidence that E2F and Sp1 can physically interact to activate transcription of two DNA replication-associated enzymes, thymidine kinase[201] and dihydrofolate reductase[202]. E2F-mediated transcription is suppressed when bound to the hypophosphorylated form of retinoblastoma (Rb), an important transcriptional regulator. When Rb is phosphorylated in mid-G1, E2F is released and can activate transcription again. The association between Sp1 and E2F is maximal in cells in mid- to late-G1, when E2F is dissociated from Rb and before it associates with cyclin A[202]. Since E2F is known to bind to TATA-binding protein, E2F may act as a bridging factor transmitting the activation signal from Sp1 to TATA-binding protein in a growth- and cell cycle-dependent manner[201].

Synergistic Interactions with the Basal Apparatus

Direct interactions of a transcriptional activator with components of the basal transcription machinery can lead to high levels of activation. These interactions can also be synergistic in nature. Previous studies have shown that the interaction of the glutamine-rich activation domains of Sp1 with drosophila $TAF_{II}110$, via hydrophobic residues present in these domains, is required for *in vitro* transcriptional activation[203]. In addition, interactions have also been shown to occur between the DNA-binding domain of Sp1 and human $TAF_{II}55$. $TAF_{II}55$ is a co-activator that can interact with multiple activators including Sp1, YY1, USF, CTF, adenoviral E1A and HIV-1 Tat proteins[204]. Recently, however, the human transcriptional cofactor complex CRSP has been shown to be required together with the TAF_{II}s for full transcriptional activation by Sp1[205]. Thus these interactions can lead to a direct linkage between Sp1 and the initiation complex. Sp1-binding sites have been shown to regulate transcriptional initiation in TATA-less promoters. By comparing fractionated preparations of human TFIID, Pugh *et al* were able to discriminate between the presence of co-activators required for Sp1 activation and another distinct factor required for TATA-less promoter activation. This factor has been referred to as a

tethering factor since its function appears to replace the TATA box, which is thought to help anchor the transcription complex to the promoter. Since Sp1 appears to be required to sequester the transcription apparatus at TATA-less promoters, the tethering activity might function to anchor the basal initiation complex to the promoter through binding to Sp1[206].

Superactivation by Sp1

Previous experiments have shown that a non-DNA binding mutant of Sp1 enhances transcriptional activation by wild-type Sp1[207]. This process, called superactivation, involves direct Sp1-Sp1 interactions and requires domains A and B[192]. Heteromeric complexes consisting of a DNA-bound Sp1 molecule and DNA binding-deficient Sp1 molecules may increase the number of activation domains at the promoter[208]. Rb can cause superactivation of Sp1- and Sp3-mediated transcription. Rb has been shown to regulate transcription of key growth control genes via its physical interaction with a number of sequence-specific transcription factors, but Rb itself does not bind specifically to DNA. Promoters responsive to activation by Rb and Sp1/Sp3 contain the core sequence 5'-GCCACC-3', and include the *c-fos*, *c-myc* and *TGF-β1* gene promoters. In the absence of functional Rb, Sp1/Sp3 are unable to mediate activation of these key growth regulatory genes[209].

1.4.6 REGULATION OF SP1

Two different types of post-translational modification of Sp1 are known to occur. Sp1 contains a number of O-linked N-acetyl-glucosamine residues clustered in the amino-terminal half of the protein. Glycosylation does not appear to affect DNA binding but has been shown to enhance the ability of Sp1 to activate transcription *in vitro*[210]. However, the most versatile and widely used form of modification appears to be phosphorylation. Sp1 phosphorylation has been described after DNA binding which might increase the stability of the DNA/protein complex[170]. On the other hand, other investigators have shown that kinase activity affects Sp1 binding and presumably transactivation. During terminal differentiation of the liver, casein kinase 2 (CK2) activity phosphorylates the zinc finger domain of Sp1 and decreases Sp1 binding[211]. In glucose responsive promoters, induced Sp1 binding is prevented by CK2 phosphorylation[212]. In contrast, increased protein kinase A activity in HL-60 cells stimulates Sp1 phosphorylation and binding[213]. Further, Sp1 phosphorylation by

extracellular signal-regulated kinase 2 (ERK 2) in a human gastric cell line stimulates DNA binding[214].

1.4.7 ANALYSIS OF SP1 FUNCTION

Despite a great deal of information on the molecular properties of Sp1, little is known about its biological function *in vivo*. Marin *et al* describe the targeted inactivation of the mouse gene encoding the transcription factor Sp1. Their results demonstrate that Sp1 is essential for normal mouse embryogenesis. Sp1-deficient embryos are found in normal numbers during early embryonic development, but they all die around day 11 of gestation. They are severely retarded in growth and show a broad range of phenotypic abnormalities[215]. However, putative target genes of Sp1 are expressed at normal levels in the Sp1-deficient embryos. For example, cell cycle-regulated genes are unaffected and methylation-free islands are maintained. Other members of the Sp1 family[180,181] may, therefore, compensate for the Sp1 knockout. In contrast, Sp1-deficient embryos expressed the MeCP2 protein at 10-fold-reduced levels. This protein binds to methylated DNA (see section 1.2.3) and is essential for normal embryonic development. It is thought that MeCP2 is required for the maintenance of differentiated cells and that Sp1 may play a role in the maintenance of differentiated cells through the regulation of genes like MeCP2. The MeCP2 knock-out results in a phenotype that is very similar to the Sp1 gene knock-out. Hence MeCP2-deficiency may contribute significantly to the phenotype of the Sp1 gene knock-out[215].

1.4.8 ACTIVATION OF CHROMATIN

Animal somatic cell DNA is characterised by a bimodal pattern of methylation: tissue-specific genes are methylated in most cell types whereas housekeeping genes have 5' CpG islands (CG-rich sequences) that are constitutively unmethylated and necessary for the activity of these genes[216]. Studies have shown that Sp1 sites in the mouse adenine phosphoribosyltransferase (aprt) housekeeping gene promoter are required to prevent *de novo* methylation of the CpG island. Mutated Sp1 elements introduced into the gene result in its *de novo* methylation in transgenic animal models[217]. As Sp1-binding sites are frequently associated with CpG islands in general[218], it is likely that these sequences are also involved in mediating the undermethylation of other CpG islands in the genome. The human phosphoglycerate kinase-1 (PGK-1) gene, for example, carries three verified Sp1-binding sites and is completely unmethylated at every CpG residue within its 530-bp island[219]. Thus Sp1 appears to be one of the

factors responsible for establishing the correct genomic methylation pattern required for regulating basal gene expression in the organism. In addition, it should be noted that despite the central CpG dinucleotide within the Sp1 binding site neither DNA binding or transactivation by Sp1 is influenced by cytosine methylation[220].

1.4.9 NEGATIVE REGULATION BY SP1

Sp1 activation can be suppressed by a variety of mechanisms. Once complexed with Sp1 or Sp3, internally initiated Sp3 isoforms may diminish the capacity of multimeric complexes to bind DNA or prevent formation of transcriptionally active protein-DNA complexes (see section 1.4.10)[221]. Activation by Sp1 can also be suppressed on formation of inactive (non-DNA-binding) complexes with other nuclear factors such as Sp1-I[222] and p107[223]. However, Sp1 can, itself, decrease transcription when bound to certain Sp1 elements. For example, the activity of the human adenine nucleotide translocase 2 (ANT2) proximal promoter is dependent on three Sp1-binding sites, two of which are adjacent to each other and interact synergistically to activate transcription. The third site situated from –7 to –2 bp inhibits transcription, probably by disrupting the recruitment and assembly of the transcriptional initiation complex[224]. A similar effect has been described in the proximal promoter of the megakaryocyte-specific α_{IIb} gene. Sp1 appears to have both a positive and negative role in the regulation of this gene depending on which site it binds to[225].

1.4.10 SP3 TRANSCRIPTION FACTOR

Several studies have reported that Sp3 stimulates transcription *in* vivo, however, this observation has not proven to be universal[226,227]. A number of laboratories have reported that Sp3 has little or no capacity to function as a transcriptional trans-activator. Instead, based on a wide variety of transient co-transfection experiments, Sp3 has been reported to function as a repressor of Sp1-mediated transcription[190,208,228,229] However, studies have now shown that Sp3 mRNA encodes at least three distinct proteins *in vivo* and that internally initiated Sp3 isoforms function as transcriptional inhibitors. Thus, these once disparate observations may be easily reconciled, i.e. experiments that employ Sp3 expression vectors that preferentially lead to biogenesis of full-length Sp3 protein will score as a transcriptional trans-activator while expression vectors that largely yield internally initiated Sp3 isoforms will score Sp3 as a transcriptional repressor. This conclusion appears to be entirely consistent with the sizes of Sp3 proteins expressed following transient transfections with Sp3 cDNAs[230].

1.4.11 SP4 TRANSCRIPTION FACTOR

Cotransfection experiments into Drosophila SL2 cells lacking endogenous Sp factors demonstrate that Sp4 is an activator protein like Sp1. However, in contrast to Sp1, Sp4 is not able to act synergistically through adjacent binding sites. Sequence comparison of the D-domain of Sp1 with the corresponding domain of Sp4 revealed no significant homologies within this region. The absence of a functionally active D-domain in Sp4 may thus account for the lack of synergistic activation. In addition, the N-terminus of Sp1 is able to superactivate Sp4-mediated transcription, suggesting that the non-DNA binding form of Sp1 directly interacts with Sp4. The implication of this finding is that the glutamine-rich domains of Sp4 and those of Sp1 are functionally related to each other. It should be noted, however, that superactivation is not a general phenomenon of glutamine-rich activation domains but rather a factor-specific property[208].

1.5 AP-1 TRANSCRIPTION FACTORS

1.5.1 INTRODUCTION

Activator protein-1 (AP-1) was first identified as a transcription factor that binds to an essential cis-element of the human metallothionein IIa (hMTIIa) promoter[231]. Subsequently, the binding site for AP-1 was found to be responsive to the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) and therefore, referred to as the TPA-response element (TRE). However, the effects of TPA have also shown to be mediated through other elements including the serum response element (SRE) and the AP-2 site[232,233], thus the TRE site is now more commonly referred to as AP-1. The consensus binding site for AP-1 is a palindromic sequence: 5'-TGA G/C TCA-3' [234,235]. In addition, certain AP-1 proteins (see below) can also bind to the cAMP-responsive element (CRE; this element is also palindromic and has the sequence: TGACGTCA)[236]. The AP-1 family of transcription factors is ubiquitously expressed and is involved in the early stage of mitogenic signalling, although these proteins also play a critical role in the differentiation of myeloid and keratinocyte cells[237,238]. The AP-1 family is also stimulated under stress situations, such as UV irradiation and heat shock [237], and some members have shown to be involved in cell-cycle arrest[237], both properties being compatible with a role in differentiation. Numerous cellular and viral genes contain AP-1 binding sites within their promoters and, accordingly, AP-1 has been shown to play a role in the regulation of transcription of these genes[234,237].

1.5.2 AP-1 DIMERISATION AND DNA-BINDING

The AP-1 family of transcription factors consists of homodimers and heterodimers of Jun (v-Jun, c-Jun, JunB, JunD), Fos (v-Fos, c-Fos, FosB, Fra1, Fra2) or activating transcription factor (ATF2, ATF3/LRF1, B-ATF) proteins[237,239]. Although Jun proteins form very stable heterodimers with Fos- and ATF-family members, they can also homodimerise among themselves[236,240-243]. ATF proteins, but not Fos proteins, also form stable homodimers[243]. Jun-Jun and Jun-Fos dimers preferentially bind to the TRE element, whereas Jun-ATF dimers or ATF homodimers prefer to bind to the CRE element. The AP-1 families belong to the basic region/leucine zipper (bZIP) class of proteins and consist of a basic DNA binding domain which is N-terminal to a hydrophobic amphipathic α-helix. Every seventh

amino acid of this domain consists of a leucine residue (leucine zipper), and due to this arrangement, the leucine side chains protrude from one side of the α-helix and form a hydrophobic surface that mediates dimerisation with structurally-related proteins[244].

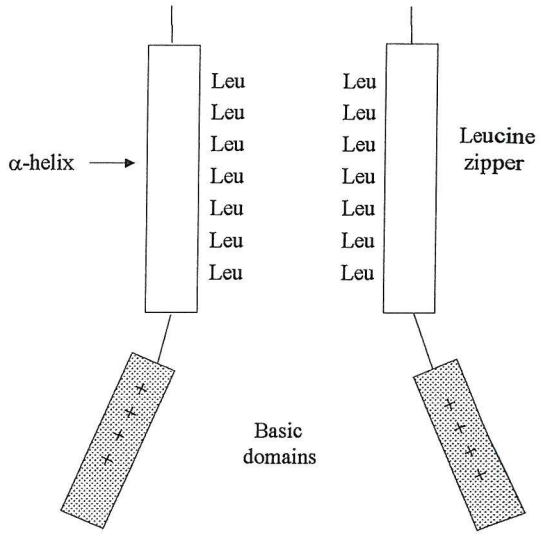


Figure 1.12 Diagrammatic representation of a basic region/leucine zipper motif. Adapted from Turner *et al.*, 1997[186].

1.5.3 JUN GENE FAMILY

The first components of AP-1 to be identified and subsequently well characterised were the proto-oncogenes *c-jun* and *c-fos*. *c-jun* is the cellular homologue of *v-jun*, carried by the avian sarcoma virus 17[245]. In addition to *c-jun*, two other related genes have been identified, *junB*[246,247] and *junD*[248,249]. The *jun* gene family are similar to each other in their gene and protein structure, particularly in the DNA-binding domain and leucine zipper regions where there is 75% amino acid homology within the Jun family[249]. However, despite these homologies in their DNA binding domains, the Jun proteins differ in their ability to transactivate AP-1 dependent genes. c-Jun homodimers exhibit strong transactivation activity, whereas JunB and JunD homodimers are poor transactivators and repress the activity of c-Jun[237,248,250,251]. On the other hand, JunB is an efficient transactivator of promoters containing multiple AP-1 sites[247,251], while JunD homodimers are not. Thus JunB may activate only a subset of AP-1 responsive genes whose promoters

contain at least two adjacent AP-1 sites. Heterodimer formation of any Jun protein with c-Fos results in the generation of potent transactivators and part of this increased activity is due to the increased stability of the heterodimer[248,250].

Transactivation activity of c-Jun (and most likely JunB and JunD) depends on at least two activation domains: one (A2) is constitutive, whereas the other (A1) is regulated. The A1 domain is in the N-terminal half of the protein[252]. The activity of this domain is regulated by extracellular signals such as UV and tyrosine kinases such as Src and Ha-Ras[253-255]. These agents increase c-Jun activity by inducing the phosphorylation of two Ser residues, S_{63} and S_{73} within A1 (see section 1.5.5). Directly N-terminal to S_{63} and S_{73} lies a sequence (δ region) required for the binding of Jun kinase that phosphorylates these two sites (see section 1.5.5)[256]. In addition, the δ region has been implicated in binding a cell-specific inhibitor that can inhibit the A1 activation domain[257]. It has been shown that this inhibitor does not bind the Jun-Fos heterodimer to the same extent as the c-Jun homodimer and may, therefore, be one reason for better activation of AP-1 sites by Jun-Fos heterodimers[258,259]. The A2 domain is also essential for activation *in vivo* and is towards the C-terminal end of c-Jun, in a region near the DNA binding domain[260].

1.5.4 FOS GENE FAMILY

c-fos is the cellular homologue of *v-fos*, a viral oncogene carried by the Finkel-Biskis-Jenkins and the Finkel-Biskis-Reilly murine osteosarcoma viruses[261,262]. In addition to *c-fos*, other related genes have been identified including *fosB*[241] and its naturally truncated form, *ΔfosB*[263], fos-related antigen 1 (*fra1*)[264], and fos-related antigen 2 (*fra2*)[265,266]. These genes are structurally-related to c-*fos* in terms of having the same number of exons and introns; however, the size of the untranslated regions of the genes is variable. The amino acid sequence between each protein is also conserved, particularly in the basic DNA and leucine zipper regions[266].

The c-Fos protein has a number of domains that activate or regulate transcription[267-269]. Three activation regions have been characterised in the C-terminus of c-Fos and individually they show little independent activity but when combined they can activate transcription synergistically[268]. Two of these regions contain homology box motifs (HOB1

and HOB2) which are also present in the A1 activation domain of c-Jun. A third region in the c-Fos C-terminus contains a TBP binding motif (TBM) which mediates interactions with TBP both *in vitro* and *in vivo* and allows interactions with the TFIID complex[270]. In addition, c-Fos contains a second HOB1 motif (HOB1-N) at its N-terminus and an inhibitory domain (ID1) which acts to silence activation domains containing the HOB1 motif[271]. Differential usage of activation domains may rely on the context of the promoter. For example, the function of ID1 may be dependent on the presence or absence of certain transcription factors on a given promoter. A particular activator may have the capacity to neutralise the effect of the ID1 and thus allow the HOB1 motif to function[271]. There is no evidence that the c-Jun inhibitor is related in sequence or function to the inhibitor domain of c-Fos[271].

1.5.5 AP-1 REGULATION

Changes in AP-1 activity, in response to extracellular signals, are regulated both at the level of transcription of the *jun* and *fos* genes and by posttranslational modification of pre-existing AP-1.

Transcriptional Regulation

c-jun is expressed in many cell types at low levels, and its expression is elevated in response to many stimuli, including growth factors, cytokines and UV irradiation[272]. Most of its inducers operate through one major cis element, the *c-jun* TRE (TTACCTCA). This TRE differs from the consensus TRE sequence by 1-base pair insertion[273], and due to this subtle change it is more efficiently recognised by c-Jun-ATF2 heterodimers than by conventional AP-1 complexes[274].

Stimulation of cells with different stimuli can induce *c-fos* transcription very rapidly and transiently[275]. Several cis elements within the *c-fos* gene promoter mediate *c-fos* induction. The cAMP-response element (CRE) is likely to be occupied by cAMP-response element-binding protein (CREB) or ATF proteins. This region mediates *c-fos* induction in response to neurotransmitters and polypeptide hormones using either cAMP or Ca^{2+} as second messengers which in turn activate protein kinase A or calmodulin-dependent protein kinases, respectively[276]. Another site that regulates c-*fos* transcription is the Sis-inducible enhancer (SIE) which is recognised by the STAT (signal transducer and activator of

transcription) group of transcription factors[277]. Stimuli that activate the Janus kinase (JAK) group of tyrosine kinases will result in activation of STATs and translocation to the nucleus[278]. The serum response element (SRE) mediates *c-fos* induction by growth factors, cytokines, and other stimuli that activate mitogen-activated protein kinases (MAPKs)[275]. MAPK family members include the ERKs[279,280], the c-Jun NH_2-terminal kinases (JNKs)[281,282] and the p38 kinases[283].

The first SRE-binding protein identified was the serum response factor (SRF)[284]. Accessory proteins such as the ternary complex factors (TCFs)[285], including Elk-1[286] and SAP-1[287] proteins, were then identified and were found to associate with the SRE-SRF complex. In response to stimulation, the TCFs undergo phosphorylation, which increases their DNA-binding or transactivation activities and thus increases *c-fos* promoter activity[288]. Other transcription factors also bind to the SRE site in the *c-fos* promoter. These factors include NF-IL-6[289], MyoD[290,291], Phox1[292], and YY1[293]. Most of these factors either potentiate or antagonise the binding of the SRF/TCF complex to the SRE site. For example, MyoD heterodimers with E12 or E47 can inhibit *c-fos* transcription by direct antagonism with SRF complexes for binding to the SRE[290]. In addition, the CBP co-activator (see below) has been shown to bind both the SRF and the TCFs and to enhance transcriptional activation of *c-fos*[294]. Experiments have shown that when exogenous c-Fos is over-expressed, the *c-fos* gene is also transcriptionally repressed. This transrepression involves the *c-fos* SRE and the C-terminal domain of c-Fos, which requires serine phosphorylation[295,296]. One possible reason for this repression could be that over-expression of c-Fos may sequester CBP or other co-activators away from the *c-fos* promoter[297].

Posttranslational Regulation

The major regulatory mechanism involved in controlling the activity of AP-1 proteins is through phosphorylation. The post-translational regulation of c-Jun has been extensively investigated. c-Jun has five amino acid (threonine and serine residues) sites whose phosphorylation controls the DNA binding and transcriptional activity of c-Jun. Phosphorylation by ERK or CK2 of three of these residues, located C-terminally to the DNA binding domain, inhibits DNA-binding of c-Jun homodimers but not c-Jun-c-Fos heterodimers[298]. Phosphorylation of the other two sites (serine 63 and 73) in the N-terminal

domain by ERK and JNK, stimulate the transcriptional activity of c-Jun homodimers[299] and heterodimers with c-Fos[300] without affecting DNA binding.

Phosphorylation of the regulatory domains in c-Fos (see section 1.5.4) may affect the level of transcriptional activation or repression. For example, phosphorylation within the HOB1 domain by MAP kinase has been shown to increase the activation capacity of c-Fos. In contrast, the transforming potential of c-Fos is down-regulated following the phosphorylation of certain residues in the C-terminal region by PKA[295].

Interactions with other proteins

A third mechanism to regulate AP-1 activity is physical interaction with other cellular proteins. Although most AP-1 protein interactions are mediated via the leucine zipper and involve heterodimerisation with other bZIP proteins[236,240-243,266], several proteins were found to interact with the amino-terminal activation domain of the Jun proteins and to modulate their activity to activate transcription. For example, Jun kinases can interact with a JNK-docking site within the δ region of the amino-terminal activation domain of c-Jun[254,256]. The enhanced transcriptional activation of c-Jun, by phosphorylation of serines 63 and 73, is most likely due to a higher affinity of JNK-phosphorylated c-Jun for the transcriptional co-activator CBP[301] (see below)[302]. As CBP is closely related to the E1A-binding protein p300, it is likely that JNK-phosphorylated c-Jun also interacts with p300[303]. Another co-activator that interacts with the amino-terminal activation domain of c-Jun is JAB1 (Jun activation domain binding protein 1)[304]. JAB1 interacts with c-Jun and JunD but not with JunB. This interaction enhances their transcriptional activation through stabilisation of the complexes to the AP-1 sites and is not affected by c-Jun phosphorylation unlike CBP[304].

The CBP adaptor protein was first characterised as a co-activator for CREB, a cAMP-responsive transcription factor[301], but was then shown to be involved in a large variety of responses. In addition to c-Jun, CBP and p300 are involved in the activation of a large variety of transcriptional enhancer elements through various transcription factors[305], including c-Fos[302], and MyoD[306,307] (see section 1.3.6). CBP directly contacts sequence-specific transactivators via one of two interaction domains located, respectively, in the N-

terminal or C-terminal part of the molecule[308]. Once recruited, CBP can modulate the transcription rate through various mechanisms. First, CBP includes two transactivation domains located in the N-terminal and C-terminal parts of the molecule[305] that contact two general transcription factors: TBP for the N-terminal transactivation domain[307,309], and TFIIB for the C-terminal transactivation domain[301]. In addition, CBP has endogenous HAT activity[310]. This enzyme destabilises the nucleosomal structure by acetylation of the N-terminal histone tails, which protrude from the nucleosome[311]. Thus, CBP may either recruit proteins of the transcriptional machinery or induce a nucleosomal remodelling process to activate transcription.

In addition to interactions with bZIP proteins, AP-1 dimers or individual Fos or Jun proteins can also interact with other non-bZIP proteins. For example, enhanced transcriptional activity is observed when AP-1 interacts with: the nuclear factor in activated T cells (NF-AT)[312], NF-κB p65[313], Fos interacting protein[314], Ets-1[315], and Smad3[316]. In contrast, c-Fos, c-Jun, and JunB suppress the transactivation of the muscle creatinine kinase (MCK) enhancer by myogenin and MyoD. JunD, however, is expressed constitutively in muscle cells and is an inefficient inhibitor of the transactivation capacity of myogenin and MyoD[134]. Transcriptional repression by Fos and Jun is specific to myogenic bHLH proteins and is not observed with the widely expressed bHLH protein E47, which recognises the same DNA sequence (see section 1.3.8). Conversely, MyoD and myogenin can suppress the transactivation by Jun and Fos of genes linked to an AP-1 site. These inhibitions require physical interactions between the two classes of transcription factors[133,134]. In addition, nuclear receptors such as the activated glucocorticoid receptor and the retinoic acid receptors inhibit both basal level and TPA induced AP-1 activity through inhibition of binding of AP-1 to the TRE[317,318]. The transcriptional activity of TPA-induced AP-1 has also shown to be inhibited by the interferon-inducible p202 protein[319].

Rb has been shown to bind and modulate the activity of a number of different transcription factors and co-activators. Modulation can take the form of repression of transcription as with E2F[320], or activation as with NF-IL6[321]. Nead *et al* have shown that hypophosphorylated Rb can bind to members of the AP-1 family, including c-Jun, and

stimulate its activity from an AP-1 consensus sequence. As mentioned previously, the AP-1 family has shown to be activated early in G1, and they are thought to have a mitogenic stimulus on the cell. In addition, AP-1 members have been implicated in the differentiation of keratinocytes and myeloid cells. The AP-1/Rb complexes were found in terminally differentiating keratinocytes and cells entering the G1 phase of the cell cycle after release from serum starvation. Thus Rb can act as a transcriptional activator in early G1 and as a potential modulator of *c-jun* expression during keratinocyte differentiation[322].

1.5.6 FUNCTION OF AP-1

Previous experiments have shown induction of *c-fos* in response to growth factors, suggesting that *c-fos* is linked to cell proliferation[237]. However, the mouse *c-fos* gene knockout showed that *c-fos* is not essential for the viability, proliferation and differentiation of most cell types, the exception being some cells that are involved in bone formation, gametogenesis and certain neuronal functions[323]. Due to the fact that experiments have shown a tight link of *c-fos* to mitogenic stimulation, it may therefore be possible that when *c-fos* is knocked out *in vivo*, other AP-1 proteins can compensate for most of its functions. It has been shown, for example, that microinjection of specific antibodies against individual Fos proteins (c-Fos, FosB, Fra1 or Fra2) has no major effect on cell proliferation, but neutralisation of all four blocked cell proliferation[324]. Interestingly, development of osteosarcomas was observed when c-Fos was over-expressed in bone tissue and in addition, the rate of osteosarcoma formation was enhanced with simultaneous over-expression of c-Jun and c-Fos[325]. Thus, these results indicate *in vivo* co-operation between c-Jun and c-Fos in tumour development. In contrast to c-*fos*, c-*jun* is essential for normal mouse development[326]. *c-jun* knockout mouse embryos die after 12-14 days at mid-to-late gestation[326,327] and show altered liver erythropoiesis, impaired hepatogenesis and generalised oedema[327]. In addition, primary fibroblasts from these embryos are completely defective in proliferation[326]. *junB* knockout mice also result in embryonic lethality[328]. In contrast, *junD* knockout mice are viable and appear healthy. However, these animals do show a reduced postnatal growth and males exhibit multiple defects in their reproductive functions. No defects in fertility are observed in females[328]. Furthermore, *fosB* knockout mice are born at a normal frequency, are fertile and present no obvious phenotypic or histologic abnormalities[329].

1.6 AIMS OF WORK

Investigate the potential involvement of the following transcriptional activities during HSC activation:

E-Box DNA Binding Proteins

1. Determine the expression of E-box DNA binding activities in the HSC during both *in vitro* (culture on plastic) and *in vivo* (CCl_4 treatment of rats) activation.

2. Characterise E-box DNA binding proteins using various techniques (e.g. Western blotting, Supershift EMSAs, Northern blotting, RT-PCR).

3. Investigate the role of nucleotides flanking the E-box motif in determining specificity of protein binding.

4. Clone the predominant E-box DNA binding protein (MyoD) in activated HSCs.

5. Investigate the factors involved in regulating MyoD binding during HSC activation.

6. Elucidate the cell-signalling pathways involved in MyoD induction.

GC-Box DNA Binding Proteins

1. Use a consensus oligonucleotide for Sp1 to examine GC-box DNA binding activities in the HSC during both *in vitro* (culture on plastic) and *in vivo* (CCl_4 treatment of rats) activation.

2. Characterise the identity of Sp1/GC-box DNA binding proteins using both Western blotting and Supershift EMSA analysis.

3. Investigate the cell-signalling pathways involved in induction of Sp1 and determine the effects of trypsin treatment on culture activated HSCs.

AP-1 DNA Binding Proteins

1. Determine the expression of AP-1 DNA binding activities in the HSC during both *in vitro* (culture on plastic) and *in vivo* (CCl_4 treatment of rats) activation.

2. Characterise the identity of AP-1 DNA binding proteins using both Western blotting and Supershift EMSA analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 ANTIBIOTICS

Ampicillin	Sigma
Gentamycin	Sigma
Neomycin	Sigma
Penicillin	Sigma
Streptomycin	Sigma
Zeocin	Invitrogen

2.1.2 ANTIBODIES

Ap1 Transcription Factor Family

Rabbit polyclonal IgG anti-human c-Fos (amino terminus)	Santa Cruz
Rabbit polyclonal IgG anti-mouse Fos B (central domain)	Santa Cruz
Rabbit polyclonal IgG anti-mouse Fra-1 (amino terminus)	Santa Cruz
Rabbit polyclonal IgG anti-human Fra-2 (amino terminus)	Santa Cruz
Rabbit polyclonal IgG anti-mouse c-Jun (amino terminus)	Santa Cruz
Rabbit polyclonal IgG anti-mouse Jun B (carboxy terminus)	Santa Cruz
Rabbit polyclonal IgG anti-mouse Jun D (carboxy terminus)	Santa Cruz

bHLH Transcription Factor Family

Rabbit polyclonal IgG anti-human E12 (bHLH domain)	Santa Cruz
Rabbit polyclonal IgG anti-human E47 (full length)	Santa Cruz
Rabbit polyclonal IgG anti-mouse MyoD (full length)	Santa Cruz
Rabbit polyclonal IgG anti-mouse MyoD (carboxy terminus)	Santa Cruz
Rabbit polyclonal IgG anti-rat myogenin (full length)	Santa Cruz

bHLHZip Transcription Factor Family

Rabbit polclonal IgG anti-human Mad 1 (full length)	Santa Cruz
Rabbit polyclonal IgG anti-human Max (carboxy terminus)	Santa Cruz

Rabbit polyclonal IgG anti-human c-Myc (amino terminus)	Santa Cruz

NF-κB *Transcription Factor Family*

Rabbit polyclonal IgG anti-human NF-κB p50 (NLS region)	Santa Cruz
Rabbit polyclonal IgG anti-human NF-κB p65 (amino terminus)	Santa Cruz

Sp1 Transcription Factor Family

Rabbit polyclonal IgG anti-rat Sp1 (internal domain)	Santa Cruz
Rabbit polyclonal IgG anti-human Sp2 (amino terminus)	Santa Cruz
Rabbit polyclonal IgG anti-human Sp3 (carboxy terminus)	Santa Cruz
Rabbit polyclonal IgG anti-human Sp4 (carboxy terminus)	Santa Cruz
Rabbit polyclonal IgG anti-rat Zf9 (amino terminus)	Santa Cruz

Conjugated Antibody

Goat anti-rabbit IgG Horse Radish Peroxidase (HRP) conjugate	Sigma

2.1.3 SIGNAL TRANSDUCTION INHIBITORS

Bisindolylmaleimide 1, Hydrochloride (BIM)	Calbiochem
Calpain Inhibitor 1	Calbiochem
Gliotoxin	Calbiochem
Herbimycin A	Calbiochem
KT5720	Calbiochem
KT5823	Calbiochem
PD98059	Calbiochem
Pyrrolidinedithiocarbamate (PDTC)	Calbiochem
Staurosporine	Calbiochem
Wortmannin	Calbiochem

2.1.4 CHEMICALS

Acetyl Coenzyme A	Sigma
Acrylamide	Scotlab
Agar	Sigma
Agarose	Life Tech.

4-(2-Aminoethyl)benzenesulfonyl Fluoride (AEBSF)	Sigma
Adenine Triphosphate (ATP)	Sigma
[α-^{32}P]dATP	Amersham
Alkaline phosphatase	Promega
Ammonium persulphate (APS)	Sigma
Aprotinin	Sigma
Avian Myeloblastosis Virus (AMV)	Promega
Blocking agent	Boehringer
Boric acid	Sigma
Bovine serum albumin (BSA)	Sigma
Bromophenol blue	Sigma
Chloroform	Sigma
^{14}C-Chloramphenicol	Sigma
Denhardts reagent	Promega
Deoxynucleoside triphosphates (dNTPs)	Sigma
Diethylpyrocarbonate (DEPC)	Sigma
Dimethyl Sulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
Dulbecco's Modified Eagle Medium (DMEM)	Gibco
Enhanced Chemiluminescent (ECL) reagent	Amersham
Ethyl Acetate	Sigma
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Ethidium bromide	Sigma
Fetal calf serum	Gibco
Ficoll 400	Sigma
Formaldehyde	Sigma
Formamide	Sigma
[γ-^{32}P]dATP	Amersham
Glycerol	Sigma
Hanks Buffered Saline (HBS)	Gibco
N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES)	Sigma
Herring sperm DNA	Promega

Iso-Amyl alcohol	Fisons
Lauryl Sulfate (sodium dodecyl sulfate, SDS)	Sigma
Luria Broth (LB)	Sigma
Magnesium chloride	Sigma
Methanol	Sigma
Milk protein	Marvel
Mineral oil	Sigma
3-[N-Morpholino]propanesulfonic acid (MOPS)	Sigma
Nonidet P-40 (NP40)	Sigma
Orange G	Sigma
Phenol: chloroform: isoamyl alcohol (25: 24: 1)	Sigma
Phosphate buffered saline (PBS)	Sigma
Poly dIdC	Sigma
Potassium chloride	Sigma
Prestained protein markers	NEB
Reagent A	Biorad-DC
Reagent C	Biorad-DC
Restriction enzymes	Promega
Sephadex G-50	Sigma
Sodium acetate	Fisons
Sodium chloride	Sigma
Sodium citrate	Sigma
Sodium fluoride	Sigma
Sodium hydroxide	Sigma
Sodium orthophosphate EDTA	Sigma
Sodium orthovanadate	Sigma
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma
Thermus Aquatus (Taq) DNA Polymerase	Promega
T4 DNA ligase	Promega
Tris	Sigma
Trypsin	Sigma
T4 Polynucleotide Kinase	Amersham
Tween 20	Sigma

Vent DNA Polymerase	NEB
Xylene cyanol	Sigma

2.1.5 OLIGONUCLEOTIDES

β-actin (For Northern)

(S) 5'-TGTACGTAGCCATCCAGGCT-3'	Helena
(A/S) 5'-TTCTCCAGGGAGGAAGAGGA-3'	Helena

MyoD (For Northern)

5'-CGGGGCCTATCAAGTCTGTGTCCCGGAGTGGCGGCGATAGTAGCTCCAT-3' Helena

MyoD (For RT-PCR)

(S) 5'-AAGACCACTAACGCTGATCG-3'	Genosys
(A/S) 5'-CTGCAGACCTTCAATGTAGC-3'	Genosys

Consensus E-box (For EMSA)

(S) 5'-CGCCGACCACGTGGTCCCTC-3'	Genosys
(A/S) 5'-GAGGGACCACGTGGTCGGCG-3'	Genosys

Mutant consensus E-box (For EMSA)

(S) 5'-CGCCGACATCGCTGTCCCTC-3'	Genosys
(A/S) 5'-GAGGGACAGCGATGTCGGCG-3'	Genosys

E-box in the αSMA gene (For EMSA)

(S) 5'-GATCATAAGCAGCTGAACTGCC-3'	Genosys
(A/S) 5'-GGCAGTTCAGCTGCTTATGATC-3'	Genosys

Mutant E-box in the αSMA gene (For EMSA)

(S) 5'-GATCATAAGTAGGCGAACTGCC-3'	Genosys
(A/S) 5'-GGCAGTTCGCCTACTTATGATC-3'	Genosys

E-box in the αSMA gene with consensus flanking ends (For EMSA)

(S) 5'-CGCCGACCAGCTGGTCCCTC-3' Oswel

(A/S) 5'-GAGGGACCAGCTGGTCGGCG-3' Oswel

E-box in the Muscle Creatine Kinase (MCK) gene (For EMSA)

(S) 5'-GATCCCCCCCAACACCTGCTGCCTGA-3' Oswel

(A/S) 5'-TCAGGCAGCAGGTGTTGGGGGGGATC-3' Oswel

Consensus E-box with BamH 1 ends (For CAT Assay)

(S) 5'-GATCCGACCACGTGGTCG-3'

(A/S) 5'-GATCCGACCACGTGGTCG-3' Helena

Consensus E-box with BamH1 ends (For CAT Assay)

(S) 5'-GATCCGACCACGTGGTCGACCACGTGGTCGACCACGTGGTCG-3'

(A/S) 5'-GATCCGACCACGTGGTCGACCACGTGGTCGACCACGTGGTCG-3'

Helena

NF-κB (For EMSA)

(S) 5'-AGTTGAGGGGACTTTCCCAGG-3' Promega

(A/S) 5'-CCTGGGAAAGTCCCCTCAACT-3' Promega

Sp1 (For EMSA)

(S) 5'-ATTCGATCGGGGCGGGGCGAGC-3' Genosys

(A/S) 5'-GCTCGCCCCGCCCCGATCGAAT-3' Genosys

Consensus AP-1 (For EMSA)

(S) 5'-TATAAAGCATGAGTCAGACACCTCT-3' Genosys

(A/S) 5'-AGAGGTGTCTGACTCATGCTTTATA-3' Genosys

TIMP-1 AP-1 (For EMSA)

(S) 5'-TGGGTGGATGAGTAATGCA-3' Genosys

(A/S) 5'-TGCATTACTCATCCACCCA-3' Genosys

2.1.6 DISPOSABLE EQUIPMENT

Eppendorf centrifuge tubes	Elkay
Filter tips	Greiner
Hybond C nitrocellulose membrane	Amersham
Hybond N nylon membrane	Amersham
Optiprep	Life Tech.
Thin Layer Chromatography (TLC) Plates	Abinghurst
Tissue culture plastics	Greiner
3MM filter paper	Whatman
X-ray film	GRI

2.1.7 SUPPLIED KITS

Effectene Transfection kit	Qiagen
Megaprime DNA labelling kit	Amersham
Plasmid maxi kit	Qiagen
QIAquick Gel Extraction kit	Qiagen
Rneasy mini kit	Qiagen
Sequenase kit	Amersham

2.1.8 SUPPLIERS ADDRESSES

Abinghurst Limited, Unit 1, Ross Road Business Centre, Northampton, NN5 5AX.

Amersham Life Science Ltd., Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA.

Autogen Bioclear UK Ltd., Holly Ditch Farm, Mile Elm, Calne, Wiltshire, SN11 0PY.

Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD.

Boehringer-Mannheim, Bell Lane, Lewes, East Sussex, BN7 1LG.

Fisons Scientific Equipment, Bishop Meadow Rd., Loughborough, LE11 0RG.

Calbiochem-Novabiochem Ltd., Boulevard Industrial Park, Padge Road, Beeston, Nottingham, NG9 2JR.

Elkay Laboratory Products Ltd., Unit 4, Marlborough Mews, Crockford Lane, Basingstoke, RG24 8NA.

Genetic Research Instumentation Ltd., Gene House, Dunmow Road, Felsted, Dunmow, Essex, CM6 3LD.

Genosys Biotechnologies (Europe) Ltd., London Road, Pampisford, Cambridgeshire, CB2 4EF.

Greiner Labortechnik Ltd., Brunel Way, Stroudwater Business Park, Stonehouse, Gloucestershire, GL10 3SX.

Helena BioSciences, Colima Avenue, Sunderland Enterprise Park, Sunderland, Tyne & Wear, SR5 3XB.

Invitrogen BV, De Schelp 12, 9351 NV Leek, The Netherlands.

Life Technologies Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF.

Marvel, Premier Beverages, Knighton, Adbaston, Stafford, ST20 0QJ.

New England Biolabs (UK) Ltd., Knowl Piece, Wilburyway, Hitchin, Hertfordshire, SG4 0TY.

Oswel DNA Service, Medical & Biological Sciences Building, University of Southampton, Boldrewood, Bassett Crescent East, Southampton, SO16 7PX.

Promega UK Ltd., Delta House, Chilworth Research Centre, Southampton, SO16 7NS.

Qiagen Ltd., Boundary Court, Gatwick Road, Crawley, West Sussex, RH10 2AX.

Scotlab, Charles Street, Luton, Bedfordshire, LU2 0EB.

Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset, BH12 4QH.

Whatman International Ltd., St Leonard's Road, 20/20 Maidstone, Kent, ME16 0LS.

2.2 METHODS

2.2.1 ISOLATION AND CULTURE OF RAT AND HUMAN HEPATIC STELLATE CELLS

Human HSCs were isolated from the livers of adult male patients following partial hepatectomy. Rat HSCs were isolated from either normal or CCl_4-treated rat liver (6 month old, male Sprague-Dawley rats, ~500g). Induction of acute liver damage in rats was achieved by administration of CCl_4 by intraperitoneal injection with 0.2ml/100g sterile CCl_4 in a 1:1 ratio with olive oil[330]. The injection was given once and the HSCs were then isolated 48 hours afterwards. The isolation of HSCs was achieved by sequential *in situ* perfusion with collagenase and pronase as described previously[16]. Buoyant HSCs were separated from the resulting cell suspension over a discontinuous one layer Optiprep gradient followed by centrifugal elutriation. Cells were then cultured on 100mm tissue culture grade dishes in DMEM supplemented with 16% fetal calf serum, penicillin (10mU/ml), streptomycin (10μg/ml) and gentamycin (32μg/ml). Plating density was 1 to 3 million cells per 100mm plate depending on length of culture. Cultures were maintained in an atmosphere of 5% CO_2, 37°C and medium was changed every 3-4 days with long-term cultures. Cells were kept in primary culture without passaging in all experiments except for those specified where effects of trypsin detachment on DNA binding activities was investigated. Trypsin detachment was achieved by washing cultures in HBS solution followed by incubation at 37°C in the presence of 1% Trypsin diluted in HBS.

2.2.2 PREPARATION OF NUCLEAR EXTRACTS

Nuclear extracts were prepared from human or rat HSCs cultured on plastic for varying periods of time by a modification of Dignam's original protocol[331]. Cells were harvested, washed twice in 1x PBS and lysed in Dignam buffer A (10mM HEPES (pH 7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.2% NP40, 0.5mM AEBSF, 2mM aprotinin, 1mM sodium fluoride and 1mM sodium vanadate). Lysates were centrifuged (13,000rpm, 10s) to collect nuclear pellets and resuspended in Dignam buffer C (20mM HEPES (pH 7.9), 25% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.5mM DTT, 0.2mM EDTA, 0.5mM AEBSF, 2mM aprotinin, 1mM sodium fluoride and 1mM sodium vanadate) and incubated on ice for 10mins with occasional vortexing. Nuclear extracts were cleared by centrifugation (13,000rpm, 1min) and stored in 5μl aliquots at -70°C until required.

Protein concentrations were determined by the Bio-Rad DC Protein Assay which is similar to the well documented Lowry Assay[332]. 5µl of extract was added to 15µl H_2O, 100µl reagent A and 800µl reagent C. Samples were mixed and left at room temperature for 15mins. Absorbances were then measured against a standard at 750nm. Concentrations were determined using a standard curve (appendix 1).

2.2.3 ELECTROMOBILITY SHIFT ASSAY

5'-end radiolabelled sense oligonucleotides were prepared for protein detection in nuclear extracts using the Electromobility Shift Assay (EMSA). End labelling was carried out at 37°C for 15mins using ~17ng sense DNA, 1.6µl 10x T4 polynucleotide kinase buffer (500mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 50mM DTT, 1mM spermidine), 5µl [γ-^{32}P]ATP (50µCi), 1µl T4-Polynucleotide kinase (10 units) and sterile H_2O in a total volume of 17µl. The volume of labelled DNA was made up to 50µl using TE buffer. Phenol extractions were carried out using phenol: chloroform: isoamyl-alcohol (25: 24: 1) and chloroform: isoamyl-alcohol (24:1). 5µg of polydIdC and 1/10 volume 3M sodium acetate were added to the second aqueous phase and the DNA precipitated at -20°C using two volumes of 100% ethanol. The DNA was recovered by centrifugation (13,000rpm, 5mins), washed in 70% ethanol and resuspended in a final volume of 10µl to give a concentration of 10ng/µl. 1.25µl of unlabelled antisense oligonucleotide (25ng/µl) was added to the sense strand. The oligonucleotides were then annealed by incubation at 85°C for 5mins followed by cooling overnight to room temperature. The volume of annealed product was then made to a final concentration of 1ng/µl and stored at -20°C until required.

Nuclear extracts (2 to 16µg) were made up to 4µl with Dignam buffer C, 1µl polydIdC (1µg/µl) was added to prevent non-specific DNA binding in a total volume of 16µl. These were incubated for 10mins on ice and then 0.2 to 0.4ng of radiolabelled probe was added to a final volume of 20µl. The mixture was incubated for a further 10mins on ice to allow oligonucleotide/protein binding to take place. Following the addition of 4µl of 6x gel loading buffer (50mM EDTA, 10% Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol and 0.25% orange G), the samples were loaded onto a 5% polyacrylamide gel containing 0.25X TBE (10X TBE: 0.9M Boric acid, 20mM EDTA, 0.9M Tris, pH 8.0).

Polymerisation of the gel was accelerated by the addition of 10% APS (1:100) and TEMED (1:1000). Electrophoresis was carried out at a constant current of 10mA for 2.5-3hrs in 0.5X TBE electrophoresis buffer. The gel was then dried on 3MM Whatman paper under vacuum for 1hr at 80°C. The [γ-^{32}P]ATP labelled DNA was visualised by autoradiography at -70°C for varying periods of time depending on the activity of the probe.

Competition EMSAs were performed by incubating the nuclear extracts with various excess concentrations of cold double-stranded oligonucleotides during the first incubation step. Supershift assays were performed by adding 1 to 4µg of supershift antibody after incubation with the radiolabelled probe and kept at 4°C for 16 hours prior to electrophoresis.

2.2.4 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Nuclear extracts (10 to 20µg) were made up to 10µl with 1x PBS. An equal volume of SDS-PAGE sample buffer (0.5% bromophenol blue, 20% glycerol, 10% β-mercaptoethanol, 4% SDS, 125mM Tris, pH 6.8) was then added. The samples were boiled for 10mins to denature fully and then electrophoresed through a 4% SDS-PAGE stacking gel (4% acrylamide, 125mM Tris, pH 6.8, 0.1% SDS, 10% APS (1:100), TEMED (1:1000)) followed by an SDS-PAGE separating gel (% acrylamide depended on size of protein but typically ranged from 9%-12%, 300mM Tris, pH 8.8, 0.1% SDS, 10% APS (1:100), TEMED (1:1000)). Prestained protein markers were run alongside the samples. Gels were then run at a constant current of 20mA for 90mins in a 1x SDS electrode buffer (25mM Tris, 192mM glycine, 0.1% SDS). After electrophoresis gels were immersed in blotting bufffer (1X SDS electrode buffer, 20% methanol) and then blotted onto Hybond C nitocellulose membranes for 1h at 35V, 0.8mA/cm^2 using a Transblot semidry protein blot apparatus.

The blot was washed in PBS to remove methanol and incubated in blocking solution (5% marvel in PBS) to block non-specific protein binding for a minimum of 30mins at room temperature with shaking. Primary antibody, diluted (1µg/ml) with 5% marvel in PBS, was added and left overnight at 4°C. The blot was then rinsed twice (5mins) in PBS/Tween and

anti-rabbit IgG-HRP conjugate, diluted (1:2000) in 5% marvel in PBS/Tween, was added for a minimum of 1h at room temperature with shaking. All concentrations of antibodies were optimised by titration. Blots were rinsed twice with PBS/Tween and then with distilled water (2x 5mins) to remove the phosphate from PBS which interferes with the ECL reagents. The HRP conjugate was then detected by a chemiluminescence reaction (ECL system) followed by exposure to film.

2.2.5 PREPARATION OF RNA

All glassware, plastics and distilled water were treated prior to use for removal of RNAase enzymes. DEPC was added to distilled water at a 1:1000 dilution, left to stand for 18hrs at room temperature and then autoclaved. All glassware was baked at 200°C for 4hrs and all plastics were immersed in 0.5M NaOH for 10mins and then in DEPC H_2O for 10mins. RNA was prepared using Qiagen's Rneasy mini kit.

2.2.6 NORTHERN BLOTS

Radiolabelled DNA was prepared using Amersham's Megaprime DNA labelling kit. Unincorporated nucleotides were removed from the probe by sephadex chromatography. Sephadex G-50 was equilibrated in H_2O at 95°C for 2hrs. Once equilibrated a 1ml syringe, plugged with a piece of siliconised glass wool, was filled with sephadex. Liquid was removed from the sephadex column by centrifugation (2,000rpm, 5mins). 50µl of DEPC water was added to the probe and then applied to the column and centrifuged (2,000rpm, 5mins). As the probe passed through the column, the free nucleotides were retained in the matrix. The purified probe was collected in a 15ml conical tube and kept at -20°C until required for hybridisation.

20µg of total RNA was made up to 10µl with DEPC H_2O and used for Northern analysis. An equal volume of RNA loading buffer (0.5% bromophenol blue, 2M formaldehyde, 0.75M formamide, 7% glycerol, 1X MOPS (1mM EDTA, 20mM MOPS, 5mM sodium acetate, pH 7)) was added to the RNA samples and incubated for 15mins at 65°C to denature the RNA and then cooled to room temperature. 1µl of ethidium bromide (1mg/ml) was added to the samples. A 1% horizontal agarose gel in 1X MOPS buffer was prepared and boiled to dissolve the agarose. When cooled to 50°C, formaldehyde at a final

concentration of 2.2M was added. The gel was then run in 1x MOPS buffer at 4-5V/cm until the bromophenol blue had migrated approximately 8cm from the wells. After electrophoresis, the gel was rinsed in DEPC water for 10mins to remove the formaldehyde and then soaked in 50mM sodium hydroxide for 20mins. Following a second 10min rinse in DEPC water, the gel was soaked twice in 10x SSC (1.5M NaCl, 150mM sodium citrate, pH 7.0) for 10mins. RNA was then transferred to a nylon membrane (Hybond N) by capillary elution using 10X SSC as the transfer medium. Transfer occurred for 16-24hrs. After transfer, the membrane was dried and exposed to low doses of ultraviolet irradiation (254nm) for 5mins, then stored at room temperature until pre-hybridisation was performed.

The filter was pre-hybridised for 3-4hrs in 20mls of hybridisation solution (0.5% Blocking agent, 2X Denhardts reagent, 50% formamide, 20μg/ml Herring sperm DNA, 1% SDS, 20X sodium orthophosphate EDTA, 5X SSC) in a rotating hybridisation oven at 42°C. Prior to hybridisation the double-stranded, radiolabelled, DNA probe was denatured by heating to 95°C for 5mins and then quickly cooled on ice. The denatured probe was mixed with 20ml of fresh hybridisation buffer and then added to the pre-hybridised filter for 16-24 hrs at 42°C. After hybridisation the filter was washed three times for 15mins in 30mls of 0.2% SSC, 0.2% SDS solution at 50°C. The location of the hybrid was then determined by autoradiography. Films were kept at -70°C for 24-72 hours.

2.2.7 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

To determine the effects of Mg^{2+}, 5μl aliquots of 10x Taq Reaction Buffer (500mM KCl, 100mM Tris-HCl (pH 9.0 at 25°C), 1% Triton X-100) were mixed with Mg^{2+} so that the final 50μl PCR reactions would contain final concentrations of 0.5 to 2.5mM Mg^{2+}. RT-PCR was carried out at 42°C for 30 mins and then at 94°C for 5 mins. The initial reaction consisted of 1μg RNA (for RNA preparation, see section 2.2.5), 1μl anti-sense MyoD primer (50pM/μl), 1μl dNTPs (1mM each), 1μl 10x Taq Reaction Buffer, 1μl AMV Reverse Transcriptase (2.5 units) and sterile DEPC H_2O in a total volume of 10μl. The cDNA produced was then used directly in PCR. 1μl sense MyoD primer (50pM/μl), 4μl 10x Taq Reaction Buffer, and 1μl Taq DNA polymerase (1 unit) were added to the 10μl cDNA products, and then made up to a final volume of 50μl with sterile H_2O. The samples

were then covered with ~40µl of PCR grade mineral oil and placed into the PCR cycler. PCR was carried out for 35 cycles at 94°C for 40 secs, 56°C for 45 secs, and 72°C for 1 min. A final step consisting of 5 mins at 72°C was performed to ensure complete extension.

2.2.8 RT-PCR CLONING

Purification of PCR product

Mineral oil was removed from the PCR product obtained at a final concentration of 1mM Mg^{2+}. Due to the overhang left by Taq polymerase, the product was blunt-ended by Vent polymerase. 2µl dNTPs (0.4mM each), 1µl Vent polymerase (1 unit), and 2µl 10x Vent polymerase buffer (100mM KCl, 200mM Tris-HCl (pH 8.8 at 25°C), 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1% Triton X-100) were made up to a final volume of 50µl with H_2O, and incubated at 72°C for 10 mins. The product was then run on a 2% agarose gel, and purified using Qiagen's QIAquick Gel Extraction kit.

Phosphorylation of PCR product

Approximately 1µg of DNA was then phosphorylated using 1µl 10x T4 polynucleotide kinase buffer (500mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 50mM DTT, 1mM spermidine), 1µl ATP (1mM), and 1µl T4 Polynucleotide kinase (10 units) in a final volume of 10µl. The reaction was incubated at 37°C for 30 mins and then the enzyme was inactivated by incubating at 70°C for 10 mins.

De-phosphorylation of pcDNA3

The expression vector, pcDNA3, was digested with EcoR V to create blunt-ends. 10µg pcDNA3, 10µl 10x EcoR V Buffer (60mM Tris-HCl (pH 7.9 at 37°C), 60mM $MgCl_2$, 1.5M NaCl, 10mM DTT), 5µl EcoR V (50 units), and 10µl 10x BSA in a final volume of 100µl were incubated for ~3 hours at 37°C. The reaction was phenol extracted and the DNA recovered by ethanol precipitation. The DNA was then resuspended in 50µl H_2O. Half of the DNA (~5µg) was used in a de-phosphorylation reaction consisting of 5µl 10x alkaline phosphatase buffer (0.5M Tris-HCl (pH 9.3), 10mM $MgCl_2$, 1mM $ZnCl_2$, 10mM spermidine), and 1µl alkaline phosphatase (1 unit) in a final volume of 50µl. This was

incubated at 37°C for 30 mins, and then another 1µl of enzyme was added for another 30 mins. The reaction was then phenol extracted and DNA recovered by ethanol precipitation. The DNA was resuspended in 20µl H_2O.

Blunt-end ligation of PCR product and pcDNA3

Ligation reactions were set up consisting of 1µl 10x ligation buffer (300mM Tris-HCl (pH 7.8), 100mM $MgCl_2$, 100mM DTT, 10mM ATP), 1µl T4 DNA ligase (1 unit), 1µl pcDNA3 (~200ng), and 4µl PCR product (~400ng) in a final volume of 10µl. Reactions were left for ~2 hours at room temperature and then transformed into competent DH5α (see section 2.2.9).

2.2.9 PREPARATION OF PLASMID DNA

Plasmid DNA was grown up by transformation of competent DH5α *Escherichia coli* (*E.coli*) by incubating 50µl of competent bacteria with 5µl of ligation mix for 30mins on ice followed by heat shocking for 45secs at 42°C and then a further incubation on ice for 5mins. The transformed bacteria were then plated out on LB agar plates containing the appropriate selective antibiotic and grown up overnight at 37°C. A single colony was inoculated into 100ml LB medium containing the appropriate antibiotic and grown up overnight at 37°C with vigorous shaking (~300rpm). Plasmid DNA was then prepared using Qiagen's plasmid maxi kit. Purity of the plasmid preparations were checked by running DNA on a 1% agarose gel with ethidium bromide and then visualising the DNA using an ultraviolet light box. DNA concentrations were determined by spectrophotometry at a wavelength of 260/280nm.

2.2.10 SEQUENCING THE PLASMIDS

Plasmids were initially sequenced using Amersham's sequenase kit. This information was then confirmed by Oswel DNA Sequencing Services.

2.2.11 CLONING E-BOX INTO PBLCAT2

Phosphorylation of E-box

Approximately 4µg of an E-box oligonucleotide with BamH 1 recognition sites on either end was phosphorylated using 2µl 10x T4 polynucleotide kinase buffer (500mM Tris-HCl

pH 7.5, 100mM $MgCl_2$, 50mM DTT, 1mM spermidine), 2µl ATP (2mM), and 2µl T4 Polynucleotide kinase (20 units) in a final volume of 20µl. The reaction was incubated at 37°C for 30 mins and then at 85°C for 10 mins to fully denature the DNA and to inactivate the enzyme. The samples were then cooled down slowly to allow the palindromic oligonucleotides to form double-strands.

De-phosphorylation of pBLCAT2

The cloning vector, pBLCAT2, was digested with BamH 1. 10µg pBLCAT2, 10µl 10x BamH 1 Buffer (60mM Tris-HCl (pH 7.5 at 37°C), 60mM $MgCl_2$, 1M NaCl, 10mM DTT), 5µl BamH 1 (50 units), and 10µl 10x BSA in a final volume of 100µl were incubated for ~3 hours at 37°C. The reaction was phenol extracted and the DNA recovered by ethanol precipitation. The DNA was then resuspended in 50µl H_2O. Half of the DNA (~5µg) was used in a de-phosphorylation reaction consisting of 5µl 10x alkaline phosphatase buffer (0.5M Tris-HCl (pH 9.3), 10mM $MgCl_2$, 1mM $ZnCl_2$, 10mM spermidine), and 1µl alkaline phosphatase (1 unit) in a final volume of 50µl. This was incubated at 37°C for 30 mins, and then another 1µl of enzyme was added for another 30 mins. The reaction was then phenol extracted and DNA recovered by ethanol precipitation. The DNA was resuspended in 20µl H_2O.

Ligation of E-box oligonucleotide and pBLCAT2

Ligation reactions were set up consisting of 1µl 10x ligation buffer (300mM Tris-HCl (pH 7.8), 100mM $MgCl_2$, 100mM DTT, 10mM ATP), 1µl T4 DNA ligase, 1µl pBLCAT2 (~200ng), and 1µl double-stranded E-box oligonucleotide (~200ng) in a final volume of 10µl. Reactions were left for ~2 hours at room temperature and then transformed into competent DH5α (see section 2.2.9). Plasmids were then sequenced (see section 2.9.10).

2.2.12 TRANSFECTION OF CELLS

$1x10^6$ HSCs were seeded on 6 well plates in 2mls of DMEM containing serum and antibiotics. Cells were incubated at 37°C, 5% CO_2 and transfected with the appropriate plasmid when 40-80% confluent. Transfections were carried out using Qiagen's Effectene

Transfection kit. After transfection, cells were left for 48 hours in culture before harvesting.

2.2.13 CHLORAMPHENICOL ACETYL TRANSFERASE (CAT) ASSAY

Following transfection, HSCs were collected by centrifugation (1000rpm, 5mins) and washed twice in 1X PBS. The pellet was then resuspended in 50µl of 0.25M Tris buffer (pH 7.4). Protein was extracted by snap freezing the pellet in liquid nitrogen for 2mins, followed by heat shocking for 5mins at 37°C and vortexing for 10secs to break open the cell pellets. This cycle was repeated three times and the cytoplasmic extract was separated by centrifugation (13,000rpm, 5mins) and recovered into a fresh tube. 5µl of the cytoplasmic extract was removed for protein measurement as previously described (see section 2.2.2).

The CAT assays were performed by making up 25µg of protein to a volume of 50µl using 0.25M Tris. 70µl of 1M Tris, pH 7.4, 20µl of acetyl-CoA and 1µl of ^{14}C-chloramphenicol were then added. After mixing well, the samples were incubated for 2hrs at 37°C. 500µl of ethyl acetate was then added to each sample and the ethyl acetate phase was recovered by centrifugation (13,000rpm, 5mins). The solvent was then evaporated by drying in a vacuum centrifuge for 30mins and the residue redissolved in 15µl of ethyl acetate followed by spotting onto a TLC plate. Separation of the ^{14}C-chloramphenicol and its products was achieved by running the TLC plate in a chromatography tank containing 100ml chloroform:methanol (95:5) for ~10mins. The TLC plate was then visualised using either a phosphoimager screen or autoradiography. Quantification of the CAT assays was achieved by analysing the screens using a phosphoimager (STORM) and then using an Image Quantifier programme to calculate percentage conversions of ^{14}C-chloramphenicol to its acetylated products.

2.2.14 DETERMINATION OF TOXICITY EFFECTS OF CELL-SIGNALLING INHIBITORS IN RAT HSCS

Various concentrations of cell-signalling inhibitors were added to rat HSCs in culture and left for 24 hours. Cultures were then washed in 1x PBS to remove DMEM and any floating HSCs. Bio-Rad DC Protein Assays were then performed. 100µl reagent A and 800µl

reagent C were added directly to the HSCs adhered to plastic tissue culture dishes, mixed and left at room temperature for 15mins. Absorbances were measured against a standard at 750nm. Concentrations were determined using a standard curve (appendix 1).

CHAPTER 3

EXPRESSION OF MYOD DURING HEPATIC STELLATE CELL ACTIVATION

3.1 INTRODUCTION

As mentioned previously, the key player in liver fibrosis is the HSC which exists in normal liver as a quiescent vitamin A storing cell, however in response to liver injury (viral, alcohol, iron, etc) the cell undergoes a progressive phenotypic transformation towards a proliferating, αSMA positive myofibroblast-like cell. In fibrotic liver these activated HSCs are directly responsible for deposition of excess extracellular matrix molecules. Transcriptional control of HSC activation is currently poorly understood although there is growing evidence for a role for several transcription factors including members of the AP-1[333-337], NF-κB[162-166] and Sp1[335,338-340] families.

Studies in other cell types have shown how bHLH proteins are important in the control of cell growth and differentiation[341]. However, until recently, the myogenic factors were shown to be expressed exclusively in skeletal muscle, with no detectable expression in cardiac or smooth muscle, despite the fact that all three muscle cell types express many of the same muscle genes[342]. Subsequently, during investigation of the contractile properties of a HSC line, Mayer *et al* showed expression of MyoD and myogenin, using immunocytochemistry[343]. This research therefore shows evidence that myogenic factors are not just restricted to skeletal muscle, and that they may have important regulatory roles in other lineages. Because of the dramatic nature of the phenotypic switch during activation of HSCs, it would be interesting to further investigate the role of these proteins in primary HSCs and to see whether changes in bHLH activity are associated. The activity of the bHLH proteins depends upon the presence of a DNA sequence containing the core motif CANNTG (E-box)[344]. Such an E-box occurs on average every 256 base pairs in the genome. Studies have shown that the nucleotides that flank the E-box binding site play an important role in dictating the specificity and affinity with which dimeric pairs interact with DNA[345]. However, although flanking sequences have shown to play an important role in determining specificity of gene regulation by different protein pairs, transcriptional activity may also be determined by other factors, such as cellular concentrations of these proteins and their co-activators[346].

The HSC is an unusual cell that shares morphological and functional features of both smooth muscle and non-smooth muscle cells. The smooth muscle genes, desmin and αSMA, contain E-box elements and have been described in HSCs (see section 1.1.8). Desmin is an intermediate filament protein and in smooth muscle cells it is considered

to interconnect actin bundles and adhere to them in the plasma membrane thus enabling contractility[347]. Niki *et al* have shown that desmin is present in freshly isolated HSCs, increases its expression between day 2 and 6 in primary culture, and then shows a tendency to decrease very slightly. αSMA however is not present in freshly isolated HSCs but is expressed after a culture period of 5 to 7 days and is then continuously increased as the cells become more activated[348]. However, in addition to smooth muscle, the E-box element has been described in many other genes. For example, Halazonetis *et al* have described the presence of E-box sites within regulatory regions of numerous genes that are induced during cell growth[349].

This study will investigate the expression of bHLH proteins in HSCs using both *in vitro* (culture activation of rat and human cells) and *in vivo* (CCl_4 treatment of rats) models of HSC activation. Various techniques (e.g.Western blotting, Supershift EMSAs, Northern blotting and RT-PCR) will then be used to characterise these proteins. In addition, the effects of nucleotides flanking the E-box motif in maintaining specificity of bHLH protein binding will be investigated.

3.2 RESULTS

3.2.1 TIME-DEPENDENT CHANGES IN E-BOX DNA BINDING ACTIVITIES DURING CULTURE ACTIVATION OF RAT HSCS (*IN VITRO*)

To determine if activation of HSCs is associated with E-box binding activities, nuclear extracts were prepared from rat HSCs cultured for various time-points (0-14 days) on plastic and then used to detect E-box DNA binding using a double-stranded, labelled consensus E-box oligonucleotide in a standard EMSA protocol. All EMSAs in this chapter were performed using 10μg of protein and 0.4ng of probe in a 20μl reaction. Figure 3.1 shows a representative timecourse. Freshly isolated HSCs expressed a single high-mobility E-box binding activity (complex C) whereas culture activated HSCs lacked expression of complex C but instead expressed two low mobility complexes (A and B). Complex A was generally more abundant than complex B with the latter complex occasionally being barely visible. Additionally, when quiescent and activated (14 day) nuclear extracts were mixed together in equal amounts, complexes A and B were reduced while complex C reappeared. Nuclear extracts from quiescent cells may, therefore, contain an inhibitory factor that prevents assembly of complexes A and B.

3.2.2 E-BOX DNA BINDING ACTIVITIES AFTER CARBON TETRACHLORIDE INDUCED ACTIVATION OF RAT HSCS (*IN VIVO*)

To establish if similar E-box binding activities were produced *in vivo*, freshly isolated HSCs were purified from rats treated with CCl_4 for 48 hours. Nuclear extracts were harvested and E-box DNA binding was detected using EMSA. These cells were similar to culture activated HSCs (*in vitro*) i.e. they expressed two complexes with identical mobility to complexes A and B and lacked expression of complex C. In addition, some of the HSCs activated *in vivo* were plated out on plastic for 7 days. Complexes A and B appeared to be much stronger than those without CCl_4 treatment that were left for the same period of time (Figure 3.2).

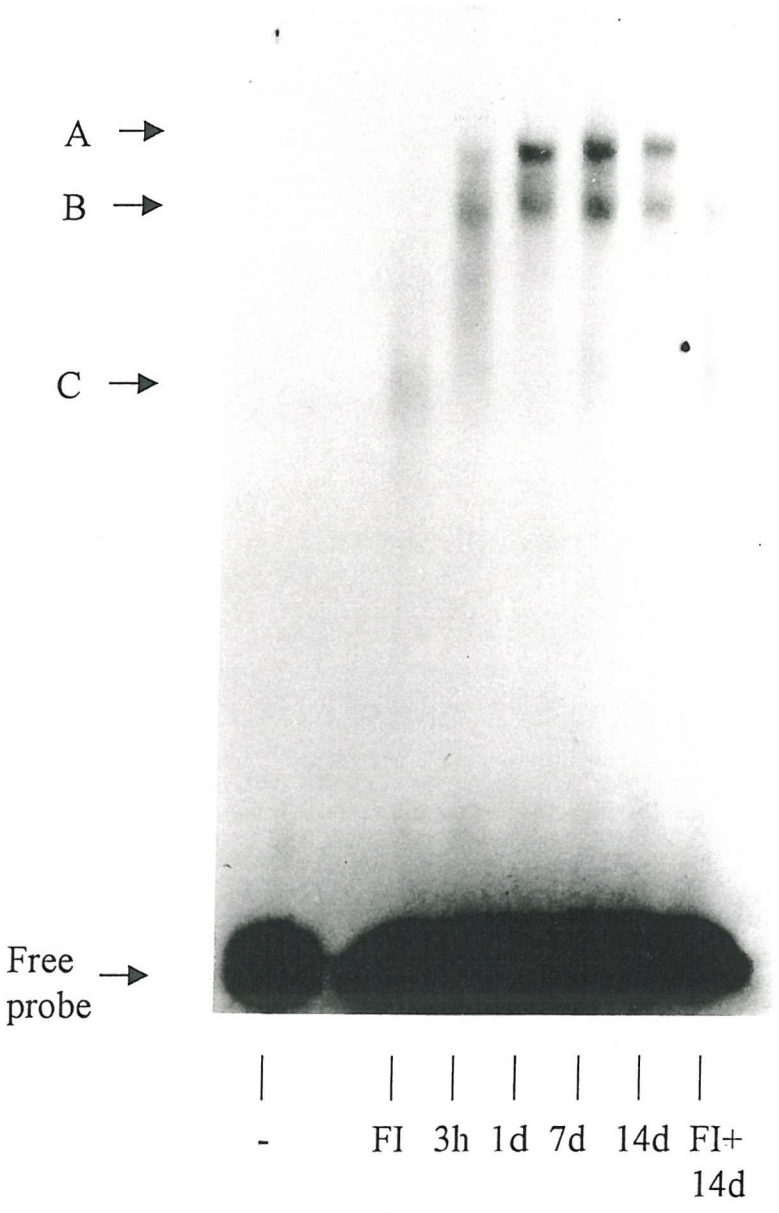


Figure 3.1 Expression of E-box binding proteins in the rat HSC during culture activation on plastic and effects of mixing freshly isolated (FI) and 14 day activated rat HSC nuclear extracts together in equal amounts. (-): Control reaction lacking nuclear extract.

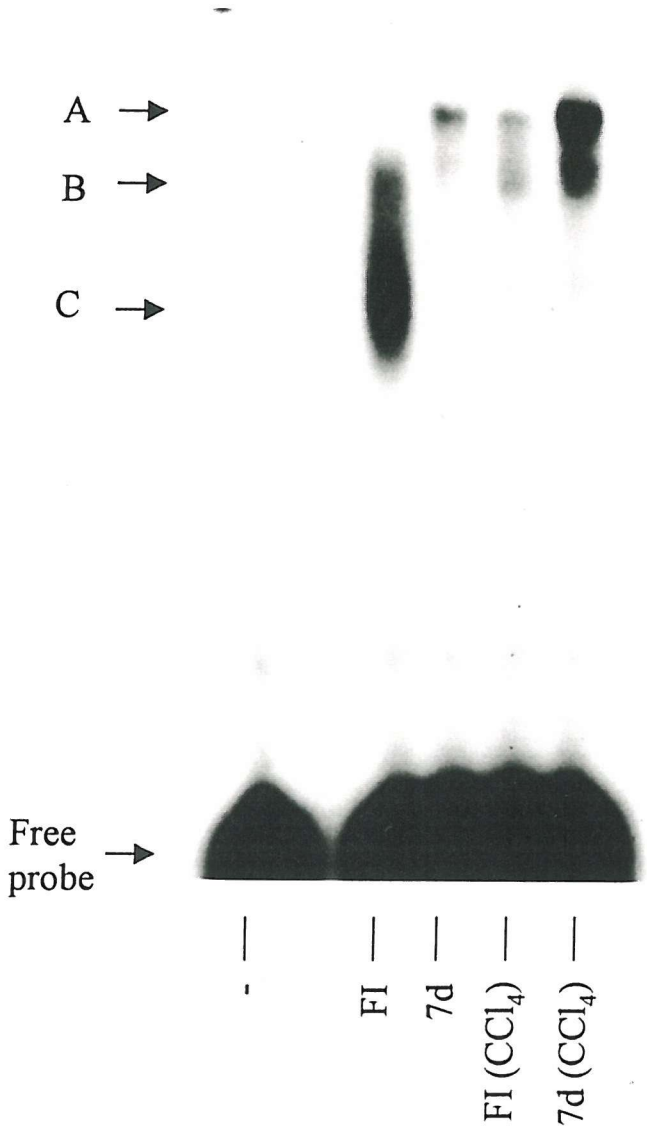


Figure 3.2 Comparison of E-box binding proteins in the rat HSC after CCl_4 treatment and from untreated control rats. (-): Control reaction lacking nuclear extract.

3.2.3 COMPARISON OF E-BOX DNA BINDING ACTIVITIES IN HUMAN AND RAT HSCS

HSCs were isolated from rat and human liver to determine if similar E-box complexes were present in the two cell types. Nuclear extracts were isolated from both freshly isolated HSCs and from those cultured on plastic for 8 days. E-box binding was then detected using the standard EMSA protocol. Results show similar patterns of expression i.e. freshly isolated human HSCs express a high mobility E-box complex whereas culture activated human HSCs express two low mobility E-box complexes (Figure 3.3).

3.2.4 CELL-TYPE SPECIFIC EXPRESSION OF E-BOX DNA BINDING PROTEINS

The E-Box DNA binding activities observed in activated rat HSCs were then compared with those in nuclear extracts purified from murine C2C12 (C2) myoblasts, monkey COS-1 kidney cells and human Jurkat T cells. E-box complexes with identical mobility to complexes A and B were seen in C2 and COS-1 cells. E-box binding appeared much stronger in C2 cells than in the other cell types. In addition, T cells formed a diffuse complex that was not seen in activated rat HSCs (Figure 3.4).

3.2.5 SPECIFICITY OF E-BOX BINDING PROTEINS

Competition assays showed that complexes A and B in activated rat HSCs were sequence specific as no competition was observed when cold double-stranded Sp1 oligonucleotide was used (Figure 3.5). Unlabelled double-stranded E-box oligonucleotides competed for both complexes completely at 80x excess. An 80x excess of cold E-box and Sp1 were then used to determine the specificity of complex C in freshly isolated rat HSCs. Results confirmed that this complex is also highly specific as no competition was observed with Sp1 (Figure 3.6).

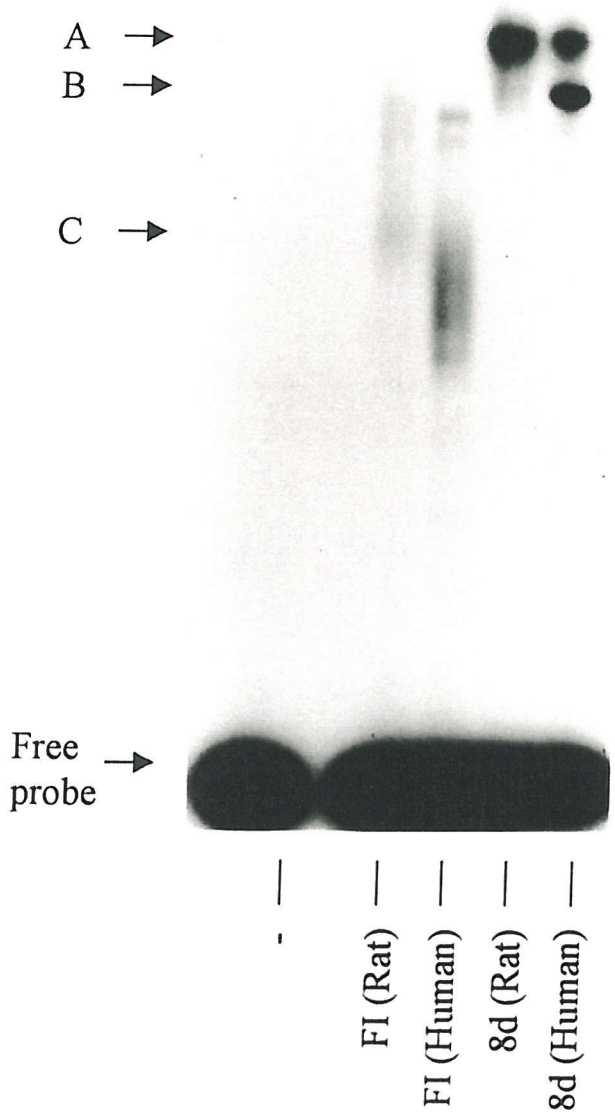


Figure 3.3 Expression of E-box binding proteins in rat and human HSCs. (-): Control reaction lacking nuclear extract.

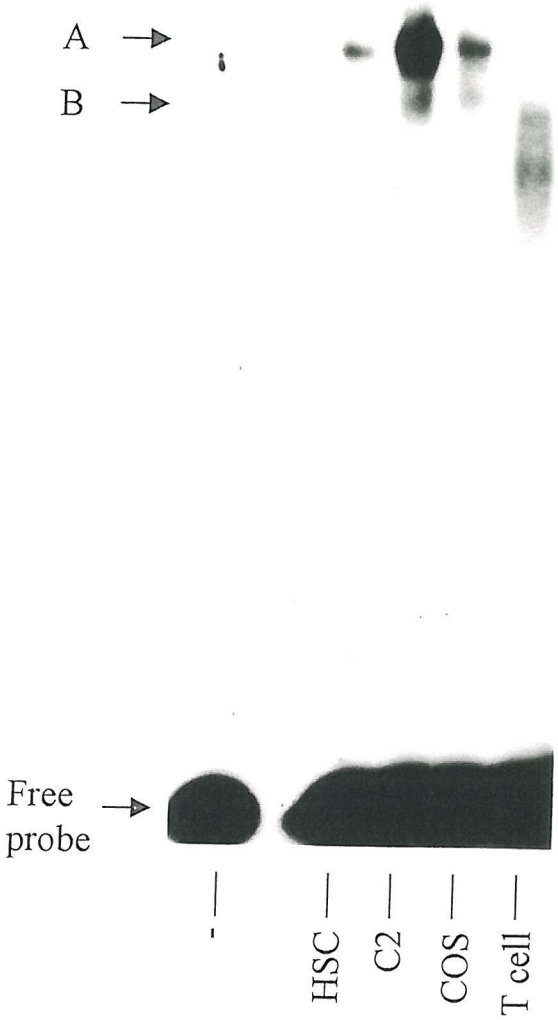


Figure 3.4 E-box binding complexes in different cell types using consensus E-box probe. (-): Control reaction lacking nuclear extract.

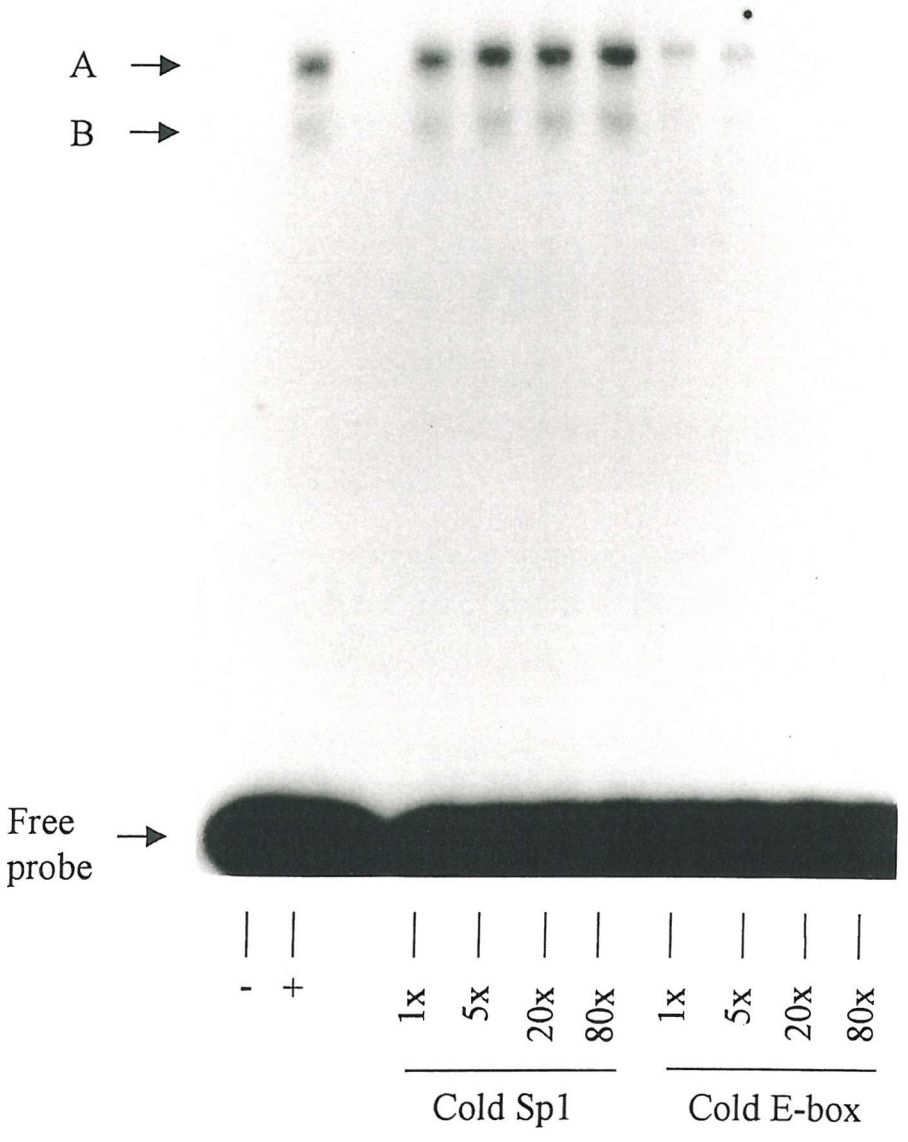


Figure 3.5 Competition assay using activated rat HSC nuclear extract with radiolabelled consensus E-box probe in the presence of increasing concentrations (1-80x excess) of unlabelled Sp1 and E-box oligonucleotides. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

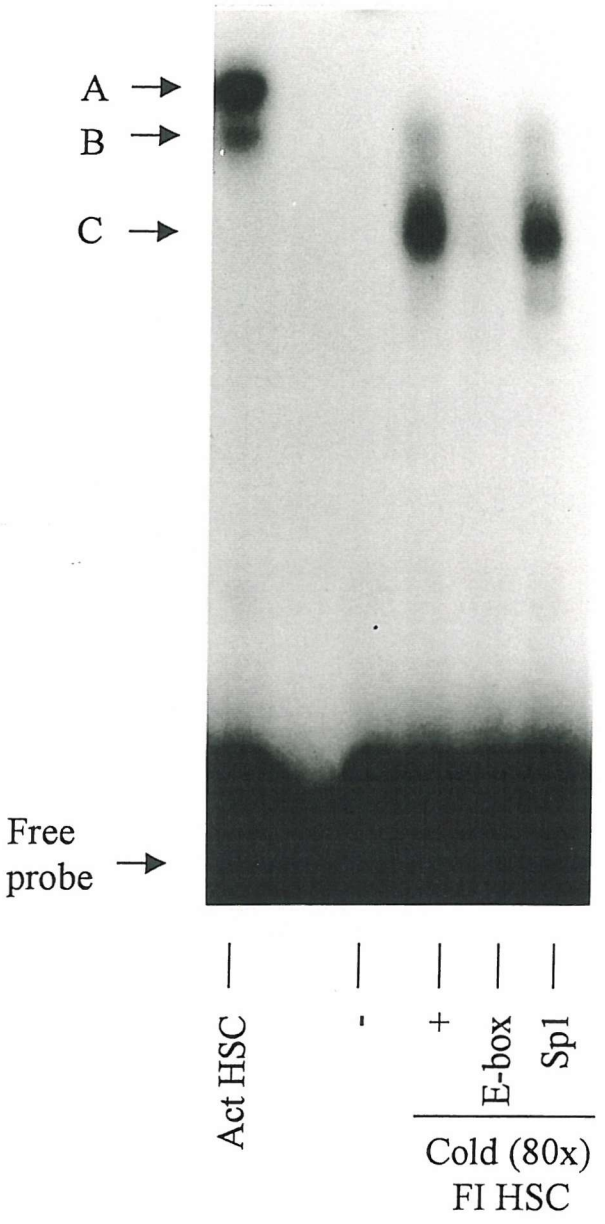


Figure 3.6 Competition assay using freshly isolated (FI) rat HSC nuclear extract with radiolabelled consensus E-box probe in the presence of (80x excess) unlabelled Sp1 and E-box oligonucleotides. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

3.2.6 ROLE OF SEQUENCES IN THE E-BOX AND FLANKING DNA

The EMSA data produced so far has been obtained using a consensus E-box oligonucleotide. It was, therefore, decided to investigate whether similar E-box complexes were produced using the E-box sequence from different genes. The E-box from the αSMA gene was used to compare binding in different cell types and with binding obtained using the consensus E-box. Binding to the αSMA E-box probe was very weak in activated rat HSCs and consisted of one low and one high mobility complex. However, binding appeared to be stronger in the other cell types (C2, COS-1 and Jurkat T cells) and multiple complexes were formed (Figure 3.7). αSMA E-box and consensus E-box have sequence differences in both the E-box (CANNTG) and the flanking DNA. Competition assays have shown that HSC E-box binding proteins to the consensus E-box probe require an intact E-box motif. For example, a mutant consensus oligonucleotide was made where the wild type consensus E-box motif 5'-CACGTG-3' was changed to 5'-CATCGC-3'. This mutant version failed to compete with wild type consensus E-box oligonucleotide. In addition, wild type and mutant versions of αSMA E-box oligonucleotides also failed to compete with the consensus E-box probe (Figure 3.8).

To investigate whether flanking DNA could influence E-box binding proteins in the HSC, it was decided to use the αSMA E-box motif (5'-CAGCTG-3') but to insert this into the consensus flanking ends rather than the αSMA genes flanking ends. This greatly enhanced E-box binding in the rat HSC producing very similar complexes as the wild type consensus E-box probe. In addition, E-box binding was determined using an E-box element from the muscle creatine kinase gene which also has sequence differences in the E-box and the flanking DNA compared to consensus and αSMA E-box probes. This probe revealed some very different complexes binding in the rat HSC (Figure 3.9). Competition assays were then performed in the presence of an excess of unlabelled wild type consensus, αSMA (with consensus flanking ends) and muscle creatine kinase E-box elements to see whether they competed with the radiolabelled wild type consensus E-box oligonucleotide. Results show that when the αSMA E-box motif is placed within the flanking ends of the consensus E-box probe, it could compete with the wild type consensus version. No competition was observed with the muscle creatine kinase E-box (Figure 3.10). These data thus show that flanking E-box DNA sequences are able to modulate recruitment of E-box binding proteins in the rat HSC.

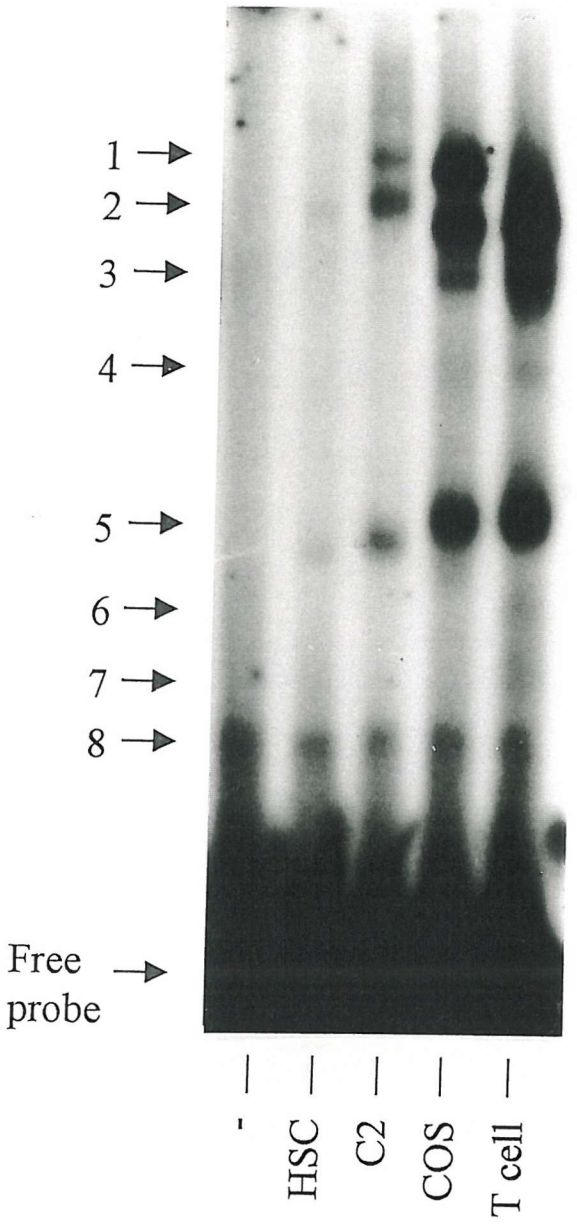


Figure 3.7 E-box binding complexes in different cell types using αSMA E-box probe. (-): Control reaction lacking nuclear extract.

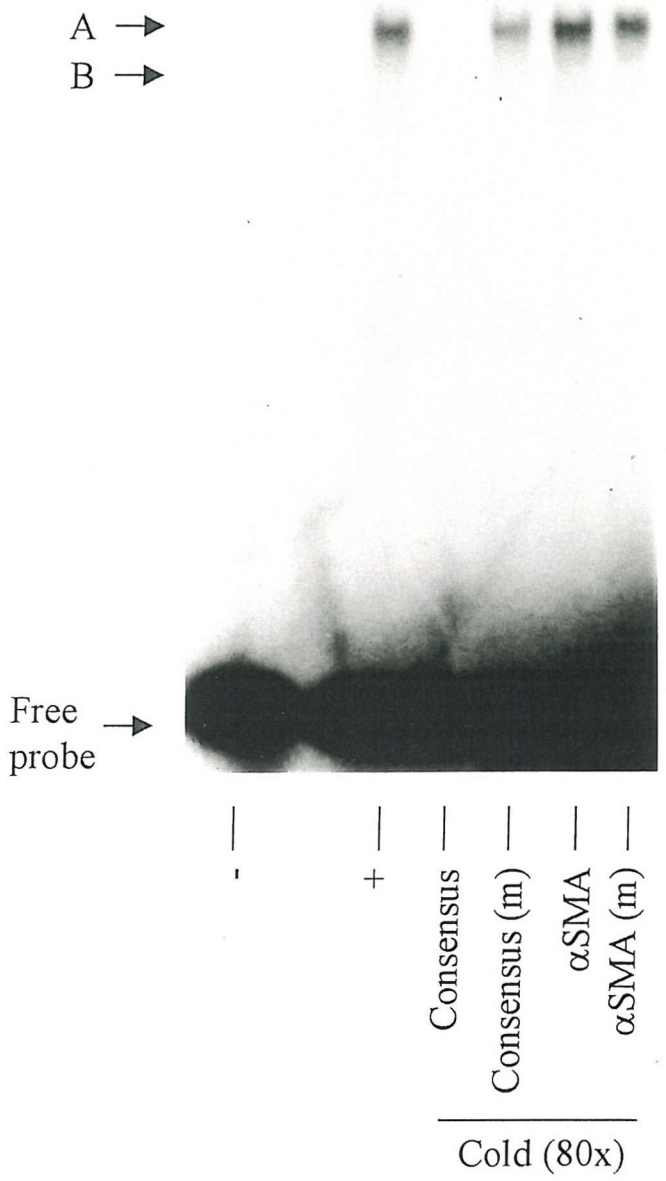


Figure 3.8 Competition assay using activated rat HSC nuclear extract with radiolabelled wild type consensus E-box probe in the presence of (80x excess) unlabelled wild type or mutant (m) versions of consensus and αSMA E-box oligonucleotides. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

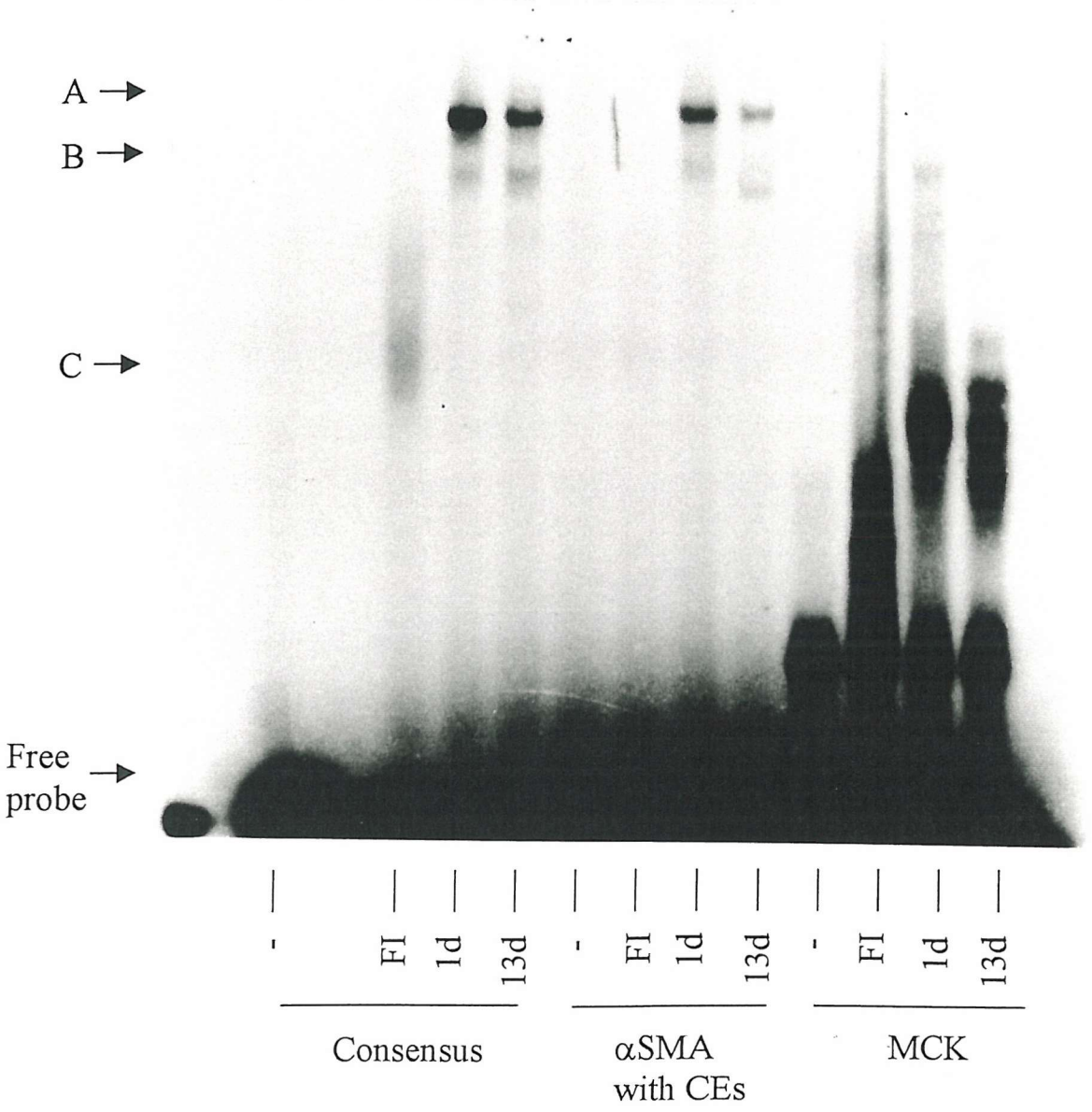


Figure 3.9 Effects of flanking DNA and use of E-box elements from different genes on E-box binding proteins in rat HSCs. (-): Control reaction lacking nuclear extract; (MCK): muscle creatine kinase; (CEs): Consensus flanking ends.

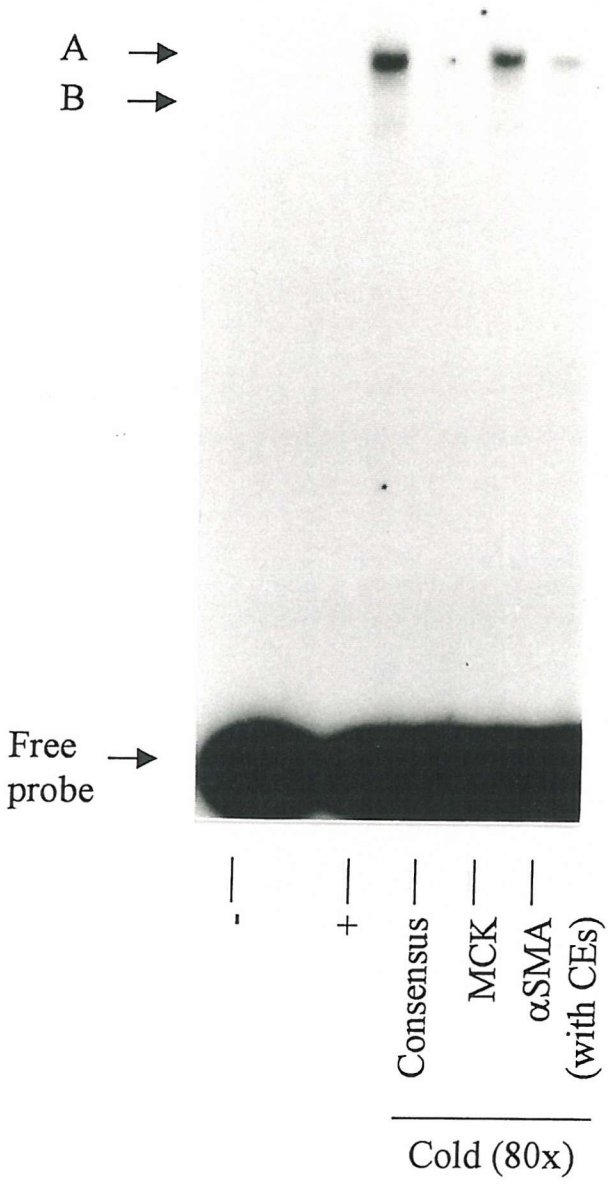


Figure 3.10 Competition assay using activated rat HSC nuclear extract with radiolabelled wild type consensus E-box probe in the presence of (80x excess) unlabelled wild type consensus, muscle creatine kinase (MCK) and αSMA with consensus flanking ends (CEs) E-box oligonucleotides. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

3.2.7 IDENTIFICATION OF THE E-BOX DNA BINDING COMPLEXES

Western blotting and supershift EMSA experiments were performed in order to identify the proteins in the E-box complexes of HSCs. Consensus E-box probe was used in all further EMSAs. For the supershifts, preformed E-box-DNA-protein complexes were incubated in the presence of antibodies (4μg/20μl EMSA reaction) recognising some of the classic bHLH proteins (E12, E47, MyoD and myogenin) and the bHLHZip proteins (c-Myc, Max and Mad1) (see figures 3.11 to 3.14). These experiments consistently showed a supershift with the Max Ab in freshly isolated cells. In addition, slight inhibition of complex C was observed with MyoD (M-318) antibody recognising full length MyoD (amino acids 1-318) and myogenin antibodies (Figure 3.11). In activated HSCs, the MyoD (M-318) antibody greatly inhibited assembly of complexes A and B (Figure 3.13) and also often re-induced a complex with similar mobility to complex C (see Figures 3.15 and 3.16). However, for reasons not yet determined, a second antibody MyoD (C-20) recognising the last 20 amino acids at the carboxy terminus of MyoD failed to either supershift, inhibit assembly of complexes A and B, or promote formation of complex C. Slight inhibition of complexes A and B was observed with the c-Myc antibody (Figure 3.13).

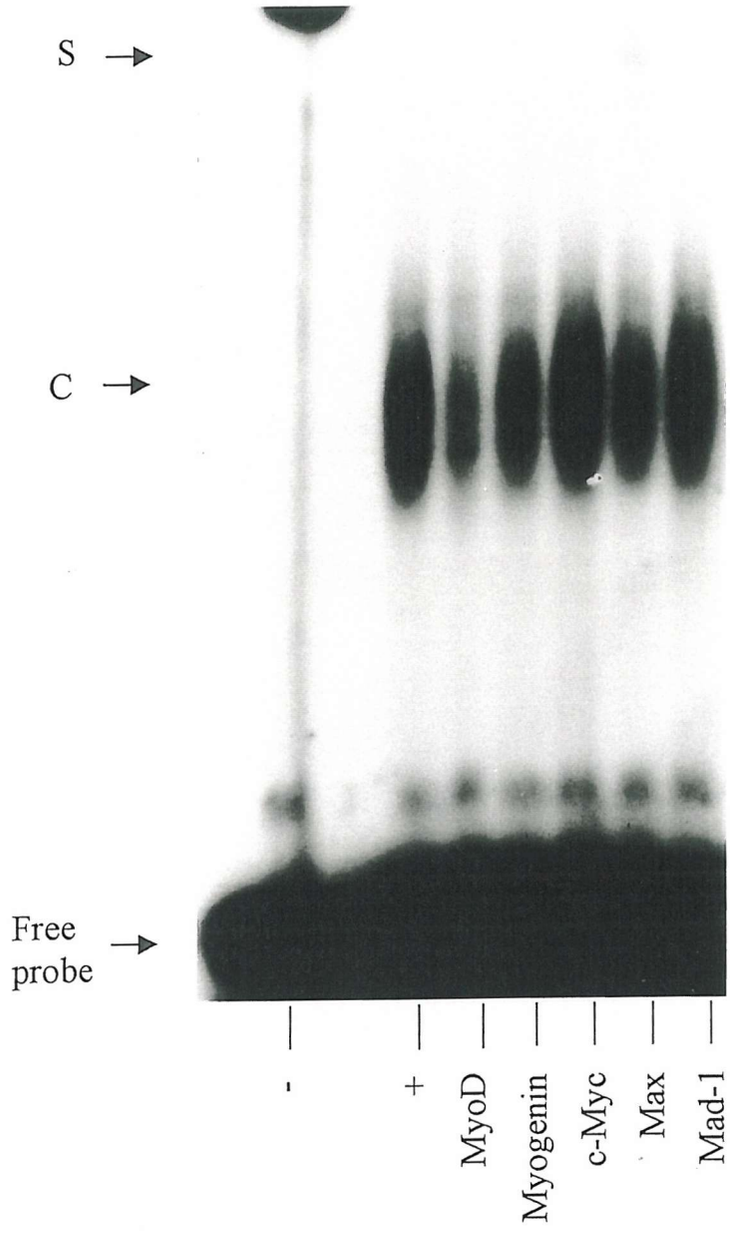


Figure 3.11 Supershift EMSA with FI rat HSC nuclear extracts using various bHLH and bHLHZip antibodies. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only; (S): Supershift.

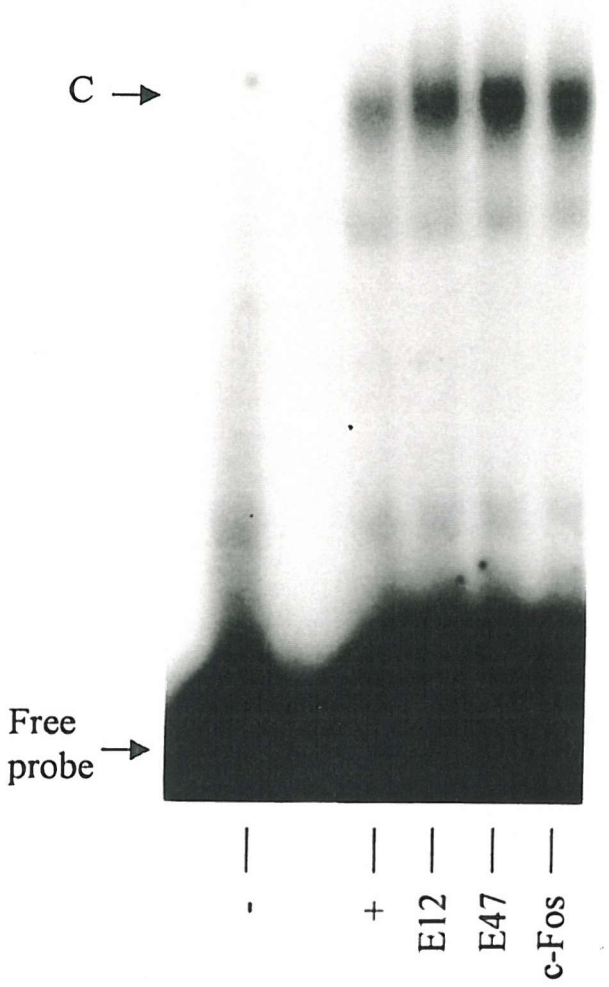


Figure 3.12 Supershift EMSA with freshly isolated rat HSC nuclear extracts using class *A* bHLH antibodies and c-Fos Ab as a control. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

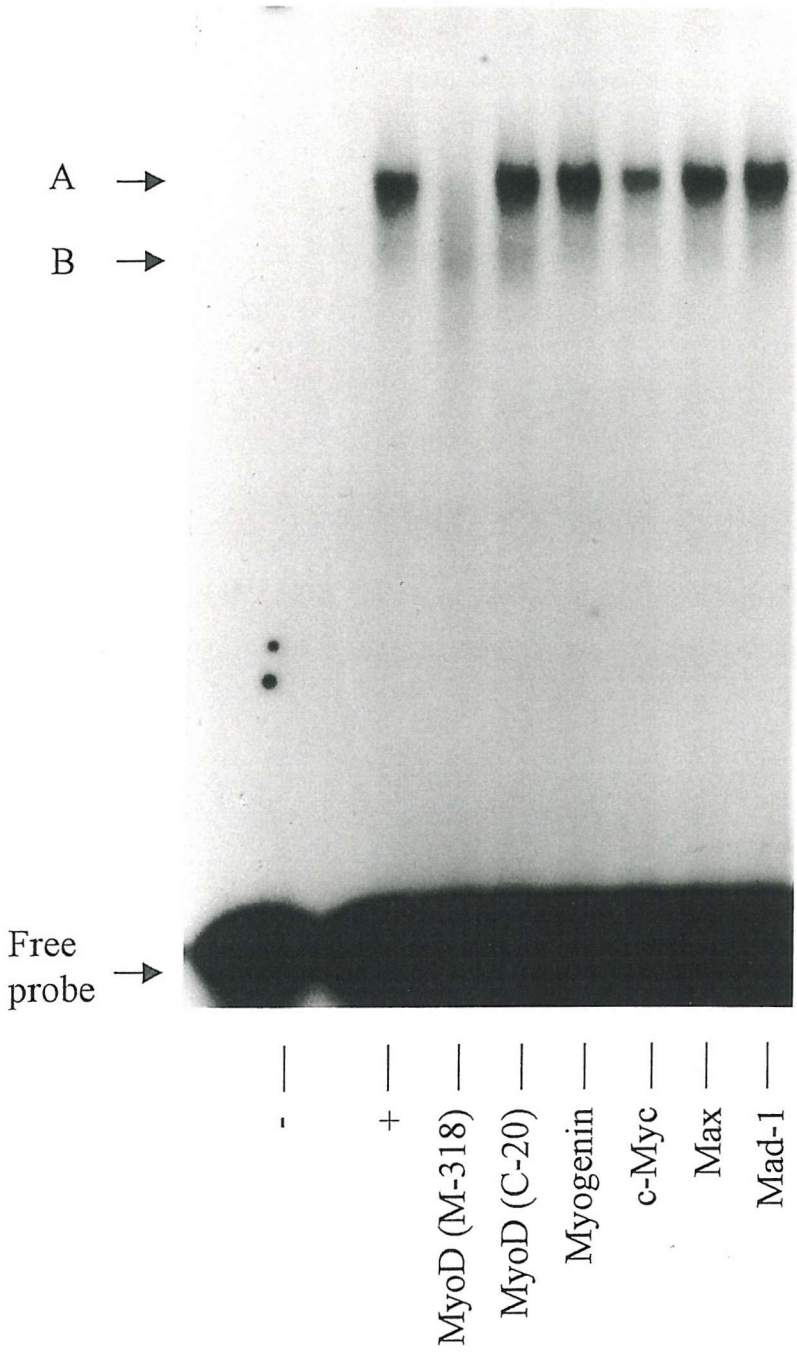


Figure 3.13 Supershift EMSA with activated rat HSC nuclear extracts using various bHLH and bHLHZip antibodies. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

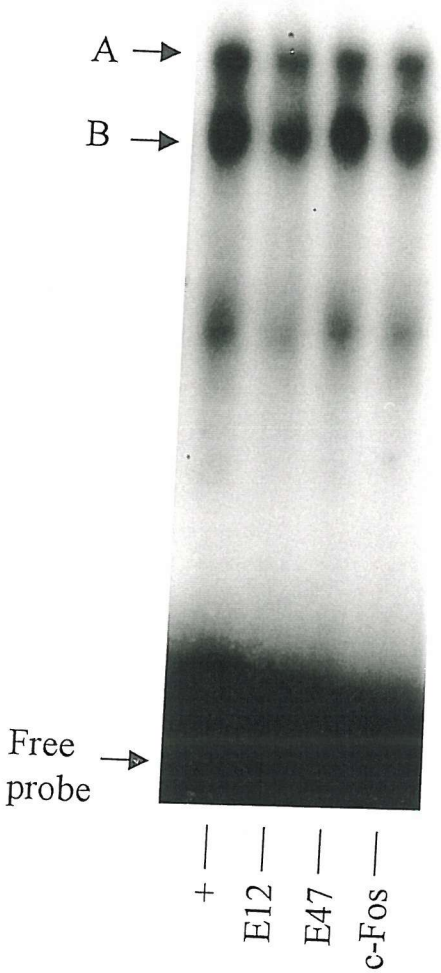


Figure 3.14 Supershift EMSA with activated rat HSC nuclear extracts using class A bHLH antibodies and c-Fos Ab as a control. (+): Control reaction with nuclear extract only.

3.2.8 EFFECTS OF MYOD (M-318) AND MYOD (C-20)

To further investigate the effects of MyoD (M-318) and MyoD (C-20) antibodies on the E-box complexes, supershift EMSAs were repeated using these two antibodies (4μg/20μl EMSA reaction) in rat HSC, C2, COS-1 and Jurkat T cells. Results show that MyoD (M-318) antibody inhibited assembly of complexes A and B in nuclear extracts from activated rat HSCs, C2 and COS-1 cells but had no effect in Jurkat T cells which formed a diffuse complex not observed in the other cell types as mentioned previously. Thus, these results show that activated rat HSCs, C2 and COS-1 cells have similar E-box binding complexes and that the effects were specific and not due to non-specific inhibition of E-box complexes. MyoD (C-20), however, had no effect on any of the cell types tested (Figures 3.15 and 3.16).

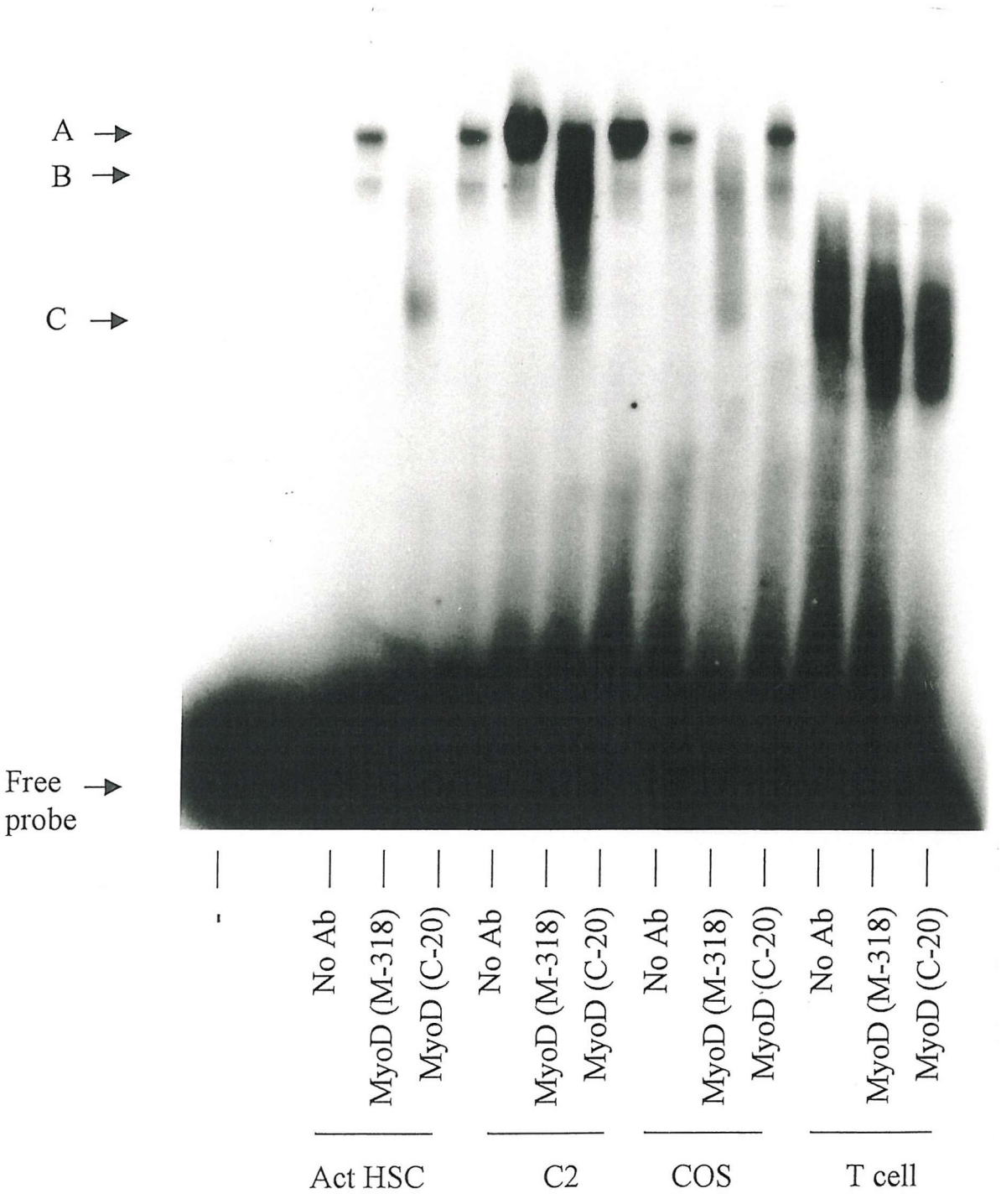


Figure 3.15 Supershift EMSA showing effects of MyoD (M-318) and MyoD (C-20) Ab in different cell types. (-): Control reaction lacking nuclear extract.

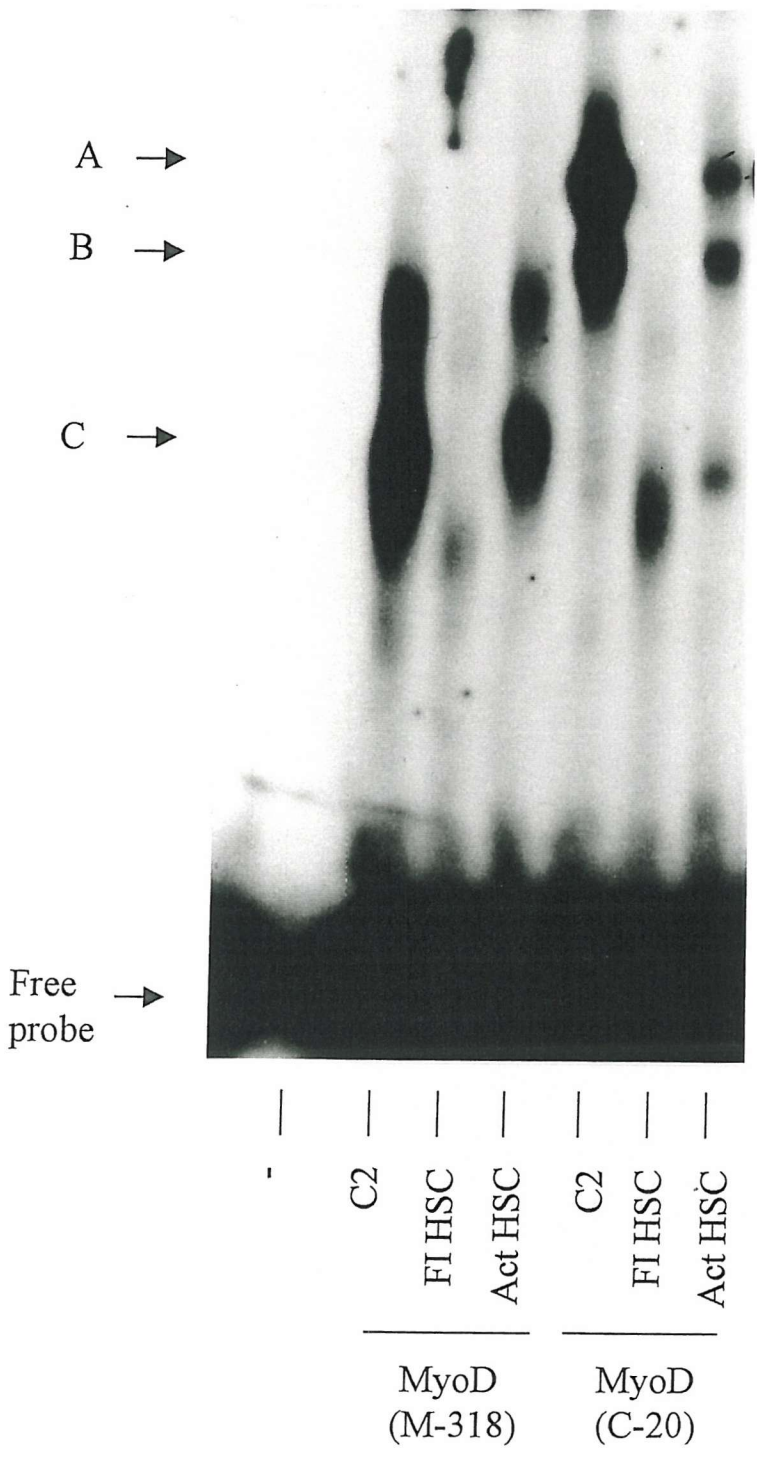


Figure 3.16 Supershift EMSA showing effects of MyoD (M-318) and MyoD (C-20) Abs in rat HSCs and C2 cells. (-): Control reaction lacking nuclear extract.

3.2.9 MYOD RNA AND PROTEIN EXPRESSION IN RAT HSCS

The inhibitory effects of MyoD (M-318) antibody on E-box binding activities in activated rat HSCs suggests that HSCs may express the myogenic transcription factor. Further analysis of MyoD protein expression in rat HSCs by Western blotting using the M-318 antibody confirmed expression of a 50kDa protein in agreement with previous studies[350]. MyoD expression was very weak in freshly isolated HSCs but significantly enhanced in activated (1 and 9 day) rat HSCs. A macrophage cell line RAW264.7 lacked detectable expression with MyoD (M-318) demonstrating cell-specific expression of MyoD (Figure 3.17). Northern blotting using a 49mer oligonucleotide representing the antisense of the first 49 coding nucleotides of the MyoD cDNA sequence[74] revealed a 2.1kb mRNA for MyoD in both freshly isolated and culture activated rat HSCs (Figure 3.18). Re-probing the filter with a 318bp fragment of rat β-actin cDNA (nucleotides 395 to 713 of β-actin cDNA sequence)[351] confirmed a roughly equal loading of RNA between samples.

RT-PCR primers were then designed that recognised a 174bp fragment of MyoD cDNA in the conserved bHLH domain (nucleotides 468 to 642 of MyoD cDNA sequence)[74]. RT-PCR was performed on activated rat HSC RNA and after titrating the Mg^{2+} to a final concentration of 1.0mM, a fragment of the expected size could be seen (Figure 3.19). Concentrations higher than 1.0mM Mg^{2+} increased the level of non-specific amplification. The fragment of DNA was then purified and cloned into pcDNA3 expression vector. Sequence analysis confirmed that the product had 100% sequence identity to the originally published sequence for rat MyoD (Figure 3.20).

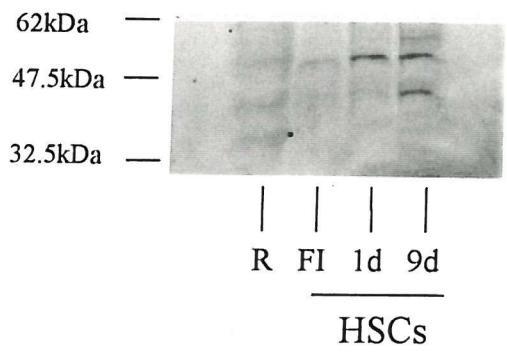


Figure 3.17 MyoD protein (50kDa) expression in rat HSCs and RAW264.7 (R) cells. Extracts (20μg) were electrophoresed through a 12% SDS-polyacrylamide gel.

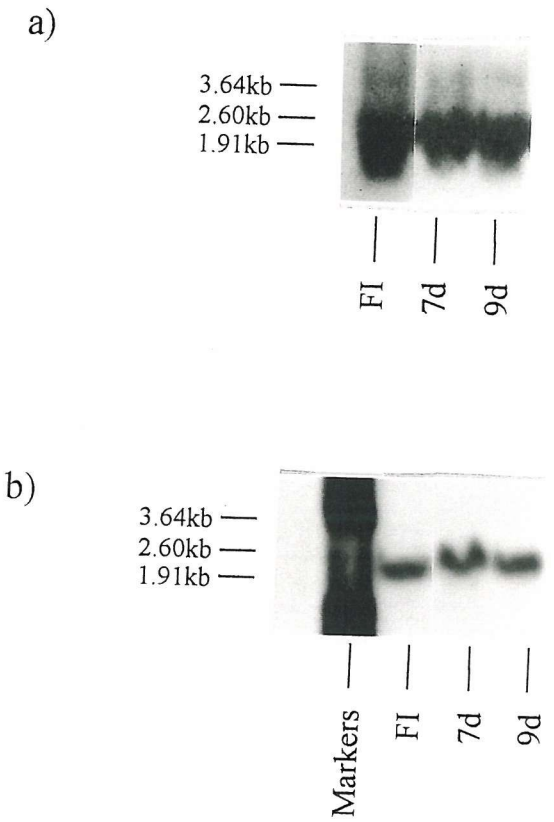


Figure 3.18 (a) MyoD mRNA (2.1kb) expression in rat HSCs. **(b)** Control showing β-actin mRNA (2.1kb) expression in rat HSCs.

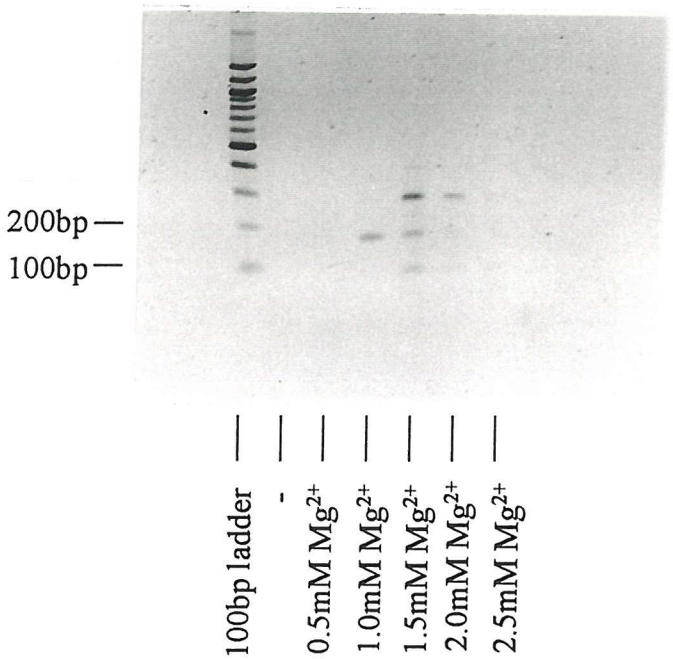


Figure 3.19 Titration of Mg^{2+} in RT-PCR using activated rat HSC RNA and MyoD RT-PCR primers. 174bp fragment can be seen with 1mM Mg^{2+}. (-): Control reaction lacking RNA.

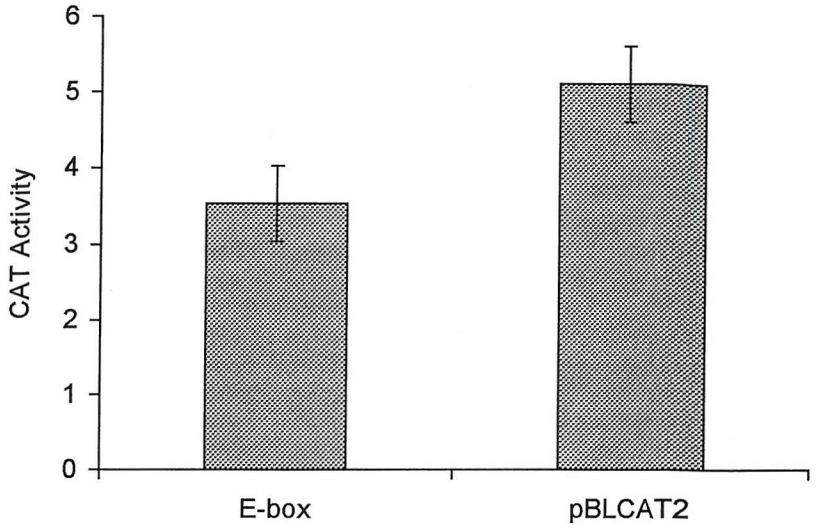
AAGACCACTA **ACGCTGATCG** CCGCAAGGCA GCCACCATGC 40
GTGAGCGGCG CCGCCTGAGC AAAGTGAACG AGGCCTTCCA 80
GACCCTTAAG CGCTGCACGT CCAGCAACCC GAACCAGCGG 120
CTACCCAAGG TGGAGATCCT GCGCAACGCC ATCC**GCTACA** 160
TTGAAGGTCT **GCAG** 174

Figure 3.20 Sequence of rat MyoD cloned by RT-PCR. Bold nucleotides are primer sequences.

3.2.10 E-BOX ACTIVITY IN ACTIVATED RAT HSCS

To determine the activity of the E-box element in HSCs, the consensus E-box oligonucleotide (5'-GACCACGTGGTC-3') was cloned into a CAT reporter plasmid, pBLCAT2 and then transfected into activated rat HSCs. After 48 hours of culture, the cells were harvested and CAT assays performed. Results demonstrated that compared to the minimal CAT activity of control pBLCAT2, the E-box element was not very active in activated rat HSCs (Figure 3.21a). CAT assays were also performed using pBLCAT2 cloned with three E-box motifs (i.e. 5'-GACCACGTGGTCGACCACGTGGTCGACCACGTGGTC-3'). Although Figure 3.21b demonstrated an increase in E-box CAT activity compared to control pBLCAT2, this was not a very reproducible effect.

a)



b)

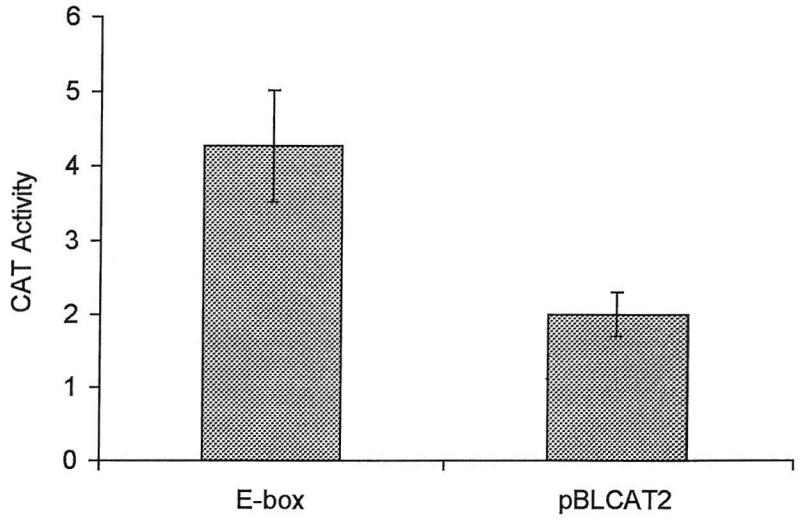


Figure 3.21 Comparison of E-box/pBLCAT2 activity to minimal activity of control pBLCAT2 in activated rat HSCs. Activities are presented as the mean of % conversion of chloramphenicol substrate to acetylated product. Error bars represent the standard error of the mean. **(a)** Consensus E-box oligonucleotide = 5'-GACCACGTGGTC-3'. **(b)** Consensus E-box oligonucleotide = 5'-GACCACGTGGTCGACCACGTGGTCGACCACGTGGTC-3'.

3.3 DISCUSSION

Analysis of the expression of E-box binding proteins in HSCs, using standard EMSA, demonstrated that both freshly isolated rat and human HSCs expressed a single high-mobility E-box binding activity (complex C). In contrast, culture activation of rat and human HSCs led to induction of two low mobility complexes (A and B) with loss of complex C. Furthermore, freshly isolated and culture activated HSCs purified from rats treated with CCl_4 revealed complexes with identical mobility to complexes A and B, and lacked expression of complex C. Thus, HSC activation *in vivo* produces similar effects on E-box DNA binding activities as *in vitro*. Specificity of all three complexes was confirmed using competition assay with specific and non-specific oligonucleotides. Competition was only observed with specific oligonucleotides. Because E-box DNA binding proteins can be either ubiquitous or cell-type specific, it was decided to further investigate the expression of these proteins in other cell types (murine C2 myoblasts, monkey COS-1 kidney cells and human Jurkat T cells). C2 and COS-1 cells had similar binding proteins to complexes A and B in the HSC. C2 cells are a transformed cell line of skeletal mouse muscle and hence results suggest that factors similar to myogenic bHLH factors may be involved in the control of HSC activation. Expression of similar complexes in COS-1 cells was initially unexpected. However these cells are a SV40 transformed monkey kidney fibroblast-like cell[352] and may be similar to activated kidney mesangial cells that have previously been reported to express the myogenic transcription factor, MyoD[343]. Alternatively, expression of similar E-box binding proteins in COS-1 cells may be due to the transformed phenotype of the cell. Different E-box binding activities were observed in Jurkat T cells compared to the E-box binding complexes assembled in the other cell-types suggesting that the complexes are cell-type specific.

To determine the identity of the proteins in the E-box complexes of HSCs, supershift EMSAs were performed on both freshly isolated and culture activated rat HSC nuclear extracts. These studies have consistently demonstrated the involvement of the bHLH leucine zipper protein, Max in freshly isolated HSCs. Antibodies against two partners of Max, Mad1 and c-Myc, had no effect on the E-box binding complexes. c-Myc expression is generally induced when quiescent cells are stimulated by mitogenic agents and repressed with addition of differentiation agents. In contrast Mad1 is induced upon differentiation

(see section 1.3.3), thus Max may bind E-box sites in the quiescent HSC as a transcriptionally inactive homodimer. In addition, slight inhibition of complex C was observed with anti-MyoD (M-318) antibody recognising full-length MyoD and with anti-myogenin antibodies. However, antibodies against their class A partners, E12 and E47, had no effect suggesting that in freshly isolated HSCs, low levels of MyoD and myogenin may bind to E-box oligonucleotides as homodimers or possibly as heterodimers with, as yet, unidentified partners.

In contrast, anti-MyoD (M-318) antibody was shown to dramatically inhibit assembly of complexes A and B in activated HSCs while sometimes also re-inducing an E-box DNA binding activity with similar mobility to complex C. A similar effect was also observed when nuclear extracts from freshly isolated cells were mixed 1:1 with extracts from activated HSCs. These data indicate that factors controlling the activity of class B bHLH proteins may regulate E-box binding activities during HSC activation. However, the MyoD (C-20) antibody recognising the carboxy terminus of MyoD had no effect on the E-box binding complexes. Considering this antibody only recognises 20 amino acids, it may be possible that when bound to E-box, MyoD is in such a conformation that prevents MyoD (C-20) antibody from recognising it. In addition, there may be other transcription factors bound to E-box that might form part of complexes A and B that could possibly mask the carboxy domain of MyoD. Another possibility for lack of recognition could be that the transcription factor recognised by MyoD (M-318) in complexes A and B is not MyoD but is very similar. However, the antibodies used in these experiments were claimed by the manufacturer's (Santa Cruz Biotech, Inc) to be specific and non cross-reactive with other muscle-specific transcription factors such as myogenin, Myf-5 and MRF-4. To date, the expression and function of MyoD has mainly been described in cells of the myogenic lineage. These preliminary findings suggest that MyoD may also be expressed in activated rat HSCs and indicate a wider role for this transcription factor outside of the muscle cell system. Further studies with the MyoD (M-318) antibody showed a similar inhibition of complexes A and B in C2 and COS-1 cells but not in T cells. The lack of inhibition with T cells therefore confirms that the inhibition effects observed with MyoD (M-318) antibody are specific. Alternatively, the MyoD (M-318) antibody could potentially have protease activity which may explain the effects on E-box DNA binding complexes described above. Furthermore, the Western blots with MyoD (M-318) antibody gave more than one band.

Additional studies should be performed to determine if this is the case, for example, one suitable control would be to repeat the EMSA but to add the MyoD (M-318) antibody to an NF-κB shift instead. However, the MyoD (M-318) antibody had no effect on the E-box DNA binding complexes observed in T cells suggesting that protease activity is probably not the reason for the observed effects on E-box DNA binding complexes in HSCs, C2 and COS-1 cells.

Apart from those indicated, all EMSAs were obtained using an E-box oligonucleotide (referred to as 'consensus E-box') designed by Halazonetis and Kandil[349]. This oligonucleotide has an optimum c-Myc DNA-binding site that is palindromic, GACCACGTGGTC, and is present within regulatory regions of genes that are expressed during cell growth. Furthermore, Solomon *et al* demonstrated the importance of the nucleotides that flank the DNA core sequence. For example, they have observed that the presence of a 5'-T or a 3'-A (e.g. TCACGTGA) inhibits DNA binding of the c-Myc/Max heterodimer, but not that of the Max/Max homodimer[345]. Preliminary studies suggested that c-Myc was expressed in activated rat HSCs and hence to further investigate this, the oligonucleotide described above was chosen for the EMSA experiments. However, supershift EMSAs using antibodies against c-Myc revealed only a very slight inhibitory effect on complexes A and B in activated rat HSCs unlike the dramatic inhibitory effect observed with MyoD (M-318) antibody. Max homodimer expression in freshly isolated HSCs may shift to c-Myc/Max heterodimer expression in culture activated HSCs due to the presence of mitogenic agents in the culture media and subsequent induction of c-Myc. This would assume, however, that Max is expressed in activated rat HSCs. The precise reason for lack of significant binding of c-Myc/Max heterodimers would require further investigative research. If Max is expressed in activated HSCs, there may be competition between MyoD and c-Myc/Max heterodimers at the E-box element although the E-box oligonucleotide was optimised for c-Myc binding. Alternatively, c-Myc and Max may be refrained from forming heterodimers by regulatory constraints. For example, Bahram *et al* have shown that c-Myc is negatively regulated by mitogen-induced post-translational modification[353].

To further determine how MyoD may bind to the E-box oligonucleotide in activated rat HSCs, supershift EMSAs were performed using antibodies against two of its possible partners, E12 and E47. No effects were seen on complexes A and B suggesting that MyoD may bind as a homodimer or possibly as a heterodimer with an, as yet, unidentified partner. Chu *et al* have shown that MyoD homodimers can bind to the promoter of the cyclin B1 gene and repress its transcription in proliferating myoblasts[117]. Cyclin B1 and its associated kinase are necessary for entry into and progression through mitosis[354]. MyoD may play a similar role in the HSC, for example, it may transcriptionally repress genes necessary for proliferation. This would be an excellent regulatory system for controlling levels of cell division so that they do not get out of control. This may then explain why HSC tumours do not occur. Alternatively, MyoD may just simply bind to E-box elements but neither transactivate or repress the genes. It may then prevent other transcription factors (e.g. c-Myc/Max) from binding and thus still play a role in controlling gene expression. In accordance with this idea, CAT assay results have demonstrated generally quite low activity with the consensus E-box element in activated rat HSCs. However, low activity may be due to technical reasons. For example, an oligonucleotide expressing more E-box elements may be required or, alternatively, the E-box motif may even work in combination with another, as yet, unidentified element. In addition to the potential control of cell-cycle regulatory genes, MyoD may be able to control the expression of smooth muscle genes expressed in the HSC. For example, desmin has E-box elements in its promoter and is expressed in the rat HSC (see section 3.1). MyoD may be able to control levels of this gene during HSC activation possibly analogous to those described above. Further research would be necessary to investigate these potential avenues.

To determine whether the E-box binding proteins in the HSC were specific for the consensus E-box probe, further experiments were performed with other E-box expressing genes. Initially, E-box binding was investigated using the E-box from the αSMA gene (region between –228 and –206 of the αSMA promoter sequence[146]). αSMA is the most abundant protein in fully differentiated SMCs[146]. However, there was very little E-box binding to this probe in the HSC. Results demonstrated very weak expression of a high and a low mobility complex. Competition assays demonstrated that αSMA oligonucleotides failed to compete with the consensus E-box probe thus inferring that the two E-box

sequences bind different bHLH proteins. Lee *et al* have performed similar experiments in the HSC and detected some binding to the αSMA E-box and suggested that the complexes may be due to the transcription factor, c-Myb[162]. Binding in the other cell types (C2, COS-1 and T cells) appeared much stronger and consisted of three or more complexes.

Possible reasons for low binding to αSMA E-box in the HSC:

Shimizu *et al*[355] have shown that a construct containing the region from –1 to –125 of the αSMA promoter (p125CAT) had high transcriptional activity in SMCs (57-fold > promoterless). Mutation of either of two highly conserved CC(AT-rich)$_6$GG (CArG) motifs at –62 and –112 abolished the activity of p125CAT in these cells. In skeletal myotubes, however, high transcriptional activity of αSMA is only observed when at least 271 base pairs of the promoter are used (-1 to ≥ -271). CArG boxes are important for transcription of αSMA in both SMCs and skeletal myotubes but in contrast to SMCs, activity of the CArG boxes in skeletal myotubes was dependent upon inclusion of the promoter region between –271 and –208. This region contains two E-boxes at –252 and -214. Thus, E-boxes may be important in the regulation of αSMA in skeletal muscle but not in smooth muscle[146]. This may account for low αSMA E-box binding in the HSC (SMC-like) and high E-box binding in the C2 cell (skeletal muscle). Although mature skeletal muscle cells do not express αSMA *in vivo*, the gene is transiently expressed in immature skeletal cells during development *in vivo*[356] and constitutively expressed in cultured skeletal muscle cells upon differentiation into myotubes[357].

It has been previously reported that MyoD preferentially binds the E-box sequence, 5'-CAGCTG-3' where Leu-122 was thought to contact the central base pairs[358]. Blackwell *et al* have shown that if Leu-122 is mutated to an arginine residue, then the preferred binding site is changed to 5'-CACGTG-3'. This would suggest that MyoD should preferentially bind to the αSMA oligonucleotide and contrasts with the above findings that show very little αSMA E-box binding yet abundant consensus E-box binding. However, data on the crystal structure of the MyoD bHLH domain-DNA complex has now revealed that there is no direct contact between MyoD (including Leu-122) and the two central base pairs[102]. In addition, data obtained from the selected and amplified binding sites (SAAB) assay have shown that MyoD homodimers prefer palindromic sequences containing E-box motifs with

purines on the 5' flanking side and pyrimidines on the 3' flanking side. Although not exactly the same, the consensus E-box oligonucleotide does follow this basic rule and contains 5' purine rich and 3' pyrimidine rich flanking DNA, and in addition the oligonucleotide is palindromic. This may explain why MyoD can efficiently bind to an oligonucleotide that was previously optimised for c-Myc binding. In contrast, the αSMA E-box oligonucleotide has purine rich flanking sequences on either side of the E-box motif, and is not palindromic, and may therefore explain lack of MyoD binding to this site.

However, when the αSMA E-box motif (5'-CAGCTG-3') was inserted into the consensus flanking ends rather than its own flanking ends, E-box binding in the HSC was greatly enhanced producing very similar complexes as the consensus oligonucleotide. Competition assays revealed that αSMA (consensus flanking ends) E-box DNA competed with the consensus version (unlike the wild-type) suggesting that the two sequences were binding similar proteins. In addition, competition assays using a mutant version of the consensus E-box motif but with normal flanking ends failed to compete with wild-type consensus E-box showing that the E-box binding proteins are also binding to the E-box motif and not just the flanking ends. Thus, the flanking ends of the E-box element provide an additional form of transcriptional regulation. Further studies were then conducted using the E-box element from the muscle creatine kinase gene. This E-box element produced some very different complexes and provides further evidence that different E-box binding proteins bind to different E-box genes. In addition, lack of competition between the E-box from the muscle creatine kinase gene and consensus E-box also confirmed that different proteins bind to the two E-box genes.

Northern and Western blotting revealed MyoD RNA and protein respectively in both freshly isolated and activated HSCs. However, MyoD protein expression was significantly enhanced in activated cells (unlike unchanged RNA levels) suggesting MyoD expression may be regulated by a post-transcriptional mechanism. Very little binding of MyoD to the E-box element was observed in freshly isolated cells and possible factors regulating this process are discussed in chapter 4. These regulatory factors have been shown to decrease in activated HSCs and hence ample MyoD: E-box binding is observed. Various studies have now shown that MyoD bound to E-box containing DNA is less susceptible to

ubiquitin-mediated degradation[359]. Thus if there are regulatory factors bound to MyoD in freshly isolated cells preventing binding to E-box, then MyoD will be more susceptible to this degradative pathway. This may explain the increase in MyoD protein expression observed in activated HSCs. Alternatively, an increased rate of synthesis and/or a decreased rate of proteolytic degradation could regulate an elevation of MyoD protein expression. RT-PCR cloning subsequently confirmed expression of MyoD in activated rat HSCs.

CHAPTER 4

REGULATION OF MYOD DURING HEPATIC STELLATE CELL ACTIVATION

4.1 INTRODUCTION

Results in the previous chapter revealed expression of the myogenic transcription factor, MyoD, in HSCs. Increased binding of this factor to the E-box element was observed as the HSCs became activated both *in vitro* and *in vivo*. Furthermore, Western blotting revealed enhanced MyoD protein expression in activated rat HSCs compared to relatively low expression in freshly isolated HSCs. Possible reasons for these observations were then discussed. Several factors have been described as important regulators of MyoD (see section 1.3.8). The following study will therefore further elucidate MyoD regulation in the HSC. Initially, the regulatory factors that prevent MyoD from binding to the E-box element in freshly isolated HSCs will be investigated. Further studies to analyse the cell-signalling pathways involved in MyoD protein induction during HSC activation will then be described.

4.2 RESULTS

4.2.1 REGULATION OF BHLH PROTEINS IN RAT HSCS BY ID PROTEINS

Activity of MyoD and its related bHLH family proteins can be negatively regulated by the Id family of HLH proteins (see section 1.3.9). To investigate whether the Id proteins may play a role in the regulation of E-box binding proteins in the HSC, Northern blot analysis was used initially to see whether the HSC expresses the Id proteins. A full length cDNA for Id1 was provided as a kind gift from Dr John Norton, Manchester. Results confirmed that Id1 mRNA (1.1 kb) was expressed in freshly isolated HSCs and down-regulated, but still at detectable levels, in activated cells (Figure 4.1a). The Northern blot filters were then re-probed with β-actin to ensure equal loading of RNA (Figure 4.1b).

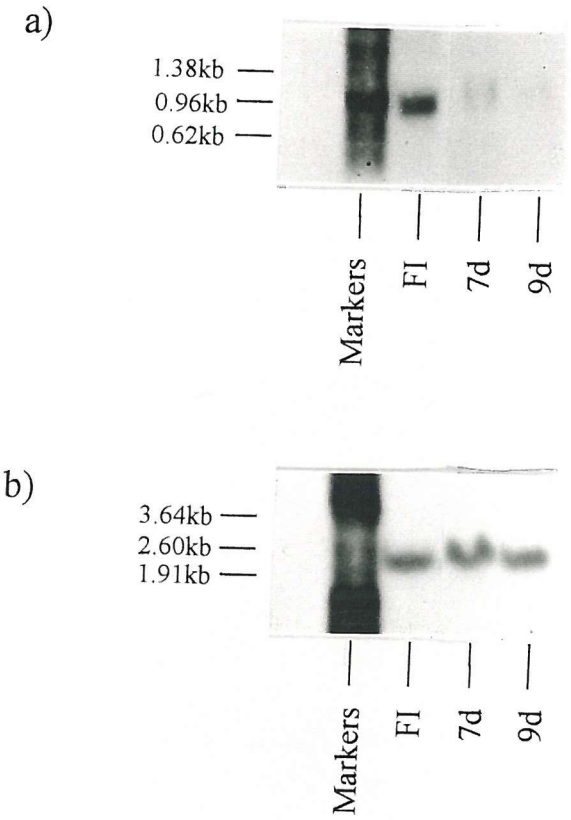


Figure 4.1 (a) Id1 mRNA (1.1kb) expression in rat HSCs using Northern analysis. **(b)** Control showing β-actin mRNA (2.1kb) expression in rat HSCs.

4.2.2 INVESTIGATION OF CELL SIGNALLING PATHWAYS THAT MAY CONTROL THE EXPRESSION OF BHLH PROTEINS IN RAT HSCS

To determine the effects of second messenger inhibitors on E-box binding proteins in the HSC, a range of inhibitors were added to freshly isolated rat HSCs in culture, left for 24 hours and then harvested. Nuclear extracts were made and run on a standard EMSA using consensus E-box probe. All EMSAs in this chapter were performed using 10μg of protein and 0.4ng of probe in a 20μl reaction. Staurosporine[360] (general protein kinase inhibitor) dose-dependently inhibited complexes A and B. Almost complete inhibition could be seen at final concentrations of 1μM and 2μM. Staurosporine can inhibit protein kinases C, A, and G. More selective inhibitors of these three kinases were therefore used. However, BIM[361] (selective PKC inhibitor), KT5720[362] (selective PKA inhibitor) and KT5823[362] (selective PKG inhibitor) had no effects on the E-box complexes. Other kinase inhibitors were then used: Herbimycin A[363] (tyrosine kinase inhibitor), PD98059[364] (selectively blocks the activity of MAP kinase kinase by inhibiting the activation of MAP kinase and subsequent phosphorylation of MAP kinase substrates) and Wortmannin[365] (PI 3-kinase inhibitor). Herbimycin A and PD98059 had no significant inhibitory effects, however Wortmannin had a slight inhibitory effect on complexes A and B at final concentrations of 1μM and 2μM (Figures 4.2-4.4). Thus Staurosporine and Wortmannin sensitive kinases may be involved in the assembly of complexes A and B in activated rat HSCs.

Interestingly, the NF-κB inhibitors: Gliotoxin[366], Calpain Inhibitor[367] (cell-permeable inhibitor of proteasome-mediated degradation of IκB-α) and Pyrrolidine Dithiocarbamate[368] (PDTC; metal chelator and oxygen free radical scavenger) all caused virtually complete inhibition of complexes A and B, suggesting that NF-κB plays a role in the induction of E-box binding proteins in the rat HSC (Figure 4.5).

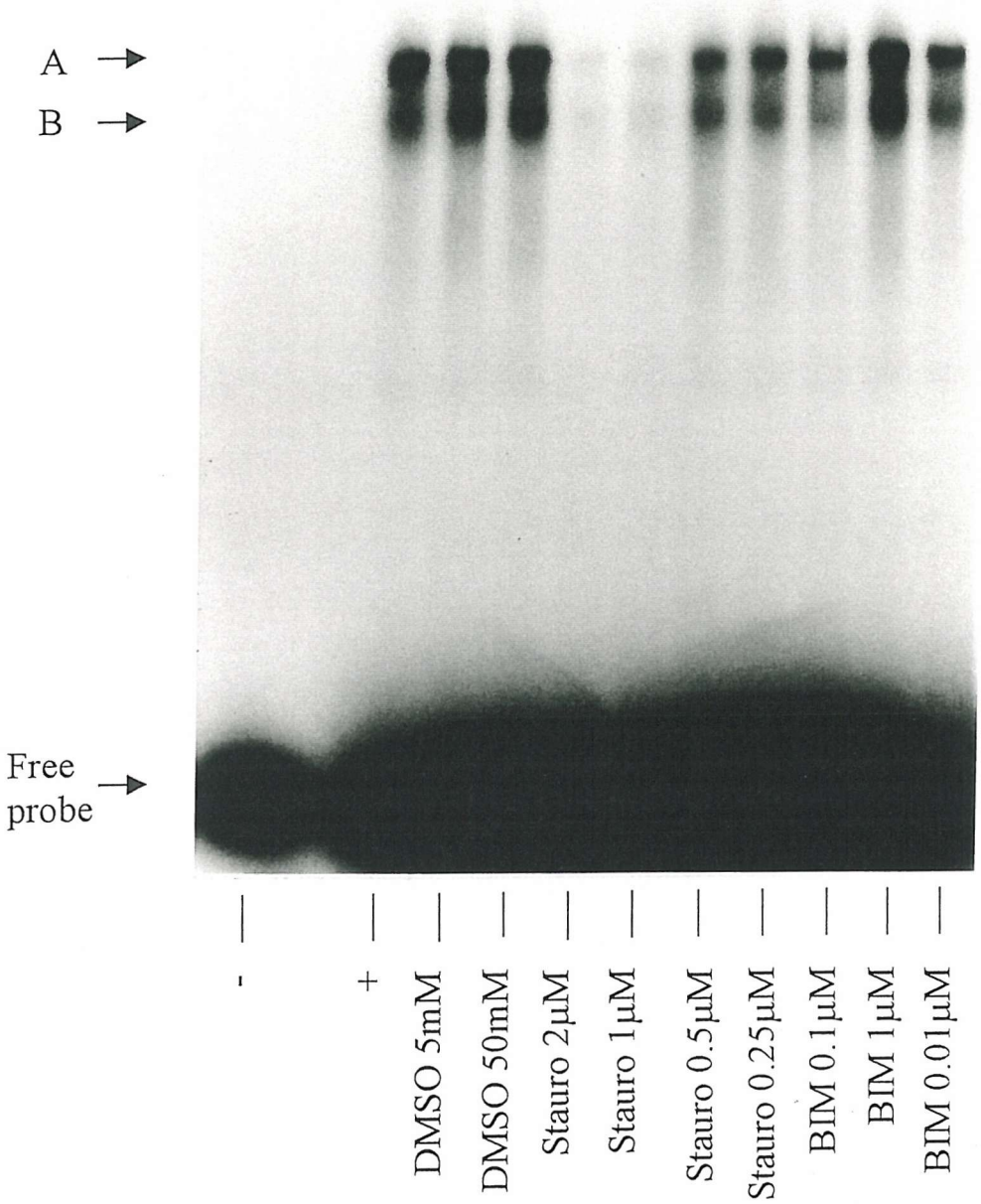


Figure 4.2 Effects of various protein kinase inhibitors on E-box binding proteins in rat HSCs. (Stauro): Staurosporine; (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

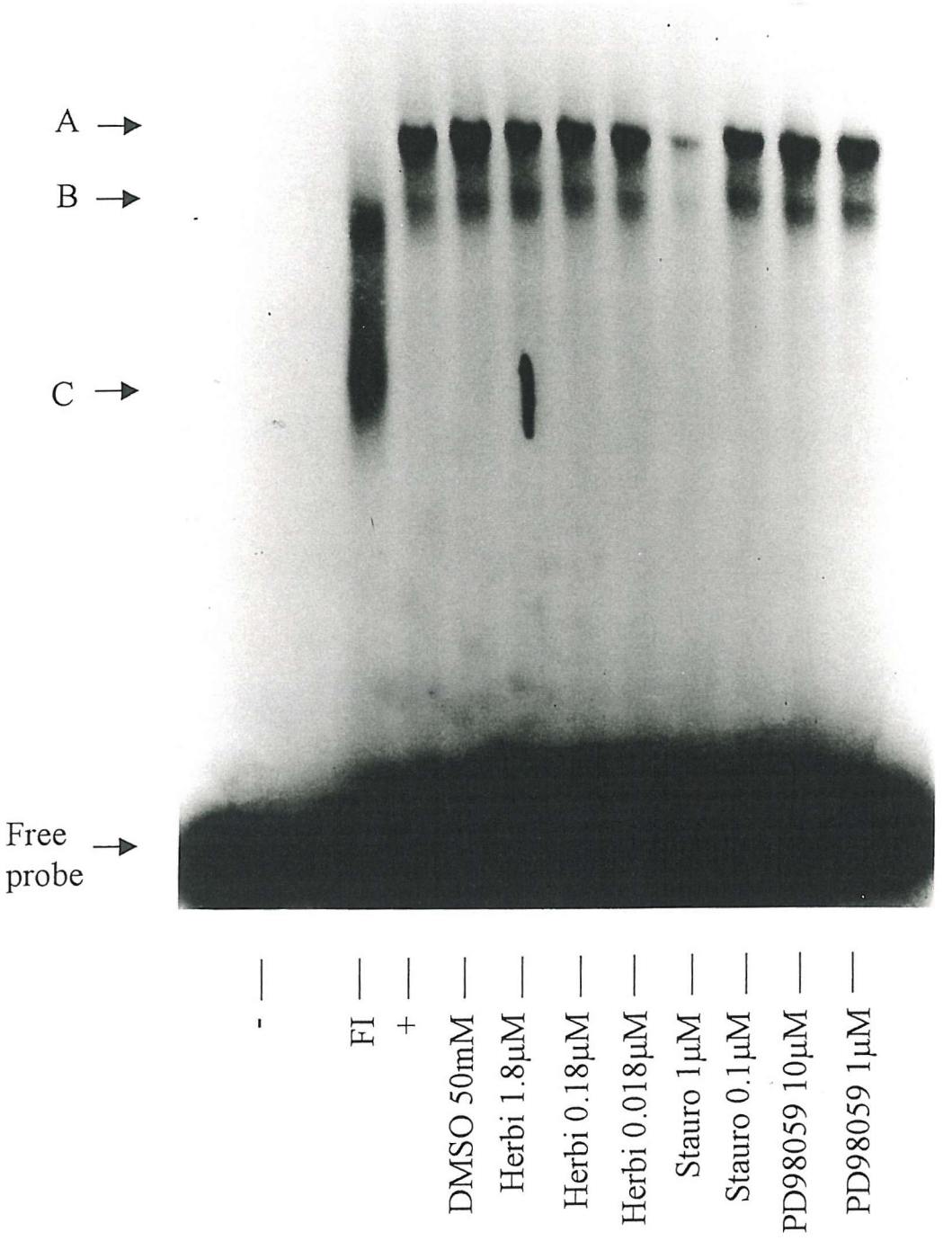


Figure 4.3 Effects of various protein kinase inhibitors on E-box binding proteins in rat HSCs. (Herbi): Herbimycin; (Stauro): Staurosporine; (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

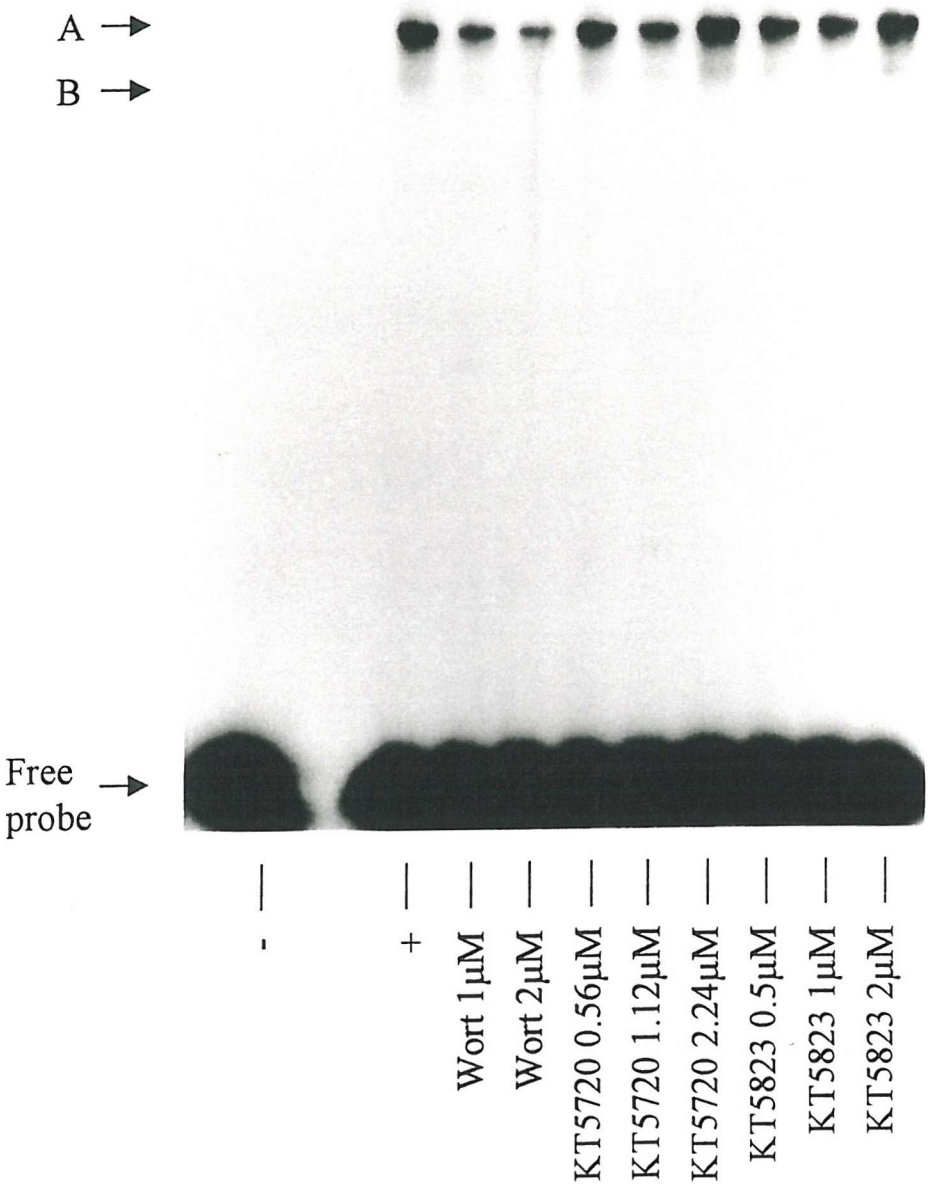


Figure 4.4 Effects of various protein kinase inhibitors on E-box binding proteins in rat HSCs. (Wort): Wortmannin; (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

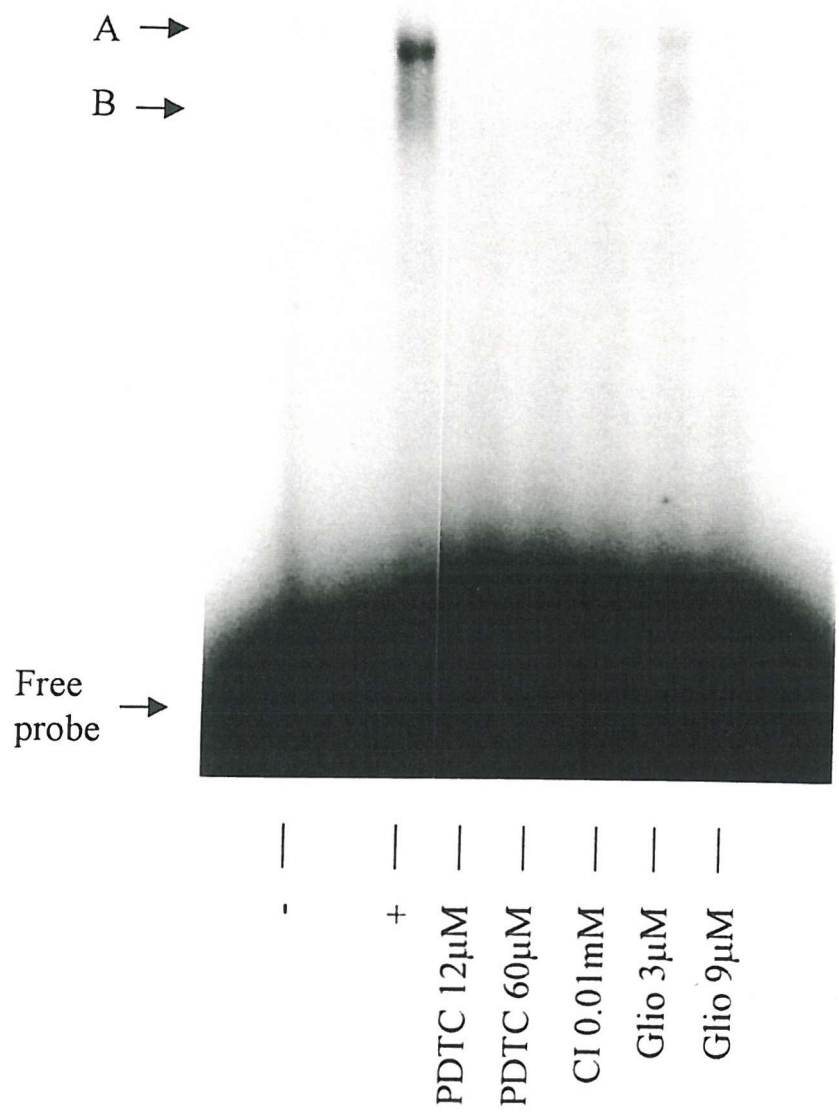


Figure 4.5 Effects of various NF-κB inhibitors on E-box binding proteins in rat HSCs. (CI): Calpain Inhibitor; (Glio): Gliotoxin; (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

4.2.3 TOXICITY EFFECTS OF CELL-SIGNALLING INHIBITORS IN RAT HSCS

Staurosporine (1μM to 2μM), PDTC (12μM to 60μM), Calpain Inhibitor (0.01mM to 0.1mM) and Gliotoxin (3μM to 9μM) all had quite dramatic inhibitory effects on complexes A and B in the rat HSC. To ensure that the downregulation of these complexes was due to inhibition of the various cell-signalling pathways already mentioned and not due to general toxicity to the HSC, it was decided to investigate the effects of these inhibitors further. The inhibitors were added to rat HSCs in culture and left for 24 hours as previously described. Protein assays were then performed. Initially, inhibitor concentrations were used that had already shown inhibitory effects. Inhibitors were dissolved and made up to relevant concentrations using DMSO. Results show that DMSO (50mM) was without significant toxicity to the HSC. Calpain Inhibitor (0.01mM) produced ~40% decrease in protein compared to untreated controls. Staurosporine (1μM), PDTC (12μM), and Gliotoxin (6μM) appeared much more toxic to the rat HSC producing ~70%, 80%, and 90% decrease in protein respectively (Figure 4.6).

These experiments were then repeated but the inhibitor concentrations were titrated. This time, Calpain Inhibitor (0.1-6μM) was much less toxic and protein levels were similar to controls. 1μM Calpain Inhibitor appeared to increase protein levels but this was not a consistent effect compared to other toxicity experiments. PDTC (1-9μM) and Staurosporine (600-900nM) produced ~60% decrease in protein thus slightly less toxic than the previous experiment. These inhibitors showed little variation as their concentrations increased. Gliotoxin, however, showed a dose-dependent decrease in toxicity. For example, a final concentration of 100nM produced ~20% decrease in protein whereas a concentration of 3μM produced ~65% decrease in protein levels compared to untreated control rat HSCs (Figure 4.7).

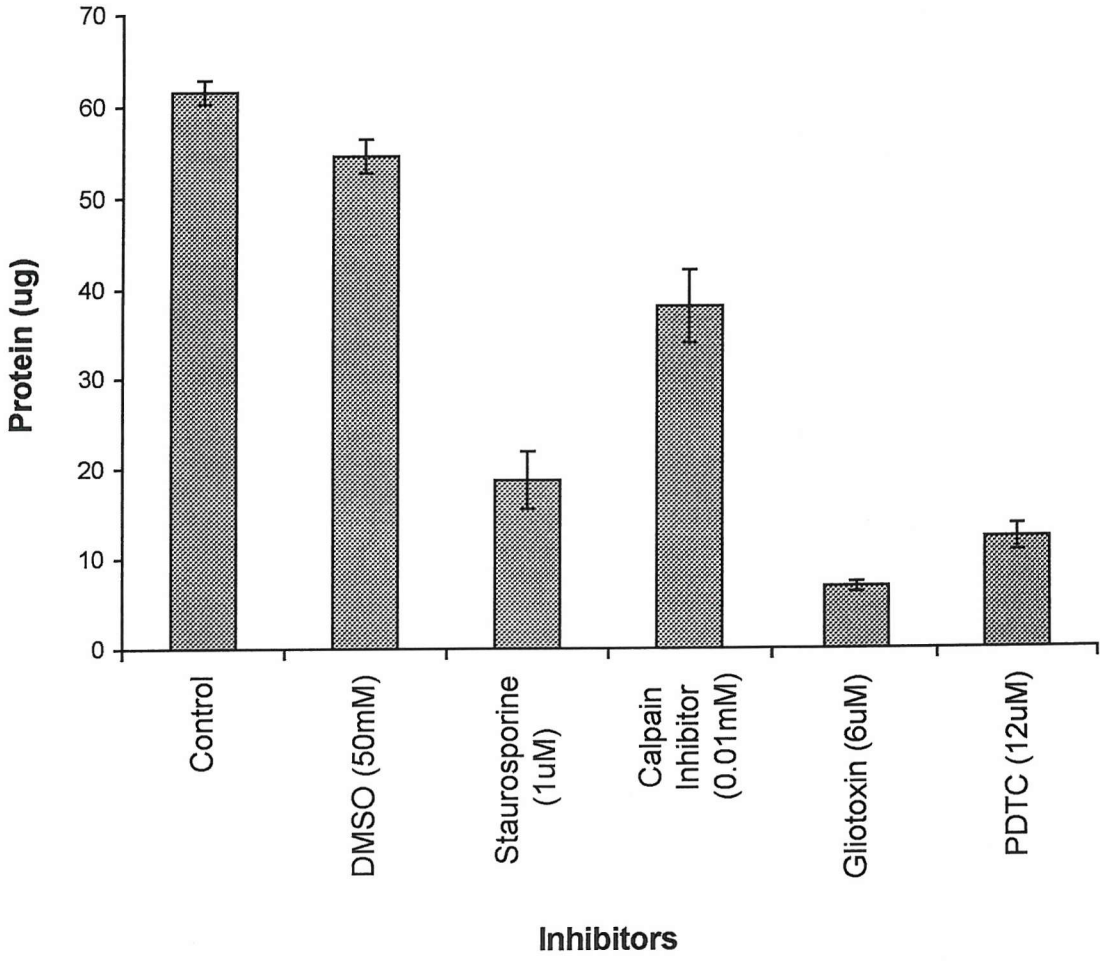


Figure 4.6 Toxicity effects of various cell-signalling inhibitors in rat HSCs. Error bars represent the standard error of the mean.

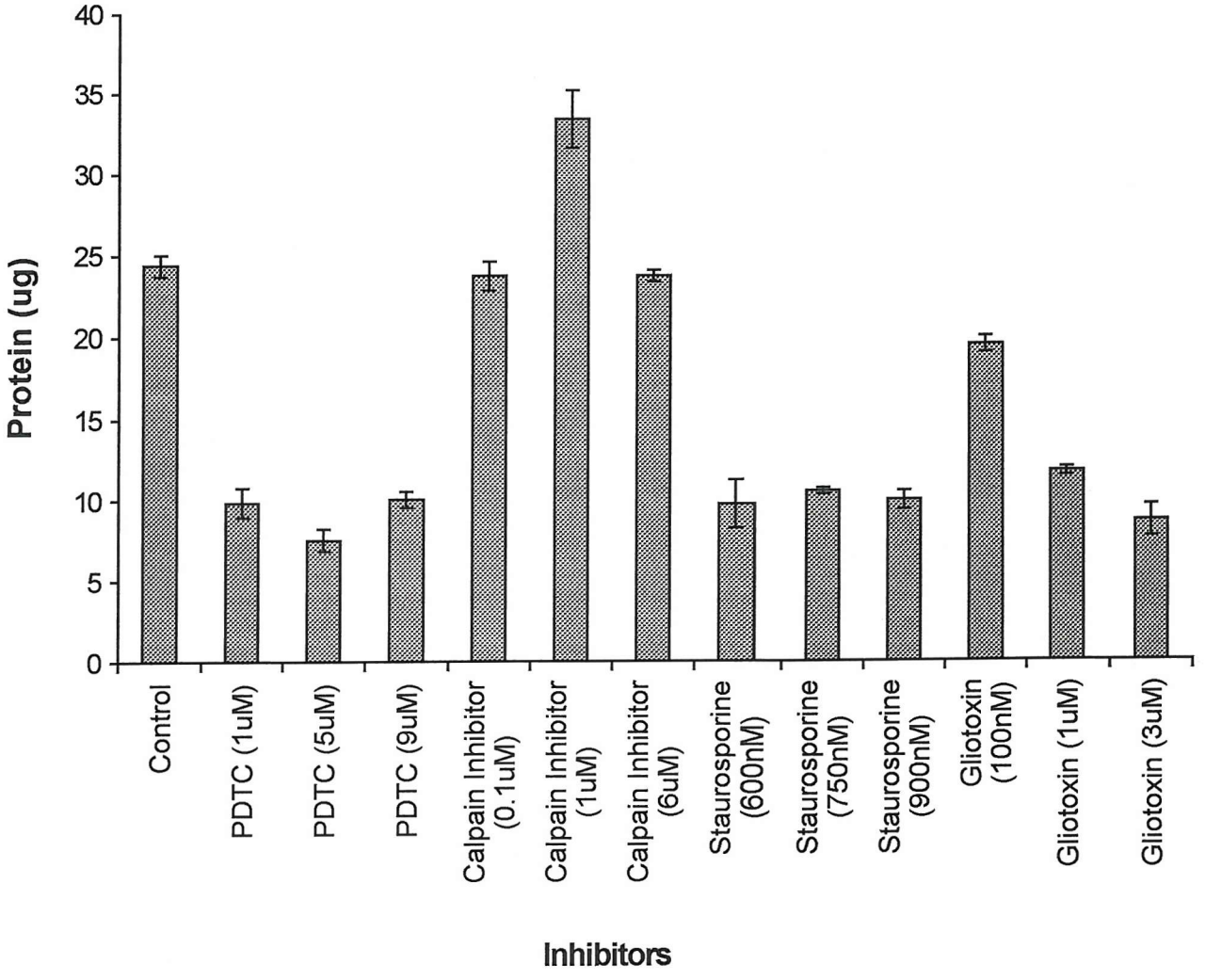


Figure 4.7 Toxicity effects of various cell-signalling inhibitors in rat HSCs. Error bars represent the standard error of the mean.

4.2.4 NF-κB EXPRESSION IN RAT HSCS

Because inhibition of rat HSC complexes A and B was observed with the NF-κB inhibitors, it was decided to further investigate NF-κB and to see whether this transcription factor was expressed in the E-box complexes by supershift EMSA using NF-κB specific antibodies, p50 and p65 (4μg/20μl EMSA reaction). No shifts were observed (Figure 4.8). Competition assays were then performed using either radiolabelled consensus E-box or NF-κB probes. Figure 4.9 shows no competition when radiolabelled consensus E-box probe was incubated with rat HSC nuclear extracts containing an 80x excess of unlabelled NF-κB. Competition was observed with unlabelled consensus E-box. In addition, figure 4.10 shows that an 80x excess of unlabelled consensus E-box failed to compete with radiolabelled NF-κB in contrast to observed competition with unlabelled NF-κB oligonucleotides. These results, therefore, suggest that NF-κB does not bind to the E-box oligonucleotide and vice versa.

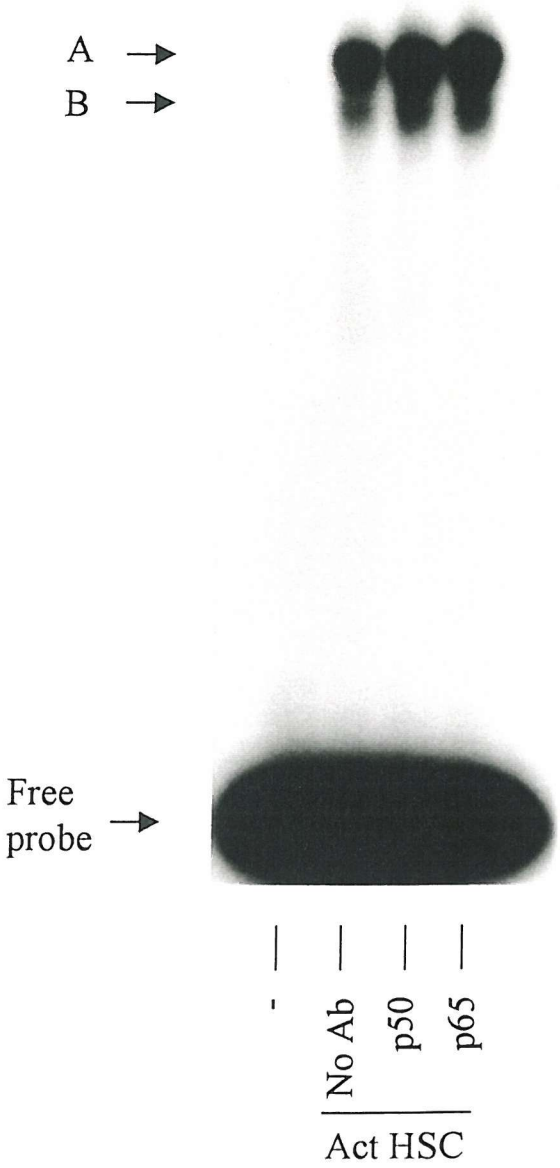


Figure 4.8 Supershift EMSA with activated rat HSC nuclear extract and consensus E-box probe in the presence of NF-κB specific antibodies. (-): Control reaction lacking nuclear extract.

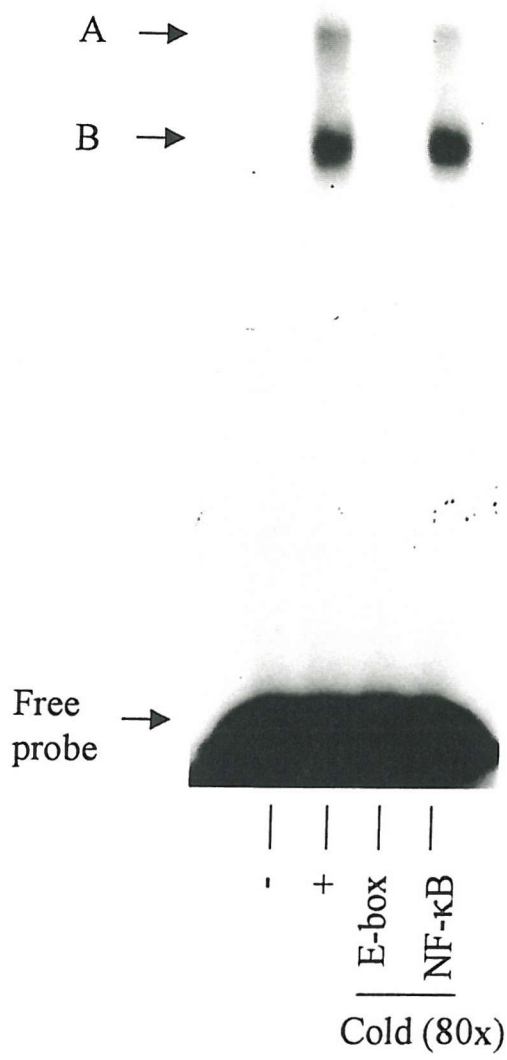


Figure 4.9 Competition assay using activated rat HSC nuclear extract with radiolabelled consensus E-box probe in the presence of (80x excess) unlabelled consensus E-box and NF-κB oligonucleotides. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

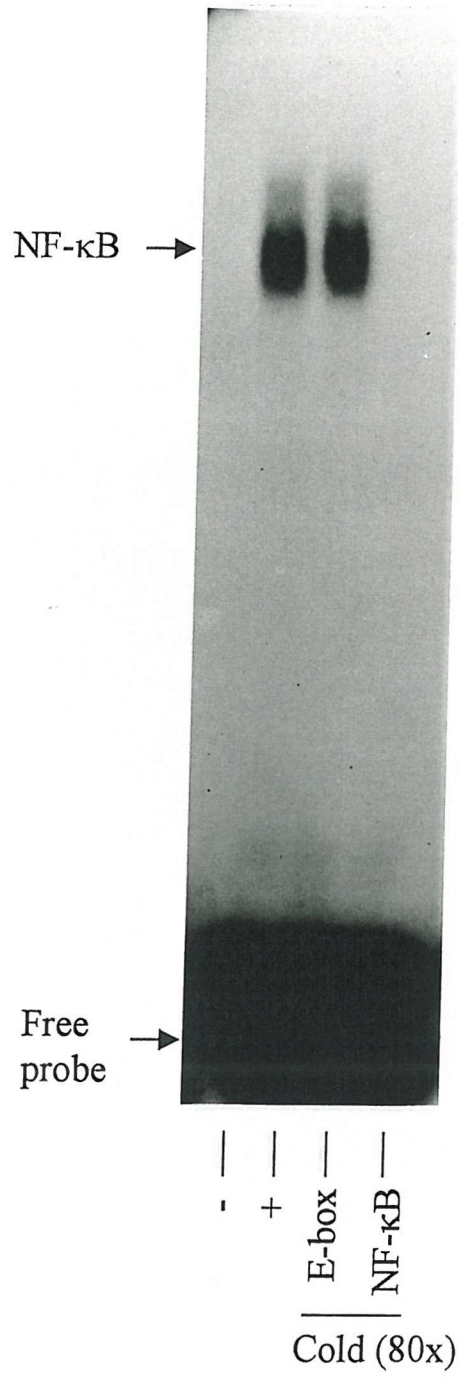


Figure 4.10 Competition assay using activated rat HSC nuclear extract with radiolabelled NF-κB probe in the presence of (80x excess) unlabelled consensus E-box and NF-κB oligonucleotides. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

4.3 DISCUSSION

To further investigate the regulatory factors present in freshly isolated HSCs that may prevent assembly of complexes A and B, it was decided to initially look at the expression of the Id proteins. Various studies have shown that these proteins act as negative regulators of differentiation (see section 1.3.9). Although Id1 has highest affinity for class A proteins it can also bind avidly to a subset of class B proteins including MyoD[139]. Northern blot analysis revealed that Id1 mRNA was abundantly expressed in freshly isolated HSCs and down-regulated as the cells became activated. This correlates with induction of E-box complexes A and B and therefore suggests that Id1 may be an important regulator of the phenotypic transition to a myofibroblast-like HSC. Simonson *et al* found very similar results in glomerular mesangial cells i.e. high expression of Id1 in quiescent cells and down-regulation as they became activated (see section 1.3.9). These cells are thought to be the kidney equivalent of the HSC. For example, in response to glomerular injury mesangial cells proliferate and undergo a phenotypic switch to an activated state characterised by increased synthesis and reorganisation of the extracellular matrix[143]. Furthermore, studies using $Id1^{+/-}Id3^{-/-}$ knockout mice have demonstrated that depletion of the Id proteins can promote increased extracellular matrix deposition and deficient metalloproteinase activity suggesting, therefore, that the Id proteins may be important regulators of fibrosis[369]. Although the studies described in this thesis were limited to Id1, it is also possible that the other Id proteins (e.g. Id2, Id3 and Id4) may associate with the E-box binding proteins expressed in HSCs.

To investigate cell-signalling pathways that may be involved in the induction of MyoD during culture activation of HSCs, a range of inhibitors were added to freshly isolated cells. Staurosporine (a general protein kinase inhibitor) and Wortmannin (PI 3-kinase inhibitor) dose-dependently inhibited complexes A and B. The other kinase inhibitors had no effect. A recent study by Jiang *et al*[137] showed that PI 3-kinase strongly enhanced myogenic differentiation (including MyoD levels) in cultures of chicken-embryo myoblasts (see section 1.3.8). Thus, the ability of Wortmannin to inhibit assembly of complexes A and B in activated HSCs further supports the idea that MyoD is a component of these complexes and that PI 3-kinase is an important signalling mediator for E-box binding proteins in the HSC. Toxicity experiments have shown that Staurosporine is ~70%

toxic at the concentrations required for inhibition of the E-box binding complexes. However, lower concentrations of Staurosporine that failed to have any effect on complexes A and B produced very similar high levels of toxicity. These results therefore suggest that the down-regulation of these complexes is probably due to the specific effects of the cell-signalling inhibitors and not due to general toxicity of the HSC. Other studies using similar concentrations of Wortmannin have shown specific effects on kinases but with no recorded effects on cell viability[365,370].

In addition, the NF-κB inhibitors: Gliotoxin, Calpain Inhibitor and PDTC all inhibited formation of complexes A and B, suggesting that NF-κB may also play an important role in the induction of MyoD. Constitutive NF-κB DNA binding has been described in culture activated rat HSCs[166]. Subsequent experiments showed that although Gliotoxin and PDTC were quite toxic to the HSC, Calpain Inhibitor was much less so. Studies in chick limb cells have shown that NF-κB can transcriptionally activate expression of the bHLH protein Twist[167,168] (see section 1.3.10). A similar role of NF-κB signalling may therefore take place in the HSC. Supershift studies using the NF-κB specific antibodies, p50 and p65, revealed that NF-κB does not form part of the E-box complexes observed in HSCs. In addition, competition assays have shown that NF-κB does not compete with other E-box binding proteins for the consensus E-box element. In contrast, Lee *et al* have shown that NF-κB can bind to the E-box in the αSMA promoter after HSC activation (see section 1.3.10)[162]. These differences observed with NF-κB: E-box DNA binding may, therefore, again reflect the importance of nucleotides flanking the E-box motif in determining E-box binding protein specificity.

As discussed above, toxicity experiments have demonstrated that the cell-signalling inhibitors used in these experiments do have high toxicity in rat HSCs. Thus, these effects should be taken into account when considering the validity of this data. Alternative techniques for elucidating significant signalling pathways involved in the induction of MyoD, for example, would be to transfect HSCs with dominant negative mutants of protein kinases, NF-κB *etc*. For further information on future work regarding cell-signalling experiments, see section 7.2.

Several kinases have been implicated in the activation of NF-κB. The best data exists for the double-stranded RNA activated kinase (PKR), which phosphorylates IκB-α *in vitro*[371]. Furthermore, *in vivo* inactivation of this kinase inhibits the ability of double-stranded RNA to activate NF-κB[372]. The involvement of PKR has also been reported in the regulation of myogenesis. *In vitro* studies using the cell line C2C12 transfected with PKR have shown that MyoD expression is increased[373]. It may be possible, therefore, that activation of PKR leads to increased expression of NF-κB and subsequent transcriptional activation of MyoD. The lack of assembly of complexes A and B in HSCs in the presence of Calpain Inhibitor (inhibits proteasome-mediated degradation of IκB-α) further supports this idea. In addition to these signalling pathways, PI 3-kinase may provide an additional means of MyoD induction. PI 3-kinase is activated by several growth factors[374] and may also lead to an induction of NF-κB with subsequent activation of MyoD. Wortmannin failed to totally inhibit formation of complexes A and B in the HSC even at quite high concentrations. These results therefore suggest that even if the PI 3-kinase pathway is inhibited by Wortmannin, some MyoD may still be induced possibly by a pathway involving PKR.

CHAPTER 5

EXPRESSION OF SP1 DURING HEPATIC STELLATE CELL ACTIVATION

5.1 INTRODUCTION

Activated HSCs are now considered to be the main source of hepatic ECM in chronic liver disease[2]. They produce a wide range of collagenous and non-collagenous ECM components (see section 1.1.8). Additionally, activated HSCs are involved in ECM remodelling by producing matrix degrading MMPs (see section 1.1.5) and TIMPs (see section 1.1.7). Very little is known, to date, about how the different expression levels of ECM components and ECM remodelling enzymes in quiescent and activated HSCs are regulated. We have shown that TIMP-1 is regulated at the level of gene transcription in the HSC[335]. Similar findings have been published for collagen α1 (I)[333,334,338,375,376], collagen α2 (I)[339,377], and stromelysin[336]. In addition, studies in other cell types have shown that interstitial collagenase[378,379] and gelatinase A[340] are regulated at the level of gene transcription. Transcription factors may, therefore, play a key role in the control of matrix deposition by activated HSCs. Studies with the HSC have demonstrated that stromelysin and interstitial collagenase are induced within the first three days of culture, and then their level of expression returns to basal. In contrast, TIMP-1, gelatinase A, and collagen type I are expressed after a culture period of 5 to 7 days and are then continuously increased as the cells become more activated[29,380]. Many of these genes have GC-rich regions in their promoters and include collagen α1 (I)[338,375], collagen α2 (I)[339], gelatinase A[340], and TIMP-1[335]. In addition, many other genes in the HSC express GC-box elements in their promoter regions, including the smooth muscle gene, desmin [381], and one of the most fibrogenic cytokines, TGF-β1[185].

In the following study, the expression of GC-box DNA binding proteins in HSCs will be investigated using both *in vitro* (culture activation of rat cells) and *in vivo* (CCl_4 treatment of rats) models of HSC activation. Western blotting and Supershift EMSA techniques will then be used to characterise the GC-box DNA binding proteins. These studies may help to further ascertain the potential role of these transcription factors in the control of genes involved in liver fibrosis. In addition, cell-signalling pathways involved in the induction of Sp1 will be examined.

5.2 RESULTS

5.2.1 INDUCTION OF GC-BOX DNA BINDING ACTIVITIES DURING CULTURE ACTIVATION OF RAT HSCS (*IN VITRO*)

Nuclear extracts were prepared from rat HSCs cultured for various time-points (0-7 days). GC-box DNA binding activity was determined by EMSA using a consensus oligonucleotide that had previously been shown to bind Sp1 transcription factor[229]. All EMSAs in this chapter were performed using 5μg of protein and 0.2ng of probe in a 20μl reaction. A diffuse GC-box DNA binding activity was consistently observed in freshly isolated HSCs. This could not be explained by poor quality or degraded nuclear extracts since distinct binding activities were detected in these extracts using other probes such as NF-κB[166]. In addition, a variety of different electrophoretic running conditions were used to try and resolve the GC-box binding activity into discrete complexes but to no avail. After 1 day of cell culture, a low mobility GC-box (LMGC) DNA binding activity was induced with loss of the diffuse complex. Subsequent culturing led to a loss of the LMGC complex and induction of two or more high mobility GC-box (HMGC) DNA binding activities. Expression of the HMGC binding activity and lack of the LMGC activity persisted throughout further periods of culture (Figure 5.1A).

5.2.2 INDUCTION OF GC-BOX DNA BINDING ACTIVITIES DURING CARBON TETRACHLORIDE INDUCED ACTIVATION OF RAT HSCS (*IN VIVO*)

To determine whether *in vivo* activation of rat HSCs was also associated with induction of GC-box DNA binding activities, freshly isolated HSCs were purified from rats treated with CCl_4 for 48 hours. Nuclear extracts were harvested and GC-box DNA binding activities were detected by EMSA. Results show that these extracts lacked the diffuse complex observed with freshly isolated HSCs from untreated rats but instead expressed a LMGC complex similar to that observed in 1 day cultured HSCs. In addition, HSCs from CCl_4 treated rats expressed a diffuse GC-box complex of slightly lower mobility than the HMGC activity observed in 7 day cultured HSCs (Figure 5.1B).

A

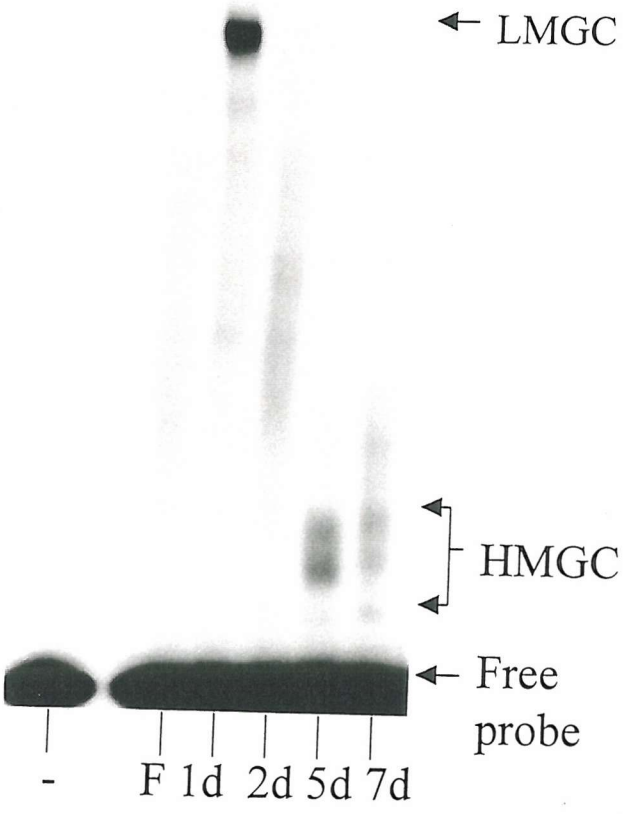


Figure 5.1 (A) Expression of GC-box DNA binding proteins in the rat HSC during culture activation on plastic. (-): Control reaction lacking nuclear extract; (F): Freshly isolated; (LMGC): Low mobility GC-box protein; (HMGC): High mobility GC-box protein.

B

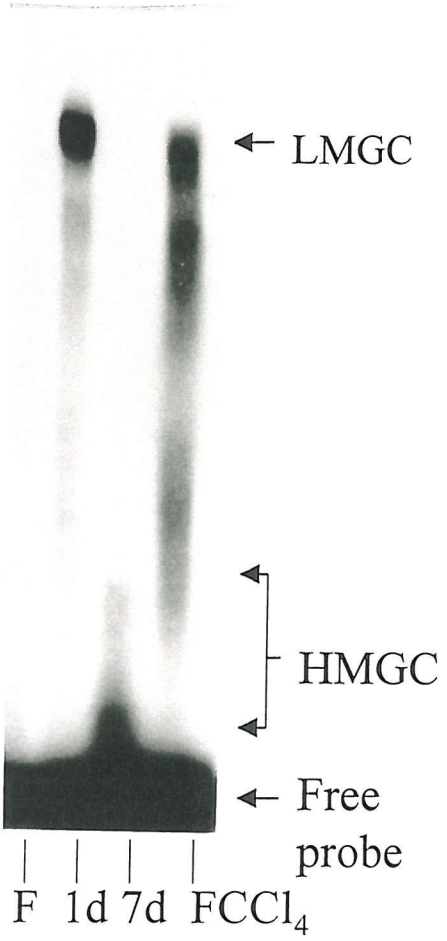


Figure 5.1 (B) Expression of GC-box DNA binding proteins in the rat HSC after CCl_4 treatment.

5.2.3 SPECIFICITY OF GC-BOX DNA BINDING PROTEINS

To determine specificity of the LMGC and HMGC binding activities in cultured HSCs, nuclear extracts from 1 day and 7 day cultured rat HSCs were incubated with either specific (GC-box) or non-specific (E-box) unlabelled oligonucleotides prior to incubation with radiolabelled GC-box probe. Figure 5.2 (A and B) shows that both the LMGC and HMGC complexes were effectively competed for using a 50-fold excess of specific oligonucleotide. In contrast, no competition was observed using up to 200-fold excess of the unrelated consensus E-box oligonucleotide.

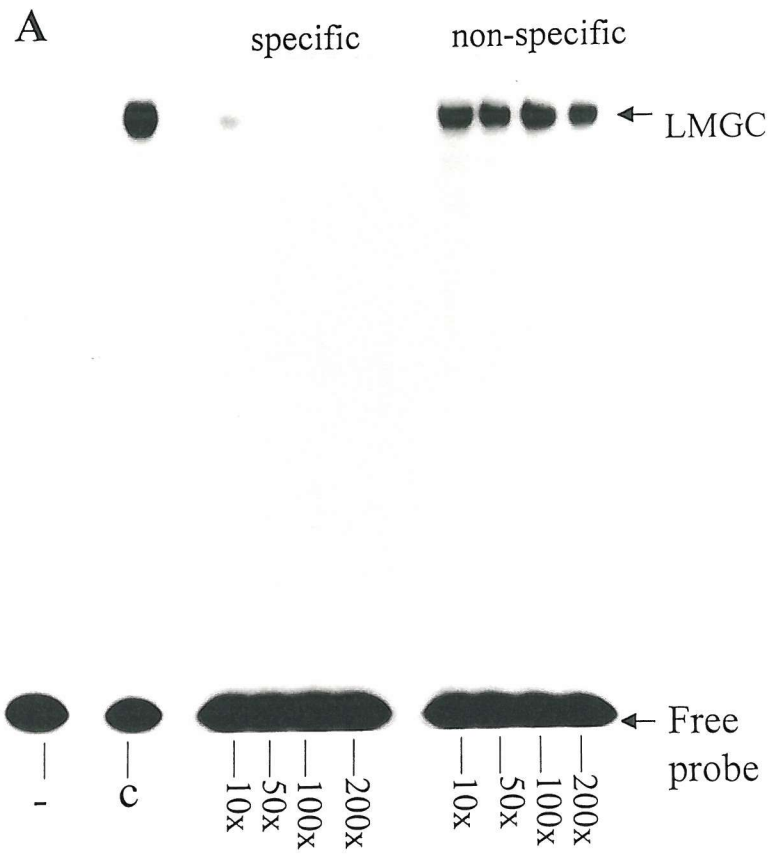


Figure 5.2 (A) Competition assay using 1 day rat HSC nuclear extract with radiolabelled GC-box in the presence of increasing concentrations (10-200x excess) of unlabelled specific (GC-box) and non-specific (E-box) oligonucleotides. (-): Control reaction lacking nuclear extract; (C): Control reaction with nuclear extract only.

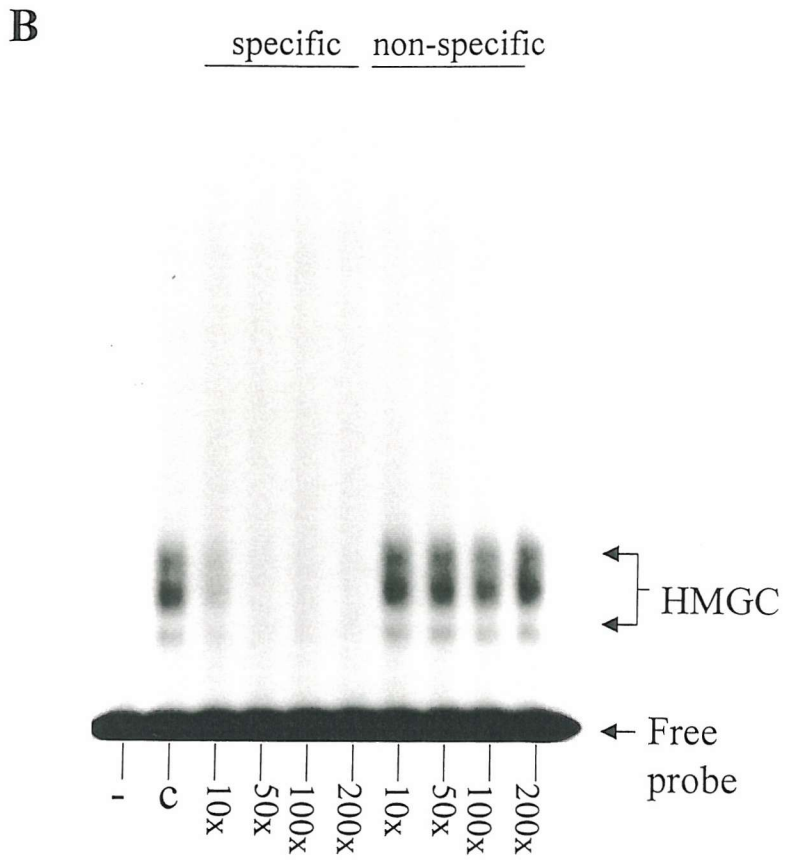


Figure 5.2 (B) Competition assay using 7 day rat HSC nuclear extract with radiolabelled GC-box in the presence of increasing concentrations (10-200x excess) of unlabelled specific (GC-box) and non-specific (E-box) oligonucleotides. (-): Control reaction lacking nuclear extract; (C): Control reaction with nuclear extract only.

5.2.4 IDENTIFICATION OF THE GC-BOX DNA BINDING PROTEINS

Western blotting and supershift EMSA experiments were performed in order to identify the proteins in the GC-box binding complexes of rat HSCs. Antibodies (2μg/20μl EMSA reaction) recognising the four members of the Sp1 protein family were used initially. The transiently induced LMGC complex was almost completely supershifted by anti-Sp1 with no reactivity observed for Sp2, Sp3 or Sp4 (Figure 5.3A). However, the four antibodies were not reactive with the HMGC complexes observed in activated HSCs (Figure 5.3B). In contrast, Rippe *et al* report that sustained Sp1 binding activity can be detected in culture-activated rat HSCs[375]. To further review this discrepancy, the expression of Sp1 protein across a broad time course of HSC activation was determined by immunoblotting. Figure 5.3C shows that freshly isolated HSCs lacked expression of Sp1. However, HSCs cultured for 1 day expressed at least two anti-Sp1 reactive proteins between the 83 and 175kDa marker proteins, probably corresponding to the phosphorylated 105kDa and unphosphorylated 95kDa Sp1 forms[375]. Cells cultured for 2 days expressed barely detectable levels of Sp1 that became undetectable by 5 days of culture and remained so throughout further periods of continuous culture.

To further characterise the HMGC complexes, an antibody (2μg/20μl EMSA reaction) recognising the GC-box binding protein Zf9 (previously shown by Ratziu *et al* to be induced in activated rat HSCs[184]) was used in supershift EMSA. However, this antibody also failed to react with the HMGC complexes (Figure 5.4). Further analysis of the GC-box activity observed in HSC nuclear extracts purified from CCl_4 treated rats demonstrated that it could be supershifted by anti-Sp1 (Figure 5.5) indicating that induction of this GC-box DNA binding activity is common to *in vitro* and *in vivo* activation of rat HSCs.

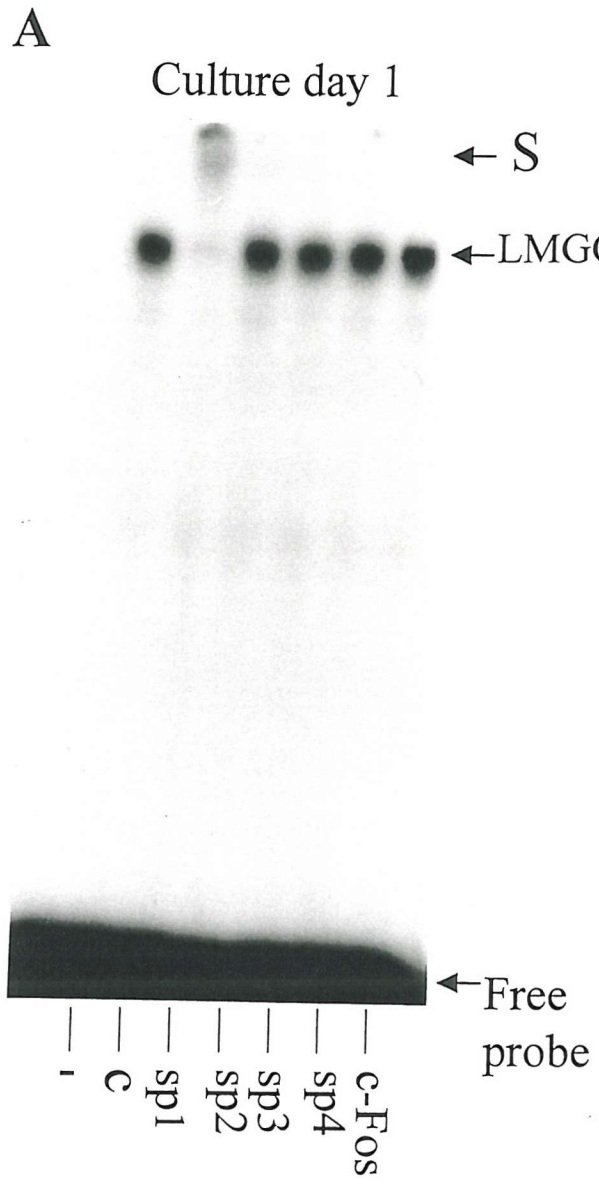


Figure 5.3 (A) Supershift EMSA with 1 day culture activated rat HSC nuclear extracts using various Sp1 protein family antibodies and c-Fos as a control. (-): Control lacking nuclear extract; (C): Control reaction with nuclear extract only; (S): Supershift.

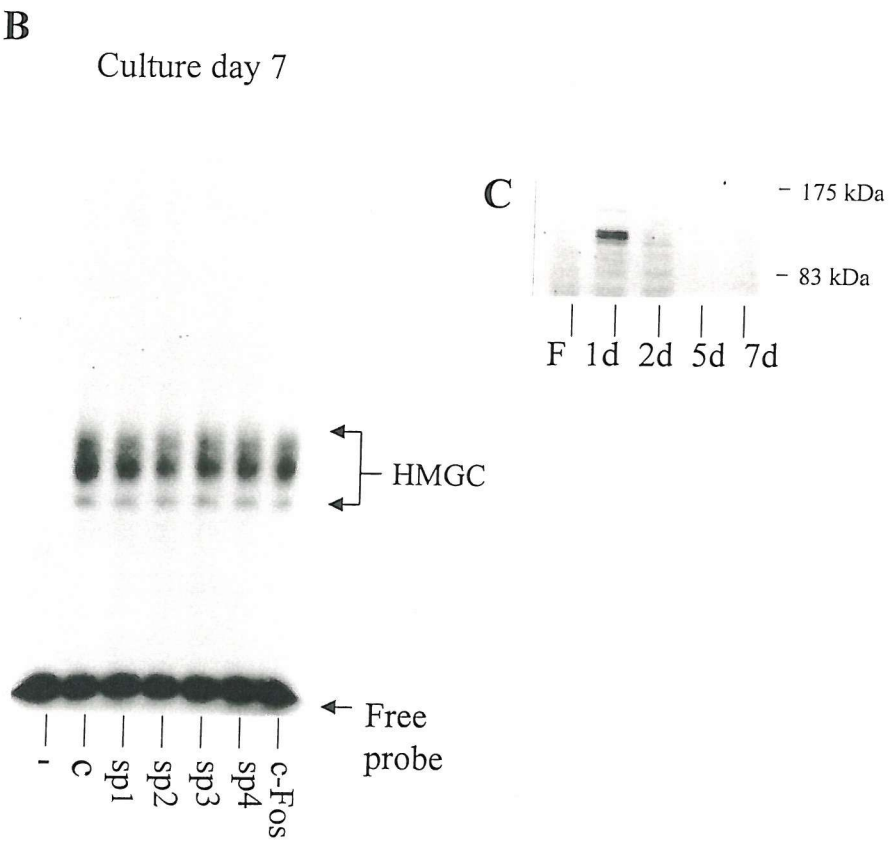


Figure 5.3 (B) Supershift EMSA with 7 day culture activated rat HSC nuclear extracts using various Sp1 protein family antibodies and c-Fos as a control. (-): Control lacking nuclear extract; (C): Control reaction with nuclear extract only. **(C)** Western blot showing transient expression of Sp1 (95 and 105kDa protein forms) in rat HSCs. Extracts (20μg) were electrophoresed through a 9% SDS-polyacrylamide gel.

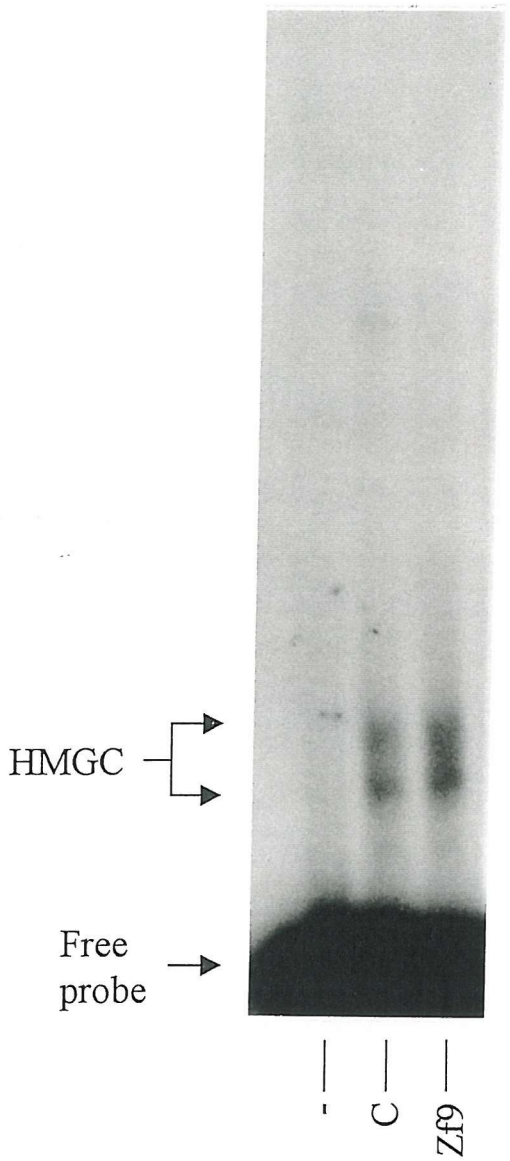


Figure 5.4 Supershift EMSA with 7 day culture activated rat HSC nuclear extracts using Zf9 antibody. (-): Control lacking nuclear extract; (+): Control with nuclear extract only.

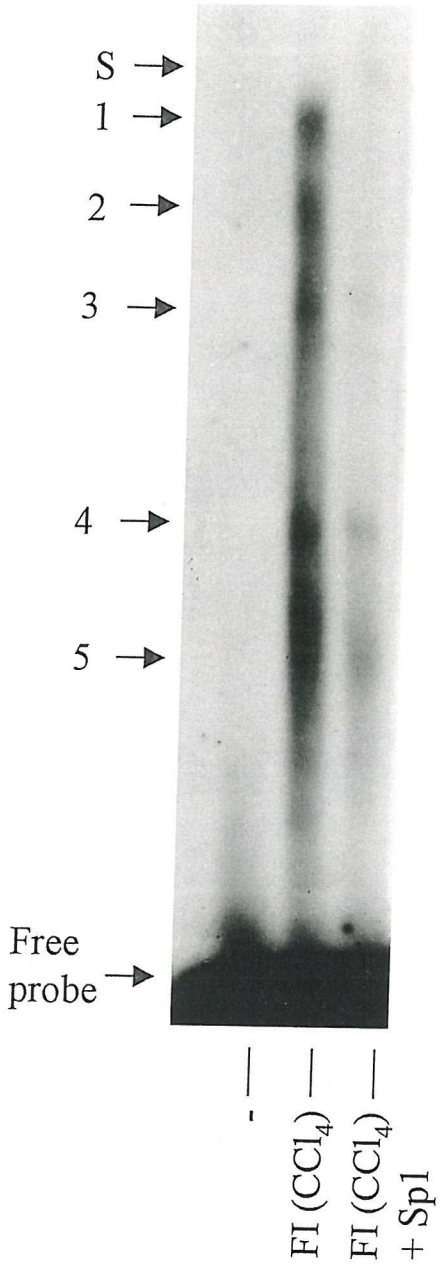


Figure 5.5 Supershift EMSA with freshly isolated (FI) HSC nuclear extracts from rats treated with CCl_4 for 48 hours using Sp1 antibody. (-): Control lacking nuclear extract; (S): Supershift.

5.2.5 EFFECTS ON GC-BOX DNA BINDING ACTIVITIES FOLLOWING TREATMENT OF RAT HSCS WITH TRYPSIN

One possible explanation for the differences observed with Sp1 binding, between the studies shown here and those published by Rippe *et al*, could be differences in the culturing of rat HSCs. For example, unlike our group, Rippe *et al* may, like many other investigators, trypsinise their HSCs during culture. It was decided, therefore, to see whether trypsin treatment could modify GC-box DNA binding activities. HSCs were cultured for 7 days and then mobilised using trypsin and were replated for another 4 hours. Nuclear extracts were then prepared and used in EMSAs. On the first day after cell isolation the characteristic Sp1 band was visible which disappeared by day 7. After trypsinisation weak expression of Sp1 binding was reinduced after 4 hours (Figure 5.6A). In addition, Western blotting analysis confirmed weak expression of p95 and p105 forms of Sp1 in trypsin treated rat HSC extracts suggesting that the protease can induce re-expression of the transcription factor (Figure 5.6B).

5.2.6 INVESTIGATION OF CELL-SIGNALLING INHIBITORS THAT MAY CONTROL THE EXPRESSION OF GC-BOX BINDING PROTEINS IN RAT HSCS

To examine signal transduction pathways involved in the induction of GC-box DNA binding activities, a range of protein kinase inhibitors were added to freshly isolated rat HSCs, left for 24 hours and then harvested. Treatment with the tyrosine kinase inhibitor Herbimycin A[363] led to a concentration-dependent inhibition of LMGC (Sp1) binding activity. Inhibition could be detected at 0.14μM and was further increased at 1.39μM and 13.90μM. Similar concentrations of Herbimycin A, however, had no effect on E-box binding proteins in rat HSCs (see section 4.2.2). Staurosporine[360] (general protein kinase inhibitor) did not significantly affect Sp1 binding, a slight decrease was seen at 1000nM. Wortmannin[365] (PI 3-kinase inhibitor) caused a slight increase in Sp1 binding activity at low concentrations (1 to 10nM) but higher concentrations (100 to 1000nM) decreased Sp1 binding again to levels approaching those observed in control cells. Thus, only Herbimycin A showed a dose-dependent suppression of Sp1 binding activity. This suggests that tyrosine kinases are involved in the induction of Sp1 in early HSC culture (Figure 5.7).

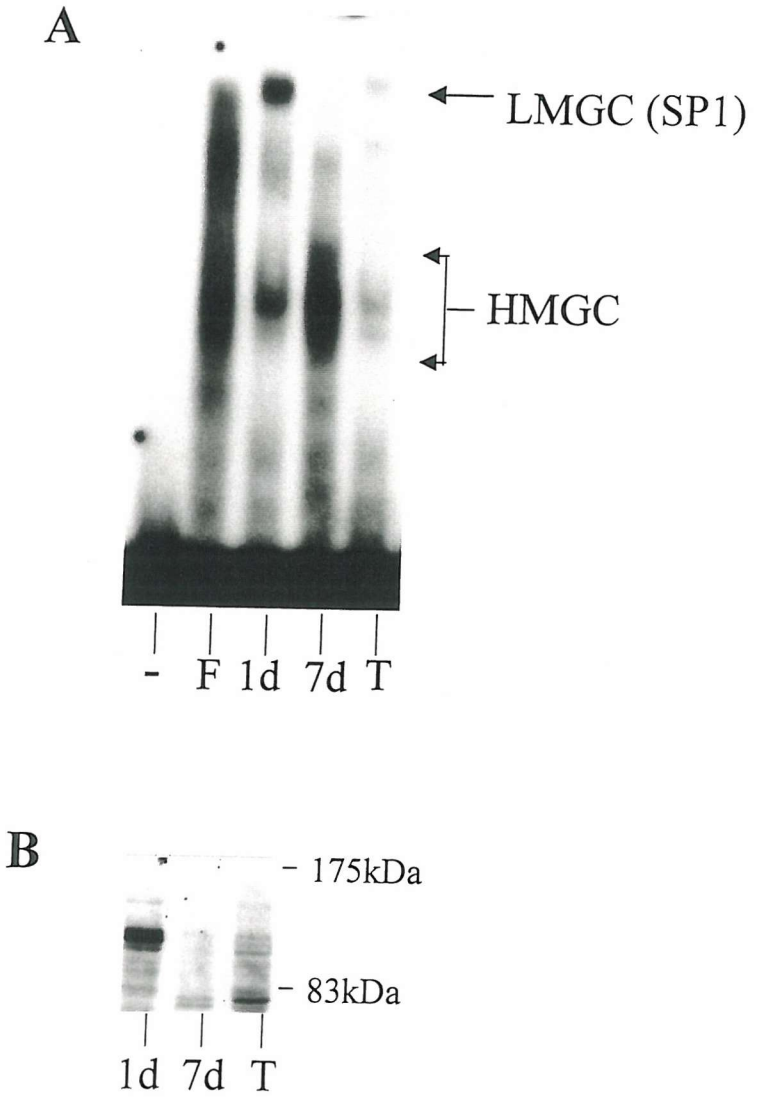


Figure 5.6 (A) Effects of trypsin treatment on LMGC (Sp1) expression in rat HSCs. (-): Control reaction lacking nuclear extract; (T): Trypsin treated extract. **(B)** Western blot analysis of Sp1 expression in primary and trypsin-passaged rat HSCs. Extracts (20μg) were electrophoresed through a 9% SDS-polyacrylamide gel.

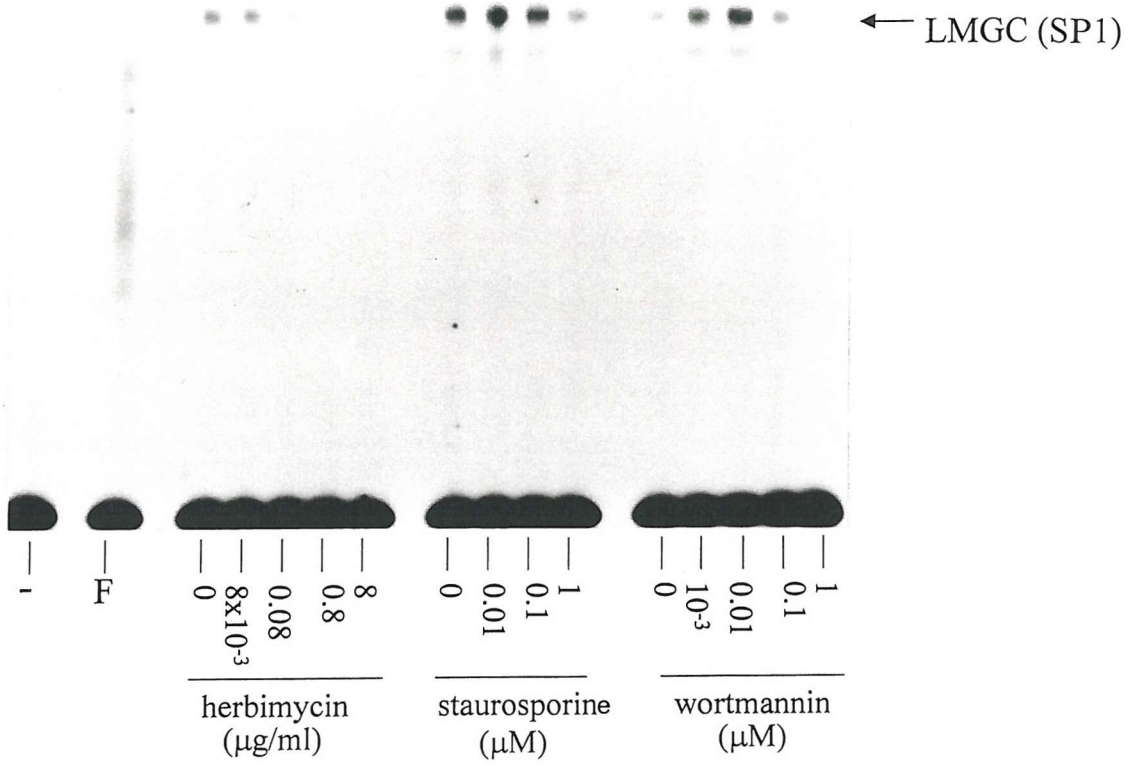


Figure 5.7 Effects of cell-signalling inhibitors that may control GC-box binding proteins in rat HSCs. (-): Control reaction lacking nuclear extract. Herbimycin (0, $8x10^{-3}$, 0.08, 0.8, 8) μg/ml = Herbimycin (0, 0.01, 0.14, 1.39, 13.90) μM respectively.

5.3 DISCUSSION

Analysis of the expression of GC-box binding factors, using standard EMSA, during *in vitro* activation of rat HSCs demonstrated strong induction of a LMGC complex in 1 day cultured cells. Supershift EMSAs subsequently identified this complex as Sp1. Further culture of HSCs demonstrated lack of Sp1 expression but instead were associated with HMGC complexes. However, antibodies against the Sp1 protein family failed to have any effect on these GC-box binding proteins. Recent studies have led to the identification of another GC-box binding protein, Zf9, which is induced during HSC activation (see section 1.4.2). However, upon further investigation, supershift EMSAs using an antibody against Zf9 and activated HSC nuclear extracts failed to have any effect on the HMGC complexes detected in these studies. This may be due to the fact that the oligonucleotides used in the two studies have differences in the nucleotides flanking their GC-box motifs. The oligonucleotide used in the studies described in this thesis were chosen for optimal Sp1 binding. Thus the identity of the HMGC complexes in activated HSCs still remains obscure. However, recent studies have described the up-regulation of BTEB in activated rat HSCs and its enhanced binding to the collagen α1 (I) gene[334]. It would be interesting to determine whether an antibody against BTEB would have any effects on the observed HMGC complexes in activated HSCs. Both LMGC and HMGC complexes were shown to be sequence specific with respect to the GC-box. Further analysis of Sp1 protein expression using Western blotting revealed similar expression as EMSA data i.e. maximal expression of Sp1 in HSCs cultured for 1 day and then barely detectable levels with subsequent culture.

Further investigations were then performed with *in vivo* models of HSC activation to determine whether similar GC-box DNA binding activities were expressed as with the *in vitro* model. These studies revealed a diffuse GC-box complex of slightly lower mobility than the HMGC activity observed in 7 day cultured HSCs and a complex that ran with similar mobility to the LMGC complex observed in 1 day culture activated rat HSCs. In addition, an antibody against Sp1 supershifted this complex suggesting that induction of Sp1 is a common event in the two models of HSC activation. However, in addition to Sp1 binding in HSCs isolated from rats treated with CCl_4 for 48 hours, similar binding was also detected in HSCs isolated from rats treated with CCl_4 for 3 weeks (personal

communication by Dr Mann). These results contrast to the transient expression of Sp1 observed in culture activated HSCs. One possible reason for these differences in temporal expression could be due to the fact that the CCl_4 method represents an acute liver injury model and requires readministration of CCl_4 every three days to achieve fibrosis. It is highly likely that at each CCl_4 injection, in addition to activated HSCs, there will be numerous quiescent and transitional HSCs. This may be due to various reasons, for example, some activated HSCs may start to revert back to a quiescent state in between dosing, and not all HSCs may be activated during each CCl_4 injury. Thus, there are likely to be HSCs at different stages of activation after each dose which would therefore create a more heterogeneous HSC population than with the cell culture model.

Rippe *et al* have reported that culture activation of rat HSCs is associated with induction of sustained Sp1 protein expression and its enhanced binding to GC-boxes in the collagen α1 (I) gene [375]. However, these results contrast to the work describing transient expression of Sp1 in early culture activated HSCs only. To further review these differences in temporal kinetics of Sp1 expression, it was decided to look at the effects of trypsin. Trypsin treatment is a standard procedure to keep cells in logarithmic growth during cell culture[382]. Various studies have been conducted in our laboratories to see whether disruption of the cellular microenvironment by trypsin and replating of rat HSCs leads to changes in the expression of certain transcription factors. Results have shown, for example, that trypsin treatment of culture activated rat HSCs leads to re-expression of a low mobility AP-1 DNA binding complex. This complex was initially only seen as a transient induction after 24 hours of culture[335,383]. Bretschneider *et al* have shown that trypsin can stimulate proteinase-activated receptor-2 (PAR-2) in smooth muscle cells which then triggers intracellular signalling events. These include an increase in intracellular Ca^{2+}, activation of NF-κB, and an increase in cell proliferation[384]. This receptor is expressed on the surface of activated rat HSCs[385] and may, therefore, be one mechanism by which trypsin alters the pattern of transcription factor expression in rat HSCs. To determine whether trypsin may also alter the expression of GC-box binding proteins, EMSAs were performed on trypsin-treated HSCs. Results confirmed that after trypsinisation of 7 day culture activated HSCs, weak expression of Sp1 was reinduced. Western blotting also showed re-induction of Sp1 after trypsinisation. Thus, if Rippe *et al* have trypsinised their HSCs during culture, this may

explain the differences in Sp1 expression between the two studies. In addition to these discrepancies, Rippe *et al* have also shown that only a portion of their observed GC-box binding complex was supershifted by an Sp1 antibody. In contrast, the results shown in this thesis demonstrate that the LMGC complex was almost totally supershifted by an Sp1 antibody. However, on further comparison of the Sp1 oligonucleotides used in the two studies, although both have the consensus GC-box, they are flanked by different nucleotides which may therefore govern differences in binding proteins to each. Despite these differences, both Western blot analysis and supershift EMSA studies clearly demonstrate that primary rat HSCs cultured on plastic for more than 48 hours lack detectable levels of Sp1.

Sp1 was initially thought to regulate expression of genes found in activated HSCs that contribute to liver fibrosis (e.g. collagen α1 (I), collagen α2 (I), TGF-β1 and its receptor gene, gelatinase A and TIMP-1). GC-boxes have been reported in all of these genes. However, these proteins are only expressed in HSCs after a culture period of 5 to 7 days (see section 5.1). The data described in this thesis, therefore, strongly opposes the role of Sp1 as a transcriptional regulator of these genes. Nevertheless, although Sp1 may not be involved, the HMGC complexes detected with further culture of HSCs may be important in the transcriptional activation of these genes. Further research is necessary to try and characterise these GC-box binding proteins. In contrast to regulation of the genes described above, Sp1 is probably important for regulating early events involved in the initiation of HSC activation. For example, it may regulate the transcriptional activation of genes required for proliferation. Sp1 has been implicated in the control of cell cycle-regulated genes including thymidine kinase, B-myb and dihydrofolate reductase[187]. In addition, Sp1 has been reported as a negative regulator of transcription and may therefore be initially involved with repression of certain genes that are only found in the activated HSC phenotype.

Finally, the signal transduction pathways involved in the induction of Sp1 binding were characterised by chemical inhibition of different protein kinase families. However, out of the cell signalling inhibitors that were investigated, only Herbimycin A dose-dependently inhibited the induction of Sp1 binding. Herbimycin A is a tyrosine kinase inhibitor and

among its targets, it has been reported to inhibit $p60^{c\text{-}src}$ and PDGF-induced phospholipase D activation[386,387]. Kim *et al*, for example, used similar concentrations of Herbimycin A in their studies and results demonstrated specific inhibition of PDGF-induced phospholipase D[363]. Further analysis and characterisation of the tyrosine kinase present in the HSC is now required to more clearly decipher the signalling pathways of Sp1 induction.

CHAPTER 6

EXPRESSION OF AP-1 DURING HEPATIC STELLATE CELL ACTIVATION

6.1 INTRODUCTION

As mentioned previously, transcriptional regulation of key genes encoding matrix molecules, matrix metalloproteinases and their inhibitors has been documented during culture activation of HSCs (see section 5.1). Many of these genes contain AP-1 binding sites in their promoters and include collagen α1 (I)[333,334], TIMP-1[315,335,388-391], interstitial collagenase[337] and stromelysin[336]. Chen *et al* have shown, for example, that UV irradiation can activate JNK and increase collagen α1 (I) gene expression in rat HSCs. The response element mediating UV induction of collagen α1 (I) gene expression is located in a GC-box in the promoter region of the collagen gene. Further studies have demonstrated that the GC-box is bound by the transcription factor, BTEB. The authors thus speculate that activation of JNK and subsequent activation of Jun directly transduces signals to the promoter of the BTEB gene through Jun-Jun dimerisation and then BTEB gene expression is stimulated. BTEB could then bind to the GC-box and stimulate collagen gene expression[334]. Another study, however, has found that an AP-1 binding site in the enhancer region of the collagen α1 (I) gene plays a critical role in the stimulation of the collagen α1 (I) gene by TGF-β[333]. In addition to these studies, Parola *et al* have investigated the effects of 4-Hydroxy-2,3-nonenal (HNE) which has been shown to stimulate procollagen type I gene expression and synthesis in human HSCs. HNE is an aldehydic end product of lipid peroxidation which has been detected in chronic liver damage (*in vivo*). These studies demonstrate that HNE directly interacts with JNKs to activate them. A subsequent increase in AP-1 DNA binding activity, associated with increased mRNA levels of *c-jun*, was then observed[392]. Studies by Poulos *et al* have shown that fibronectin and inflammatory cytokines increase MAPK activity, stimulate AP-1, and increase stromelysin gene expression in rat HSCs. Thus, signal transduction pathways involving the MAPK family may play an important role in the regulation of matrix metalloproteinase expression by cytokines and fibronectin in HSCs.

Culture activated HSCs demonstrate a marked increase in synthesis of TIMP-1[29,38], which becomes a sustained feature of the activated HSC. Expression of TIMP-1 mRNA is not observed in freshly isolated HSCs and is also not detected in HSCs cultured for 3 days. In contrast, high levels of TIMP-1 mRNA is detected in HSCs cultured for longer than 3 days[29]. Studies in our laboratory have shown that culture activation of HSCs is associated with a dramatic induction of TIMP-1 promoter activity. In addition, after

mapping the minimal region of the promoter required for this response, studies have shown that an AP-1 binding site appears to be a critical regulatory element in the TIMP-1 promoter[335]. Furthermore, a novel regulatory sequence named Upstream TIMP-1 Element-1 (UTE-1) has been identified and appears to be essential for transcriptional activity of the TIMP-1 promoter. These studies have demonstrated that UTE-1 directly interacts with a 30kDa nuclear protein that is induced in activated HSCS, with maximal expression at 5 days of culture and which persists for at least a further 9 days[393]. Previous work in other cell lineages has established a key role for Pea3/Ets binding proteins in AP-1 mediated transactivation of the TIMP-1 promoter[315,389]. However, Bahr *et al* have demonstrated that HSCs express relatively low levels of Ets-1 and Ets-2, and furthermore mutagenesis of the Pea3 DNA binding site in the TIMP-1 promoter had less than a twofold effect on its activity in activated HSCs[335].

Due to ongoing work in the laboratory concerning AP-1 and its role in TIMP-1 regulation, initial studies will be very much focused on this avenue. Although various studies have reported the induction of AP-1 DNA binding activities during HSC activation[333,334,336], they do not describe the nature of the induced Jun and Fos family proteins. A more detailed study concerning the expression of AP-1 DNA binding proteins during HSC activation will therefore be performed[335]. First, the expression of AP-1 DNA binding proteins in HSCs will be examined using both *in vitro* (culture activation of rat cells) and *in vivo* (CCl_4 treatment of rats) models of HSC activation. Supershift EMSAs and Western blotting techniques will then be used to further characterise the AP-1 proteins.

6.2 RESULTS

6.2.1 TRANSIENT INDUCTION OF A LOW MOBILITY AP-1 COMPLEX AND C-FOS/C-JUN PROTEINS DURING CULTURE ACTIVATION OF RAT HSCS

Nuclear extracts were prepared from rat HSCs cultured for various time-points (0-5 days). AP-1 DNA binding activity was determined by EMSA using a double-stranded radiolabelled consensus AP-1 probe. Results show that nuclear extracts from freshly isolated HSCs (time point 0) displayed weak, barely detectable levels of AP-1 binding. In contrast, after 1 day of culture, HSC extracts contained elevated levels of a low mobility AP-1 (LMAP-1) complex. This induction was transient with a marked reduction in levels of LMAP-1 by day 2 and complete loss by day 5 (Figure 6.1A). Additional studies have shown that even up to day 13 of culture, LMAP-1 is still not detected[335]. Similar results were also obtained when using a TIMP-1 AP-1 probe[335]. For detection of LMAP-1 complexes, all EMSAs were performed using 2μg of protein and 0.4ng of consensus AP-1 probe in a 20μl reaction.

Because initial studies focused on AP-1 and its role in TIMP-1 regulation (see section 6.1), the expression of c-Fos (62kDa) and c-Jun (39kDa) was investigated by Western blotting. Previous studies have suggested that c-Fos and c-Jun are important regulators of TIMP-1 promoter activity[315,389,390]. However, results show similar transient kinetics as observed for LMAP-1 in EMSA studies. c-Fos and c-Jun were absent in freshly isolated cells, but were induced by 4 hours of culture with maximal expression at 24 hours which with further culture to 48 and 120 hours fell to undetectable levels (Figure 6.1B). These results are therefore in contrast to those expected for TIMP-1 regulation in HSCs i.e. sustained expression after ~5 to 7 days of culture.

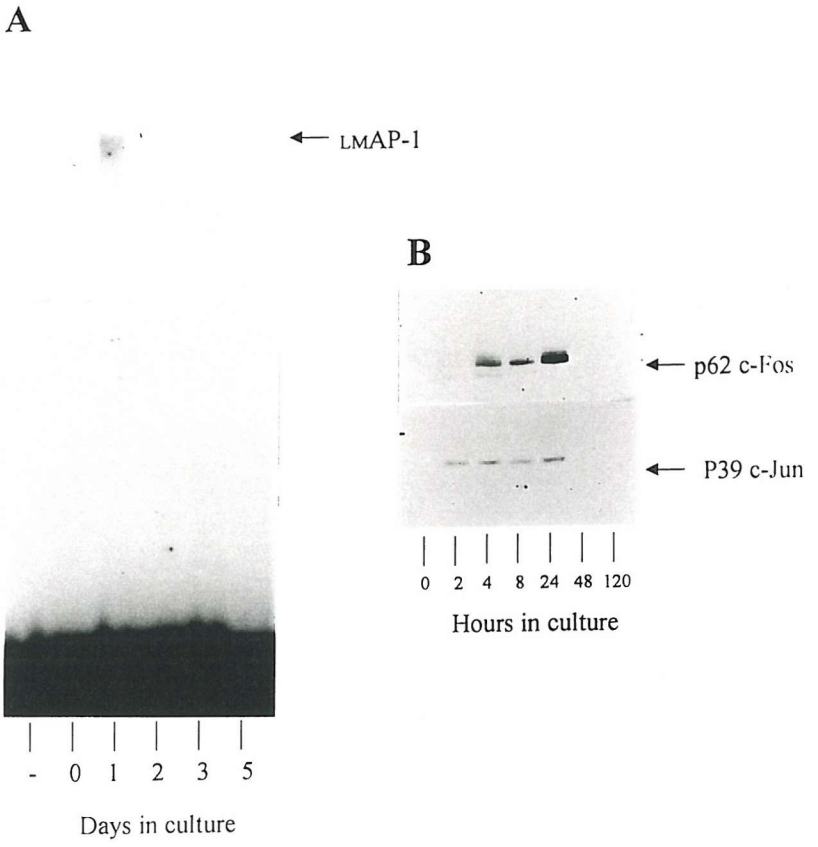


Figure 6.1 (A) Transient induction of a low mobility AP-1 (LMAP-1) complex in rat HSCs. (-): Control reaction lacking nuclear extract. **(B)** Western blot showing transient induction of c-Fos and c-Jun during culture activation of rat HSCs. Extracts (10μg) were electrophoresed through a 12% SDS-polyacrylamide gel.

6.2.2 SPECIFICITY OF LMAP-1 COMPLEX IN RAT HSCS

To determine specificity of the LMAP-1 binding activity in 1 day rat HSCs, nuclear extracts were incubated with either specific (AP-1) or non-specific (E-box) unlabelled oligonucleotides prior to incubation with radiolabelled AP-1 probe. Figure 6.2 shows that LMAP-1 was effectively competed for using a 10x excess of specific oligonucleotide. In contrast, no competition was observed using up to 200x excess of the unrelated consensus E-box oligonucleotide.

6.2.3 DETECTION OF A SEQUENCE-SPECIFIC HIGH MOBILITY AP-1 COMPLEX IN CULTURE ACTIVATED RAT HSCS

Longer exposure of EMSA gels to radiograph film (3 to 7 days) revealed that nuclear extracts from 5 to 13 day activated rat HSCs expressed higher mobility AP-1 (HMAP-1) complexes although still lacking LMAP-1 binding activity. An increased amount of nuclear extract was therefore used to observe the HMAP-1 binding proteins and thus explains lack of detection of these complexes in previous gels. Titration of 7 day HSC nuclear extracts (from 0 to 16μg total protein/EMSA reaction) revealed a diffuse high mobility AP-1 binding activity that could be resolved into at least two complexes. Increased formation of HMAP-1 was observed with addition of increasing amounts of nuclear extract. A similar titration in freshly isolated HSCs showed very low, almost undetectable levels of HMAP-1 showing that they are induced during culture activation (Figure 6.3A). For detection of HMAP-1 complexes, all further EMSAs were performed using 8μg of protein and 0.4ng of consensus AP-1 probe in a 20μl reaction. Similar results were also observed with TIMP-1 AP-1 probes[335]. Competition assays show that HMAP-1 was completely competed for using an 80x excess of specific (AP-1) unlabelled oligonucleotide (Figure 6.3B).

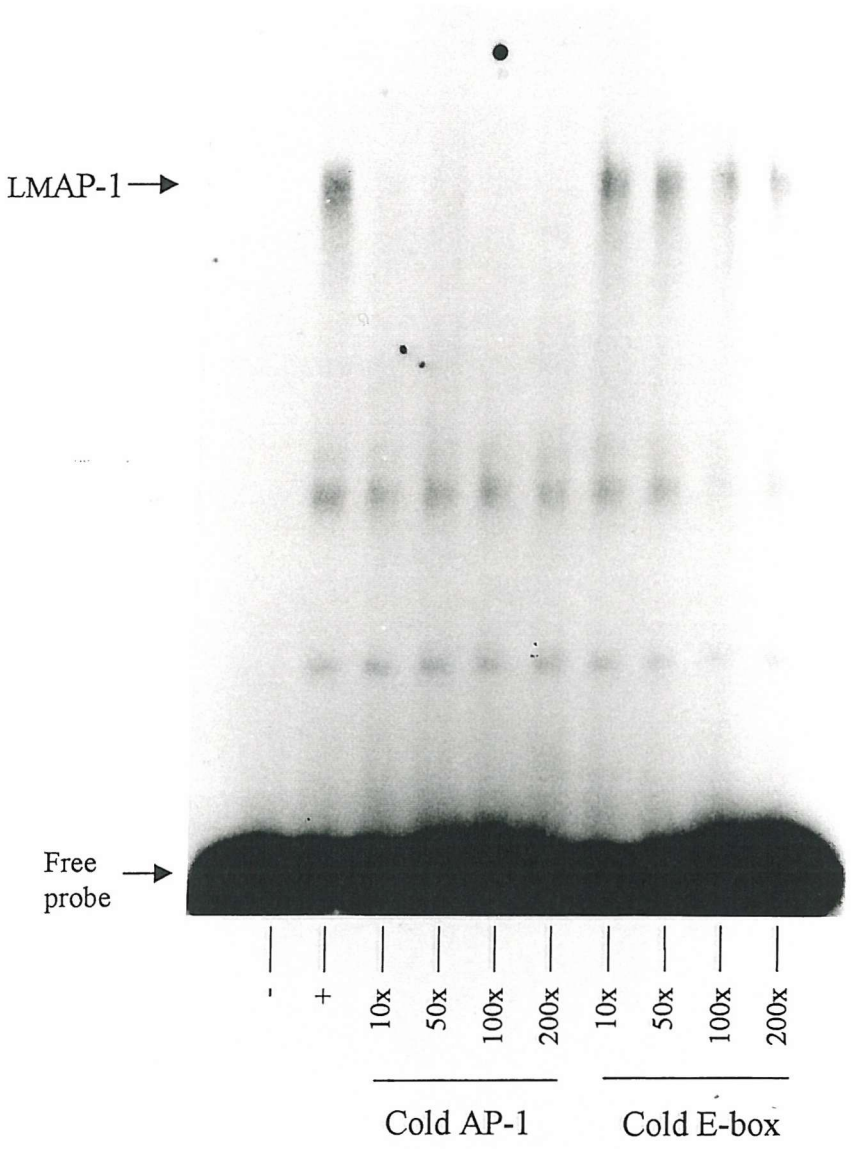


Figure 6.2 Competition assay using 1 day rat HSC nuclear extract with radiolabelled AP-1 in the presence of increasing concentrations (10-200x excess) of unlabelled specific (AP-1) and non-specific (E-box) oligonucleotides. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only.

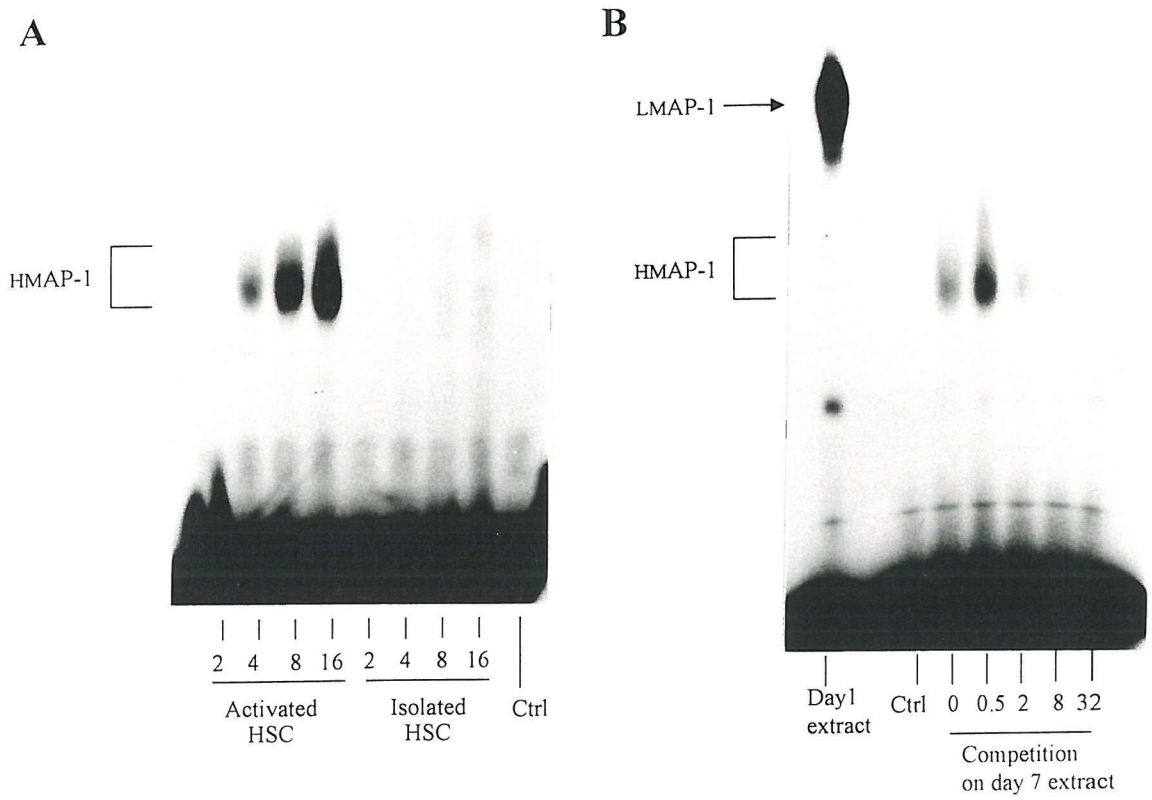


Figure 6.3 (A) Detection of a high mobility AP-1 (HMAP-1) complex in activated rat HSCs. Nuclear extracts were used at 2 to 16μg final protein. (Ctrl): Control reaction lacking nuclear extract. **(B)** Competition assay using 7 day HSC nuclear extract with radiolabelled AP-1 in the presence of increasing concentrations (0.5, 2, 8, 32ng = ~1, 5, 20, 80x excess respectively) of unlabelled specific (AP-1) oligonucleotide. 1 and 7 day nuclear extracts were used at 2μg/20μl and 8μg/20μl EMSA reaction respectively.

6.2.4 CHARACTERISATION OF AP-1 PROTEINS IN HMAP-1 COMPLEXES IN RAT HSCS

Because TIMP-1 expression is not detected in HSCs until culture day 5 to 7 and is thereafter sustained at a high level, initial studies were therefore mainly focused on HMAP-1. LMAP-1 is at undetectable levels by this stage. Supershift EMSAs were used to identify the proteins in the HMAP-1 complex. Antibodies (2μg/20μl EMSA reaction) recognising various AP-1 proteins were used. Antisera to c-Jun and c-Fos were unable to alter expression of HMAP-1. A pan-Jun-specific antibody that recognises the DNA binding domain of c-Jun, JunD, and JunB gave rise to a supershift complex and also caused inhibition of HMAP-1 complex. Western blot studies by postdoctorate fellow (Matthew Wright) showed that activated HSCs express JunD, Fra2 and FosB[335]. Therefore the presence of these three proteins in HMAP-1 complexes was tested by supershift assay. Anti-JunD antibody gave rise to a strong supershift and reduced levels of HMAP-1, anti-FosB antibody caused almost complete loss of HMAP-1 although no supershift was seen and anti-Fra2 antibody generated a weak supershift and reduced levels of HMAP-1. In contrast, Fra1, which by Western blot studies is only expressed in 24hour culture activated HSCs[335], was not detected by this method (Figure 6.4).

6.2.5 CHARACTERISATION OF AP-1 PROTEINS IN LMAP-1 COMPLEXES IN RAT HSCS

It was decided to further investigate the transient LMAP-1 complexes because other important genes in the HSC contain AP-1 sites and demonstrate similar kinetics as LMAP1. For example, interstitial collagenase and stromelysin are induced during the first few days of culture and then return to basal levels again. Supershifts were therefore performed and antibodies (1μg/20μl EMSA reaction) recognising various AP-1 proteins were incubated with 1 day rat HSC nuclear extracts. Antibodies recognising c-Fos and c-Jun caused quite dramatic inhibition of the LMAP-1 complex, in accordance with the Western blotting data demonstrating maximal expression of c-Fos and c-Jun in 1 day culture extracts (see Figure 6.1B). Inhibition was also observed using anti-FosB. In addition, supershifts were produced using JunB and JunD antibodies (Figure 6.5). Anti-Fra1 and anti-Fra2 had no effects on LMAP-1 (Figure 6.6). The control antibody, Sp1, also had no effect on LMAP-1 (Figures 6.5 and 6.6).

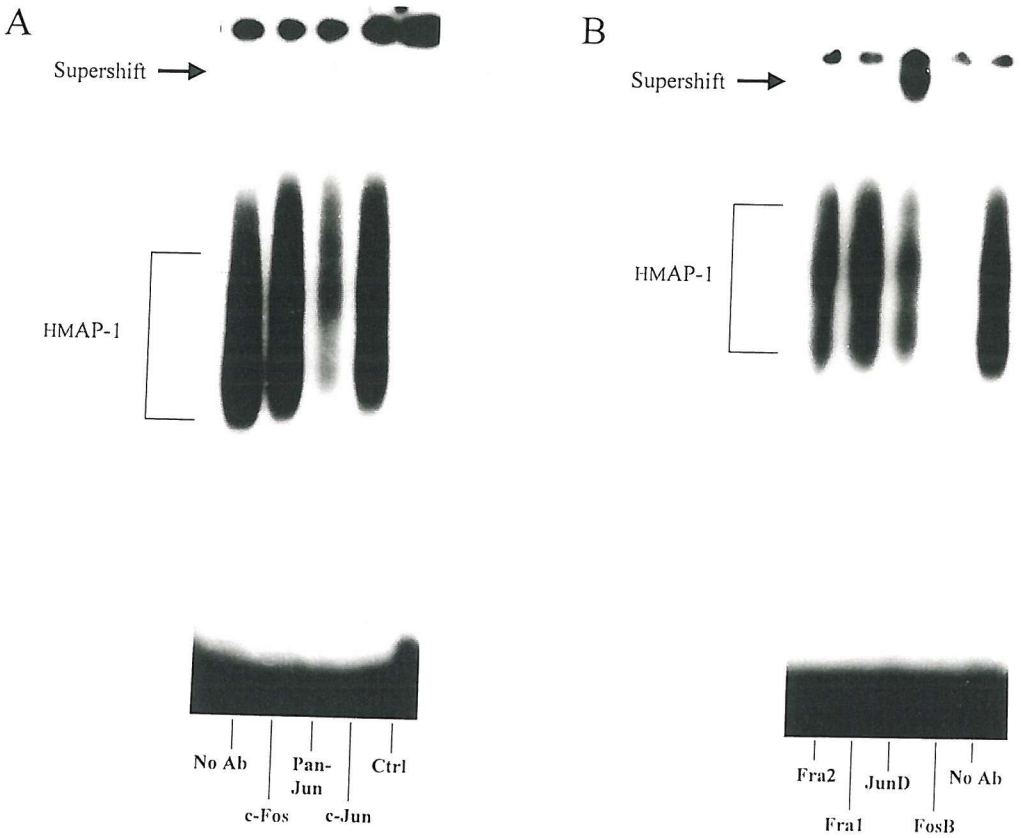


Figure 6.4 (A and B) Supershift EMSAs with 13 day culture activated rat HSC nuclear extracts and antibodies recognising various AP-1 proteins. (Ctrl): Control reaction lacking nuclear extract.

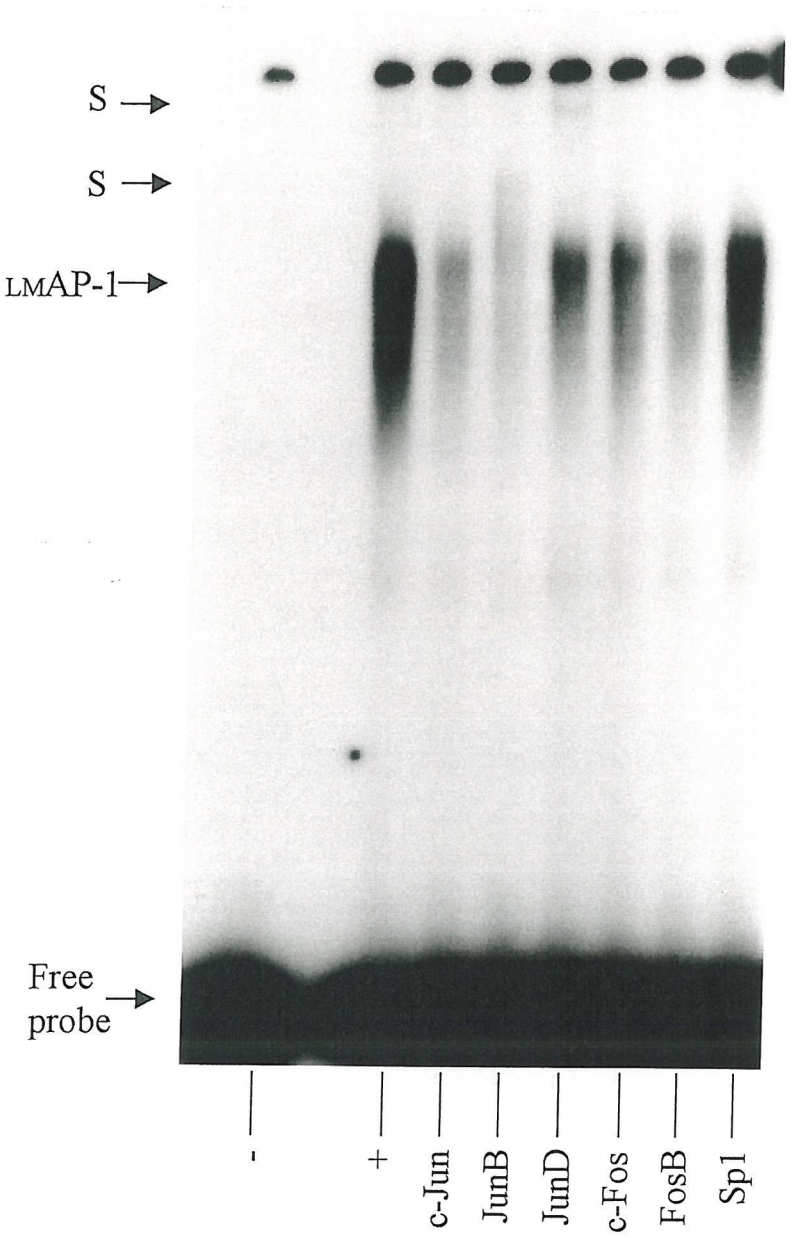


Figure 6.5 Supershift EMSAs with 1 day culture activated rat HSC nuclear extracts and antibodies recognising various AP-1 proteins and Sp1 as a control. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only; (S): Supershift.

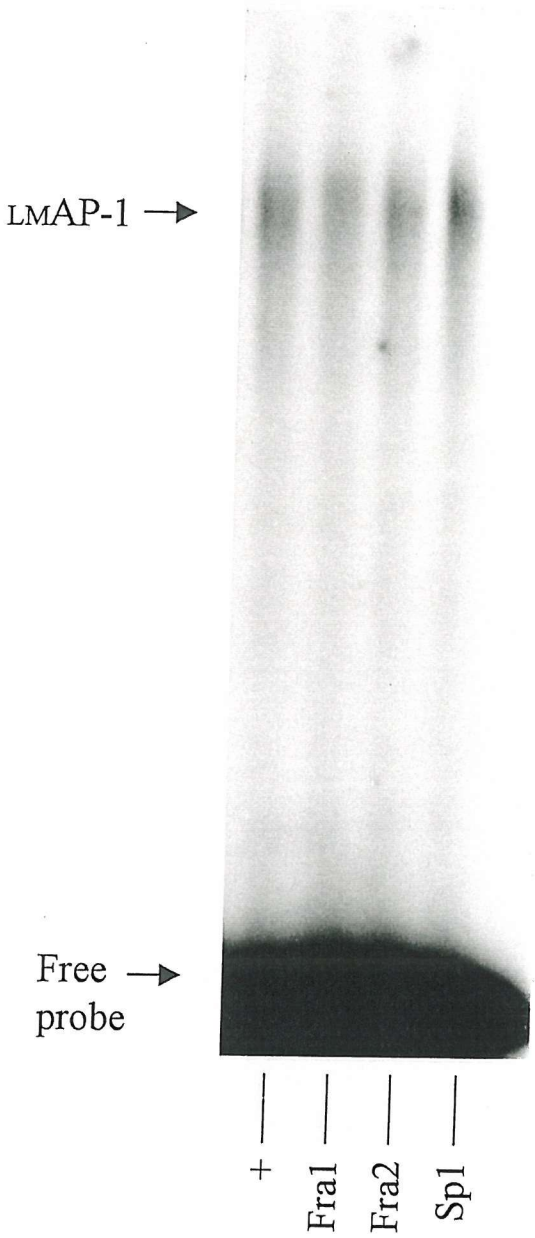


Figure 6.6 Supershift EMSAs with 1 day culture activated rat HSC nuclear extracts and antibodies recognising various AP-1 proteins and Sp1 as a control. (+): Control reaction with nuclear extract only.

6.2.6 EXPRESSION AND CHARACTERISATION OF AP-1 DNA BINDING PROTEINS AFTER CARBON TETRACHLORIDE INDUCED ACTIVATION OF RAT HSCS (*IN VIVO*)

To determine whether *in vivo* activation of rat HSCs was also associated with expression of AP-1 DNA binding activities, freshly isolated HSCs were purified from rats treated with CCl_4 for 48 hours. Nuclear extracts were harvested and AP-1 DNA binding activity was detected using EMSA. These EMSAs were performed using 5μg of protein and 0.4ng of consensus AP-1 probe in a 20μl reaction. Results show that AP-1 DNA binding was induced in freshly isolated HSCs after CCl_4 treatment (Figure 6.7). To further characterise the proteins expressed in these complexes, supershift EMSAs were performed using antibodies (2μg/20μl EMSA reaction) against various AP-1 proteins. Antibodies recognising JunB and JunD consistently resulted in supershifts (Figure 6.8). In addition, slight inhibition of the AP-1 complex was observed with antibodies against Fra1 and Fra2. Western blotting was then used to further examine the expression of the 39kDa protein, JunD, in freshly isolated HSCs from CCl_4 treated and untreated rats. Results show increased expression of JunD in rat HSCs after CCl_4 treatment (Figure 6.9).

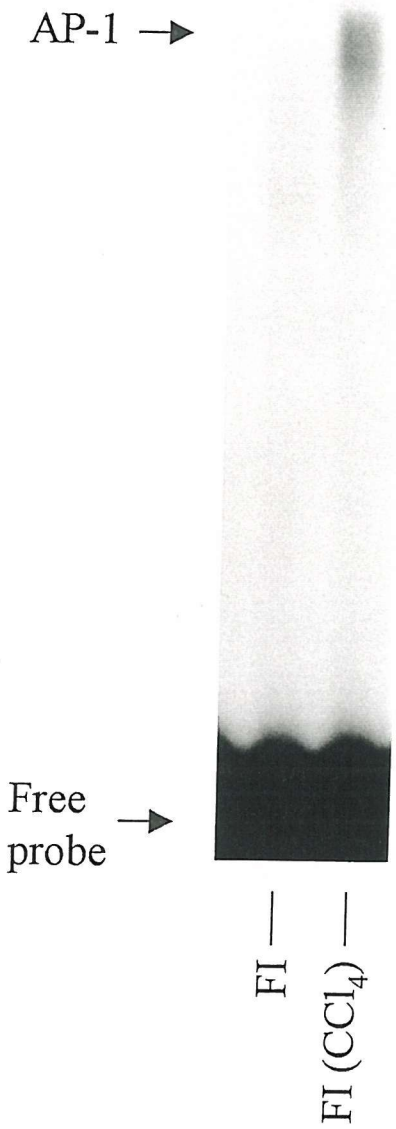


Figure 6.7 Induction of AP-1 DNA binding proteins in the rat HSC after CCl_4 treatment. (FI): Freshly isolated.

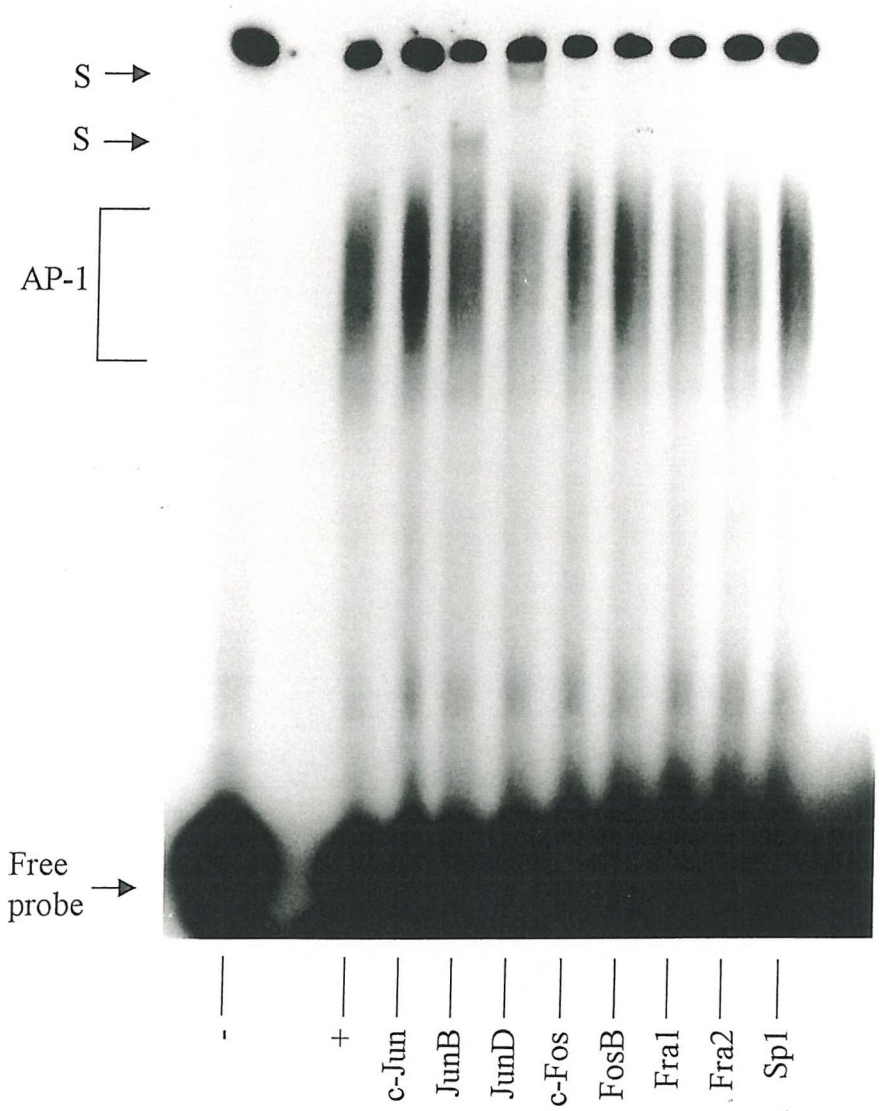


Figure 6.8 Supershift EMSA with freshly isolated HSC nuclear extracts from rats treated with CCl_4 for 48 hours using various AP-1 antibodies and Sp1 as a control. (-): Control reaction lacking nuclear extract; (+): Control reaction with nuclear extract only; (S): Supershift.

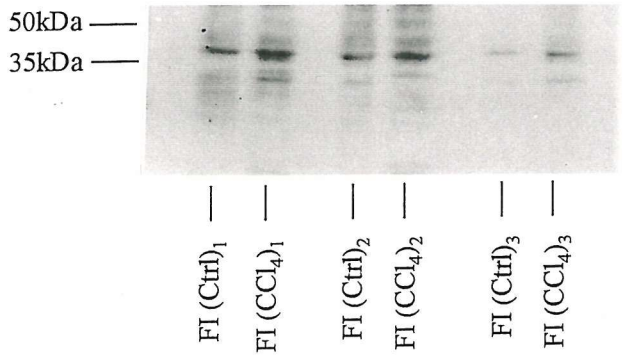


Figure 6.9 Western blot analysis of JunD (39kDa) expression in CCl_4 treated and untreated freshly isolated (FI) rat HSCs. Extracts (15μg) were electrophoresed through a 12% SDS-polyacrylamide gel.

6.3 DISCUSSION

Analysis of AP-1 protein expression during culture activation of rat HSCs has demonstrated transient induction of LMAP-1 binding proteins during early culture of HSCs. Supershift EMSAs have implicated c-Jun, JunB, JunD, c-Fos and FosB as components of these LMAP-1 complexes. Previous studies have suggested that c-Fos and c-Jun are important regulators of TIMP-1 promoter activity (see section 6.2.1). However, additional analysis of these two transcription factors by Western blotting revealed that they are only transiently expressed during early culture of HSCs with similar kinetics as the LMAP-1 complexes detected by EMSA studies. Further culture of HSCs was associated with loss of the LMAP-1 complexes and subsequent assembly of HMAP-1 complexes. Analysis of the HMAP-1 complexes by supershift EMSAs demonstrated its likely components as JunD, Fra2 and FosB. Competition assays demonstrated that both the LMAP-1 and HMAP-1 complexes were sequence-specific with respect to the AP-1 consensus oligonucleotide. Both Western blot and EMSA studies have demonstrated similar results concerning expression of c-Fos, c-Jun, and JunB[335] in 24hour cultured HSCs. In addition, an anti-JunD antibody generated a weak supershift using EMSA techniques and in parallel with this, Western analysis[335] has revealed weak expression of JunD in 24hour HSCs although expression of this protein is significantly increased with further culture. Furthermore, analogous results have been determined for the expression of JunD, Fra2 and FosB in long-term culture activated HSCs when using either Western blot[335] or EMSA studies. However, there have also been disparate observations between the two methods. For example, an anti-FosB antibody reduced levels of LMAP-1 in 24hour cultured HSCs using the EMSA technique whereas according to Western blot studies[335], FosB is only expressed in long-term activated HSCs. Possible reasons for these discrepancies could be due to the following:

1) The detection of proteins in EMSA requires a radiolabelled probe whereas in Western analysis, a chemiluminescence reaction is required to detect protein expression. The EMSA methodology is therefore probably a more sensitive assay when compared to the Western blot technique.
2) The state of the protein differs considerably in the two assays. For example, in EMSA, the protein is in its native state, bound to DNA, and probably other proteins will also

interact with it. In contrast, in Westerns, the protein is fully denatured and immobilised on nitrocellulose. It is possible, therefore, that the antibodies may behave differently in the two systems.

3) The concentration of the antibodies used in EMSA and Western blot techniques also differs. For example, the antibody used in EMSA is highly concentrated and may, therefore, possibly cross-react with other proteins.

Transient induction of c-Fos and c-Jun is coincident with the transient expression of classic AP-1 dependent genes such as collagenase and stromelysin (see section 5.1). In contrast, genes expressed later on in the HSC, such as TIMP-1 and type I collagen, are unlikely to be regulated by these early AP-1 binding proteins. One possible reason for lack of responsiveness of these promoters to c-Fos and c-Jun could be simply due to the absence of additional transcription factors necessary for full transcriptional activity of these genes. For example, studies in F9 embryonic carcinoma (F9) cells have shown that c-Fos and c-Jun can act synergistically with members of the Pea3/Ets transcription factor family to regulate TIMP-1 promoter activity[315]. However, studies in our laboratory have shown that although rat HSCs express Ets-1 and Ets-2, levels of these proteins are relatively low and are difficult to detect in Pea3/Ets DNA binding assays[335]. Absence of TIMP-1 expression in early cultured HSCs may, therefore, be due to inability of the Ets factors to act in synergy with c-Fos and c-Jun. This may be due to lack of an appropriate post-translational modification required for Ets function (e.g. phosphorylation) in early cultured HSCs[394] or alternatively, competition for limiting amounts of Ets proteins. Furthermore, recent studies by Trim *et al*[393] have demonstrated the importance of the regulatory element UTE-1 for TIMP-1 promoter activity and its interaction with a 30kDa nuclear protein (see section 6.1). However, this protein is not expressed in early cultured HSCs and may explain lack of TIMP-1 expression during these early time-points. In addition, another recent study has described a new family of transcription factors, the Smad family. Smads can act as both DNA-binding factors and as transcription factor-binding proteins. Smad3 and Smad4 have been reported to bind directly to all three Jun family members. These studies have shown that Smad3 is critical for the ability of TGF-β to activate AP-1 sites independent of Smad DNA binding, thus suggesting that TGF-β activates AP-1 mediated transcription through the induction of Smad/AP-1 complex formation[316]. Furthermore, studies by Oberthur *et al*

have demonstrated that Smads are expressed in HSCs, and that their protein synthesis is elevated during myofibroblastic transdifferentiation of HSCs[395]. Smads may therefore provide an alternate speculation as to why c-Fos and c-Jun fail to up-regulate genes such as TIMP-1 and type I collagen during early culture of HSCs. Alternatively, another study has shown that BTEB DNA binding activity is up-regulated in activated HSCs and binds to GC-boxes in the collagen α1 (I) gene. This study has speculated that Jun homodimers can transactivate the BTEB gene which may then stimulate transcription of the collagen α1 (I) gene[334]. Again, this would provide an additional explanation for lack of type I collagen during early culture of HSCs. However, in addition to possible lack of appropriate transcription factors necessary for full transcriptional activity of certain genes, it may be possible that other, as yet, unidentified transcription factors or certain AP-1 members (see below) may act to transcriptionally repress these genes during early culture.

The temporal kinetics of the HMAP-1 complexes in HSCs suggests that they are much more likely to regulate genes such as TIMP-1 and type I collagen. In fact, other studies in the laboratory have actually demonstrated that mutation of the AP-1 site in the TIMP-1 gene located at nucleotides –93 to –87 blocked formation of HMAP-1 and dramatically reduced promoter activity[335]. Hence, the HMAP-1 proteins are likely to be important regulators of TIMP-1 promoter function. Previous reports have demonstrated that JunD:c-Fos heterodimers can transactivate the TIMP-1 promoter in F9 cells[315]. However, as c-Fos is not expressed in activated HSCs, JunD may bind either as a homodimer or as a heterodimer with Fra2, FosB or to nuclear proteins belonging to other transcription factor families. Further studies by postgraduate student (David Smart) have involved co-transfection of activated HSCs with the TIMP-1 promoter and various AP-1 expression vectors. Results have demonstrated that JunD has a positive stimulatory effect on the TIMP-1 promoter activity. In contrast, c-Jun and JunB have an inhibitory effect whereas c-Fos, FosB, Fra1 and Fra2 have no significant effects on TIMP-1 promoter activity. The following speculation may provide one possible explanation for the role of these transcription factors in liver fibrosis. During liver injury, nearby HSCs will migrate into the wound, proliferate and secrete extracellular matrix. In order to migrate, the HSCs will express MMPs, such as collagenase and stromelysin. High protein levels of c-Fos and c-Jun may therefore be responsible for expression of these MMPs and for proliferation.

During this initial stage, c-Jun and JunB may have an inhibitory effect on TIMP-1 expression or lack of UTE-1 binding protein may prevent TIMP-1 transcription. However, after the HSCs have migrated to the wound, protein levels of JunD are significantly increased concomitant with increased UTE-1 binding protein and hence may have a positive stimulatory effect on TIMP-1 expression. HSCs may then repair the damaged wound. Further studies are thus needed to fully elucidate the potential functions of these AP-1 binding proteins in the HSC. The different AP-1 complexes formed during culture activation of HSCs are probably due to the different homo- or hetero-dimers forming during transition of HSC activation. Changes in AP-1 activity are regulated both at the level of gene transcription and by post-translational modification of pre-existing AP-1 (see section 1.5.5). Sonobe *et al* have shown, for example, that c-Fos/c-Jun heterodimers have strong stimulatory effects on *fra2* promoter activity in F9 cells[396]. Analogous to these studies, the up-regulation of Fra2 in fully activated rat HSCs may also be due to the initial effects of c-Fos and c-Jun in early cultured rat HSCs. Further research would be necessary to fully decipher the regulatory factors involved in controlling such a complex network of AP-1 binding factors.

Finally, it was decided to examine the expression of AP-1 binding proteins in HSCs from rats treated with CCl_4 for 48 hours (*in vivo*) to see whether their profile was similar to culture activated rat HSCs (*in vitro*). An AP-1 DNA binding activity was induced in freshly isolated HSCs from CCl_4 treated rats. Further analysis of this complex revealed expression of JunB, JunD, Fra1 and Fra2. Thus, there are some similarities between the two models of HSC activation, for example, JunB, JunD and Fra2 have been detected in both systems and are probably therefore very important transcription factors involved in HSC activation. Possible reasons for observing AP-1 proteins *in vivo* that have previously been detected in both early and long-term culture activated HSCs are discussed in section 5.3. However, there are also some differences between the *in vitro* and *in vivo* models. For example, HSCs isolated from rats treated with CCl_4 fail to express AP-1 DNA binding complexes inclusive of c-Jun, c-Fos and FosB which have been detected in HSCs cultured on plastic. Possible reasons for this could be that the *in vitro* model is a very simplified system where purified HSCs are all cultured under identical conditions and all go through the same stages of activation. In contrast, the *in vivo* model is a more complex system where HSCs will be activated at different times, for example, CCl_4 is re-administered every

three days. In addition, various other cells in the liver, such as Kupffer cells, endothelial cells and injured hepatocytes will secrete a variety of cytokines which have been shown to increase the fibrogenic capacity of the HSC (see section 1.1.8). Thus, the *in vivo* model is a more physiological representation of liver injury. It is therefore probably not surprising that there are some differences in the expression of AP-1 proteins observed between the two systems. Further analysis of JunD by Western blotting revealed increased expression of this protein in rat HSCs after CCl_4 treatment, analogous to the observed induction of the AP-1 complex in EMSA studies.

CHAPTER 7

GENERAL DISCUSSION

7.1 SUMMARY

Activation of HSCs is a key step in liver fibrosis and involves the progressive transformation of the quiescent retinoid-storing cell towards a proliferating myofibroblast-like phenotype, this latter cell is responsible for production of the excess extracellular matrix associated with liver fibrosis. The transcriptional control of HSC activation is currently poorly understood but is likely to involve several key factors that help orchestrate the phenotypic transformation. In accordance with this theory, the studies described in this thesis have shed new light on the potential regulation of key genes involved in liver fibrosis by MyoD, Sp1 and AP-1 transcription factors. The nominal findings of this thesis are as follows:

1. Both *in vitro* and *in vivo* models of HSC activation were associated with expression of bHLH protein: DNA binding activities. The expression of these proteins changed as the HSC switched from a quiescent to an activated phenotype. Further studies identified MyoD as the predominant bHLH transcription factor in activated HSCs. EMSA studies using E-box expressing oligonucleotides demonstrated that the nucleotides flanking the E-box motif were important in maintaining specificity of bHLH protein binding.

2. Further analysis of the regulatory factors involved in regulating MyoD: E-box DNA binding revealed the expression of the inhibitory protein, Id1 in quiescent HSCs. Subsequently, the cell-signalling pathways involved in MyoD induction demonstrated the involvement of PI 3-kinase and the transcription factor, NF-κB.

3. Additional studies in both *in vitro* and *in vivo* models of HSC activation demonstrated the expression of GC-box DNA binding proteins. Sp1 was shown to be transiently induced in early cell culture models of HSC activation in contrast to, as yet, uncharacterised GC-box binding activities in long-term culture activated HSCs that were independent of Sp1 expression. Trypsin treatment of HSCs in culture revealed that Sp1 expression could be re-induced. Investigation of cell-signalling pathways involved in the induction of Sp1 demonstrated the involvement of an, as yet, uncharacterised tyrosine kinase.

4. Both *in vitro* and *in vivo* models of HSC activation demonstrated the expression of AP-1 DNA binding proteins. Western blot[335] and EMSA studies demonstrated transient expression of c-Fos, c-Jun and JunB during early culture of HSCs. In contrast, JunD, Fra2 and FosB were up-regulated and sustained in long-term culture activated HSCs. *In vivo* studies revealed that the AP-1 DNA binding activities were slightly different to those observed *in vitro*. These HSCs expressed AP-1 DNA binding activities that were composed of JunB, JunD, Fra1 and Fra2.

Further studies are needed to fully elucidate the potential role of these transcription factors on regulation of genes expressed during liver fibrosis. It has been speculated in this thesis, for example, that MyoD may transcriptionally repress genes necessary for proliferation in order to regulate levels of cell division. In contrast, the transient expression of Sp1 in early cultured HSCs may regulate the transcriptional activation of genes required for proliferation[187]. Furthermore, the GC-box binding proteins detected in long-term culture activated rat HSCs may be important in the regulation of genes expressed later on in the HSC i.e. collagen α1 (I), collagen α2 (I), TGF-β1 and its receptor gene, gelatinase A and TIMP-1. Transient induction of the AP-1 binding proteins, c-Fos and c-Jun, are coincident with the transient expression of classic AP-1 dependent genes such as collagenase[337] and stromelysin[336]. In contrast, expression of JunD, FosB and Fra2 in long-term culture activated HSCs coincides with up-regulated levels of genes such as type I collagen and TIMP-1. Previous studies in our laboratories have shown the importance of the latter AP-1 proteins in the regulation of TIMP-1 promoter activity[335]. Another study, however, has shown that AP-1 binding proteins can transcriptionally activate the GC-box binding protein, BTEB, which can then activate the collagen α1 (I) gene[334]. This study therefore highlights the idea of the transcription factors being interconnected and controlled by one another.

Future therapies for treating liver fibrosis may involve the targeting of certain transcription factors. Drugs, for example, may be used to interfere with these proteins, and thus result in altered gene expression. There are many ways that this may be achieved[397]:

1. Inhibit translation of genes encoding transcription factors by using anti-sense RNA.

2. Use natural cytoplasmic inhibitors, for example, the natural inhibitors of MyoD and NF-κB, could be engineered so that they no longer dissociate from their targets in response to external stimuli. To ensure that the modified inhibitors are only expressed in target tissue, they could be brought under control of tissue-specific promoters.
3. Interfere with post-translational modifications of transcription factors, such as phosphorylation or dephosphorylation. Thus, kinases and phosphatases would be ideal drug targets.
4. Modulate translocation of transcription factors into the nucleus by targeting phospho-proteins involved in this process.
5. Interfere with transcription factor dimerisation and DNA recognition.
6. Interfere with transcription, for example, by using oligonucleotides that can bind to the DNA and form a triple helix and subsequently inhibit transcription.

This thesis has therefore provided further insight into the transcriptional events occurring during HSC activation and hopefully, with further research, will help lead to the development of new therapeutic strategies for treating liver fibrosis, possibly such as those described above.

7.2 FUTURE WORK

MyoD

1. A 174bp fragment of MyoD cDNA in the conserved bHLH domain has been successfully cloned (see section 3.2.9). Further experiments should therefore be aimed at cloning the full- length cDNA. This may be achieved by either RT-PCR as before or alternatively the cloned PCR fragment could be used to screen a cDNA expression library.

2. Further analyse the signalling pathways involved in the induction of MyoD. For example, culture HSCs in the presence of a PKR inhibitor and then look for both MyoD and NF-κB binding using EMSA. If results demonstrate lack of binding for both transcription factors, then it would be interesting to transfect HSCs with PKR cDNA but in the presence of either dominant-negative mutant NF-κB proteins or NF-κB cell-

signalling inhibitors to see whether PKR can activate MyoD expression independent of NF-κB.

3. Investigate the post-transcriptional mechanisms of MyoD expression (see below). An increase in MyoD protein has been observed in activated HSCs. This may be due to various reasons, for example, Hatoum *et al* have demonstrated that MyoD bound to E-box containing DNA is less susceptible to ubiquitin-mediated degradation[359]. Alternatively, an increased rate of synthesis and/or a decreased rate of proteolytic degradation could regulate an elevation of MyoD protein expression (see section 3.3).

Rate of Protein Synthesis

Compare the protein synthesis of MyoD in both freshly isolated and activated HSCs by metabolically labelling proteins with ^{35}S methionine. To keep HSCs in a freshly isolated state during this procedure, culture them on matrigel. After labelling, prepare lysates and normalise by protein assay. Then immunoprecipitate the labelled MyoD with an anti-MyoD antibody and run on an SDS-PAGE gel. MyoD protein levels could then be compared in freshly isolated and activated HSCs by either autoradiography or a phosphoimager.

Rate of Proteolytic Degradation

The above experiments could then be repeated but in the presence of proteolytic inhibitors to see whether the rate of proteolytic degradation has any effect on changes in MyoD protein expression. For example, there may be a higher rate of proteolysis in freshly isolated HSCs.

Effects of the Ubiquitin Pathway on MyoD Degradation

In addition, the above experiments could be repeated but in the presence of both proteolytic and proteasome inhibitors. Western blots could then be performed with these gels using antibodies against ubiquitin to look at its effects on MyoD degradation. Comparisons could be made, for example, between the amount of ubiquitinated MyoD in freshly isolated and activated HSCs. In addition, antibodies against the Id proteins could be used to see whether they do actually conjugate to MyoD.

4. Investigate the functions (*in vitro*) of MyoD in the HSC by interfering with its E-box binding and then look at its effects on the phenotype of the cell (e.g. proliferation, apoptosis, gene expression, matrix production etc.). RNA expression of potential genes regulated by MyoD, for example, could be determined by Northern analysis to see whether expression levels are changed. There are various ways that MyoD binding could be modified:

   a) Repress MyoD expression by transfection of HSCs with anti-sense mRNA, anti-sense oligonucleotides or dominant-negative mutant MyoD proteins. In addition, a MyoD antibody could be microinjected into HSCs and the effects on phenotype could be monitored by time-lapsed photography. Furthermore, Id cDNAs could be transfected into HSCs to further evaluate the effects of these proteins on MyoD expression.
   b) Induce MyoD expression by transfection of HSCs with MyoD cDNA expression vectors.

5. To further evaluate the role of MyoD *in vivo*, MyoD knockout mice could be used. Studies have shown that the abrogation of MyoD alone has no effects on muscle development (see section 1.3.2)[87]. To determine the effects of MyoD depletion on HSC activation, histology and functional assays could be performed before and after CCl_4 treatment.

6. Id knockout mice could be used to examine the effects of Id depletion on HSC activation. Mice with disruption of individual Id genes are essentially normal. However, double knockouts are embryo lethal (see section 1.3.9)[150,369,398,399].

Sp1

1. Characterise the tyrosine kinase involved in induction of Sp1 and further elucidate the signalling pathways.

2. Further characterise the HMGC complexes observed in long-term culture activated HSCs and determine the signalling pathways involved in induction of these proteins.

3. Prevent induction of Sp1 by transfecting HSCs with anti-sense oligonucleotides, anti-sense mRNA or dominant-negative mutant Sp1 proteins. To enhance induction of Sp1 proteins, transfect HSCs with Sp1 cDNA expression vectors. Then look at effects of increasing or decreasing Sp1 on the phenotype of cultured HSCs as described for MyoD.

4. In addition, it would be of interest to investigate the functions of Sp1 proteins *in vivo*. However, Marin *et al* have shown that Sp1-deficient embryos all die around day 11 of gestation (see section 1.4.7)[215]. Alternatively, conditional knockouts or over and under expressive transgenics could be developed.

AP-1

1. Previous studies in our laboratory have shown that the LMAP-1 complexes are induced through a tyrosine kinase-signalling pathway. However, the signalling pathways involved in triggering induction of the HMAP-1 complexes still need to be determined.

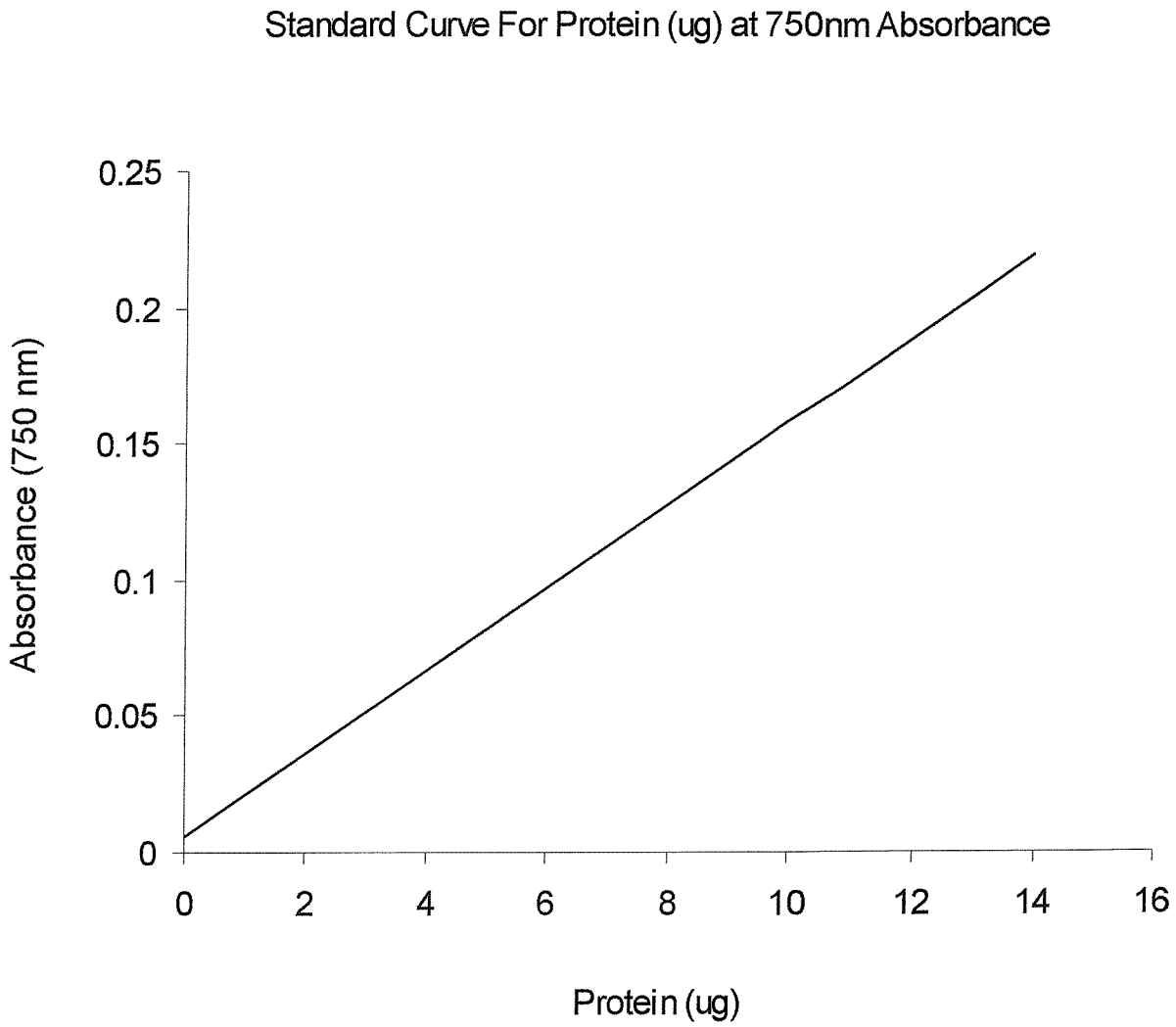
2. Furthermore, it would be interesting to culture HSCs on matrigel to prevent their activation. If AP-1 induction is subsequently blocked this would indicate that disruption of normal HSC: matrix interactions are a trigger. The components of the matrix responsible for this could then be identified. For example, antibodies against integrins/matrix molecules could be used to disrupt interactions on matrigel. HSCs could be cultured on different matrix substrates (i.e. collagens, laminin, fibronectin *etc*) and then AP-1 EMSAs could be performed to monitor effects on transcription. This could also be applied to the MyoD and Sp1 transcription factors.

3. Prevent induction of specific AP-1 proteins by transfecting HSCs with anti-sense oligonucleotides, anti-sense mRNA or dominant-negative mutant Fos/Jun proteins. To enhance induction of AP-1 proteins, transfect HSCs with AP-1 cDNA expression vectors. Then look at effects of increasing or decreasing AP-1 on the phenotype of cultured HSCs as described for MyoD.

4. Investigate the functions of AP-1 proteins *in vivo*. A *c-fos* knockout mouse exists and has both biochemical and phenotypic abnormalities including lack of induction of

collagenase and stromelysin in response to growth factors[323,400]. It would be interesting to see if these mice have a lower susceptibility to liver fibrosis. To determine this, CCl_4 could be administered followed by histology and collagen assays. A *c-jun* knockout mouse however is lethal[326,327]. In contrast, *junD* knockout mice are viable and appear healthy (see section 1.5.6)[328]. These mice could be used to further investigate the role of JunD during HSC activation.

APPENDIX 1


Standard Curve For Protein (ug) at 750nm Absorbance
Absorbance (750 nm)
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
14
16
Protein (ug)

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