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The interaction between Brn-3a POU proteins with LIM-1 proteins and their role on HSV genome

By

ANGELA VERNADAKI

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Department of Biochemistry & Molecular Biology

Faculty of Science

Supervised by Dr. Karen Lillycrop

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ABSTRACT

The repeated recurrence of epithelial lesions following initial infection with Herpes Simplex Virus (HSV) is dependent upon the establishment of asymptomatic latent infections of sensory neurons from which the virus can repeatedly emerge to attack susceptible epithelial cells. The failure of the viral lytic cycle and consequent establishment of latent infections in sensory neurons is due to a lack of viral immediate-early (IE) gene expression following initial infection.

The lytic infection of the viral genome is expressed in three stages, resulting in the sequential transcription of the immediate-early, early and late genes. The temporal regulation of HSV-1 gene expression during permissive infection commences with the induction of the immediate-early genes (IE) by the virion protein VP16. VP16 activates transcription by binding, along with the cellular factors Oct-1 and host cell factor (HCF), to the TAATGARAT (R=purine) elements present in all IE promoters.

IE1 (ICP4) is the major regulatory protein of the virus, and it is necessary for the transition of viral gene transcription from the IE to the early (E) phase.

IE3 (ICP0) is a potent trans-activator of viral and cellular promoters in transient assays, and it provides for efficient viral gene expression and growth, *in vitro* and *in vivo*.

Regarding Oct-2, the single RNA transcript produced from the Oct-2 gene is subject to alternative splicing to produce different forms of the mRNA in B-lymphocytes compared to neuronal cells. The predominantly B cell form Oct-2.1 has a stimulatory effect on gene expression, while the predominant neuronal forms Oct-2.4 and Oct-2.5 have an inhibitory effect on gene expression by repressing promoters such as those of the HSV immediate early genes and the cellular tyrosine hydroxylase gene.

Another family of POU proteins -Brn-3- has also revealed to play a critical role in IE gene expression. Brn-3 family of transcription factors, are the most closely related to the unc-86 within the POU domain. Following the identification of the founder members of the Brn-3 family, known as Brn-3a, two other members of the family have been identified and they are known as Brn-3b and Brn-3c and are expressed at high levels in sensory neurons. The three different Brn-3 factors are encoded by three distinct genes and show only restricted homology to one another outside the POU domain. Those transcription factors have also been shown to bind to several octamer/TAATGARAT-related sequences and to modulate the activity of artificial test promoters containing such proteins.

LIM-HD proteins are a major class of transcriptional activators that cooperate with other activators to direct cellular differentiation. LIM-HD proteins contain a DNA binding homeodomain and two N-terminal zinc-binding LIM domains. Lim-1 is a LIM-HD protein. In previous studies it has been shown that LIM-HD proteins exhibit transcriptional synergy when they interact with basic helix-loop-helix proteins.

In our studies we investigated the role between the Lim-1 protein and Brn-3a/Brn-3c proteins in the IE gene expression of HSV-1.

It was demonstrated that LIM-HD proteins are expressed in SNS and expression is regulated by NGF. Brn-3a, Brn-3c in conjunction with Lim-1 synergise, in the induction of ICPO expression. It was also shown that Isl-1 and Brn-3 proteins interact synergistically and induce ICP0 gene expression. Similar experiments in ICP4 gene expression revealed no induction in gene expression

It was also evident that the induction of the ICPO expression happens via the octamer/TAATGARAT motif when ND7 and BHK cell lines were used. Furthermore, we demonstrated the roles of the two domains of Lim-1 in this interaction with Brn-3 proteins. Results from deletion mutations revealed that both domains of Lim-1 are required for the synergism to happen.

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I dedicate this project to the memory of my father who left me so early. I would like to thank him for always believing in me.

ABBREVIATIONS

a.a amino acid

bp base pair

BHK Baby Hamster Kindey cell line

^{0}C Celcius

C- Carboxyl terminal

cDNA complementary DNA

CAT Chloramphenicol Acetyl Transferase

Da Daltons

dCter LIM-1 mutant lacking the activation domain

dLIM LIM-1 mutant lacking the LIM-HD

DMEM Dulbecco's Modified Eagle Medium

DNA Deoxynucleic acid

DRG Dorsal Root Ganglia

E Early genes

EDTA Ethylene-Diamine-Tris-Acetate

FCS Fetal Calf Serum

HCF Host Cellular Factor

HD Homeodomain

HSV Herpes Simplex Virus

IE Immediate Early genes

Kb Kilobase

Kbp Kilobase pair

KDa KiloDalton

L Late Genes

LB Millers Luria Broth

M Molar

mins minutes

MoMLV-RT Moloney Murine Leukaemia Virus-Reverse Transcriptase

mRNA	messenger ribonucleic acid
N	Amino terminal
NGF	Nerve Growth Factor
Oct	octamer binding protein
OD	Optical Density
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEG	Polyehtylene Glycol
P-LIM	Pituitary LIM homeodomain factor
RNA	Ribonucleic Acid
rpm	revolutions per minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SNAP-25	Synaptosome associated protein of 25Kda
SNS	Sympathetic Nervous System
SSC	Sodium Saline Citrate
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
V	Volts
w/v	weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Herpes Simplex Virus (HSV)

The herpes viruses comprise a very large group of animal viruses each member of which is usually specialised in nature to infect a particular species of mammal, fish, bird, reptile, and amphibian. (Roizman et al.,1992). The three typical characteristics shared by all studied herpes viruses include: a) a particle morphology, b) the possession of a large genome consisting of a single molecule of double stranded DNA ranging in size between 120 and 250 kbp, and c) the ability following productive infection to produce disease, as well as enter a latent phase in some cells of the infected natural host. This latent phase ensures survival of the viral genome throughout the lifetime of the particular infected individual and the ability to renter the productive phase from time to time (Subak-Sharpe et al., 1998).

The morphological structure of the infectious virus has been well studied. Located centrally in the virion is its double stranded DNA genome (McGeoch et al., 1988). The folded DNA molecule lies enclosed in an icosahedral capsid assembly of 162 capsomeres, which are approximately 100nm in diameter and composed of a number of HSV structural proteins. The nucleocapsid and enclosed envelope is embedded in an apparently amorphous matrix called the tegument (Fig. 1.1).

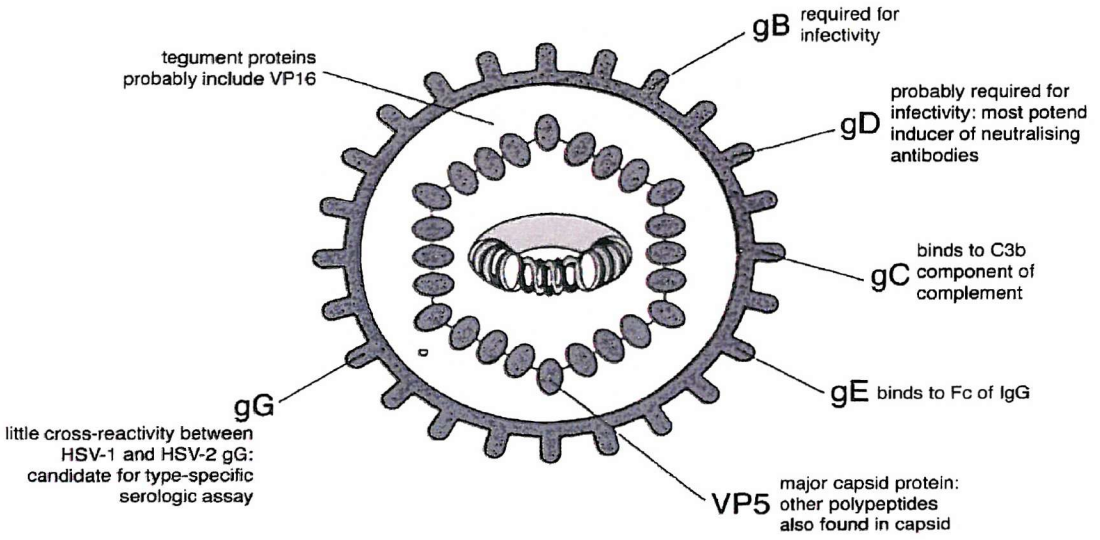


Fig.1.1: Schematic diagram of the HSV virion

Approximately 20 different HSV proteins are known to be constituents of the tegument, some present in major, others in quite minor amounts (McGeoch et a., 1993; Roizman et al., 1993). The outer surface of the tegument is enclosed in the viral envelope, a lipid membrane of host origin decorated with 12 or more different HSV membrane proteins. Embedded in the lipid bilayer of the envelope are five or six viral glycoproteins, which mediate attachment of virus to the cell (Corey and Spear 1986).

1.1.1 HSV Replication

The HSV genome arrangement and recognised gene content was initially defined in terms of open reading frames which were found to be transcribed and expressed (with the apparent exception of the latency-associated transcripts: LATs) (Fraser et al., 1992).

Fig. 1.3 illustrates the HSV life cycle from the initial infection of a cell by a virion to the final release from the infected cell of newly formed virus progeny particles. The

virion, contains HSV genetic material and therefore is capable of infecting another cell to reinitiate the whole cycle (Subak-Sharpe and Dargan 1998).

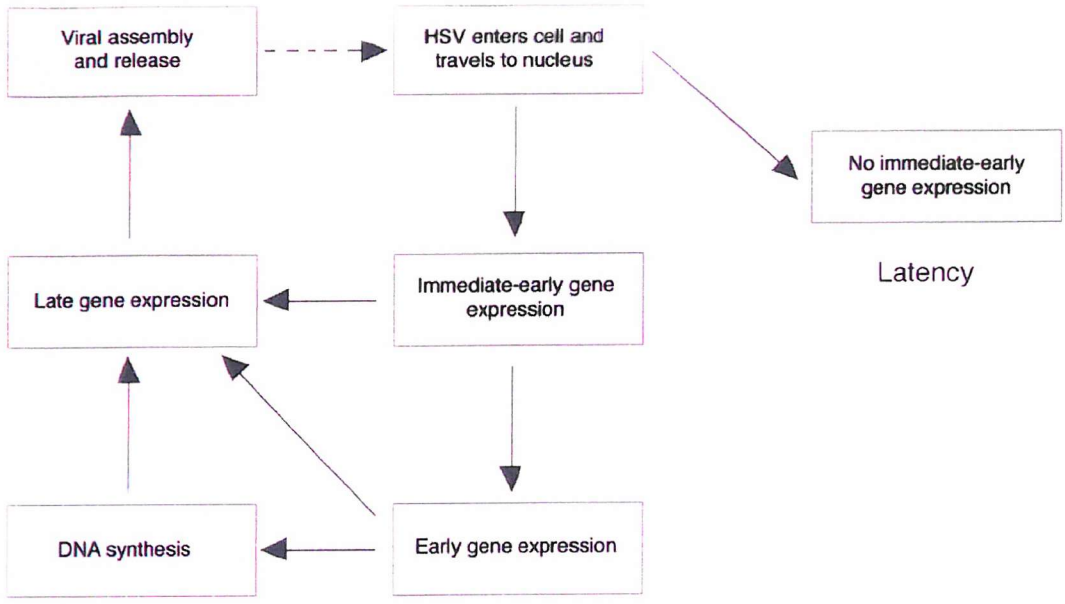


Fig.1.2: Lytic cycle and latency

HSV has a board range of hosts and infect many tissue-culture cell lines that have been derived from vertebrate species. Efficient replication will not always be followed by infection. Infection of permissive cell lines usually results in replication of virions and cell death is due in part to an inhibition of cell macromolecular synthesis. Little is known about the initial steps of viral attachment to the cell surface. Although the wide range of hosts suggests that the receptors for HSV on cell surfaces, are widely distributed, these receptors have not been identified. Some evidence suggests that HSV-1 and HSV-2 bind to different receptors (Vahlne et al., 1979, Vahlne et al., 1980).

After attachment, fusion between the viral envelope and the cell membrane occurs and the nucleocapsid is liberated into the cytoplasm of the cell. This is followed by

controlled disassembly of the nucleocapsid to release the viral DNA. Once the viral genome reaches the nucleus of the cell, expression of viral genes occurs in a highly regulated fashion. The genes designated alpha (or immediate early- IE), are expressed earliest in infection, without any requirement for prior viral protein synthesis. The second class of HSV genes, designated beta (or early- E) requires prior synthesis of an alpha protein but no replication of viral DNA. The beta proteins include regulatory proteins and enzymes required for replication of DNA. The third class of HSV genes is designated gamma (or late-L), and the expression of these genes is dependent upon the replication of viral DNA. Most of the structural proteins specified by the virus are gamma proteins (Fig.1.2).

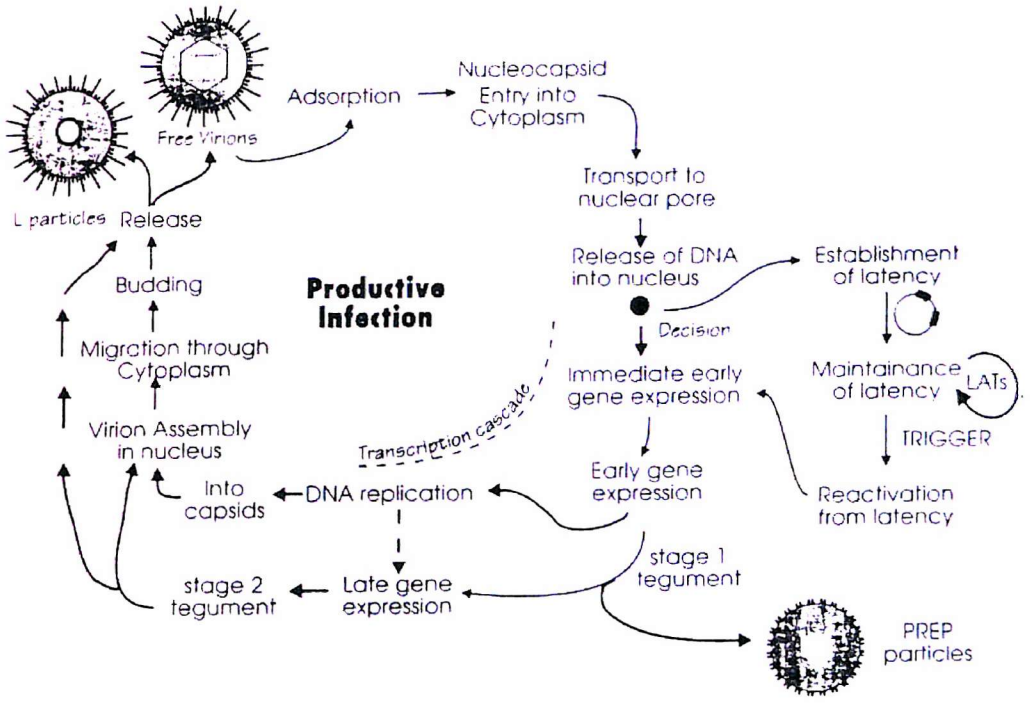


Fig.1.3: HSV life cycle

Once the viral genome has been replicated and structural proteins have been synthesised, nucleocapsids are assembled in the nucleus of the cell. Envelopment

occurs as the nucleocapsids bud through the inner nuclear membrane into the perinuclear space. Virions are then transported through the endoplasmic reticulum and the Golgi apparatus to the cell surface. Viral glycoproteins are found on the surfaces of infected cells as well as in the envelopes of the virions. These glycoproteins carry antigenic determinants that can be recognised in cytolytic immune reactions that can abort the release of infectious virus from cells.

As mentioned above the lytic infection of the viral genome is expressed in three stages, resulting in the sequential transcription of the immediate-early, early and late genes. Therefore, the temporal regulation of herpes simplex virus type 1 (HSV-1) gene expression during permissive infection commences with the induction of the immediate-early (IE) genes by the virion protein VP16. VP16 activates transcription by binding, along with the cellular factors Oct-1 and host cell factor (HCF), to the TAATGARAT (R= purine) elements present in all IE promoters. The products of the first phase of this process are the five immediate-early proteins, ICP0 (or IE3), ICP4 (or IE1), ICP22, ICP27, and ICP47. With the exception of ICP47, these IE proteins are known to have regulatory functions demonstrated to affect the co-ordinated expression of the HSV genome. ICP4 and ICP27 are absolutely essential for virus replication, while the growth of ICP0 mutants is significantly impaired. These three IE proteins have the most profound effects on subsequent viral gene expression.

However, ICP4 is the major regulatory protein of the virus, and it is necessary for the transition of viral gene transcription from the IE to the early (E) phase. It functions as a repressor or activator of transcription by forming multiple contacts with basal transcription factors (Samaniego et a.,1998).

ICP0 is a potent trans-activator of viral and cellular promoters in transient assays, and it provides for efficient viral gene expression and growth, *in vitro* and *in vivo*. ICP0 enhances gene expression, in part by increasing the transcription rates of viral genes, although it is not still clear how it is accomplished. It is possible that those

functions of ICP0 underlie its requirement in lytic viral growth and in the efficient reactivation of the virus from latent infections (Samaniego et al., 1998).

ICP27 regulates the processing of viral and cellular mRNAs. It may modulate the activities of ICP4 and ICP0, as well as, the modification state of ICP4. The combined activities of ICP27 contribute to efficient to late-gene expression. Also recent studies have demonstrated that ICP27 significantly contributes to elevated levels of early-gene expression, and this actually explains its requirement in viral DNA synthesis (Samaniego et al., 1998).

Finally, ICP22 and ICP47 can be deleted from the genome without greatly affecting the viral growth and viability in most cell types. ICP22 promotes efficient late-gene expression in a cell type-dependent manner. The function of ICP47 may be more relevant *in vivo*, where it may help the virus escape immune surveillance (Samaniego et al., 1998).

1.1.2 Latency

Even before the nature of the infectious agent became known, studies suggested an association of recurrences with nerve trauma and, by extension, with the peripheral nervous system (Stevens 1989). After initial infection and many rounds of viral replication at the body surface, the following series of events occurs. The virus ascends by retrograde axonal transport in nerve axons to the cell bodies of the neurons in the sensory ganglia. There, one of two mutually exclusive events occurs: either viral replication with neuronal destruction or establishment of a latent infection and neuronal survival. In cases of neuronal survival, the viral genome is reactivated in later episodes and the genome passes anterograde in axons, crossing from axon to epithelium, where a productive infection and lesions develop. As was the case in the initial infection, the reactivated infection may not produce obvious disease.

The form in which the virus travels has not been firmly established, but there is some ultrastructural evidence that it passes as a nucleocapsid. This mechanism makes sense, since there is now good evidence that infection of cultured cells involves fusion of the viral envelope with the cellular plasma membrane, followed by entry of the nucleocapsid into the cytoplasm. In the case of neurons in vivo, the envelope would be left at the site of entry into the cell, most probably at intraepithelial nerve endings. It is now universally accepted that sensory neurons harbour the latent virus and although these particular neurons have been of greatest interest, the fact that many neural tissues have been shown to harbour latent virus suggests that a variety of neurons can become latently infected (Stevens 1989).

Because of the limited material available from latently infected ganglia, studies on the state of latent viral DNA have concentrated on measuring the levels of specific fragments of the viral genome and in particular on determining the amount of the genome termini detectable compared to that of internal fragments. Fraser and colleagues who used latent infections in the mouse brain stem (pons-medulla) have carried out the majority of those studies as a model system (Rock & Fraser 1983, 1985). Although the latent infections of the CNS differ from those of peripheral ganglia in being very difficult to reactivate (Fraser et al., 1984), insights into the structure of viral DNA obtained in this system have been confirmed with studies of ganglionic DNA in both animal and human systems (Efstathiou et al., 1986).

In those studies, the termini of the viral DNA could not be detected in the latently infected brain stem (Rock & Fraser 1983) or in similarly infected human ganglia (Efstathiou et al., 1986). From these studies then, it can de deducted that unlike the DNA of the infecting virion, the DNA in latently infected cells is not in a simple linear form. More studies also indicated that the termini of the latent genome are joined together to create an internal head -to tail joint fragment and that junctions of viral and cellular DNA cannot be detected.

There is evidence (Deshmane & Fraser 1989) that the viral DNA in latently infected cells is packaged with histones into the nucleosomal structure characteristic of inactive cellular genes. In the brain stem model it seems that despite the transcriptional inactivity, the viral genome is not extensively methylated in the manner seen for many cellular genes that are inactive in a particular cell type. Therefore, the viral genome may be directly regulated by specific cellular transcription factors present in the neuron rather than by blanket long-term repression that shuts down the entire genome.

Viral genetic expression during the latent phase of infection, both in experimental animals and in humans, has been the object of considerable study. This followed the initial observation by Stevens et al. (1987) that a unique transcript (LAT) is synthesised in latently infected murine sensory neurons.

RNA corresponding to one region of the viral DNA was observed in the latently infected cells. This latency associated transcript (LAT) is derived from a region of the genome overlapping that encoding the immediate-early protein, ICP0, but is transcribed in the opposite direction of the ICP0 mRNA, being produced from the complementary strand of DNA to that encoding ICP0. Further studies have identified two LAT transcripts. The major transcript is approximately 2.2 kilobases (kb) in length. Another transcript is a spliced derivative of the first and is about 1.5 kb long (Wagner et al., 1988). As a group, the transcripts are minimally polyadenylated or nonpolyadenylated. In addition to these two transcripts, a third, very minor transcript some 500 bases shorter than the second has been reported and probably represents another splicing variant (Spivak and Fraser 1987).

Following the discovery of the LATs, speculation has centred on their function -if any- in the establishment and maintenance of latent infection. The fact that these transcripts are predominantly nuclear suggests that they do not encode a protein. This conclusion is also supported by the fact that anti bodies raised against synthetic

peptides predicted from the DNA sequence of the gene do not detect a protein in latently infected ganglia .

Theoretically the transcript could function in any of the stages of establishment, of maintenance or of reactivation from the latent state. It was reported in a study (Javier at al., 1988) that a spontaneous deletion mutant of an HSV-1-HSV-2 recombinant virus that did not synthesise the LAT, was able to establish, be maintained in and reactivate from the latent state (reactivation being defined here as induction of viral replication initiated by in vitro cultivation of ganglia from mice). These results indicate that expression of the LAT is not an absolute requirement for completion of any of the phases in the latency cycle.

Other studies employed an HSV-1virus in which the deletion was engineered and gave identical results. Further quantitative studies indicated that the transcripts played absolutely no role in establishment or maintenance of the latent infection in mice (Stevens 1989). These results show that HSV-1 infection may be maintained in neurons in the complete absence of viral genetic expression; no virus-encoded transcripts can be detected in the ganglia of mice infected with these deletion mutants.

The results found with deletion mutants suggested that if the LAT plays a role in the latent state, it does by facilitating reactivation, a phenomenon that may well be different *in vivo* from *in vitro*. During reactivation *in vitro*, mature infectious virus is produced by the neurons. Synthesis of virions in all systems studied results in cell death, which also occurs in reactivating neurons maintained in vitro (Stevens 1978). This destruction of neurons is difficult to reconcile with multiple reactivations involving similar if not identical anatomic sites and no increasing anesthesias over time of the area where lesions appear. As an alternative to replication of virions and neuronal destruction it may be suggested that reactivation in vivo involves expression, in neurons, of a subset of viral genes (with replication of the viral DNA obviously being essential) and that these gene products do not destroy the neuron. The DNA would

then pass intra-axonally to the epithelium, infect epithelial cells by transfection through intraepithelial nerve endings, and initiate a replication cascade, leading ultimately to vesicles. Whatever the physical form taken, it seems almost certain that the viral genome does pass intra-axonally to the neuroepithelial junction, where infection of the body surface follows (Stevens 1989).

Over the years, there have been several attempts to study latency in in vitro systems, and the results obtained must be tempered by the knowledge that latency as it exists in nature may not have been reproduced as yet.

To date, studies have concerned genes involved with establishment of and reactivation from latent infection (Russell et al., 1987). It was found that superinfection at 42^0C with a virus defective in expression of the immediate-early gene ICP4 resulted in the rescue of "latent" virus presumably by complementation, suggesting that ICP4 expression is not necessary for reactivation. In addition, HSV-1 mutants defective in expression of immediate-early genes ICP0 and ICP4 were shown to persist, but the ICP0 mutant could not "reactivate" a virus already present when it was used in superinfection experiments.

The contribution of different systems to an understanding of latency may or may not be a direct one. Such systems may not be particularly informative with respect to latency, but they will certainly contribute to an understanding of viral gene expression in non-permissive cells (Stevens 1989).

It is important therefore to know at which stage the lytic cycle is aborted in latently infected cells and also whether the immediate-early proteins are synthesised in such cells. Studies complicated by the reactivation of the virus resulted in the detection of numerous viral transcripts. Also by using in-situ hybridisation, it was shown that RNAs encoding the immediate-early proteins are not detectable in latently infected trigeminal or spinal ganglia of humans and mice and in the latently infected mouse brain stem. Therefore, the failure of the lytic cycle in latently infected cells is due to

the absence of the immediate-early mRNAs and thus of their corresponding proteins (Latchman 1990).

1.2 POU Domain Proteins- Structure & Function

Studies have shown that there is a complex network of interacting developmental regulators that are expressed in overlapping temporal and spatial arrangements leading to the activation of different regulatory genes, specific for organ development and cell phenotypes (Akam 1987; Ingham1988; Nusslein and Wieschaus 1980; Olson 1990; Scott et al 1989). Many of those regulators encode sequence-specific DNA-binding proteins, presumably transcription factors, which share specific DNA-binding motifs conserved throughout evolution. A gene family that contains a novel DNA-binding motif, which is called **POU domain**, seems to play a critical role in development and provides insights into the mechanisms by which distinct cellular phenotypes emerge during organogenesis (Rosenfeld 1991).

The POU domain was first recognised after comparison of cDNAs encoding three mammalian transcription regulators, **P**it1 (Bodner et al., 1988), **O**ct1 (Sturm et al., 1988), Oct2 (Clerc et al., 1988; Ko et al 1988; Scheidereit et al., 1988), and a *C. Elegans* developmental control gene, ***Unc86*** (Finney et al., 1988), hence its name.

Pit1 and Oct2 are tissue specific transcription factors and are predominantly expressed in pituitary-and lymphoid-cell types, respectively. Oct1 is ubiquitously expressed and activates a number of housekeeping genes. The *unc86* gene product is required for determination of cell fate in sensory neurons in *C. elegans* (Verrijer C., et.al., 1993). Subsequently, studies have identified four new mammalian POU-domain genes, referred to as *brain-1 (Brn-1), Brain-2 (Brn-2), Brain-3 (Brn-3)* and *Testes-1 (Tst-1)*, that are expressed in the nervous system and, in some cases, a limited number of other organs. These new genes, as well as those encoding Pit-1, Oct-1 and Oct-2, are widely expressed in the developing neural tube, and all (but the

Pit-1 encoding gene) exhibit differential, overlapping patterns of restricted expression in the adult brain (He et al., 1989). Many more members of this family have now been identified.

POU domain proteins form a subfamily of the homeodomain proteins since they contain a divergent POU-type homeodomain (60 aa). At the amino-terminal of the POU-type homeodomain there is a second conserved domain, the POU-specific domain (75-82aa). The POU-specific domain and the POU-type homeodomain are connected by a short linker region and together form the150-160 aa POU domain (Fig.1.4). Therefore, POU domain is bipartite in nature.

Based upon the sequence of the linker region and the main basic cluster at the amino-terminus of the POU homeodomain, the POU domain family can be divided into six classes, I-VI. Pit-1 belongs to POU I family, Oct-1 and Oct-2 belong to POU II, Brn-1, Brn-2 and Tst-1 belong to POU III family and Brn-3 and unc-86 belong to POU IV family (He et al 1989). Although the POU-specific domain is a unique feature of this family of transcription factors, the POU homeodomain is unambiguously related to the DNA-binding homeodomain of various developmental regulators first described in *Drosophila*. Therefore, the variant POU homeodomain is composed of three α-helices. The first two are relatively variable between members of the gene family, whereas the third helix is highly conserved. It is predicted that this third helix is the one that actually contacts the major groove of DNA directly and has therefore been termed the recognition helix (Wegner et al., 1993).

Classic homeodomain proteins generally bind to short A/T-rich sequences with a TAAT core. On the other hand, POU-domain proteins seem to recognise longer DNA motifs, which in the case of class II members Oct-1 and Oct-2 resemble the octamer ATGCAAAT, and in the case of class I member Pit-1 resemble the related motif ATGNATAT. The interaction of POU-domain proteins with DNA does not merely happen to bases within the binding motif. Flanking sequences are also involved, therefore a degree of degeneracy within the DNA-binding motif is allowed. The

divergent POU homeodomain on its own cannot guarantee high affinity binding of the POU-domain protein to its target sites. Therefore, the addition of the POU specific domain is required.

In the case of Oct-1, major groove contacts with the 5' half of the octamer motif are provided by the POU-specific domain, whereas major groove contacts with the 3' half are established by the POU-homeodomain. Similar contacts are observed for Pit-1 binding to its target sites. Multidimensional nuclear magnetic resonance spectroscopy, shown that the POU-specific domain has a structure that consists of four α-helices grouped around a hydrophobic core (Fig.1.4).

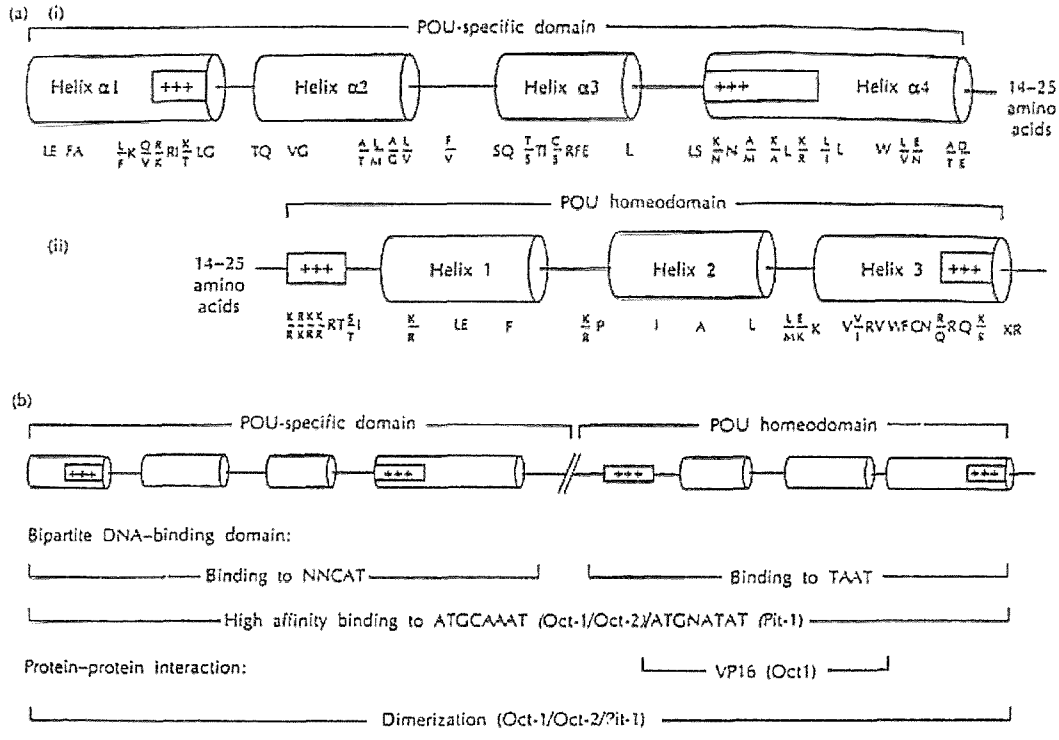


Fig.1.4: Structure of the POU domain

These helices form a helix-turn-helix motif similar to the one found in the DNA-binding domain of the bacteriophage λ-repressor. Comparison with bacteriophage λ-repressor suggests that helix α3 of the POU-specific domain contacts the major groove of DNA in a manner similar to helix 3 of the POU homeodomain. Because of the bipartite nature of the DNA-binding POU domain, the binding sites for POU domain and classic homeodomain proteins are essentially non-overlapping, avoiding possible functional interference between both groups of developmental regulators (Wegner et al., 1993).

1.2.1. Oct-1 transcription factor and its role in HSV

The POU domain as well as acting as a DNA-binding domain can also provide an interface for functionally important interaction with other proteins. One of the most characteristic examples of such an interaction is the activation of the immediate early genes of Herpes Simplex Virus (HSV) by Oct-1. Oct-1 is recruited by the viral promoter as part of a complex that contains, Oct-1, the multisubunit host cell factor HCF/C1 and the virus-encoded VP16. In this complex, the Oct-1 POU domain directly contacts the HSV transactivator VP16 (Wegner et al., 1993), (Fig.1.5a-i).

(a) (i)

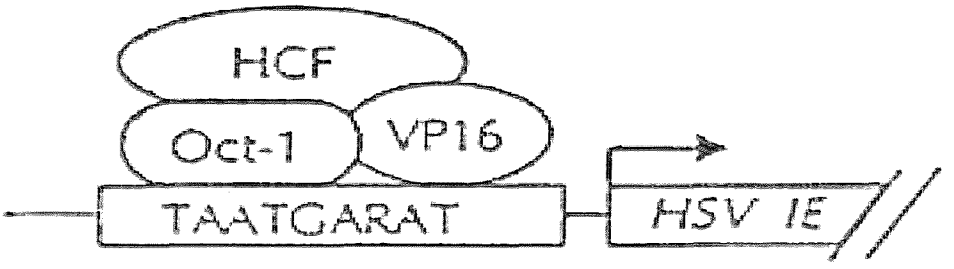


Fig.1.5a-i: The herpes simplex virus (HSV) VP16, Oct-1 and the host cell factor, HCF, form a complex that activates transcription of the viral immediate early (HSV-IE) genes.

The specific interaction has been mapped to specific amino acids within the first two helices of the POU homeodomain. Oct-1 and VP16 are in direct contact with DNA. In order to modifying the site recognised by Oct-1, VP16 supplies its strong acidic transactivation domain. This domain is much more potent than the glutamine-rich transcriptional activation domain present in the amino-terminal part of Oct-1, which is only capable of activating a subset of octamer containing promoters. There are many lymphoid-specific promoters that cannot be activated by Oct-1 alone. These promoters require the B cell specific co-activator OCA-B as well (Wegner et al., 1993).

Analysis of positive control mutations in Oct-1 has been aided by the existence of its close relative Oct-2, which displays the same DNA-binding specificity but fails to respond to VP16 in vivo, or to associate with VP16 in vitro. By being able to compare and contrast Oct-1, Oct-2 and their interaction with VP16 this gives the opportunity to investigate how homeodomains can affect differential positive control while recognising the same DNA sequence. It was found that a single amino acid was the difference between the Oct-1 and Oct-2 domains (a glutamic acid for alanine

difference), and this difference is enough to direct the differential transcriptional response of two homeodomain proteins to a VP-16 regulatory co-factor (Lai et al., 1992).

1.2.2 Oct-2 and its role in IE gene expression

The octamer motif (consensus ATGCAAATNA) is found in the promoters of a number of cellular genes, including those encoding histone H2B, the immunoglobulins and the small nuclear RNA's. The motif binds to different transcription factors, including Oct-1 and Oct-2, and plays a crucial role in the expression of cellular genes that contain it.

The upstream regions of the HSV immediate-early genes contain multiple copies of the sequence TAATGARAT that is essential for their transcativation by the virion protein VP16. As shown in Fig.1.6, this sequence is related to the octamer consensus sequence, the right-hand half of the octamer motif showing close sequence similarity with the left-hand half of the TAATGARAT element. This similarity allows Oct-1 and Oct-2 to exhibit weak binding to the TAATGARAT element.

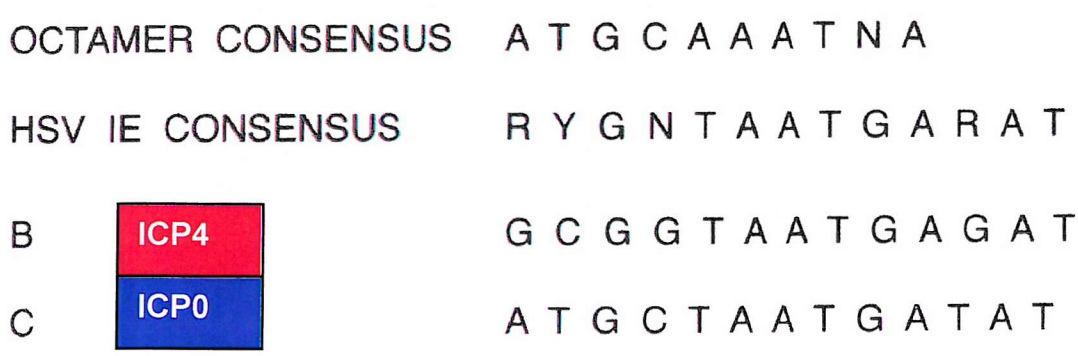


Fig.1.6: Comparison of the octamer motif consensus and the viral TAATGARAT consensus

Different studies shown that when specific bases of either the octamer or TAATGARAT were destroyed by mutation, each of the individual elements could bind a single molecule of octamer-binding protein. A complex of identical mobility is obtained in DNA-mobility-shift assays carried out with the individual elements and the composite motif. Also, the composite motif binds only a single molecule of octamer-binding protein as the individual elements do, and maybe this is due to the fact that the central six bases (TAATGA) are common to both motifs and cause steric hindrance to the binding of two molecules to the overlapping motifs.

As mentioned before the binding to the composite motif is of high affinity. However, mutations in either the octamer or the TAATGARAT portions dramatically decrease binding affinity or the levels of gene expression driven by the motif. This high-affinity model though, raises questions like why the occurences of these motifs in the HSV-1 IE1 promoter are more complicated, since cellular genes have simple octamer motifs and the other HSV immediate-early genes have simple TAATGARAT motifs (Dent and Latchman 1991).

Expression of IE1 also appears to be critically important for re-activation from latent infection. Therefore, viral mutants lacking this gene fail to re-activate both in latency models *in vitro* and after latent infection of animals. Hence the overlapping octamer/TAATGARAT motif may represent a response to the need for the IE1 gene to be expressed at high levels in both the presence and the absence of VP16.

However, whatever its precise role, it is clear that this motif represents a high-affinity binding site for cellular octamer-binding proteins, and offers a unique opportunity to study a composite motif, each half of which can bind the same transcription factor under different conditions (Dent and Latchman 1991).

1.2.3 The overlapping octamer/TAATGARAT motif

Oct-2 was originally thought to be exclusively expressed in B lymphocytes where it was shown to bind to and activate the immunoglobulin heavy chain promoter and never Oct-2 transcripts have since been detected in neuronal cells. A single RNA transcript produced from the Oct-2 gene is subject to alternative splicing to produce distinct forms of the mRNA in B lymphocytes compared to neuronal cells. The different forms produced in B cells and neuronal cells have distinct effects on gene expression. The predominant B cell form Oct-2.1 has a stimulatory effect on gene expression, while the predominant neuronal forms Oct-2.4 and Oct-2.5 have an inhibitory effect on gene expression (Latchman 1996). In co-transfection assays, both Oct-2,4 and Oct-2,5 were able to repress IE genes only, whereas Oct-2,1 activated IE gene expression. These observations explain the differences between different observations suggesting that an Oct-2 binding octamer motif stimulated gene expression in B cells but inhibited gene expression in neuronal cells (Latchman 1996).

The difference in activity between Oct-2.1 and Oct-2.4 or Oct-2.5 arises from the fact that the alternative splicing event results in Oct-2.4 and Oct-2.5 lacking the strong C-terminal activation domain that is present in Oct-2.1. However all three isoforms contain a transcriptional inhibitory domain which is located at the N-terminus of the molecule. The strong C-terminal activation domain of Oct-2.1 overcomes the effects of the N-terminal inhibitory domain resulting in activation of transcription. On the other hand, in Oct-2.4 and Oct-2.5 where the C-terminal activation domain is absent, the N-terminal domain is able to repress transcription (Latchman 1996).

By using deletion analysis, it was shown that the inhibitory domain comprises the 40 amino acids from 142 to 181 of the Oct-2 molecule. This region constitutes a separable inhibitory domain which, when linked to the DNA binding domain of the yeast GAL4 transcription factor can repress promoters containing GAL4 binding sites. It is thought that the Oct-2 inhibitory domain acts by interacting with the basal transcriptional complex to inhibit its activity (Latchman 1996).

These findings suggest that the presence of Oct-2.4 and Oct-2.5 in neuronal cells might be responsible for the inhibition of HSV IE gene expression following initial infection of neuronal cells leading to the observed failure of the lytic cycle. This hypothesis is supported by the observation that ectopic repression of Oct-2,4 or Oct-2,5 in normally permissive cells blocked IE gene expression and inhibited the HSV lytic cycle (Fig.1.7), (Lillycrop et al., 1994).

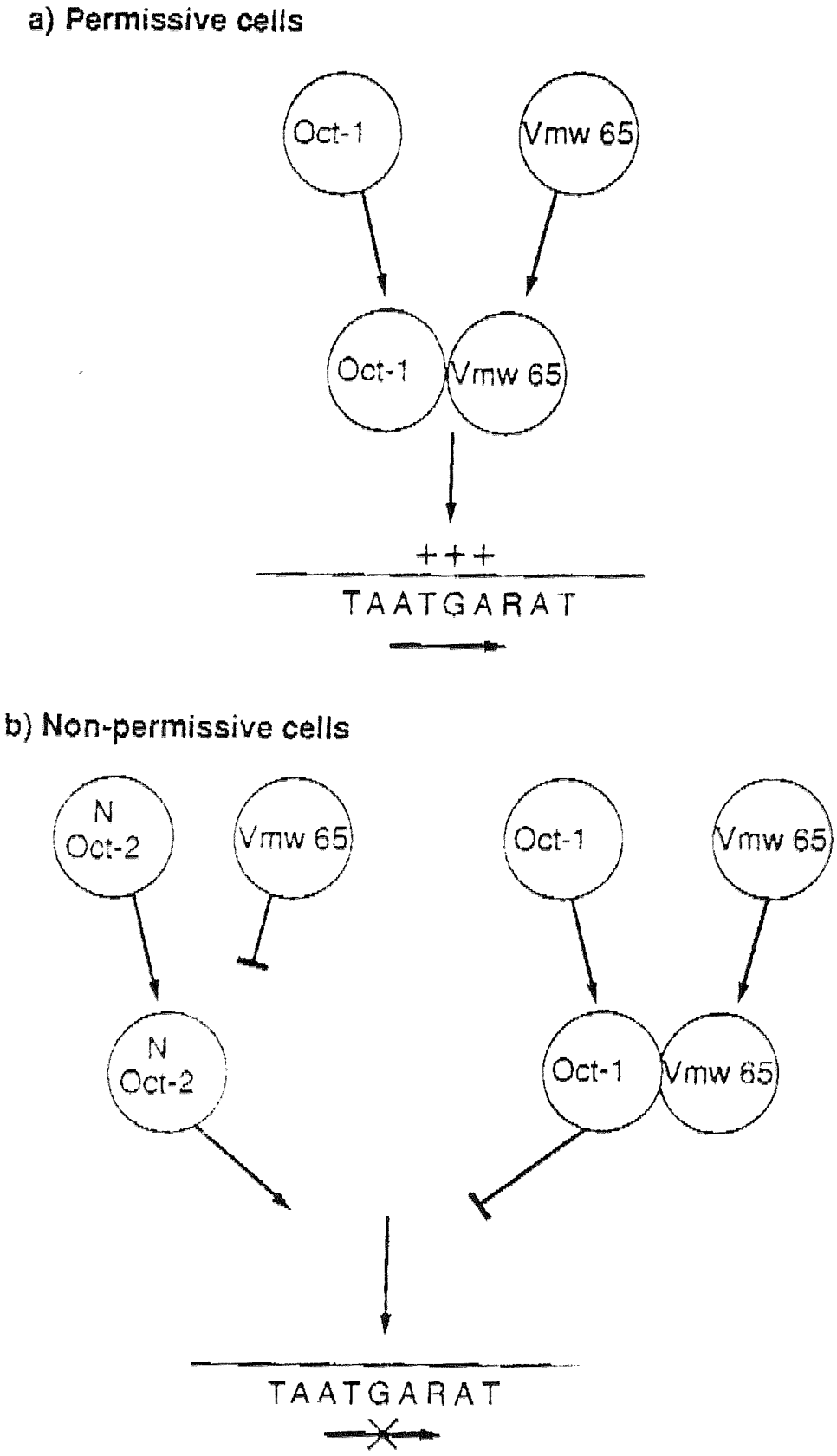


Fig.1.7: Ectopic repression of Oct-2,4 or Oct-2,5 in normally permissive cells blocked IE gene expression and inhibited the HSV lytic cycle.

1.3 Brain-3 POU family transcription factors

In addition to Oct-1 and Oct-2, sensory neurons have also been shown to express the Brn-3 subfamily of POU domain proteins.

Among the mammalian POU proteins, the Brn-3 family of transcription factors are the homologs of *unc-86* within the POU domain. Following the identification of the founder members of the Brn-3 family, known as Brn-3a (or Brn-3.0), two other members of the family have been identified and they are known as Brn-3b (or Brn-3.2), and Brn-3c (or Brn-3.1). The three different Brn-3 factors are encoded by three distinct genes and show only restricted homology to one another outside the POU domain and are expressed at high levels in sensory neurons in DRG, trigeminal and spinal ganglia as well as in sensory nuclei in the brain stem (Lillycrop et al., 1992).

Earlier work has shown that targeted deletion of the Brn-3b and Brn-3c genes produce, respectively, defects in the retina and the inner ear. Targeted deletion of the Brn-3a gene results in defective suckling and in uncoordinated limb and trunk movements, leading to early postnatal death. Brn-3a (-/-) mice show a loss of neurons in the trigeminal ganglia, the medial habenula, the red nucleus, and the caudal region of the inferior olivary nucleus but not in the retina and DRG. In the trigeminal and DRG, but not in the retina, there is a marked decrease in the frequency of neurons expressing Brn-3b and Brn-3c, suggesting that Brn-3a positively regulates Brn-3b and Brn-3c expression in somatosensory neurons. Thus, Brn-3a exerts its major developmental effects in somatosensory neurons and in brainstem nuclei involved in motor control. The phenotypes of Brn-3a, Brn-3b and Brn-3c mutant mice indicate that individual Brn-3 genes have evolved to control development in the auditory, visual, or somatosensory systems and that despite

differences between these systems in transduction mechanisms, sensory organ structures, and central information processing, there may be fundamental homologies in the genetic regulatory events that control their development (Xiang, et. al., 1996)

The role of Brn-3 transcription factors in sensory neurons has been studied extensively using the ND7 cell line, which was obtained by fusing primary dorsal root ganglion (DRG) neurons with C1300 neuroblastoma cells and which retain many of the characteristics of sensory neurons (Wood et al., 1990). These cells proliferate indefinitely in serum-containing medium. Removing of the serum or treatment with cyclic AMP ceases dividing and the cells differentiate to a mature neuronal phenotype bearing long neurite processes.

During the process of differentiation, changes occur to the Brn-3 family of POU domain transcription factors. The level of Brn-3a increases from very low in the proliferating ND7 cells to high in the differentiated cells. Also, the levels of the closely related Brn-3b factor fall from high in the proliferating cells to low in the undifferentiated cells, while the levels of Brn-3c remain unchanged (Smith et al., 1997).

However, although Brn-3a and Brn-3b are closely related to one another, they have antagonistic effects on the activities of specific gene promoters. These findings raise the possibility that the rise in Brn-3a and fall in Brn-3b expression that occurs upon ND7 differentiation plays a crucial role in the differentiation process. Specific genes whose protein products are required for differentiation can be activated by the enhanced expression of the activating Brn-3a factor and a fall in the level of the Brn-3b repressor (Smith et al., 1997).

Furthermore it has been shown that members of the Brn-3 subfamily can modulate IE gene expression. Theil et al (1994) showed that Brn-3a could bind with low affinity to various octamer-related motifsincluding overlapping with an octamer/TAATGARAT motif from the IE1 promoter. Moreover Brn-3a and Brn-3c were reported to weakly

activate IE3 promoter while Brn-3b inhibited expression. Interestingly however in co-transfection assays, neither Brn3a or Brn-3c were able to relieve Oct-2,4 /Oct-2,5 repression of IE gene expression.

This data suggests that Brn-3 proteins are unlikely to act as sole transactivators of IE gene expression *in vivo*, and may require additional proteins to bind with high affinity and facilitate gene expression.

Interestingly the *C. Elegans* POU domain protein unc-86 , has been shown to co-operatively interact with a LIM-homeodomain protein mec-3, and this interaction is vital for unc-86 function. As the Brn-3 proteins are orthologs of unc-86 and LIM-HD proteins have been shown to be evolutionary conserved, this raises the possibility that Brn-3 proteins like unc-86, may function as heterodimers with LIM-HD proteins to influence gene expression, but whether they can influence IE gene expression in the absence or presence of LIM-HD proteins remains to be determined.

1.4 LIM- proteins

The LIM domain contains a cysteine-rich motif that was first identified in the protein products of three genes, *Lin-11* (Freyd et al., 1990), from *Caenorbabditis elegans*, *Isl-1* (Karlsson et al., 1990) from rat, and ***M**ec-3* (Way et al., 1988) from *C.elegans*. Two main classes of LIM proteins are known. One class contains proteins that, like the founder members have two LIM domains in association with a homeodomain, and these are designated as LIM-HD proteins. The second class has no homeodomain, but contains only LIM domains and these are designated as LIM-only proteins (Sanchez-Garcia and Rabbitts 1994). Another recently described protein has two LIM domains linked to a protein kinase domain and most probably represents a third type of LIM protein (Mizuno et al., 1994), (Fig.1.8).

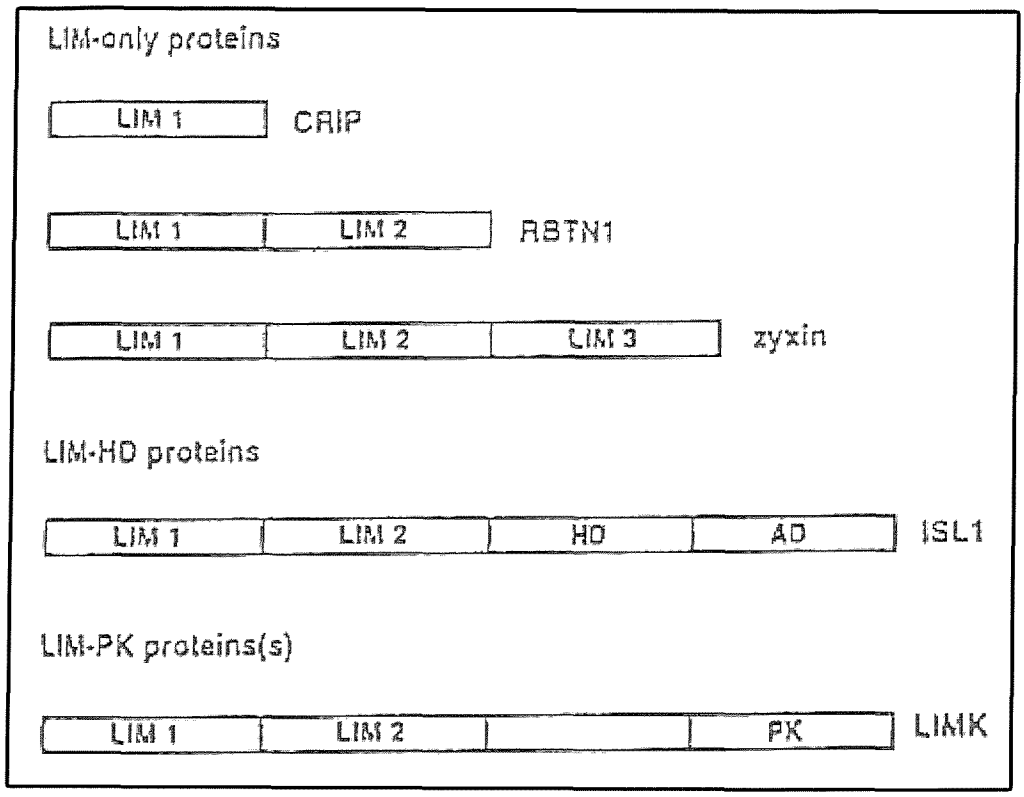


Fig.1.8: The structural variation of LIM proteins

The LIM domain has been found in more than 18 proteins and the sequences of LIM domains are highly conserved between proteins found in very different tissues and across a wide range of species.

LIM-HD proteins have been revealed either by mutational or by direct biochemical analysis, in the control of differentiation of specific cell types. The *Lin-11* gene encodes a protein that controls certain asymmetric cell divisions during vulval development in *C.elegans*. The rat *Isl-1* gene is expressed in developing and mature islet cells, and encodes a LIM-HD protein that binds to the enhancer of the insulin gene and is probably involved in the cell specific expression of this gene. There is

also increase evidence that Isl-1 may be involved in establishing motoneurone fate in the developing brain. *Mec-3* is required for the differentiation of certain C.elegans mechanosensory neurons (Sanchez-Garcia and Rabbitts 1994).

The LIM domain is a specialised double-zinc finger motif. LIM domains interact specifically with other LIM domains and with many different protein domains. LIM domains are thought to function as protein interaction modules by mediating specific contacts between members of functional complexes and modulating the activity of some of the constituent proteins. Nucleic acid binding by LIM domains, while suggested by structural considerations, remains an unproven possibility. Many LIM proteins are associated with the cytoskeleton, having a role in adhesion-plaque and actin-microfilament organisation. Among nuclear LIM proteins, the LIM homeodomain form a major subfamily with important functions in cell lineage determination and pattern formation during animal development (Dawid et al., 1998).

The N-terminal LIM domains of the nuclear LIM homeodomain and LIM-only proteins are closely related. The convention of numbering Lim domains by their location in proteins from N to C termini has been adopted, i.e., LIM1 or LIM2 are more similar than LIM domains found at different positions in a single protein (Dawid et al., 1995). The most N terminal LIM domains equal LIM1. The nomenclature is still being developed but by convection the nuclear-LIM only proteins are now designated LMO (Greenberg et al., 1990). No convection has yet been adopted for the extra-nuclear LIM-only proteins such as cysteine-rich protein (CRP), CRIP and PINCH (Rearden 1994).

1.4.1 LIM-HD Proteins (or LHX)

LIM-HD genes act in a wide variety of developmental contexts and display functional similarities in organisms that are as distantly related as nematodes, arthropods and vertebrates. All LIM-HD genes have expression domains in the nervous system, and the analysis of the LIM-HD mutant animals has revealed a neural function for each individual LIM-HD gene examined. These functional studies indicate that LIM-HD genes confer subtype-specific cell identities in the developing embryo. There is no indication that they are involved in the determination of pan-neural features. Rather, most LIM-HD genes appear to be active at a step in neural development when cells leave the cell cycle and begin to express determinants that convey specific identities (Hobert and Westphal, 2000). This is possibly best demonstrated in *C. elegans*. Based on complete genome sequence data, *C. elegans* encodes a total of seven LIM-HD genes, six of which have been functionally characterised. Each of these fulfills a neural regulatory role and each is expressed in postmitotic neurons. Moreover, LIM-HD gene function is required to determine the correct axonal arrangement of diverse groups of LIM-HD -expressing neurons, such as sensory, motor and interneurons. These observations suggest that individual LIM-HD group members in *C. elegans* share overlapping functions in distinct cell types and might regulate common sets of downstream target genes. Conversely, the regulation of touch-neuron-specific genes by mec-3 (Duggan et al., 1998), or a GABA ergic marker and a sensory-neuron-specific receptor by lim-6, also point to highly specialised functions of LIM-HD genes in *C. elegans* (Hobert et al., 1999).

In more complex nervous systems, such as those of flies and vertebrates, LIM-HD-encoding genes appear to affect terminal differentiation at a more fundamental level because manipulations of LIM-HD gene expression convey distinct identities to neurons. This has been well illustrated by studies that examined the role of members

of the Lim-3 and Islet group of LIM-HD encoding genes in motor neuron development. Results suggest that LIM-HD encoding genes are required in a relatively short time window to affect neural subtype identities in a rather global way (Hobert and Westphal, 2000).

LIM-HD proteins are highly conserved. For example, a high level of conservation appears to exist in interneurons, which express non-overlapping sets of LIM-HD proteins in vertebrates and invertebrates. Human LHX2 (Rincon-Limas et al., 1999) appears to rescue the mutant phenotypes of *Drosophila apterous* completely, suggesting a conservation of LIM-HD function in interneurons.

During the course of evolution, LIM-HD gene duplication led to the diversification not only of the coding region of the duplicated genes but also, and most notably, of the regulatory sequences surrounding these genes. For example, the function of the vertebrate Lhx2 gene in erythroid development serves as a co-option in a new context, as *C. elegans* does not contain a hematopoietic system.

LIM-HD proteins appear to harbor a significant discriminatory power of DNA binding in vitro. For example, rat Isl-1, mouse Lhx2 and hamster Lhx1a were colned as sequence specific enhancer binding proteins (Karllson et al., 1990; German et al., 1992; Roberson et al., 1994). Nevertheless, it is likely that in vivo DNA-binding specificity is modulated by the interaction with transcriptional regulators. With the exception of the POU protein UNC-86 and the LIM-HD protein MEC-3 whose interaction is not dependent on LIM homeodomains (Xue et al., 1993), all LIM-HD interactions reported so far depend on the LIM domains of LIM-HD proteins. LIM domain-dependent interactions with co-factors can be classified into several types, including direct interactions between two different transcription factors or indirect interactions that are mediated by members of the Ldb co-factor family. The intrinsic dimerization capacity of Ldb allows LIM-HD proteins to ineract with distinct transcriptional factors, such as Otx proteins (Bach et al., 1997) and allows any given

LIM-HD to form homomeric and heteromeric complexes with other LIM-HD proteins (Jurata at al., 1998).

Another example is the LIN-11 group of LIM-HD proteins that has invertebrate members detected in *Drosophila* and *C.elegans*. The *Drosophila* gene *bk87* was identified in a DNA-target-binding screen (Kalionis and O'Farrell 1993). The *C. elegans* genes lin-11 and mec-3 the founder members of LIM-HD gene family, are both required for the terminal differentiation of a subset of specific, non-overlapping sensory, motor neurons and interneurons (Hobert and Westphal 2000).

The Lhx1 and Lhx5 proteins (also known as Lim1 and Lim5) are the two known vertebrate members of the LIN-11 group. Gain of function experiments using *Xenopus* embryos strongly suggests that the Lhx1 gene functions in neural induction. In mouse mutant the lack of Lhx1 gene functions, head formation is severely compromised (Hobert and Westphal 2000). However, because Lhx1 null mutant embryos die at mid-gestation, an analysis of Lhx1 function at later stages of neural development has been precluded. The closely related Lhx5 gene is similarly expressed during gastrulation (Hobert and Westphal 2000).

The examples are endless and cover almost all the LHX gene family. In general all the studies have revealed a prominent involvement of LIM-HD proteins in tissue patterning and differentiation, and their function in neural patterning is evident in all organisms studied to date.

1.5 Aim of the project

Our overall aim was to:

- Determine which LIM-HD proteins are expressed in SNS.
- Investigate the role of Brn-3 proteins in HSV gene expression.
- Investigate whether Brn-3 interacts with LIM-HD proteins

CHAPTER 2

MATERIALS & METHODS

2.1 Laboratory chemicals

The laboratory chemicals were purchased from Sigma U.K. and solvents were purchased from Fisher. Unless otherwise stated, tissue culture grade products were purchased from GIBCO BRL. Radiochemicals and membranes (Hybond-C/N) were purchased from Amersham International. Where necessary, solutions were sterilized by autoclaving at 15 pounds per square inch for 20 minutes. Antibiotics and other heat sensitive or low volume solutions were sterilized by filtration through a 0.2μm filter (Millipore U.K.).

2.2 Buffers

The following buffers were used:

1) Phosphate buffered saline (PBS): 140mM NaCl, 2.7mM KCl, 9.2mM Na_2PO_4 1.84mM KH_2PO_4 (pH 7.2).
2) Tris-borate-EDTA (TBE): 450mM Tris, 400mM boric acid, 10mM EDTA (pH 8.0)
3) Tris-glycine buffer: 25mM Tris, 250mM glycine, 0.1% (w/v) SDS
4) .Agarose gel loading dye: 0.25% (w/v), bromophenol blue, 0.25% (w/v) xylene cyanol, and 15% (w/v) ficoll in distilled water and stored at room temperature.
5) DNA 1Kb ladder (GIBCO BRL): 0.1μg/lane

2.3 Equipment

The general equipment that was used included Beckman J2-21 Rpmx1000 centrifuge for 50-400 ml volumes. Sigma 3K10 Howe centrifuge for 5-50ml volumes, MSE microcentaur centrifuge for <1ml volumes. UVP transilluminator and UVP camera, R100 Luckham Rotatest shaker, Sartorious balance, Hook and Tucker rotamixer, Biohit Biological Safety Class II cabinet, Biohit CO2 incubator and other general laboratory equipment.

2.4 Plasmids

PJ7Ω: The PJΩ vector series has been designed to allow easy insertion and subsequent expression of exogenous genes in a wide variety of mammalian cells. PJ7Ω is a mammalian expression vector conferring resistance to ampicillin Fig2.1 (Morgenstern and Land 1990).

CATC: Complementary pairs of oligonucleotides with the sequences indicated in Fig.1.6 (Chapter 1) were synthesized on an Applied Biosystems model 381A oligonucleotide synthesizer. All oligonucleotides were synthesized so that, when annealed, the double-stranded molecule would have a 5'-GATC single-stranded extension at either end to facilitate cloning. The TAATGARAT element is included. Annealed oligonucleotides were cloned into the BamH1 site of PBL_2CAT (Luckow and Schutz 1987). The sequence of oligonucleotides is given below (Dent, et.al., 1991).

5' RYGNTAATGARAT **3'**

5'GCGGTAATGAGAT **3'**

5'ATGCTAATGATAT**3'**

5'ATGCAAATAA**3'**

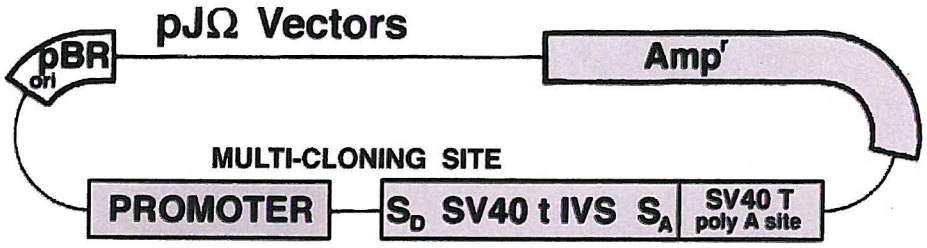


Fig.2.1: Schematic diagram of the PJΩ vectors

2.5 LIM-1 mutants

Deletion of the activation domain of LIM-1 gave rise to a mutant so called M1 (or dLim) and the activation of the LIM-domain gave rise to a mutant so called M2 (or dCter). Glycerol stocks of both mutants were used for large scale DNA preparation.

2.6 Large Scale DNA Preparation

DH5a bacteria containing plasmid vector were grown in 400ml of LB/ampicillin (100μg/ml ampicillin) overnight at 37°C. Plasmids recovered by centrifugation (5000 rpm for 20 mins) and resuspended in 4ml of 25% (w/v) sucrose, 50mM TrisHCl (pH 8.0) and 1mg/ml lysozyme for 15 mins on ice. EDTA was added to a final concentration of 10mM and left on ice for 15mins. Three volumes of triton buffer (3% (w/v) triton-100, 150mM TrisHCl (pH8.0), 187.5mM EDTA (pH8.0)) was added and left for 30 mins on ice and then centrifuged at 18000 rpm for one hour. The supernatant was then recovered, and made to a final concentration of 0.5M NaCl. An equal volume of phenol/chloroform (1:1) was added, mixed and centrifuged at 3000 rpm for 10 mins. The top aqueous layer was then removed and added to an equal volume of chloroform. After thorough mixing and centrifugation at 3000 rpm for 10 mins, the aqueous top layer was removed and transferred to a fresh falcon tube. 10% (w/v) PEG 6000 was dissolved in the aqueous layer at 37°C for 10 mins. The mixture was left for 1 hr on ice and subsequently subjected to centrifugation (12000 rpm for 10 mins). The resulting pellet was resuspended in 500μl of 0.1M TrisHCl (pH8.0) with 10μg RnaseA and incubated at 37°C for 1hr. An equal volume of 10mM TrisHCl (pH8.0), 1mMEDTA, 1M NaCl, 20%(w/v) PEG 6000 was added and left for 1 hour on ice. After centrifugation (12000 rpm for 10 mins) the pellet was redissolved in 500μl of 10mM TrisHCl (pH8.0), 0.5NaCl. The DNA was subsequently extracted with an equal volume of phenol/chloroform (1:1) and centrifuged at 12000 rpm for 10 mins. A 10% (w/v) volume of 4M NaCl was added, and the DNA precipitated with four volumes of ethanol for 10 mins at -70°C. The DNA was recovered by centrifugation (12000 rpm for 10 mins), washed in 70% (w/v) ethanol and allowed to air dry before being resuspended in H_2O to 1μg/μl.

2.6.1 Quantitative Analysis of DNA

The concentration of DNA in a given aqueous solution (in distilled water) was determined by measuring the absorbance at 260nm using quartz cuvettes and the Gene Quant (Pharmacia Biotech) DNA/RNA calculator. Using distilled water as a reference blank, double stranded DNA with an OD_{260} of 1.0 had a concentration of 50μg/ml. Single stranded DNA with an OD_{260} of 1.0 had a concentration of 33μg/ml. If the ratio of $OD_{260/280}$ was >1.7 the DNA was considered to be of acceptable quality. Measurement of DNA on the Gene Quant spectrophotometer was as per manufacturer instructions.

2.6.2 Phenol Chloroform Extraction & Ethanol Precipitation

DNA was extracted from aqueous solution and restriction digest mixtures by addition of an equal volume of phenol/chloroform (1:1), thorough mixing and centrifugation at 12000 rpm for 10 mins. The aqueous top layer was then transferred to a fresh tube or eppedorfe and a 10% (w/v) volume of 4M NaCl added. The DNA was precipitated by addition of four volumes of ethanol and incubation for 10 mins at -70^0C. DNA was recovered by centrifugation at 12000 rpm for 10 mins, washed in ethanol (70% w/v) and allowed to air dry prior to resuspension in the appropriate volume of H_2O.

2.6.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for the separation and characterization of DNA. Agarose gels of between 0.6 and 2.0% (w/v) agarose containing 0.5μg/ml ethidium bromide in Tris-acetate-EDTA (TAE) buffer were set directly into electrophoresis trays with plastic combs inserted to form loading wells (Hybaid). Samples were loaded with one-sixth the volume of agarose gel loading dye and electrophoresis performed as Hybaid instruction with TAE buffer at between 70V for 45 mins. One well containing 1μg of a 1Kb DNA marker was used to assess the size of the electrophoresed DNA. Post DNA electrophoresis, the DNA was visualized by ultra violet illumination.

2.6.4 Purification of DNA by Gene Clean™II

DNA was purified from aqueous solution (for DNA sequencing) or from agarose gel (0.6-2% (w/v) agarose) with gene clean kit (BIO 101inc.). This kit was used for the rapid purification of double stranded DNA between 1 and 15kb. The DNA band of interest was excised from agarose gel and its weight ascertained. DNA was purified as per manufacturer instructions.

2.7 Cell Line Cultures & Media

The ND7 neuronal cell line (Wood et al., 1990) was maintained in Lebovitz's (L15) media containing 10% (w/v) Fetal Calf Serum (FCS), 0.3% (w/v) d-glucose, 5% (w/v) $NaHCO_3$, 12mM glutamine. BHK cell line (Macpherson et al., 1962) was maintained in Dulbecco's modified eagle medium (DMEM) containing 10% (w/v) FCS, 10 units/ml penicillin/streptomycin and 1mM glutamine. All cells were maintained using a class II biological safety cabinet and a 50% CO_2 incubator at 37^0C.

2.8 DNA transfection

1) DNA

The plasmid DNA used for transfection was made from a large scale DNA preparation and suspended in H_2O under sterile conditions.

2) Preparation of cell lines by stable DNA transfection

ND7 and BHK cells ($5x10^5$ cells) were plated onto $92x17mm^2$ tissue culture grade dishes (Nunclon), Day 1 in 5ml DMEM containing 10% (w/v) FCS, 10 units/ml, penicillin/streptomycin and 1mM glutamine. On Day 2, the cells were stably transfected with 10μg of plasmid DNA by the calcium phosphate method of Gorman (Gorman 1985). Stable transfectants were selected by supplementing the media with G418 (Geneticin) to a concentration of 1.5mg/ml, 48 hours after transfection. Independent clones were isolated after 2 weeks of selection when individual foci of cells were evident. Independent clones were grown in full media (L15 Lebovitz media, 10% (w/v) FCS) supplemented with 1.5mg/ml G418.

3) Transient DNA Transfection

Cells (ND7, BHK) were transfected by the same method as for stable transfection (as described above), on Day 1 and Day 2. In addition to transfection of experimental and control plasmid DNA constructs, plasmid constructs containing a control promoter whose activity would be unaltered were also transfected in parallel into the cells. Transiently transfected cells were harvested in ice cold PBS 60 hours later with a cell scraper.

4) Chloramphenicol Acetyl Transferase (CAT) assay

Cells (ND7,BHK) were seeded at $5x10^5$/cell culture dish ($92x17mm^2$) in DMEM with 10% (w/v) FCS on Day 1of transient transfection. Cells were transfected with 10μg plasmid DNA containing the chloramphenicol acetyl transferase (CAT) gene by the calcium phosphate method of transfection (Gorman 1985) on Day 2. Cells were harvested 60 hours post transfection in ice cold PBS with a scraper and equalized for protein (Pierce BCA assay as per manufacturer instruction) and assayed for CAT activity as (Gorman 1985) with an incubation time of 2 hours prior to chloramphenicol extraction with ethyl acetate.

2.9 Polymerase Chain Reaction (PCR)

1) RNA Extraction

Primary cultures of adult DRG (Wood et al,1992) neurons harvested in ice-cold PBS and centrifuged at 1000 rpm for 5mins. The cells were suspended in Trizol (Gibco) (1ml/10^6 cells) and incubated for 5mins at room temperature. 0.2ml of chloroform/ml of Trizol was added and the sample shaken and left to stand at room temperature for 2mins. After centrifugation at 10000 rpm for 10mins, the top layer, containing RNA was removed and transferred to a fresh tube. 0.5ml of isopropanol was added and incubated at room temperature for 10mins. The sample was centrifuged at 10000 rpm for 10mins, the isopropanol removed and the RNA allowed to air dry for 2mins before resuspension in 30μl of distilled water (0.1%DEPC). RNA was stored at -70^{0}C.

2) cDNA preparation

The concentration of RNA was measured by spectrophotometry at 260nm using a Gene Quant (Pharmacia Biotech) spectrophotometer. Each DNA reaction of 50μl total volume contained 0.1μg RNA, 25mM $MgCl_2$, PCR reaction buffer (10mM KCl, 10mM TrisHCl (pH 9.0), 0.1% Triton X-100), 20 units Rnasin, 12.5mM dNTP, 1μg random hexamers and 200 units of Moloney Murine Leukaemia Virus Reverse Transcriptase (MoMLV RT). The RNA was preheated to 65^0 C for 2mins prior to addition to the reaction. Samples were incubated at 37^0 C for 1 hour and subsequently stored at -70^0 C.

3) PCR Conditions

Adult DRG cultures were treated in the absence or presence of NGF (5ng/ml) and the levels of *isl-1* and *Lim-1* determined by quantitative RT-PCR. Quantitative RT-PCR was used to analyse the levels of LIM-homeodomain mRNA due to the small amounts of RNA available from these primary cultures. Total cellular RNA was extracted from these cultures and used as template to prepare cDNA which was amplified using Taq polymerase with specific primers for *Isl-1, Lim-1* and *Brn-3a* as shown below.

Isl-1: 5' CGCTCTAAGGTGTACCACATCG 3' and

5' CATCTTCCACCATGCGTAGAC 3'

Lim-1: 5' GACTTCTGACAGCTCTAC 3' and

5' ACCCGTACTGTCAAATC 3'

Brn-3a: 5' GCTGCAGAGCAACCTCTTCGC 3' and

5' CATGAGCGCGAGCGACGGC 3'

Cyclophilin: 5' TTGGGTCGCGTCTGCTTCGA 3' and

5' GCCAGGACCTGTATGCTTCA 3'

The comparison of the homeodomains of the LIM-homeodomain genes and their sequence of degenerate primers is shown below.

Lin11: KRR GPR TTI KAK QL E TL K NAF AAT PKP TRH I RE QLA AET GLN MRV IQV WFN

Mec3: KRR GPR TTI KQN QLD VLN EMF SNT PKP SKH ARA KLA LET GLS MRV IQV WFN

Isl1: KTT RVR TVL NE K QL H TL R T CY AAN PEP DAL MKE QLV EMT GLS PRV IRV WFN

$K^R/_T\ ^R/_T\ ^G/_R\ ^P/_V$ RT^T_N **RVI$^Q/_R$ VWFN**

Primer 1 ***Primer 2***

As controls, the expressions of the POU domain gene Brn-3a and the house keeping gene cyclophilin were analysed. The PCR conditions in which the cDNA was linearly proportional to the PCR product was initially established for each primer pair by taking aliquots at 15, 20,25,30 and 35 cycles to locate the exponential phase of the PCR cycle. One tenth of the cDNA was amplified for 25 cycles with *Isl-1*, *Lim-1*, and *Brn-3a* primers and 20 cycles with the cyclophilin primers.

Samples were analysed on 2% agarose gels, southern blotted and probed with radiolabelled probes. In each experiment, the identity of the PCR product was further confirmed by digestion with restriction endonucleases.

2.10 DNA Sequencing

DNA was prepared as Wizard plus SV minipreps kit (Promega) and the DNA resuspended in 100μl of water. 20μl of double stranded DNA was used in the sequencing reaction. Double stranded DNA templates were first denatured by the addition of 20μl 0.4M NaCl for 10mins at room temperature. 4μl 0.3M Na-acetate was then added together with 800μl ethanol. The DNA was incubated at -20^0 C for 10mins and subsequently centrifuged at 12000 rpm for 10mins. The DNA was pellet and washed in 70% (w/v) ethanol and then resuspended in 7μl of nuclease free distilled water. Sequencing was carried out as stated in the t7/t3 DNA polymerase United States Biochemical Corporation protocol (manufacturer instructions).

2.11 Southern Blot and Hybridisation

DNA was agarose gel electrophoresed as described in 2.6.3 section. After visualisation by ultraviolet illumination, the gel was denatured with 0.5M NaOH, 1.5M NaCl for 30 mins and subsequently neutralised with TrisHCl (pH 8.0) 1M and NaCl 1.5M for 30mins. DNA was transferred from the gel to Hybond-N membrane by southern blot over night in 20xSSC. The membrane was subsequently marked with pencil to confer the orientation of the sample and the DNA cross-linked to the Hybond-N membrane by ultraviolet illumination for 3mins. The membrane and DNA was then prehybridised with 6xSSC, 1x Denhrardts, 0.1% (w/v) SDS and 0.1mg/ml of denatured double stranded salmon DNA in a Hybaid prehybridisation bottle at 65^0 C for 2 hours in a hybridisation oven (Hybaid). Hybridisation was carried out in 6xSSC, 1xDenhardts,0.1% (w/v) SDS, 10% (w/v) Dextran Sulphate and labelled probe ($1x10^7$cpm/ml). Labelled probe was added to the hybridisation mixture and incubated at 65^0 C over night with the DNA/membrane filter. The filter was washed in 2xSSC, 0.1% (w/v) SDS and subsequently in 0.1xSSC, 0.1% (w/v) SDS. Blue sensitive X-ray film (Genetic Research Instrumentation Ltd.) was exposed with the filters over night at -70^0 C in order to visualise the data.

2.12 Oligo Labelling

Probes for southern blotting were oligo labelled using the method of Feinberg and Vogelstein 1983. DNA fragments containing specific cDNAs (50ng) were denatured at 100^0 C for 5mins with 20μCi

90u/ml random hexamers and 5mM each od dTTP, dGTP, and dATP. The reaction was incubated for 5 hours before being stopped with 20mM NaCl, 20mMTrisHCl

(pH7.5), 2mM EDTA, 0.25%SDS, 1μm dCTP and subsequently passed down a G50 sephadex column.

2.13 Restriction Digests of DNA

All restriction enzymes were used with the buffers provided and as recommended by the manufacturer. 0.2-2 μg of DNA (100ng/μl) was digested with 10 units of enzyme in a total volume of 20μl at 37^0 C for 4 hours.

2.14 DNA Ligation

1) Typical DNA Ligation

100ng of vector and insert DNA in a molar ratio of vector:insert, 1:3 was ligated using two units of T4 DNA ligase and 1xDNA ligase buffer (as per manufacturer instructions) in a total volume of 10μl. The ligation reaction was incubated at room temperature overnight. 5μl of ligation reaction was used to transform bacteria.

2) In Gel Ligation

The gel slice containing the insert DNA was incubated at 50^0 C until an aqueous solution was apparent. 100ng of vector and insert DNA in a molar ratio of vector: insert, 1:3 was ligated with two units of T4 DNA ligase and 1xDNA ligase buffer (as per manufacturer instructions) in a total volume of 100μl. The ligation reaction was incubated at room temperature overnight. 50μl of ligation reaction was used to transform bacteria.

2.15 Bacterial Storage

Long-term storage of bacteria was as a 50% bacterial culture in Millers Luria Broth (LB) (Gibco BRL, made as per manufacturer instructions in distilled water and autoclaved), with 50% glycerol (w/v) placed at -70^0 C. For short-term storage, 10ml LB plates (LB, 1.5% select agar) were made. Using a flame sterilised glass spreader or loop, bacterial culture was transferred to LB plate. The plate and bacteria were subsequently incubated at 37^0 C over night to allow bacterial cell growth. Afterwards bacterial/LB agar plates were sealed with parafilm and stored at 4^0 C.

2.16 DH5α bacterial cell growth

Routinely, DH5α (Gibco BRL) bacteria were grown from a bacterial/LB agar plate. Using a flame sterilised pipette tip a single colony of bacteria was picked from the plate and transferred to a 5ml culture of LB media. Bacteria were subsequently grown at 37^0 C over night with constant agitation. Bacteria from a glycerol stock (long term storage conditions) were grown by dilution of the bacteria 1:1000 with LB media to a final volume of 10ml. The cell suspension was subsequently incubated at 37^0 C over night with constant agitation.

2.17 Preparation of Competent DH5α and Bacterial Transformation

A single colony of DH5α bacteria was picked using a sterilised pipette tip, from an LB agar plate and grown in LB media over night at 37^0 C with constant agitation, as a 10ml culture. 100μl of the over night culture was diluted into 10ml of LB and grown for 2.0-2.5 hours at 37^0 C until the exponential phase of growth (OD 0.4-0.6 at 550nm) was reached. Bacterial cells were harvested by centrifugation at 3000 rpm, 4^0 C for 10mins and resuspended in 5ml of 100mM, ice cold $CaCl_2$. The bacteria were centrifuged at 3000 rpm, 4^0 C for 10mins and resuspended in 500μl of ice cold $CaCl_2$. DNA (1-10ng) or 50μl of ligation mix was added to 150μl of competent DH5α bacteria and the mixture incubated on ice for 30mins. The cells were heat-shocked at 42^0 C for 2mins and returned to ice for a further 30mins. 200μl of LB was added to the DNA/cell mixture and incubated at 37^0 C for 20mins. The mixture was then spread under sterile conditions onto LB agar plate containing 100μg/ml ampicillin using a sterilised glass spreader. The plate with the bacterial cell/DNA mixture was then incubated over night at 37^0 C to allow bacterial growth. Individual colonies were picked the next day and grown up in 10ml LB containing 100μg/ml ampicillin over night at 37^0 C with constant agitation.

CHAPTER 3

Regulation of HSV IE gene expression by Brn-3 POU proteins/Interaction between Brn-3 POU protein family and LIM proteins

3.1 Introduction

Herpes simplex virus (HSV) establishes life-long latent infections of sensory neurons from which the virus can be re-activated to allow repeated cycles of productive infection elsewhere in the body (Roizman et.al., 1987, Latchman,D., 1990). It has been shown that latently infected cells do not express the viral-immediate (IE) RNAs and proteins (Stevens et al., 1987, Croen et al., 1987). These findings have led to the idea that the establishment and maintenance of latent infection is likely to involve a failure of IE gene expression hence preventing the lytic cycle to occur.

During normal lytic infection, IE gene expression is dependent on the formation of a complex between the HSV protein, VP16, the Host Cell Factor (HCF) and the cellular octamer-binding transcription factor, Oct-1, which binds to the octamer related TAAATGARAT motif in the IE promoters (O'Hare et al., 1988; Preston et al., 1988). It has been previously shown that both neuronal cell lines and primary sensory neurons express an additional octamer-binding protein Oct-2 (Lillycrop et al., 1991) which is capable of binding to the TAATGARAT motif and inhibiting both the basal activity of the IE promoter and its transactivation by VP16 (Lillycrop et al., 1995).

The critical role of the TAATGARAT motif in controlling IE gene transcription during both lytic infection and the onset of latent infection suggests that it may also be involved in the reactivation. However, this effect is unlikely to be dependent on the activation of this motif via the Oct-1-VP16 complex, and this is because the virion transactivator VP16 is not present in latently infected neurons. It is normally produced late in the viral lytic cycle after the prior expression of the IE genes (Spear and Roizman, 1980). Similarly the Oct-1 protein is a weak transactivator in the absence of VP16 and would have a minimal stimulatory effect on the IE promoter when VP16 is absent (Tanaka et al., 1992).

The lack of effect of Oct-1 in the absence of VP16 and the inhibitory effect of Oct-2 suggest that other neuronally expressed members of the POU family of transcription factors might be able to stimulate viral IE gene expression and therefore play a role in viral re-activation from sensory neurons (Lillycrop et al.,1995). One obvious candidate in this regard is the Brn-3 POU transcription factors that have been shown expression at high levels in sensory neurons. Recent studies have shown that there are three closely related forms of Brn-3, known as Brn-3a, Brn-3b, and Brn-3c, each of which are encoded by a distinct gene (Theil et al., 1994). POU proteins may act in the development of the mammalian nervous system in a manner similar to the *C.Elegans* POU protein Unc-86. The POU-type homeodomain protein UNC-86 and the LIM-type homeodomain protein MEC-3, which specify neuronal cell fate in the nematode *C.Elegans*, bind cooperatively as a heterodimer to the mec-3 promoter. Heterodimer formation increases DNA binding stability and, therefore, increases DNA binding specificity. The in vivo significance of this heterodimer formation in neuronal differentiation is suggested by (i) a loss-of- function mec-3 mutation whose product in vitro binds DNA well but forms heterodimers with UNC-86 poorly and (ii) a mec-3 mutation with wild-type function whose product binds DNA poorly but forms heterodimers well (Xue et al., 1993). Unc-86 is the homolog of Brn-3a. These transcription factors have also been shown to bind to several octamer/TAATGARAT-related sequences including those found upstream of the ICPO gene. It has been shown that Brn3a and Brn-3c can activate the transcription of the ICPO promoter while Brn-3b inhibits the transcription (Morris et al., 1994; Lillycrop et al., 1995; Budhram-Mahadeo et al., 1995). In those studies both Brn3a and Brn3c are weakly activators (induction of ICPO expression by 1.5 fold) The fact that Brn3a and Brn-3c binding to the TAATGARAT motif was very weak in combination with the fact that the Brn-3a/Brn-3c induction of ICPO expression was only 1.5 fold, suggest that if Brn-3a or Brn-3c play a critical role in viral reactivation, they do so in combination with other factors (Lillycrop et al.,1995).

3.2 LIM-HD & POU proteins

A number of studies have shown that members of the POU-domain transcription factors family can interact synergistically with members of the LIM-HD family. Unc-86, the *C.Elegans* POU homologue of Brn-3, interacts with the LIM-HD transcription factor Mec-3 (Chalfie et al., 1989). The differentiation of six touch receptor neurons in the nematode *C.Elegans* requires two homeobox genes, unc-86 and mec-3. The unc-86 gene encodes a POU-type homeoprotein required in touch cell precursors to generate the touch receptor neurons. The mec-3 gene, which encodes a LIM-type homeoprotein, is needed to specify the touch cell fate once the cells have been produced. The presence of unc-86 protein (UNC-86) in the touch cells, the binding of UNC-86 to the mec-3 promoter, and the requirement for some of the UNC-86 binding sites in vivo for mec-3 expression, suggest that unc-86 directly initiates mec-3 expression. Furthermore, mutation of one UNC-86 binding site in the mec-3 promoter reveals that unc-86 is also required to maintain mec-3 expression, a role consistent with the finding that UNC-86 is present in the touch cells throughout the life of the animals. The mec-3 gene is required for proper expression of genes such as mec-4 and mec-7 that are needed for touch receptor function and it is required for the maintenance of its own expression. Because mec-3 protein binds to its own promoter and mutations in some MEC-3 binding sites affect the maintained expression of *mec-3lacz* fusions, such autoregulation is likely to be direct.

The proteins UNC-86 and MEC-3 bind to overlapping regions in the mec-3 promoter, two of which (CS2 and CS3) are needed for mec-3 expression. The binding of UNC-86 and MEC-3 to oligonucleotides of this region has been examined in gel-mobility-shift assays (Xue et al; 1993). UNC-86 bound differently to the two oligonucleotides, yielding one retarded band with CS2 and two retarded bands with CS3. The two CS3 bands represent the binding of UNC-86 as a monomer and a dimer. Homodimer

formation has been observed with three other POU-type homeoproteins, Pit-1, Oct-2, and Cfl-a (Xue et al; 1993). As for MEC-3, it bound to both oligonucleotides and resulted in a single retarded species, although the binding of MEC-3 to CS2 was weak. When both MEC-3 and UNC-86 were added to each of the oligonucleotides, the MEC-3 band was reduced and a more intense band (the UM complex) appeared at a position near the UNC-86 monomer band. The UM complex contains both MEC-3 and UNC-86 because it can be supershifted by either an antibody to MEC-3 (anti-MEC-3) or an antibody to an influeza virus eitope added to UNC-86. In the UM complex MEC-3 bound to at least 24 times as much oligonucleotides (CS2 or CS3) as it did without UNC-86. The binding of oligonucleotide by UNC-86 in the UM complex increased at least six fold over the binding of DNA to UNC-86 alone. Those results suggest that the binding of both proteins to DNA especially the binding of MEC-3 to DNA is greatly increased by a cooperative protein interaction (Xue et al; 1993). Interactions between UNC-86 and MEC-3 were also detected by chemical cross-linking, which showed that heterodimer is formed, and by immunoprecipitation, which showed that the proteins can interact without DNA. Brn-3 POU proteins are orthologs of UNC-86 POU proteins

3.2.1 Identification of LIM-HD genes expressed in primary sensory neurons

As LIM-HD proteins have been evolutionary conserved, we initially tried to identify members of the LIM-HD family that were expressed in primary sensory neurons.
Adult sensory neurons in the DRG are known to express high levels of the LIM-HD gene Isl-1 (Thor et al., 1991), however to determine what other LIM-HD genes they expressed, total cellular RNA was extracted from primary DRG using Trizol and

Moloney Murine Leukemia Virus Reverse Transcriptase. The RNA was then used as template to prepare cDNA. This was then amplified by polymerase chain reaction (PCR) using a pair of degenerate PCR primers designed to anneal to two highly conserved regions at either end of the DNA binding domain of LIM-homeodomain transcription factors (Fig.3.1). A PCR product of 162 bp was amplified, which is consistent with the expected size of the LIM-homeodomain DNA binding domain, and the PCR product was then cleaved with ECOR1 and subcloned into ECOR1 cut PBluescript vector (SK-)

Sequencing revealed that the clones fell into four categories as shown in Table 1. The largest class did not show any homology to any known LIM-HD gene, beyond the sequence of the primer, these were therefore not studied further. The remaining 3 classes all showed sequence homology characteristic of LIM-HD genes. The largest of these classes, class 2, contained sequences identical to rat *Isl-1*, a LIM-HD gene shown previously to be expressed in sensory neurons of the trigeminal and dorsal root ganglion (Thor et al., 1991). Class 3 contained clones identical to *Rlim* (Furuyama et al., 1994) and class 4 contained clones with sequence homology to mouse *Lim-3* (Seidah et al., 1994), suggesting that these clones are likely to be derived from rat *Lim-3* mRNA. From these results it was revealed which LIM-HD genes are expressed in primary sensory neurons.

Lin11: KRR GPR TTI KAK QL E TL K NAF AAT PKP TRH I RE QLA AET GLN MRV IQV WFN

Mec3: KRR GPR TTI KQN QLD VLN EMF SNT PKP SKH ARA KLA LET GLS MRV IQV WFN

Isl1: KTT RVR TVL NE K QL H TL R T CY AAN PEP DAL MKE QLV EMT GLS PRV IRV WFN

$K^{R}I_{T}{}^{R}I_{T}{}^{G}I_{R}{}^{P}I_{V}RT^{T}{}_{N}$ $RVI^{Q}{}_{R}VWFN$

Primer 1 **Primer 2**

Fig. 3.1 The comparison of the homeodomains of the LIM-homeodomain genes and their sequence of degenerate primers

Table 1

The PCR products from the amplification of cDNA prepared from DRG cultures using the following degenerate LIM-homeodomain primers (5' GATCGAATTCGGC/AGNC/AGNGGNCCNC/AGNAC-NACNAT 3') and (5' GATCGAATTCGGGA/GAACCANACT/CTGT/G/AATNACNCT/GC 3') were cloned and sequenced.

Class	No of clones	Comment
I	21	Unrelated to LIM-homeodomain genes
II	19	Identical to rat *Isl-1*
III	15	Identical to *Rlim*, a rat homeodomain gene
IV	4	Closely related to mouse *Lim-3*

3.3 Regulation of LIM-HD gene expression by NGF

Therefore, having established that LIM-HD proteins are expressed in SNS, we next investigated whether their expression was modulated by factors known to influence viral reactivation. In particular, we looked at the effect of NGF on LIM-HD expression, since NGF withdrawal shown to lead to the rapid reactivation of latent virus. Brn-3a has previously shown to be unaffected by NGF in sensory neurons (Wood et al., 1992).

In order to determine whether NGF has any influence to the expression of the LIM-homeodomain genes in sensory neurons, adult DRG cultures were treated in the absence or presence of NGF (5ng/ml) and the levels of Isli-1 and Lim-1 determined by quantitative RT-PCR. Total cellular RNA was extracted from these cultures and used as a template to prepare cDNA that was amplifying using Taq polymerase with specific primers for Isli-1 and Lim-1.

As controls, the expressions of the POU domain gene Brn-3a and the house keeping gene cyclophilin were analysed. The PCR conditions in which the cDNA was linearly proportional to the PCR product was initially established for each primer pair by taking aliquots at 15, 20,25,30 and 35 cycles to locate the exponential phase of the PCR cycle. One tenth of the cDNA was amplified for 25 cycles with Isl-1, Lim-1, and Brn-3a primers and 20 cycles with the cyclophilin primers.

It was found that the addition of NGF led to a rapid increase in Isl-1 and Lim-1 expression after 24 hours of treatment. In contrast, NGF had no effect on the expression of Lim-3 or the housekeeping gene cyclophilin or on the expression of the POU homeodomain transcription factor Brn-3a as previously shown (Fig. 3.2).

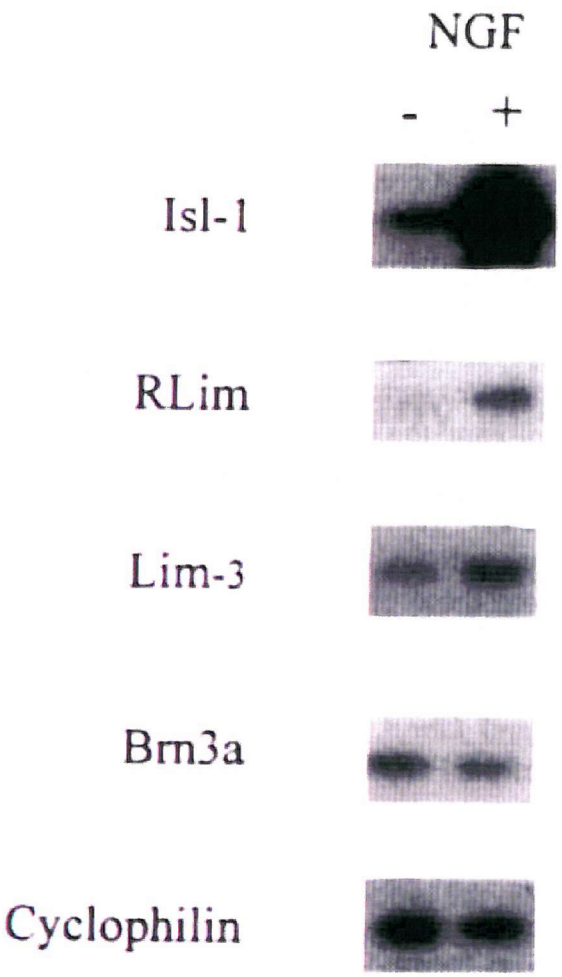


Fig.3.2: Elevation in *Isl-1* and *Lim-1* mRNA expression upon NGF addition to DRG cultures. Representative RT-PCR assay showing the amplification of cDNA from DRG cultures grown in the absence of NGF (-NGF) or in the presence of NGF (+NGF) for 24 hours. CDNA from the cultures were amplified with specific primers *for Isl-1, Lim-1,Lim-3, Brn-3a*, and *cyclophilin*.

3.4 LIM-HD proteins and their role on ICPO expression

Having shown that LIM-HD proteins are present in SNS and are induced by NGF withdrawal, we next investigated whether the expression of LIM-HD proteins could alter the expression of the IE genes alone or in conjunction with members of the Brn-3 family. To assess this a promoter construct containing the ICP0 promoter (-300 to +33) was fused upstream of the reporter -CAT-gene pBL_3CAT and transiently transfected into ND7 cells with either an empty expression vector (V), or expression vectors carrying Lim-1, Brn-3a, Brn-3b or Brn-3c (Fig.3.3). We found that Lim-1, Brn-3a and Brn-3c weakly induced ICPO expression (1.25, 1.5, 1.0 respectively) while Brn-3b inhibited expression. All transfections were normalised by co-transfection with pcmV-βgal. Interestingly, Lim-1 also affected ICP0 expression and weakly activated ICP0.

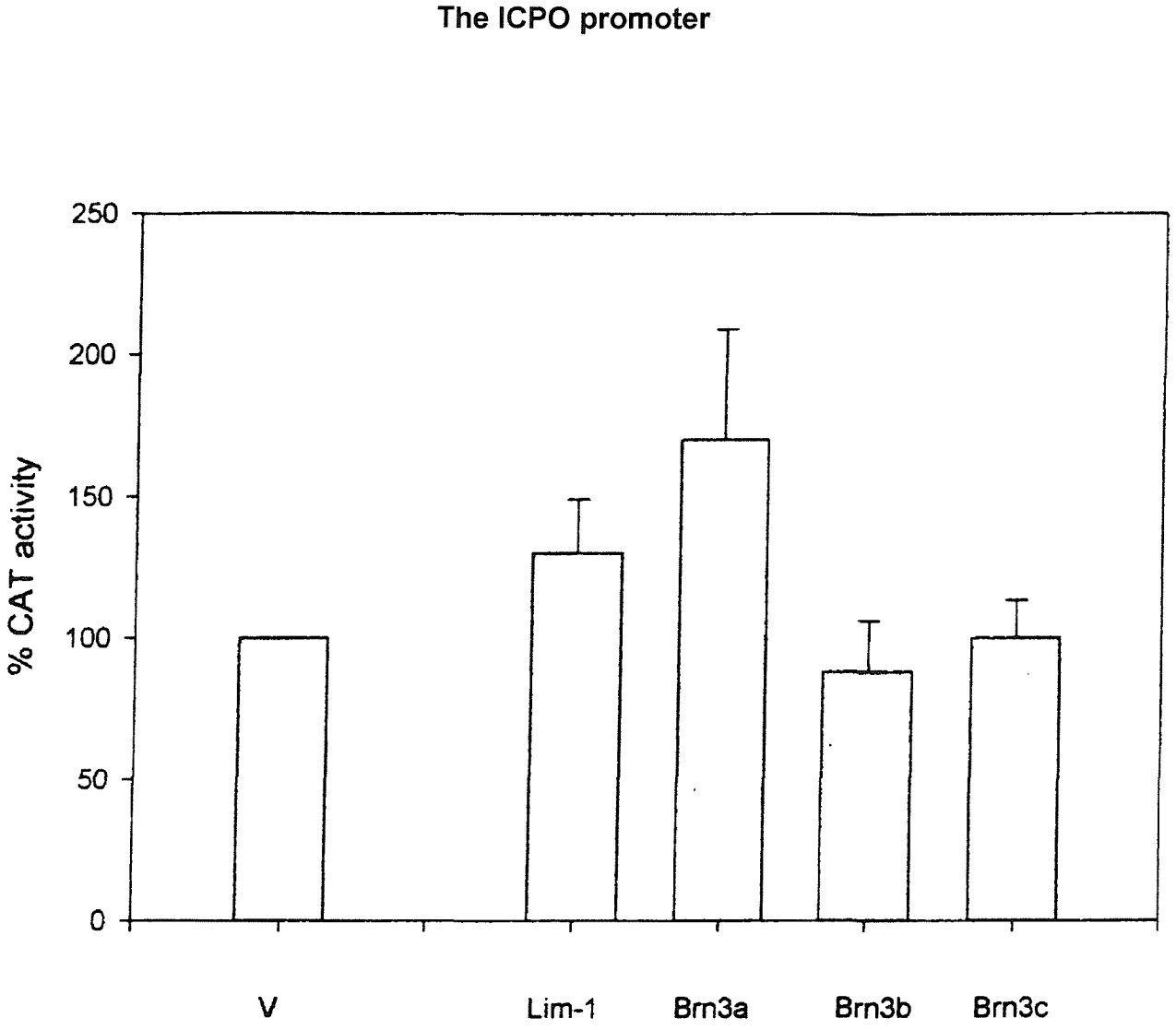


Fig. 3.3: ICPO promoter (5μg) was fused with CAT reporter gene and transiently transfected into ND7 cells along with an empty expression vector (V) (10μg) or Lim-1(10μg), Brn-3a (10μg), Brn-3b (10μg) and Brn-3c (10μg) expression vectors alone. Values are the mean of 3 independent experiments +/- SEM

3.5 LIM-HD proteins/Brn-3 family on ICP0 expression

Since Lim-1 and Brn-3a, appeared to activate weakly ICPO expression alone, we next investigated whether Lim-1 in combination with Brn-3a, Brn-3b or Brn-3c could enhance the expression of ICPO. The ICP0-CAT promoter (-300 to +33) construct was transiently transfected into ND7 cells with expression vectors carrying Lim-1, Lim-1 and Oct-2.5, Oct-2.5, Lim-1 and Brn-3c, Brn-3c, Lim-1 and Brn-3b, Brn-3b, Lim-1 and Brn-3a and finally Brn-3a. As shown in Fig.3.4, co-transfection of Lim-1 with Brn-3c led to a large induction in ICP0 expression. In the presence of Lim-1 and Brn-3c, expression was far more than the sum of Lim-1 and Brn-3c alone, suggesting that Lim-1 and Brn-3c synergistically interact to induce ICP0 expression. Similarly, when the ICP0 promoter was co-transfected with Lim-1 and Brn-3a, a large synergistic increase in ICP0 gene expression was seen. Interestingly, the combimation of Lim-1 and Brn-3b also led to an increase in ICP0 gene expression, despite the fact that Brn-eb alone inhibits ICP0 expression. Lim-1 had no effect on the ability of the POU-domain protein Oct-2.5 to inhibit ICP0 gene expression. These results clearly suggest that Lim-1 can synergistically interact with members of the Brn-3 subfamily of POU-domain proteins leading to the induction of the ICP0 gene expression.

Activation of ICP0 promoter due to synergism between Lim-1 and Brn-3 POU proteins

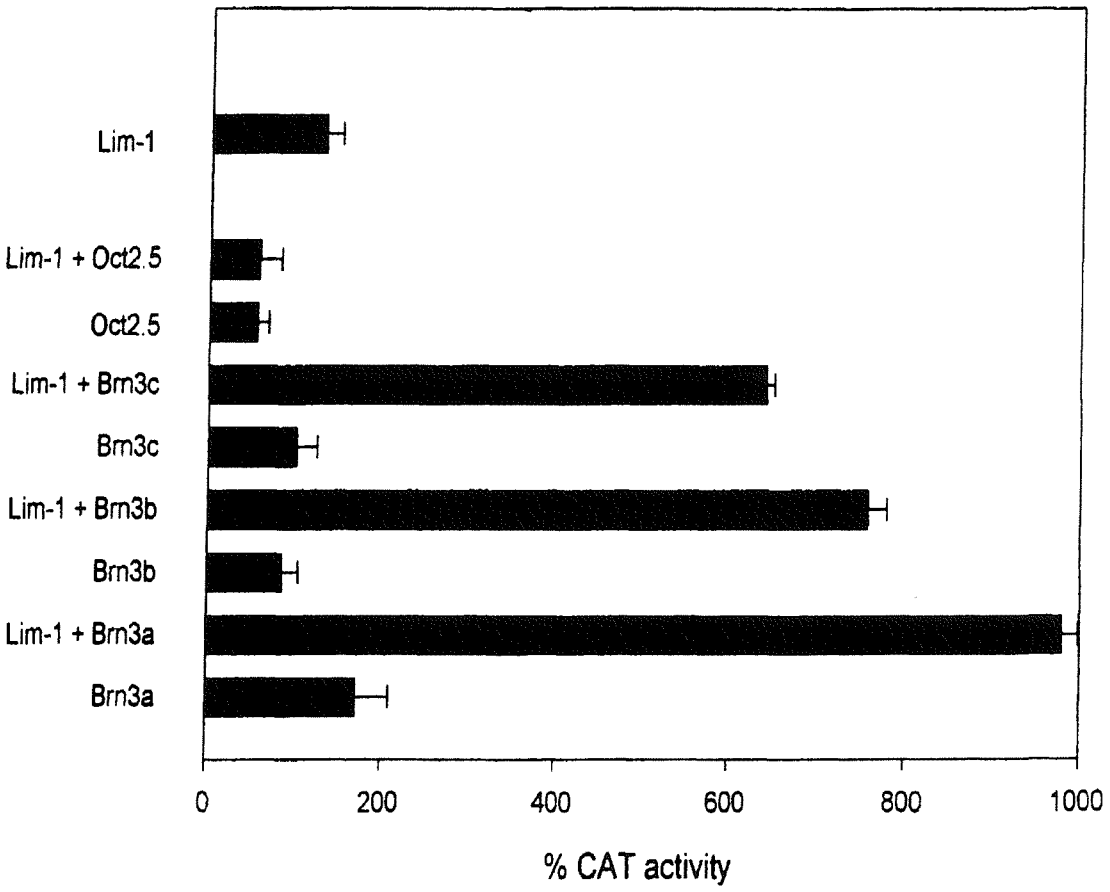


Fig.3.4: ICP0 promoter (5μg) was fused to CAT reporter gene and transiently transfected into ND7 cells with Oct-2.5 (10μg), Lim-1 (10μg), Brn-3a (10μg), Brn-3b (10μg), and Brn-3c (10μg) expression vectors in combination and alone. Synergism between Brn-3a, Brn-3c and Lim-1 activates the promoter and leads to gene expression.

Values are expressed relative to the ICP0 promoter transfected with the empty vector which is set to 100%.Values are the mean of 3 independent experiments +/- SEM

3.6 LIM-HD proteins/Brn-3 family on ICP4 expression

As Lim-1 and Brn-3a/Brn-3c appeared to enhance ICPO expression, we also investigated whether Lim-1 in combination with Brn3a/Brn3c could also enhance the expression of the ICP4 promoter. The ICP4 promoter (-300 to +33) was fused upstream of the reporter -CAT- gene and transiently transfected into ND7 cells with expression vectors carrying Lim-1, Lim-1 and Oct-2.5, Oct-2.5, Lim-1 and Brn-3c, Brn-3c, Lim-1 and Brn-3b, Brn-3b, Lim-1 and Brn-3a and finally Brn-3a. No synergism was established between Lim-1/ Brn-3a, Lim-/Brn-3c Lim-1/Brn-3b and Lim-1/Oct-2.5. (Fig.3.5). This might be due to the fact that ICP4 contains a simple TAATGARAT motif in its promoter, whereas, ICP0 contains an overlapping octamer/TAATAGARAT motif.

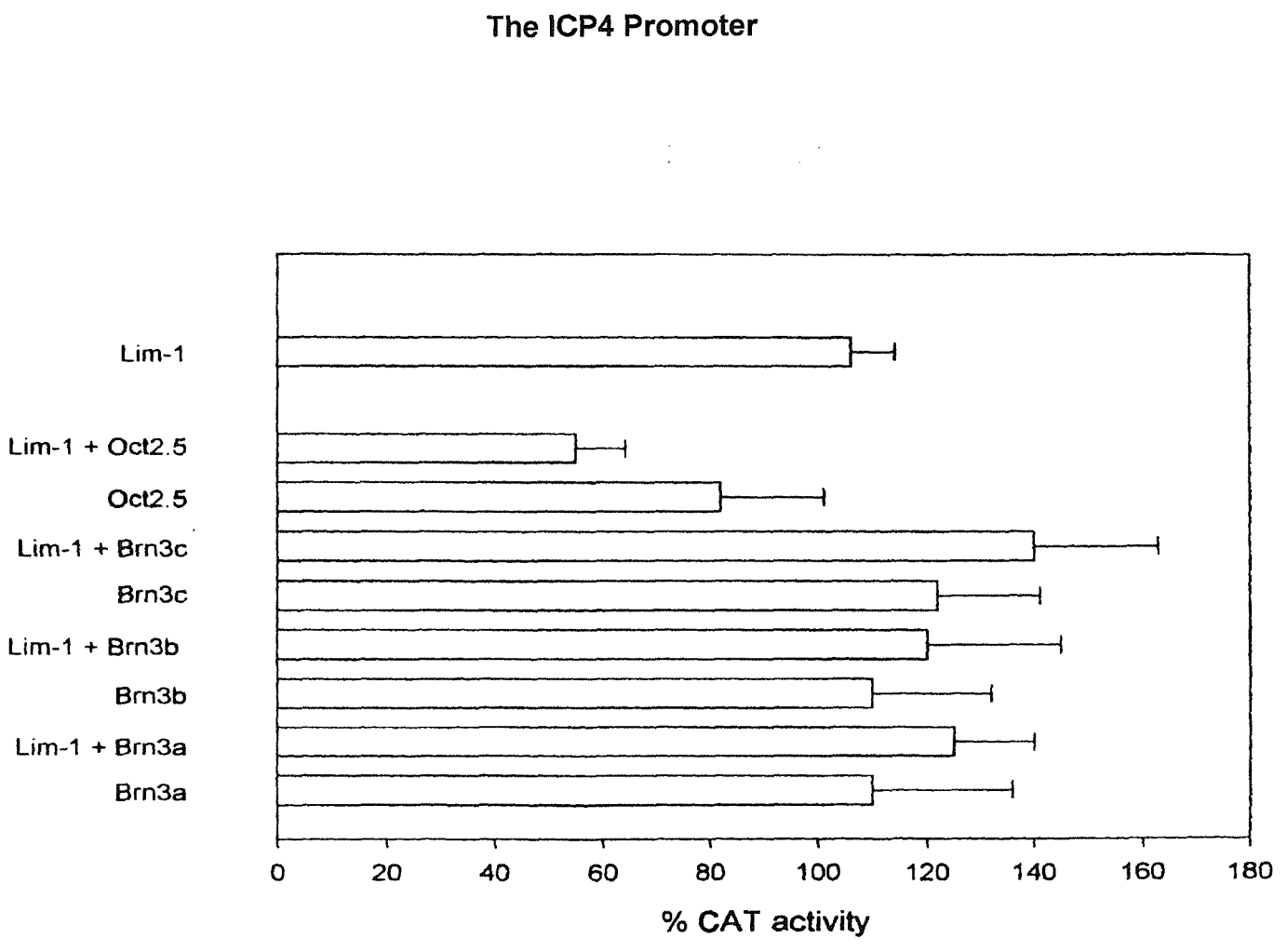


Fig. 3.5: ICP4 (5μg) promoter was fused into a CAT reporter gene and transiently transfected with Lim-1 (10μg), Oct-2.5 (10μg), Brn-3a (10μg), Brn-3b (10μg) and Brn-3c (10μg) expression vectors in combination or alone. Values are expressed relative to the ICP4 promoter transfected with the empty expression vector which is set to 100%.

Values are the mean of 3 independent experiments +/- SEM

3.7 Isl-1 regulation of IE gene expression

To determine if Isl-1 like Lim-1 can also regulate IE gene expression, the ICP0 promoter fused to the reporter gene CAT and was co-transfected with expression vectors containg Isl-1 alone or with Oct-2.5 Brn-3a, Brn3b or Brn-3c. Isl-1 weakly enhances the expression of the ICP0 promoter (Fig.3.6).

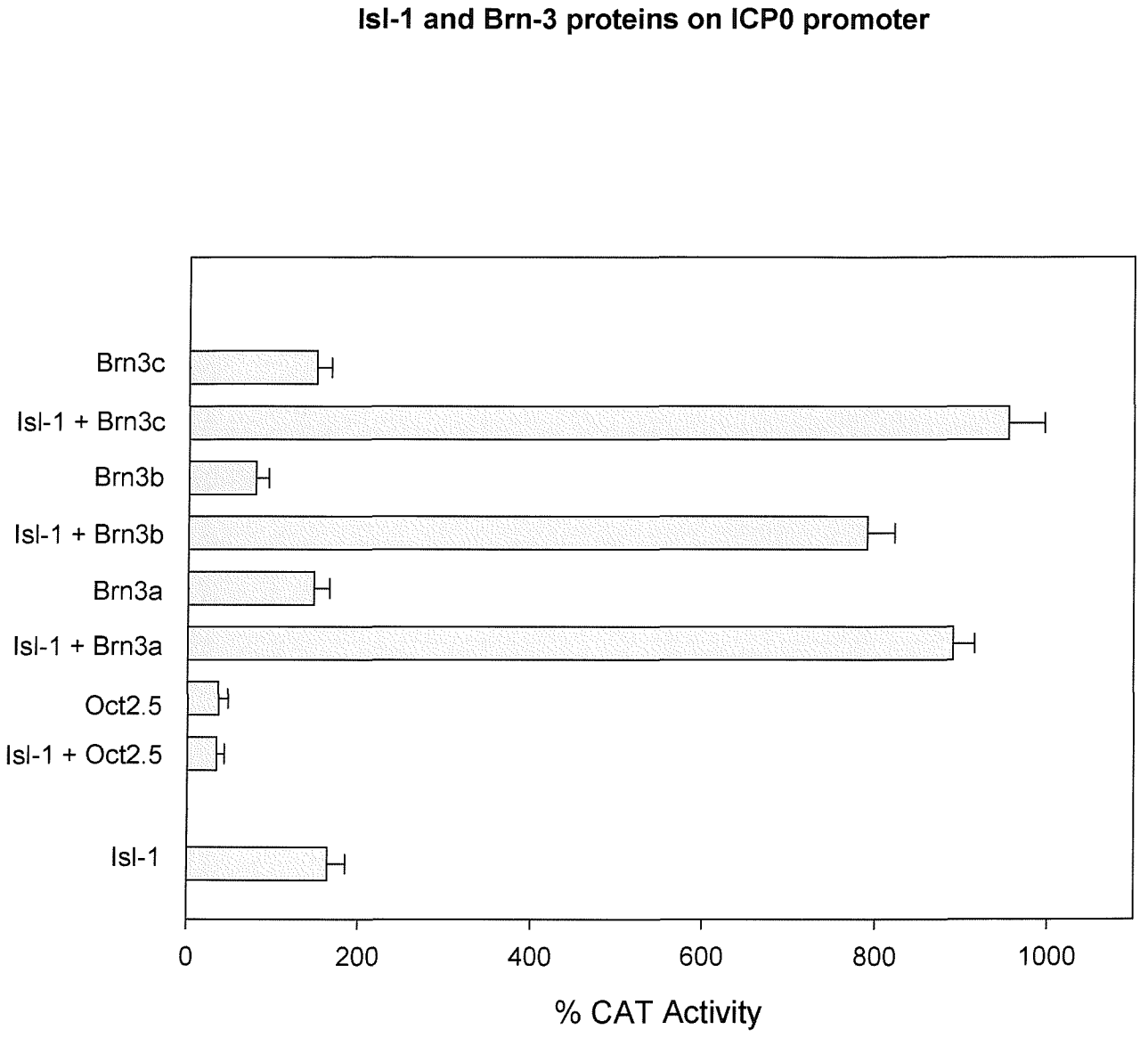


Fig. 3.6: ICP0 (5μg) promoter was fused into a CAT reporter gene and transiently transfected with Isl-1 (10 μg), Brn-3a (10 μg), Brn-3b (10 μg), Brn-3c (10 μg), and Oct-2.5 (10 μg) expression vectors in combination or alone. Values are expressed relative to the ICP0 promoter transfected with the empty expression vector PJ7 when is set to 100%. Values are the mean of 3 independent experiments +/- SEM

However, when Isl-1 is co-transfected with Brn-3a, Brn-3b or Brn-3c expression of ICP0 greatly increases, suggesting that Isl-1 like Lim-1 can synergistically interact with the Brn-3 proteins. Isl-1 like Lim-1 however does not appear to interact with Oct-2.5 as when Oct-2.5 and Isl-1 were co-transfected with ICP0 no increase in expression was observed. The synergistic interaction between Isl-1 and the Brn-3 proteins only occurred on the ICP0 promoter, no synergistic interactions were observed between Isl-1 and Brn-3 proteins on the ICP4 promoter (Fig. 3.7).

Isl-1 and Brn-3 proteins on ICP4 promoter

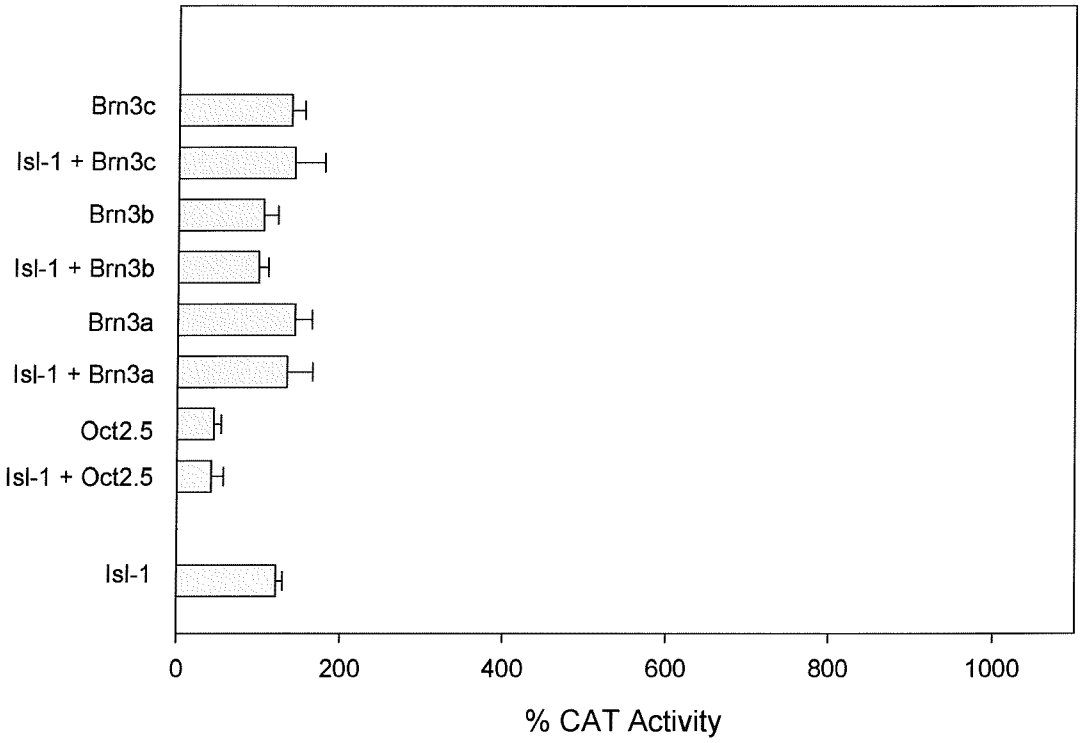
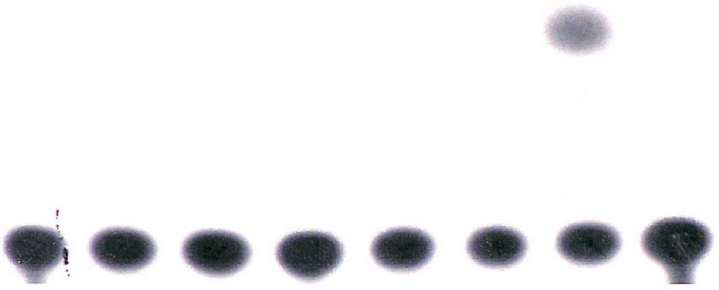


Fig. 3.7: ICP4 (5μg) promoter was fused into a CAT reporter gene and transiently transfected with Isl-1 (10 μg), Brn-3a (10 μg), Brn-3b (10 μg), Brn-3c (10 μg), and Oct-2.5 (10 μg) expression vectors in combination or alone. Values are expressed relative to the ICP4 promoter transfected with the empty expression vector PJ7 when is set to 100%. Values are the mean of 3 independent experiments +/- SEM

3.8 Brn-3a and Lim-1 act synergistically via an octamer/TAATGARAT motif

In order to confirm further that the induction of ICPO expression by Lim-1 and Brn-3a was occuring via the octamer/TAATAGARAT motif in the ICPO promoter, an oligonucleotide containing the octamer/TAATGARAT sequence for ICPO was fused upstream of the thymidine kinase promoter of CATC vector.

As shown in figures 3.8/3.9, in the presence of both Brn-3a and Lim-1 the expression of the CATC construct was far greater than that observed with Brn-3c or Lim-1 alone. This strongly suggests that Brn-3a and Lim-1 can synergistically interact via an octamer/TAATGARAT motif.



BHK cells were transiently co-transfected as follows

Lane 1: PBL_2CAT (10μg) and PJ7 (10μg), Lane2: PBL_2CAT (10 μg) with Brn-3a (10 μg), Lane 3: PBL_2CAT (10 μg) with Brn-3a (5 μg) and Lim-1 (5 μg), Lane 4: PBL_2CAT (10 μg) with Lim-1 (10 μg), Lane 5: CATC (10 μg) with PJ7 (10 μg), Lane 6: CATC (10 μg) with Brn-3a (10 μg), Lane 7: CATC (10 μg) with Brn-3a (5 μg) and Lim-1 (5 μg), Lane 8: CATC (10 μg) with Lim-1 (10 μg)

Fig.3.8 Induction of the ICP0 promoter via the Octamer/ TAATGARAT motif

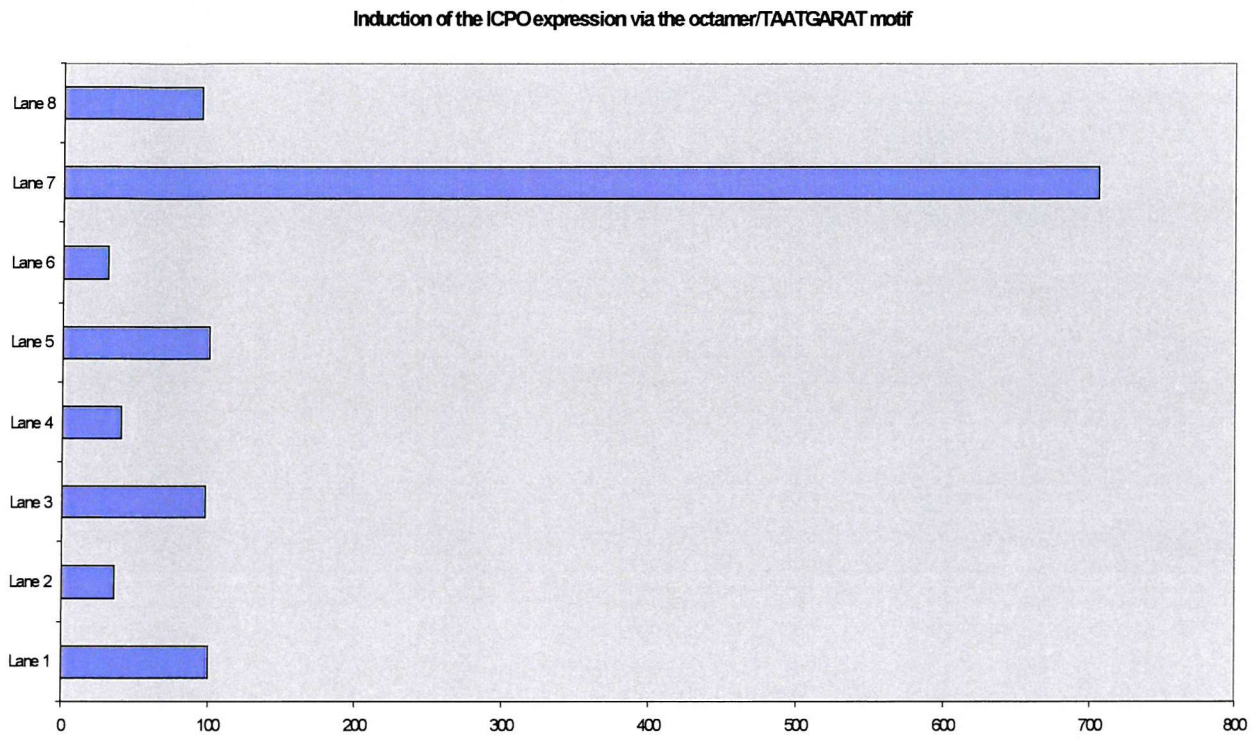


Fig 3.9: The above displayed bar chart illustrates the induction of ICP0 gene expression by Brn-3a and Lim-1 via the TAATGARAT motif

3.9 Deletion Mutations of LIM-1

In order to detect which domain of Lim-1 is involved in the synergistic interaction with Brn-3 proteins, a series of deletion mutants of Lim-1 were created.

Deletion of the LIM domain of Lim-1 gave rise to the M_2 mutant (or dLim) while deletion mutation of the activation domain of Lim-1 gave rise to the M_1 (or dCter) mutant.

For this experiment the ICP0 promoter (-300 to +33) was fused to a reporter -CAT-gene and transiently transfected into ND7 cells with either an empty expression vector or expression vectors carrying Brn-3a, Brn-3b, Brn-3c, Lim-1, and its mutants. Figure 3.10 shows that deletion of the Lim domain abolishes the synergistic interaction between the Lim-1 and the Brn-3 proteins. In contrast, deletion of the C terminal activation domain does not abolish this interaction, but does marginally reduce the level of transactivation.

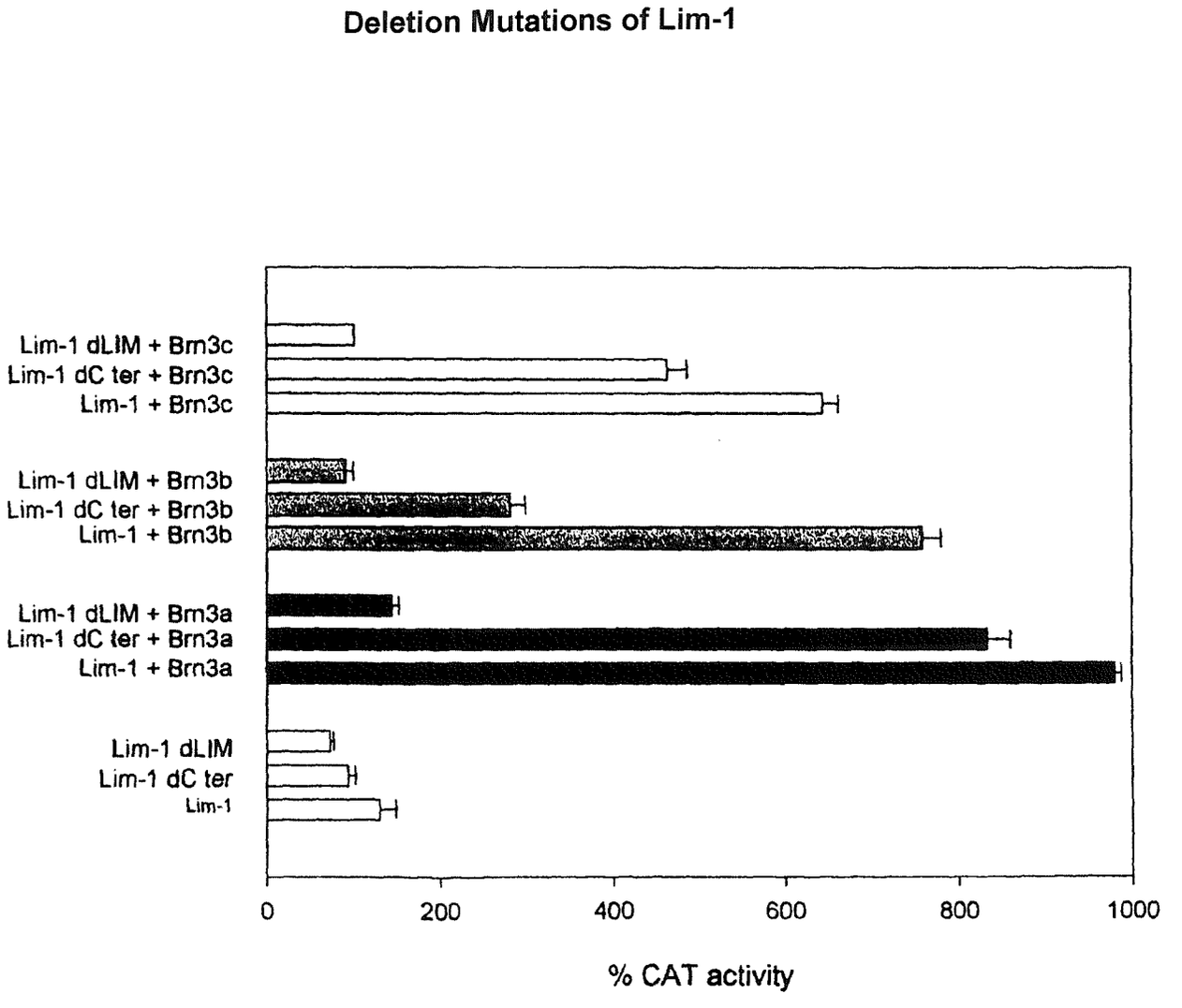


Fig.3.10: Deletions of the two domains of Lim-1 illustrate that both domains are needed for the synergism with Brn-3a for gene expression of the ICP0 promoter. Values are were normalised to the activity (100%) of an empty vector (PJ7).

Values are the mean of 3 independent experiments +/- SEM

We also investigated whether the deletion mutants of Lim-1 affected the expression of the reporter plasmid CATC. CATC was then co-transfected into BHK cells together with the Lim-1 mutants and Brn-3a. It was found that in the presence of Lim-1 and Brn-3a alone there was no increase in CATC expression. However, a large increase in CATC expression was shown when Lim-1 and Brn-3a were co-tranfected with CATC. However, this increase in CATC expression was abolished when Brn-3a was co-transfected with either Lim-1 mutant.

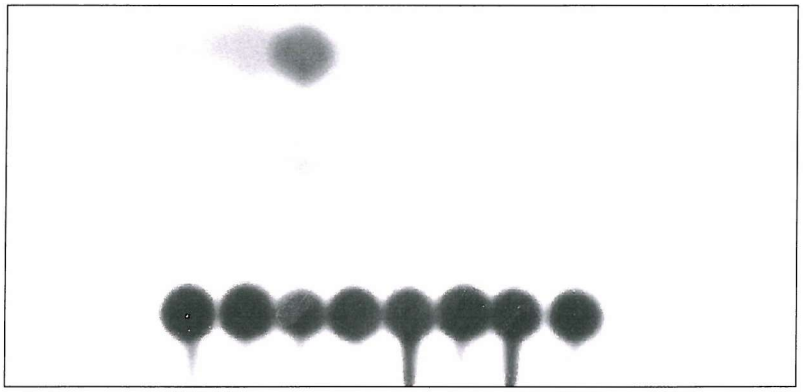


Fig 3.11 Chloramphenicol Acetyl Transferase (CAT) assay

BHK cells were transfected as follows: **Lane 1:** CATC (5 μg) with PJ7 (10 μg), **Lane 2:** CATC (5 μg) with Brn-3a (5 μg) and PJ7 (5 μg), **Lane 3:** CATC (5 μg) with Brn-3a (5 μg) and Lim-1 (5 μg), **Lane 4:** CATC (5 μg) with Brn-3a (5 μg) and M_1 (5 μg), **Lane 5:** CATC (5 μg) with Brn-3a (5 μg) and M_2 (5 μg), **Lane 6:** CATC (5 μg) with Lim-1 (5 μg) and PJ7 (5 μg), **Lane 7:** CATC (5 μg) with M_1 (5 μg) and PJ7 (5 μg), **Lane 8:** CATC (5 μg) with M_2 (5 μg) and PJ7 (5 μg)

Values were normalised to the activity (100%) of an empty vector

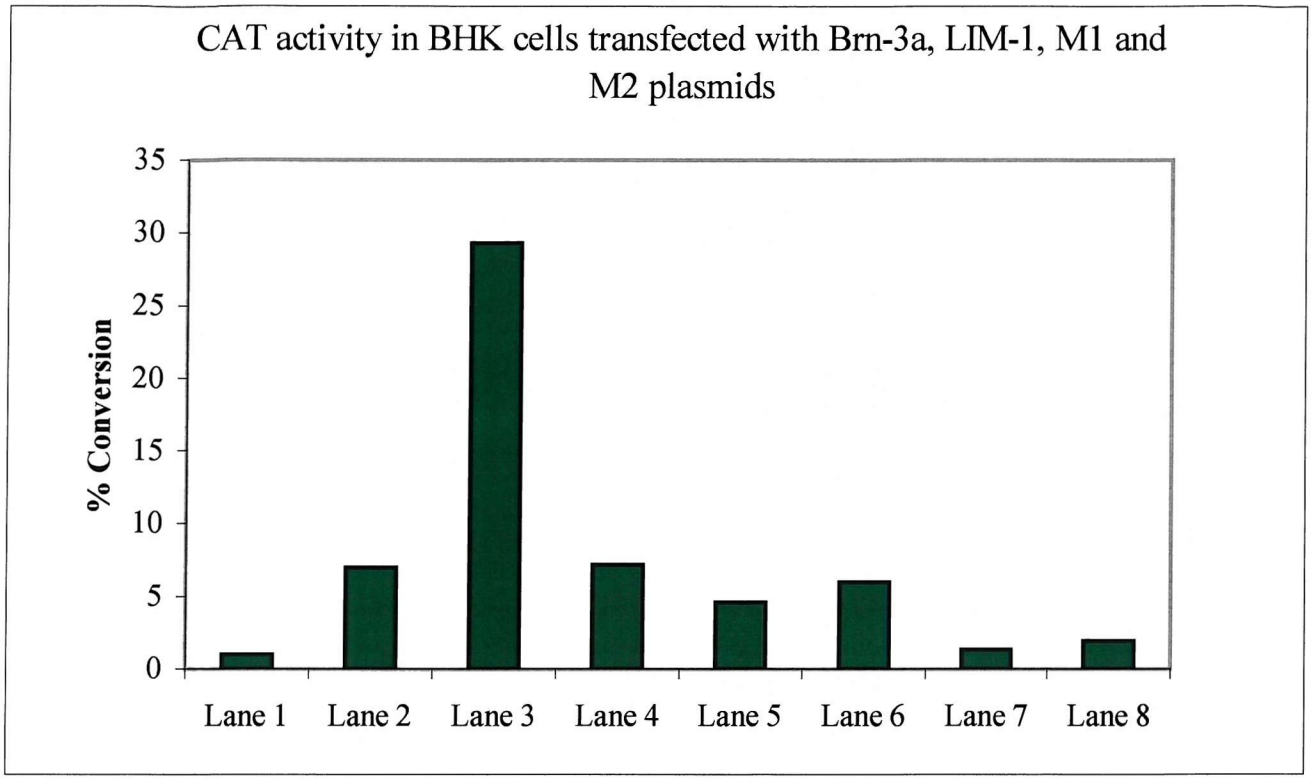


Fig 3.12: The above-displayed bar-chart illustrates the synergistic effect of Brn-3a and Lim-1 in inducing gene expression of the CATC promoter.

3.10 Summary

We found that primary sensory neurons express the LIM-HD proteins Lim-1 and Lim-3 as well as Isl-1, as previously reported. It has been shown that the combination of LIM-HD and Isl-1, Isl-2, Lim-1 and Lim-3 defines subsets of motor neurons. It has also been shown that the combination of LIM-HD genes Isl-1, Isl-2, Lim-1 and Lim-3 which are expressed in motor neurons, define specific subsets of motor neurons. It would be interesting therefore to determine whether these LIM-HD genes define specific subsets of sensory neurons by using in situ hybridization to monitor the expression of these genes within the sensory ganglia. Interestingly, the expression of the LIM-HD genes, Isl-1 and Lim-1 was influenced by NGF-upon NGF withdrwal, levels of Isl-1 and Lim-1 mRNA expression increased. As NGF withdrwal has also been shown to lead to HSV reactivation, and a fall in Oct-2.5 expression, then this result raises the possibility that LIM-HD proteins may play a role in viral reactivation also due to their ability to activate the ICP0 promoter. It was also revealed that while Lim-1 could only weakly activate ICP0 expression, in the presence of members of the Brn-3 subfamily of POU-domain proteins, ICP0 expression was greatly enhanced. Interestingly the expression of ICP4 was unaffected. This may be due to the difference between ICP0 and ICP4 promoters as ICP0 contains an overlapping TAATGARAT motif while ICP4 promoter contains only a single TAATGARAT motif. It would be interesting to determine using DNA binding assays the difference in binding affinity of Lim-1/Brn-3a for an overlapping TAATGARAT motif versus a single TAATGARAT motif or indeed to determine whether a heterodimer can form on a single TAATGARAT motif.

CHAPTER 4

DISCUSSION

4.1 Discussion

It is now universally accepted that the sensory neurons harbor the latent HSV virus and although these particular neurons have been of greatest interest, the fact that many neural tissues have been shown to harbor latent virus suggests that a variety of neurons can become latently infected (Stevens 1989). The lytic infection of the viral genome is expressed in three stages, resulting in the sequential transcription of the immediate-early (IE), early (E) and late genes (L).

The temporal regulation of HSV-1 gene expression during permissive infection commences with the induction of the IE genes by the virion protein VP16. VP16 activates transcription by binding, along with the cellular factors Oct-1 and host cellular factor (HCF), to the TAATGARAT (R=purine) elements present in all IE promoters.

In sensory neurons IE gene expresion is blocked. Sensory neurons express a number of POU-domain proteins including Oct-1, Oct-2 and the Brn-3 family of proteins. In sensory neurons the Oct-2 transcription factor is alternatively spliced to produce predominantly Oct-2.4 and Oct-2.5. Both of these proteins have been shown to bind to the TAATGARAT motifs within the IE promoters and repress expression. This has laed to the hypothesis that the presence of Oct-2.4 and Oct-2.5 in sensory neurons blocks the IE gene expression and HSV latent infection. Sensory neurons also express the Brn-3 subfamily of POU domain proteins. These like Oct-2.4 and Oct-2.5 can bind to the TAATGARAT motif in the IE promoter, actually with lower affinity than Oct-2.4 and Oct-2.5. However, the expression of Oct-2.4 and Oct-2.5 is decreased upon NGF withdrawal leading to the theory that the Brn-3 proteins may play a role in viral reactivation. However, as previous studies have shown that the Brn-3 proteins only bind to the TAAATGARAT motifs with low affinity, if the Brn-3 proteins play a role in reactivation then they are likely to do so in combination with other transcription factors. Interestingly, the nematode POU domain protein unc-86

has been shown to interact with the LIM-HD protein mec-3 and this interaction increases the binding affinity of unc-86 for its octamer binding site. As LIM-HD proteins are also like POU domain proteins evolutionary conserved, we initially determined which LIM-HD genes were expressed in primary sensory neurons. using degenerate PCR

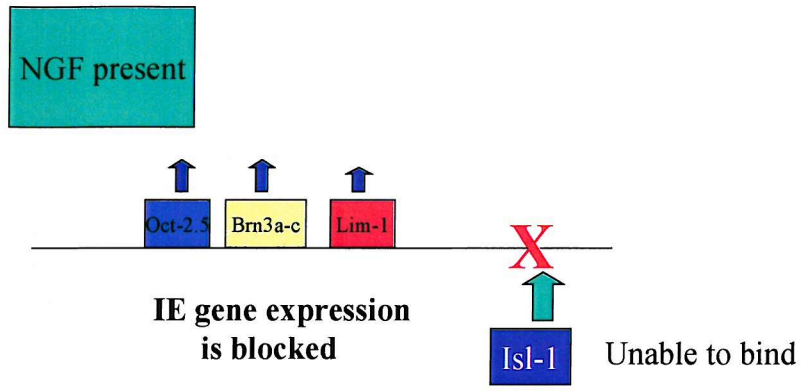
Sequencing results revealed that clones fell into four categories as shown in Table 1. The largest class did not show any homology to any known LIM-HD gene, beyond the sequence of the primer, these were therefore not studied further. The remaining 3 classes all showed sequence homology characteristic of LIM-HD genes. The largest of these classes, class 2, contained sequences identical to rat *Isl-1*, a LIM-HD gene shown previously to be expressed in sensory neurons of the trigeminal and dorsal root ganglion (Thor et al., 1991). Class 3 contained clones identical to *Rlim* (Furuyama et al., 1994) and class 4 contained clones with sequence homology to mouse *Lim-3* (Seidah et al., 1994), suggesting that these clones are likely to be derived from rat *Lim-3* mRNA.

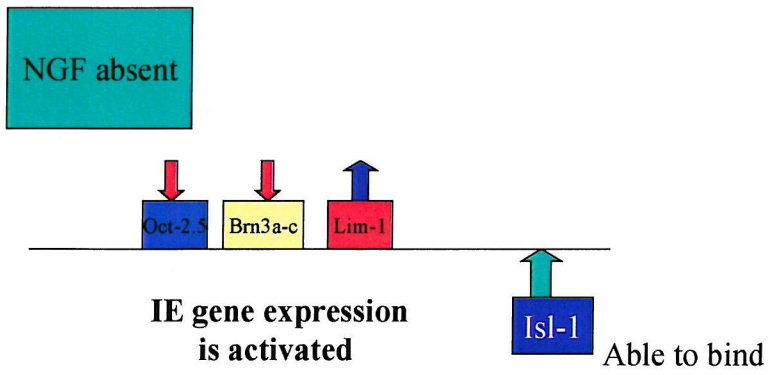
. It was also shown that this expression is modulated by NGF, since both Lim-1 and Isl-1 expression was induced by NGF withdrawal, while results with cyclophilin and Brn-3a alone remained constant. It was further illustrated that Brn-3a, Lim-1 and Brn-3c alone are poor activators of ICPO expression. Conversely, Brn-3a and Brn-3c in conjunction with Lim-1 synergise in the induction of ICPO expression. Similar experiments in ICP4 gene expression revealed no induction in gene expression and therefore no synergism between Lim-1 and Brn-3 POU proteins. This is possibly due to the fact that ICP4 contains only a simple TAATGARAT motif whereas ICPO contains an overlapping octamer/TAATGARAT motif that is critically required for the synergism to happen. It was also evident that the synergism between Brn-3 proteins and Lim-1 leads to induction of the ICPO and CATC gene expression via the octamer/TAATGARAT motif when ND7 and BHK cell lines were used.

Furthermore, we demonstrated the roles of the two domains of Lim-1 in this interaction with Brn-3a. Results from deletion mutations revealed that both domains of Lim-1 are required for the synergism with Brn-3a, although the Lim domain appears to play a more critical role.

From these results, one can speculate the possibility that viral reactivation could be caused by the synergistic interaction between Brn-3a and Lim-1. To prove this further would need to investigate the role of the octamer/TAATGARAT motif sequence in the ICPO promoter within HSV-1 genome.

A potential model for the activation of IE gene expression is shown below:





CHAPTER 5

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