CHARACTERISATION OF NOVEL NEURONAL CELL LINES

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To my sister, Helen.

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Abstract

Novel hybrid neuronal cell lines, derived from neonatal rat primary dorsal root ganglion neurons and a neuroblastoma, have been studied to ascertain the similarity of some of their properties to those of primary sensory neurons.

Particular attention has been paid to the non-permissive interaction between these cells and Herpes Simplex Virus, as the natural site of HSV latent infections is the sensory ganglion. The permissivity of the ND cells has therefore been established with respect to a permissive cell line.

The mechanism of this non-permissive interaction has also been investigated. Elements from the HSV IE gene promoters that bind the HSV IE gene transcription activating complex containing oct-1 and VP16, are shown to also bind an inhibitor of viral IE gene transcription. Such sequences, when introduced into a cell containing IE genes, are able to increase the level of transcription of the IE genes that is normally low. A model of transcriptional inhibition of the viral immediate-early genes in neuronal cells is proposed.

The effect on HSV IE gene expression of cyclic AMP is shown to be either stimulatory or repressive, depending on the concentration. It is shown that the transcription of the IE genes is stimulated at low levels of cyclic AMP via a cyclic AMP responsive element in the IE-1 gene promoter, which is likely to be responsible for the subsequent induction of at least IE-3 gene transcription. This may represent a molecular mechanism of reactivation from latent HSV infections, particularly as cyclic AMP has been observed by others to reactivate latent HSV infections.

ND cells can be differentiated *in vitro* by modulation of the culture medium. Little change in gene expression occurs during the morphological differentiation, suggesting that ND cells are mature in the undifferentiated form. However, the pathways of exocytosis mature as neurites are extended and this is demonstrated by a redistribution of proteins associated with exocytotic vesicles to the neurite tips.

The ND cells represent many aspects of the dorsal root ganglion neuron whilst maintaining the immortal phenotype from the neuroblastoma. These cells therefore represent an *in vitro* model of the cells of dorsal root ganglion as they exhibit a non-permissive interaction with HSV and on differentiation acquire the pathways of regulated exocytosis.

Abbreviations

A	Adenosine
AP1	Activating Protein 1
ATF	Activating Transcription Factor
ATP	Adenosine Triphosphate
bp	base-pair(s)
C	Cytosine
cAMP	Cyclic adenosine monophosphate
DNA	Deoxyribonucleic acid
G	guanosine
HCMV	Human Cytomegalovirus
Hepes	Hydroxyl piperazineethanesulphonic acid
HSV	Herpes Simplex Virus
ICP	Infected Cell Protein
IE	Immediate-Early
Kb	Kilo-base pair(s)
LTR	Long Terminal Repeat
min	Minutes
MOI	Multiplicity of Infection
mRNA	Messenger ribonucleic acid
oct-1	Octamer motif binding protein
pfu	Plaque forming unit
pi	Post Infection
RNA	Ribonucleic acid
sP1	Stimulatory Protein 1
T	Thymidine
Tris	Hyroxymethyl aminomethane
ts	Temperature sensitive
VZV	Varicella Zoster Virus
wt	Wild Type

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Chapter 1 Introduction

1.i. Herpes Simplex Viruses

Herpes Simplex Viruses (HSVs) are linear double-stranded (ds) DNA alphaherpesviruses which naturally infect only humans and chimpanzees (McClure *et al*, 1980). However under experimental conditions, they can infect a wide range of mammalian cells.
Two separate serotypes (types 1 and 2) were identified by Schneweiss in 1962 and these may be distinguished by subtype-specific anti-glycoprotein G antibodies (Whitley, 1990). They are now correctly designated Human Herpesviruses1 and 2 according to the International Committee on the Taxonomy of Viruses (Roizman and Sears, 1990).

On infection with HSV, two outcomes of infection are possible. A lytic cycle of infection can occur in the mucosal epithelia of the mouth, nose (HSV-1) and genitalia (HSV-2) and this results in the production of infectious virions and host cell death. A latent infection may be subsequently established, in the sensory ganglia which innervate the initial sites of infection. Here, non-dividing viral genomes are maintained. In response to a variety of stimuli, these have the ability to periodically reactivate and produce infectious progeny at the initial site of infection. If a latent infection is established in the central nervous system (CNS) and it is induced to reactivate, fatal encephalitis can ensue (Kibrick *et al*, 1965, Leider

et al, 1965). HSV-induced encephalitis has also been established in experimental animals (Good and Campbell, 1948, Stevens, 1975). The study of HSV is therefore of both academic and clinical interest as the virus is capable of lying dormant for decades in a neuronal cell in the body of the host or of totally destroying an epithelial cell within one day.

Observations in the past have also suggested a role for HSV in the aetiology of cervical cancer. However, experimental work to date has not provided conclusive evidence supporting this theory and together with the association of the disease with Papilloma Virus, HSV is not now generally considered to be a factor in the progression of this disease (Whitley, 1990).

1.ii. HSV Structure

HSVs 1 and 2 are structurally similar. The virion measures 120 nm in length and contains 30-35 proteins (Heine *et al*, 1974). The core is electron-opaque and may be described as a torus of DNA spooled around a cylindrical mass containing protein (Furlong *et al*,1972). Surrounding this core is an icosadeltahedral capsid consisting of 162 capsomeres which is in turn covered by an amorphous tegument. The outer envelope covering the virus consists largely of spikes which contain glycoproteins.

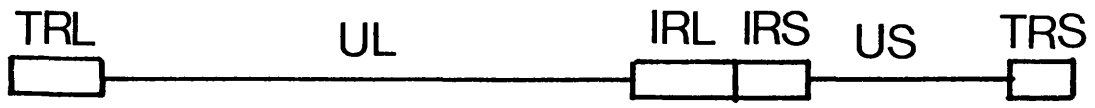
1.iii. HSV Genome

The genome of HSV is a linear ds DNA molecule, approximately 150 Kb in length (Roizman and Sears, 1990). It is divided into two covalently-linked segments, unique-long (U_L) and unique-short (U_S), each of which is flanked by inverted repeats (Sheldrick and Berthelot, 1974), see **Figure 1.1a**. Because of this genomic structure, four isomers of sequence orientation of the DNA are possible (Delius and Clements, 1976).

The genes encoded by HSV are divided into three temporal classes, immediate-early (IE), early (E) and late (L). The IE genes map near the terminii of the unique components, two of them being positioned in the inverted repeats and therefore being diploid, see **Figure 1.1.b**. The gene lay-out of the related herpesvirus Varicella Zoster Virus (VZV) is very similar to that of HSV in the U_L segment (McGeogh *et al*, 1988). The HSV genome encodes at least 70 polypeptides (McGeogh *et al*, 1985,1988) and some of these have been classified as non-essential, at least in tissue-culture systems. The thymidine kinase gene is a classic example of such a gene (Kit and Dubbs, 1963). Genes dispensable for growth appear to be clustered in the short segment (Longnecker and Roizman 1987).

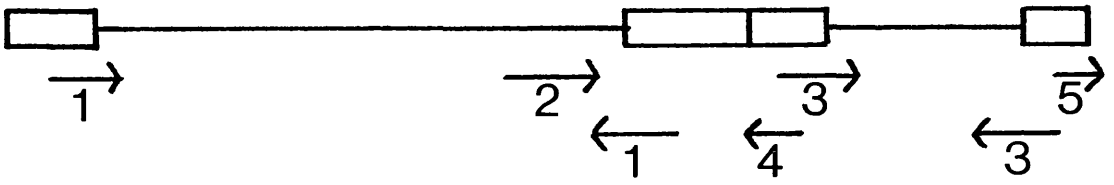
Figure 1.1

a) Structure of the HSV Genome



The genome consists of approximately 150 Kb of DNA divided into the long (L) and the short (S) components. Each component is flanked by inverted repeats. (T: terminal, R: repeat, L: long segment, I: inverted, S: short segment.)

b) Map Positions of the Immediate-Early Genes



The IE genes encoding ICP0 and ICP4 are located in the inverted repeats and are therefore diploid.

1.iv. HSV Life-Cycle: Lysis

a) Host Cell Binding

HSV first encounters the host cell by binding to the glycosaminoglycan heparin sulphate (WuDunn and Spear, 1989, Lycke *et al*, 1991). Recent work has suggested that glycoprotein B (gB), gC and gD all play a role in binding and penetration (Campadelli-Fiume *et al*, 1988, Kuhn *et al*, 1990, Muggeridge *et al*, 1990). gH may be important in penetration through the plasma membrane (Buckmaster *et al*, 1984, Bzik *et al*, 1984). It has also been suggested that HSV binds to the high affinity receptor for fibroblastic growth factor (FGF) (Kaner *et al*, 1990). However this observation has not been repeated by other investigators (P. Spear, personal communication, Mirda *et al*, 1992).

Following binding, the virus penetrates the cell membrane. The theory of viral envelope fusion with the plasma membrane (Morgan *et al*, 1968) still retains greatest favour, especially with the observation that entry of virus by endocytosis results in no release of progeny (Campadelli-Fiume *et al*, 1988). Once inside the cell, the capsid containing the viral DNA moves to the nuclear membrane, mediated by the cytoskeleton, where it migrates through a nuclear pore (Tognon *et al*, 1981, Batterson *et al*, 1983). The release of capsid proteins and viral DNA into the nucleus requires a viral function (Batterson *et al*, 1983). In the nucleus the DNA circularises so that viral transcription is probably from a circular episome-like structure.

b) Gene Expression

On circularisation of the viral DNA in the host cell nucleus, viral gene expression proceeds in a sequential co-ordinately regulated fashion (Honess and Roizman,1974). This results in a cascade of gene expression occurring in the three interdependent temporal phases designated IE, E and L. The IE genes encode mainly regulatory proteins and are expressed 3-4 hours post-infection (p. i.). They require no prior protein synthesis (Roizman and Sears, 1990). The E genes produce proteins involved in DNA replication and appear 5-7 hours p. i. and the L genes encode structural proteins which appear after 12 hours of infection. These phases have also been defined using RNA and protein synthesis inhibitors such that IE proteins are required prior to E and L gene expression (Clements *et al*,1977, Watson and Clements,1980) and the E proteins prior to L gene expression. The appearance of E proteins during the infection accompanies the decline of the IE proteins, as the appearance of L proteins accompanies E protein production decline (Honess and Roizman, 1974, 1975). The L proteins have been further divided into two classes on the basis of their dependence on DNA replication; 'leaky' late proteins are expressed before 'true' late proteins, which are dependent on DNA replication for their expression.

Initiation of IE Gene Expression

The IE genes are five in number and are designated IE-1, IE-2, IE-3, IE-4 and IE-5. They encode infected cell proteins ICP0, ICP27, ICP4, ICP22 and ICP47 respectively. The host RNA polymerase is necessary for all viral transcription (Costanzo *et al*, 1977). Together with the

TATA box and the proximal promoter needed for basal transcription (Gelman and Silverstein, 1987, Kemp *et al*, 1990), high level activated transcription of the IE genes is mediated by a *cis*-acting sequence present in one or more copies in the far upstream regions of the IE promoters (Mackem and Roizman, 1982, Cordingley *et al*,1983, Whitton and Clements, 1984, Kristie and Roizman, 1984, Gaffney *et al*, 1985, Bzik and Preston, 1986). This *cis*-acting motif, TAATGARATTC (R=purine), can enhance transcription up to 100 fold (Cordingley *et al*, 1983) and is active in either orientation (Bzik and Preston, 1986). Other enhancer-like sequences also exist in the IE promoters. For example, sequences which bind the transcription factor Sp1 (Jones and Tjian, 1985, Gelman and Silverstein, 1987), SV40 enhancer 'core'-like sequences and sequences homologous to important motifs in the adenovirus E1a and Polyoma Virus enhancers (Bzik and Preston, 1986). However, together these enhance transcription only 10 fold (Bzik and Preston, 1986). A factor carried in the tegument of the virus is responsible for the induction of IE gene expression via the TAATGARATTC sequence (Batterson and Roizman, 1983). Now identified as the late protein Vmw65 (VP16, α-TIF) (Campbell *et al*, 1984, Pellet *et al*, 1985), this protein is a highly potent transcriptional activator (Sadowski *et al*, 1988). However it has no affinity for DNA (Marsden *et al*,1987, Post *et al*,1981) but instead exerts its effect on the IE promoters by forming into a multi-protein complex with a cellular factor which binds to the TAATGARATTC motif (Kristie and Roizman, 1987, McKnight *et al*, 1988,Triezenberg *et al*,1988a,1988b). This cellular factor is the ubiquitous octamer-binding protein, oct-1 (OTF-1, NFIII) (Preston et al, 1988, O'Hare and Goding, 1988, Gerster and Roeder, 1988, Kristie

et al, 1989), which normally directs the transcription of cellular genes which contain the octamer motif (ATGCAAATNA) in their promoters (Kemp and Latchman, 1988, Preston *et al*, 1988). Such genes include the snRNA genes and the histone genes (Reviewed by Falkner *et al*, 1986).

Oct-1 is one of a number of octamer-binding proteins which belong to the POU family of transcription factors, the members of which all share a highly conserved domain (the POU domain) (Sturm and Herr, 1988). This has been sub-divided into the POU-specific and the POU-homeobox. The POU-homeobox is related to the homeobox found in the homeodomain genes of *Drosophila* and many of the POU proteins discovered to date all have roles in development, particularly of the nervous system (Rosenfeld, 1991). The POU-homeo domain and POU-specific domain are responsible for the high-affinity and sequence-specific DNA-binding activity of oct-1 (Sturm and Herr, 1988), whilst the POU-homeo domain is employed in the protein:protein interaction with Vmw65 (Stern *et al*, 1989). At least two other cellular proteins also bind in the oct-1-Vmw65 complex (Kristie et al, 1989). At this point it is noteworthy to state that the TAATGARATTC sequence and the octamer sequence are highly related in their ability to bind octamer-binding proteins. The TAATGARATTC sequence may be considered as a degenerate octamer motif.

Observations have also been made to suggest that ICP4 is involved in negatively regulating the transcription of some of the IE genes, including itself (O'Hare and Hayward, 1985). In these experiments the promoter from the gene encoding ICP4 when linked to the gene

encoding the readily assayable enzyme chloramphenicol acetyl transferase (CAT) was repressed by an ICP4-expressing plasmid. It was also shown that ICP4 bound at the CAP site, whereas in the other IE genes it binds further upstream. When present as a temperature-sensitive allele in the mutant virus tsK, over-production of the IE proteins occurs at the non-permissive temperature (Preston, 1979). ICP4 has been shown to down-regulate ICP0 and ICP27 in a co-transfection assay (Gelman and Silverstein, 1987). However recent observations have disputed that ICP4 can down-regulate the ICP0 gene (Orr and Everett, 1991).

Initiation of E and L Gene Expression

High levels of IE proteins are required for the induction of the E and L gene transcription and the subsequent production of progeny virions. In particular ICP4 is required for transcription of the E genes encoding proteins such as thymidine kinase (tk), the major DNA binding protein ICP8 and the viral DNA polymerase (Preston, 1979, Watson and Clements,1980, O'Hare and Hayward,1984). ICP4-induced genes contain the consensus sequence ATCGTCNNNNYCGRC (Faber and Wilcox, 1986) in their promoters, this sequence also being present in the IE promoters which are down-regulated by ICP4 (ICP0 and ICP27). ICP0 has also been shown to be involved in E gene transcription (O'Hare and Hayward, 1985, Quinlan and Knipe, 1985, Gelman and Silverstein, 1985) in one case by augmenting the response of a gD promoter to ICP4 (Everett, 1984). It has also been shown that a virus mutant in ICP0 is reduced in its ability to produce E proteins and in its plating efficiency (Cai and Schaffer, 1991). It was also shown in these experiments that a cellular protein could substitute for

the ICP0 function.

ICP27 is an essential protein that stimulates the transcription of the E protein gB and that of other E and 'leaky' late proteins such as gD, although to a lesser extent than do the other IE proteins (Sacks *et al*, 1985, Rice and Knipe, 1988). Mutant viruses containing a temperature-sensitive allele of ICP27 show the dependence on ICP27 of gB and L protein expression (Sacks *et al*, 1985). ICP27 is active on E genes to a more limited degree than ICP0 and ICP4 (Everett, 1986), although it acts in concert with these other two IE proteins. ICP27 can also act in a negative manner (Rice *et al*, 1989) again in combination with both ICP0 and ICP4 (Sekulovich *et al*, 1988).

Only ICP4 and ICP27 have been shown to be essential for productive infection in tissue culture (Dixon and Schaffer, 1980, Post and Roizman, 1981, Sacks *et al*, 1985, Sacks and Schaffer, 1987). By using viral deletion mutants, it has been shown that ICP22 is required late in infection and has been found to be dispensable in some tissue culture systems (Sears *et al*, 1985), whilst ICP47 is dispensable at least in some systems (Mavromara-Nazos and Roizman, 1986).

'True' late gene expression comprising genes such as gC and Vmw65 is dependent on DNA replication (Johnson et al, 1986). There is a also a role for the major viral DNA binding protein ICP8 in late gene expression, a role that is distinct from its role in DNA replication (Gao and Knipe,1991).

Other Gene Regulatory Mechanisms

Viral RNAs may be selectively held in the nucleus and thus prevented from being translated (Latchman, 1990 Roizman and Sears, 1990). There is however little regulation of gene expression at the level of

RNA splicing, although spliced viral transcripts are produced. The Latency-Associated Transcripts (LATs, see later) are an example of spliced viral transcripts (Wagner *et al*,1988).

The virus-host shut-off function (VHS, see later) can down-regulate translation of the IE proteins as this has been shown to occur in enucleated cells where proteins normally involved in such processes are absent (Roizman and Sears, 1990). It has also been observed that in the change from IE to E/L protein production, the IE RNAs stay associated with the polyribosomes, suggesting a translational control is at work to down-regulate IE protein production. In a similar fashion, as the rate of gD protein production decreases, the RNA nevertheless remains abundant.

Post-translational modifications may regulate the activity of the viral proteins and these include phosphorylation, particularly of ICP4 (Papavassilou *et al*, 1991), sulphation, glycosylation (the glycoproteins) and poly-ADP ribosylation. It has been suggested that these changes may accompany translocations across the membrane (Matthews *et al*, 1983).

c) DNA Replication

There are three origins of replication (Locker *et al*, 1982, Stow, 1982, Deb and Doelberg, 1988) and the virus itself expresses many functions necessary for the replication event. Replication occurs for 9-12 hours beginning at 3 hours p.i., probably by a rolling circle mechanism, and is such that concatemers of viral genomes are cleaved

into monomers and packaged into capsids (Ben-Porat and Tokazewski, 1977, Jacob *et al*, 1977). During the processing of the DNA, part of the repeated sequences (the 'a' sequences) are amplified and viral DNA lacking free ends (concatemerised DNA) is cleaved. Isomerisation of the viral genome is linked to this stage (Roizman and Sears, 1990).

d) Host Cell Macromolecular Synthesis Shutdown

As the viral infection proceeds, major structural and biochemical changes occur in the host cell. The structural changes include enlargening, displacement and fragmentation of the nucleolus and margination of the host chromosomes (Roizman and Sears, 1990). The cell membranes thicken, possibly due to tegument proteins on the inside of the nuclear and cytoplasmic membranes, and the cells round up and clump (Morgan *et al*, 1959).

Biochemical changes include decreases in the rates of cellular DNA, RNA and protein synthesis (Roizman *et al*, 1965). By 3 hours p. i., ^{3}H U incorporation into RNA is decreased by 50 % and protein synthesis is decreased to 70 % of the control. Disaggregation of those polyribosomes translating host mRNAs also occurs. It is thought that such changes are brought about by a protein(s) carried in the tegument (the VHS protein[s]) as UV light treatment of virions before infection results in no disaggregation. Furthermore, actinomycin D does not prevent this disaggregation (Read and Frenkel,1983). A second wave of destruction of host cell functions occurs 6 hours p. i., after which time an E/L-dependent *de novo*-synthesised protein can have been made (Sydiskis and Roizman,

1966, 1967, Read and Frenkel, 1983). This second wave correlates with a second burst of polysome breakdown.

Host cell mRNA sequences are degraded after infection with HSV (NIshioka and Silverstein, 1977, 1978a). The decrease in cellular DNA and RNA is also thought to be brought about by a function carried by the virus (Fenwick and Walker, 1978) but it has also been shown that the degradation of cellular RNA requires the expression of the viral genome (Nishioka and Silverstein, 1978b).

Whilst the majority of host cell macromolecular synthesis is down-regulated, certain genes have been shown to be up-regulated. For example, the 90 Kd heat shock protein is elevated on infection (LaThangue and Latchman, 1988) as is the p40 protein (LaThangue and Latchman, 1988), and the Sm auto-antigen (Bachmann *et al*, 1976, Sharpe *et al*, 1989). These increases are likely to be accounted for by changes in the transcription rate (Patel *et al*, 1986, Latchman *et al*, 1987). Changes in transcription also account for the increase in abundance of the U3 snRNA (Estridge *et al*, 1990) and transcripts from the *Alu* repeats (Jang and Latchman, 1989). The increase in abundance of these transcripts may indicate sequences in the promoters of the genes encoding these proteins that are similar to the TAATGARATTC sequence. The promoter may therefore be *trans*-activated by the oct-1/VP16 complex. This idea is borne out by the studies of Kemp and Latchman (1988) which showed that some of the genes which are transcriptionally induced on HSV infection have near-perfect octamer sequences, related to the TAATGARATTC sequences found in the IE promoters.

e) Envelopment and Release of Virions

Virions form in the nucleus and appear as particles with a single membrane around a dense nucleoid body (Roizman and Sears. 1990). At the inner nuclear membrane immature glycoproteins are acquired which mature during the transit to the extracellular space (Torrisi *et al*, 1992). The mature extracellular virus has two membranes (Sears and Roizman,1990). Viruses leaves the cell by bulging as buds throught the cell and vacuolar membranes. This budding is a relatively slow process (Epstein, 1962). It has also been noted that particles with double membranes occur in the nucleus, suggesting that development can be completed there (Morgan *et al*, 1959).

f) Anti-HSV Treatment

The drug Acyclovir (acycloguanosine) has been shown to be effective against the lytic HSV cycle. It is phosphorylated by the viral thymidine kinase and is subsequently incorporated into newly-synthesised DNA, resulting in chain termination (Elion *et al*, 1977). However, it is therefore ineffective against latent viral genomes (see below), and also does not relieve well-established viral lytic infections.

1.v. HSV Life Cycle: Latency

a) Historical Background

(Reviewed by Baringer, 1975 and Stevens, 1975)

The phenomenon of a latent HSV infection has been observed since the beginning of this century. Vidal established the infectious nature of the disease in 1873 when he re-inoculated herpetic lesion material back into the same patient. After experiments at the turn of the century, Cushing noted in 1904 that an eruption of herpetic vesicles occured after trigeminal rhizotomy, suggesting an association of HSV with the nervous system. The observations by Doerr and Vochting in 1920 that rabbits with Herpetic Keratitis had nervous symptoms further compounded this link. Histological lesions were observed in the trigeminal ganglia after corneal inoculation of the virus by Friedenwald in 1923, as were lesions in the optic system and vagal nuclei after inoculations in the retina and trachea respectively by Goodpasture and Teague in the 1920s. These results led Goodpasture to speculate that HSV was most likely harboured in the trigeminal ganglion and that the most likely route of travel was the axon. The association of the disease with the nervous system was further corroborated after the observation by Findlay and McCallum in 1943 that the recurrent disease was highly localised. Observations by Carton and Kilbourne in 1952 that injury to the centrally projecting fibres of the trigeminal ganglia resulted in reactivation led to the idea again that HSV was being harboured in the olfactory bulb and trigeminal ganglia. Carton in 1953 and Ellison *et al* in 1959 also documented again that HSV lesions on the lip and mouth arose in

patients who had had trigeminal rhizotomy. That the lesions were often preceded by parasthesias or neuralgic pain was noted in subsequent years by Behrman and Knight in 1954, Constantine *et al* in 1968 and Layzer and Conant in 1974. It is now generally accepted that HSV resides in a latent form in the sensory ganglia and more specifically in the trigeminal and dorsal root ganglia (HSV-1) and sacral ganglia (HSV-2).

b) Detection of HSV Genomes in Sensory Ganglia

By using the technique of explant co-cultivation, it has now been well corroborated that HSV indeed resides in a latent form in the sensory ganglia (Cook and Stevens, 1971, Bastian *et al*, 1972, Baringer and Swoveland, 1973, Baringer, 1974, Warren *et al*, 1978) and autonomic ganglia (Reaves and Heath, 1975) of humans and experimental animals. Virus particles have since been observed in the trigeminal ganglia of latently infected rabbits (Baringer and Swoveland, 1973) and viral DNA has also been detected by *in situ* hybridisation in latently infected murine trigeminal, dorsal root and autonomic ganglia (Stroop *et al*, 1984, Cook *et al*, 1974) and CNS of mice (Cabrera *et al*, 1980, Rock and Fraser, 1983) and in the trigeminal ganglia and brainstem of humans (Efstathiou *et al*, 1986). The site of viral latency has also been unequivocally established as the neuron (Cook *et al*, 1974, McLennan and Darby, 1980, Stroop *et al*, 1984).

Estimations of the number of viral genomes maintained in a single neuron have been made and these more or less agree with the estimation of 0.1 genome equivalent / cell (Puga *et al*, 1978, Cabrera *et al*, 1980).

It is now generally accepted that the latent HSV genome is present in the neuron in an extrachromosomal episomal form (Mellerick and Fraser, 1987), although the idea of an integrated concatemer has not been ruled out. While joining of the terminii was detected in a mouse latency model (Rock and Fraser, 1985), this has not been observed in other *in vitro* systems although these employed drugs, supraoptimal temperatures and interferon and therefore may not represent the true latent state. The transcriptional silence of the viral genome (see below) has led investigators to study the methylation state of the latent virus, as methylation is important in the regulation of eukaryotic genes (Doerfler, 1981). To this end, a persistent infection has been established in B cells which could be switched to 'latency' using Concanavlin A (Youssoufian *et al*,1982). In this situation, the genome was present at 0.1 copies / cell and was extensively methylated. The genome was transcriptionally silent. The infection could also be switched to a lytic type using Phytohaemagglutinin, which caused the DNA to become non-methylated and active. The nucleotide analogue 5-Azacytidine (5-AZC) has also been used to study the methylation of latent HSV genomes. 5-AZC reduces cytidine methylation when it is incorporated into newly-synthesised DNA in place of cytidine, but is unable to be methylated. When added to explant cultures of latent HSV-2 infected guinea pig DRGs and spinal cords (SC), the rate of HSV recovery was enhanced (Stephanoupoulos *et al*, 1988). This was due to an increased induction of reactivation rather than increased DNA replication. 5-AZC, L-ethionine and dimethyl sulphoxide (DMSO) were also used successfully to increase the efficiency of isolation of HSV from intact or disaggregated murine ganglia (Whitby *et al*, 1987). However it has recently been

shown by Dressler *et al* (1987) that latent virus found in the brainstem of the mouse is not extensively methylated, suggesting that another type of regulation is present.

c) RNA Expression in Latently Infected Neurons

No transcripts from the IE genes of HSV nor indeed from the E or L genes have been detected in latently infected ganglion cells (Stevens *et al*, 1987). It seems likely therefore that the lytic cycle is aborted at the stage of IE gene transcription resulting in a latent infection being subsequently established.

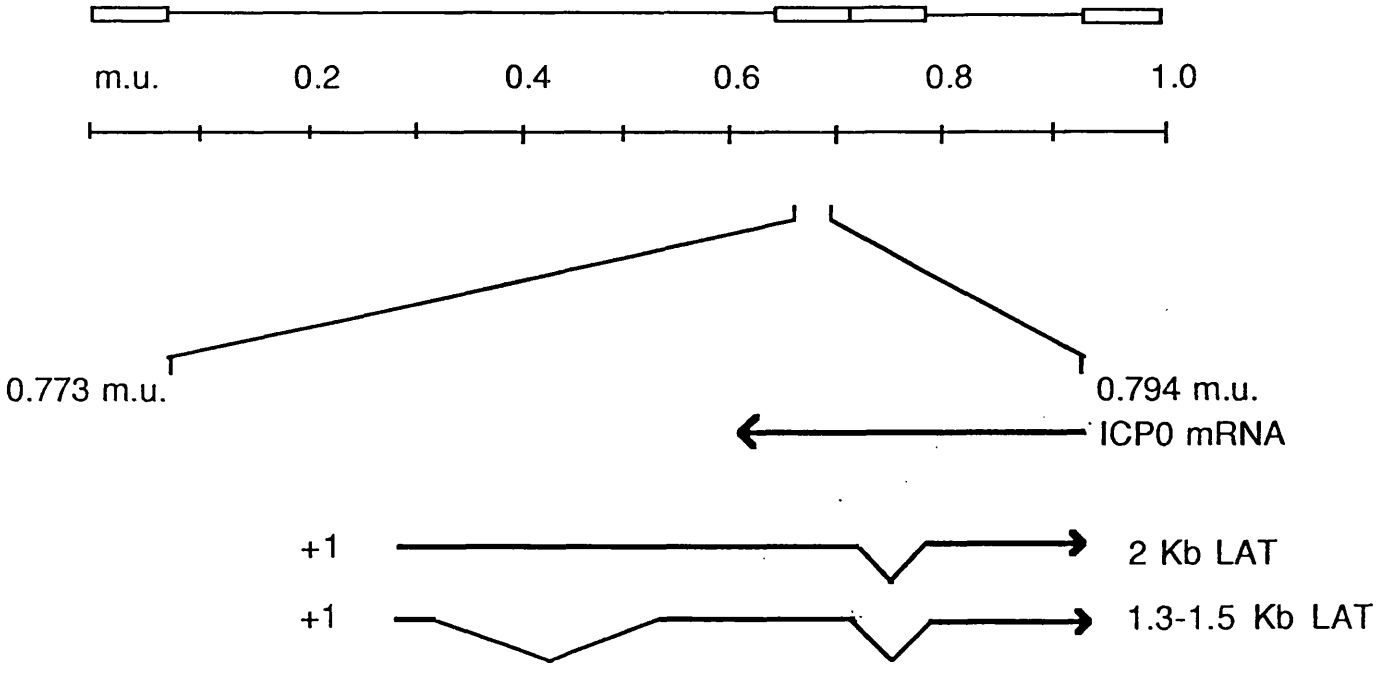
That a small section of the genome was transcribed during a latent HSV infection was suggested in 1979 (Galloway *et al*). Subsequently it was proposed that specific sequences were transcribed (Galloway *et al*, 1982). Even later experiments showed that a gene co-linear with that encoding ICP0 is transcribed in latently infected trigeminal ganglia (Puga and Notkins, 1987, Deatly *et al*, 1987), and using single-stranded probes more specifically that the RNA produced was anti-sense to the ICP0 mRNA (Stevens *et al*, 1987, Croen *et al*, 1987, Gordon *et al*, 1988).

Three such Latency-Associated Transcripts (LATs) were originally described, of 2.0, 1.5 and 1.45 Kb in length, which overlap the 3'end of the transcript derived from the gene encoding ICP0. They are transcribed from the opposite strand to that from which ICP0 mRNAs are transcribed (Spivack and Fraser,1987), see **Figure 1.2.** Transcripts of 1.85 and 1.35 Kb were later reported (Krause *et al*, 1988).

Figure 1.2

Relative Positions of the Genes Encoding the ICP0 mRNA and the Latency-Associated Transcript

The 3' ends of the ICP0 gene and that encoding LAT are overlapping.



Although originally identified as poly-adenylated (Puga and Notkins, 1987), the LATs have since been characterised as being far less poly-adenylated than actin (Wagner *et al*, 1988a). In fact, further investigation by Wagner *et al* (1988a) found 95 % of the LATs in the poly A- fraction and they also detected hybridisation in an area outside the previously mapped LATs.

The different LATs appear to be spliced from the same primary transcript, that is they share their 5' and 3' ends, and they also have two potential open reading frames (Weschler *et al*, 1988). Slightly different sizes of transcripts were reported by Wagner *et al* (1988b) who detected transcripts of 2.1-2.2 Kb and of 1.4 Kb and they suggested that the smaller was a spliced variant of the larger. It has also been suggested that the 2 Kb transcript is a stable intron (Farrell *et al*, 1991).

On sequencing a short distance upstream of the 5' end of the LATs, no consensus promoter sequences were found. However, a TATA box was discovered at -687, a CAAT box at -817 and Sp1 sites at -887, -863 and -589 upstream from the start point of transcription. It was therefore suggested that a 5' intron may exist and that the primary transcript is synthesised from a promoter much further upstream (Weschler *et al*, 1988). A much larger but minor transcript was later detected as a weak signal on a Northern Blot of approximately 8.3 Kb. This was found to be co-linear with the smaller and major transcripts and extended both 5' and 3' to the previously cha racterised LATs (Mitchell *et al*, 1990, Zwaagstra *et al*, 1990).

Recently a novel 5' structure of the LAT introns has been identified (Spivack and Fraser, 1991). This may represent a control mechanism such that splicing only occurs in sensory neurons during latency.

Splicing results in a 276 bp leader sequence being juxtaposed next to open reading frames and removes an intron that inhibits translation.

A Cyclic AMP-responsive element (CRE) has also been identified in the LAT promoter (Leib et al, 1991) as has a Latency Promoter Binding factor (LPBF) site which if deleted in non-neuronal or neuronal cells decreases the activity of the promoter 8-30 fold (Zwaagstra *et al*, 1991).

The Role of the LATs

The role of the LATs has been the subject of intense investigation. The LATs may encode a protein to either maintain latency or to regulate the lytic cycle. It has also been suggested that the LATs may act as anti-sense inhibitors to the ICP0 mRNA (Croen *et al*, 1987, Stevens *et al*, 1987) and that their levels may decline on reactivation, facilitating expression of the ICP0 gene which is essential for reactivation (Stow and Stow, 1986). The idea that the LATs encode proteins has recently been vindicated, as an antibody raised against a LAT-β-galactosidase fusion protein has detected a protein of approximately 80 Kd in latently infected sensory ganglia (Doerig *et al*, 1991) although the size does not agree with the size predicted by Weschler *et al* (1988). The role of this protein in latency however remains elusive.

The 2.0 Kb transcript has been shown to be present in far greater abundance in latently infected ganglia than in lytically infected tissue culture cells (Spivack and Fraser, 1987). These investigators also measured a steady increase of the LATs over 60 days and a decrease during reactivation even when progression through the

reactivation event was blocked (Spivack and Fraser, 1988). This suggested that a change in abundance of the LATs is an early event in reactivation.

To further try to assign a role to the LATs, mutant viruses have been constructed which lack the LAT gene. The mutant 1704 (Steiner *et al*, 1989) has deleted most of the IR_L copy of the LAT coding region and also a 1.2 Kb deletion immediately upstream of the coding region of the TR_L copy. On explant co-cultivation, reactivation was impaired when compared with the parent 17+, but detectable levels of the LATs were not required for the establishment and maintenance of latency. The LAT mutant virus, X10-13, has been used in a rabbit animal model. After iontophoresis of 0.01 % epinephrine to induce reactivation, an increased efficiency of reactivation resulted, compared with the parent XC-20 (Hill *et al*, 1990). These experiments were further substantiated using the 1704 mutant in an animal model (Trousdale *et al*, 1991).

d) Functions Involved in the Mechanisms of Latency

Much interest has focused on the genes that are involved in the mechanisms of latency. Two herpes-encoded proteins have been detected in latently infected Trigeminal ganglia. The major viral *trans*-activator ICP4 has been detected at low levels in contrast to the high levels found in a lytic infection (Green *et al*, 1981). However it is not known whether this protein is newly synthesised in latently infected cells, or if it is carried by the infecting virion. It has been suggested however that this protein may play a role in the establishment or maintenance of latency, perhaps in a

different phosphorylated state (Green *et al*, 1981).

The viral thymidine kinase protein has also been identified in latently infected ganglia (Yamamoto *et al*, 1977), although its function remains unknown.

Latency has been established in murine trigeminal ganglia using viral mutants such as in1814 which does not express Vmw65 (Sears *et al*, 1991, Valyi-Nagi et al, 1991). These results prove that the establishment of latency is not precluded by the absence of Vmw65 and suggest an early divergence of the latent and lytic pathways. Viruses mutant in the thymidine kinase gene will also establish latency in murine trigeminal ganglia (Coen *et al*, 1989) and express LAT (Tenser *et al*, 1989), suggesting that viral replication is not a prereqisite to establishing latency.

1.vi. Genetic Background and Immunity

Although genetic background plays a role in determining the susceptibility of cells to the lytic cycle of HSV (Lopez, 1975, Carrit and Goldfarb, 1976), there is no documentation of a susceptibility locus for latent infection. However, the amount of interferon (IFN) produced by macrophages is genetically controlled (Zawatky *et al*, 1982, Pedersen *et al*, 1983) and this may play a role in determining the outcome of an HSV:neuronal cell interaction. IFN has a specific inhibitory effect on IE RNA translation (Oberman and Panet, 1988) and this may limit the extent of the lytic cycle in neuronal cells.

Anti-HSV antiserum will restrict the number of infected cells in sensory ganglia (Walz *et al*, 1976) and protect against viral

spread in the peripheral and central nervous systems and against death (McKendall *et al*, 1979) although it is not clear if IFN has any role in this protection. Activated T lymphocytes will also limit HSV spread as they are cytotoxic to syngeneic target cells (Igietseme *et al*, 1991).

1.vii. *In Vitro* Models of Latency

In vitro models of latency are of great value as the cells are easier to access, in order to investigate the molecular events by biochemical and molecular means.

a) Primary Neuronal cultures *In Vitro*

Primary neurons in culture have been utilised to study the non-permissive interaction of HSV with the cells. However when grown *in vitro* it nevertheless seems necessary to modulate the environment in order to prevent an initial lytic destruction of the monolayers, or at least the spread of a secondary infection through the monolayers. Thus Wigdahl *et al* (1983) established latency in neurons of rat spinal ganglia by treating with bromo-vinyl-deoxyuridine (BVDU) and human IFN. The cells were infected at a MOI of 2.5 and after 7 days the inhibitors were removed. Subsequently no detectable virus was observed for 2-7 days. If the latent infection was maintained at 40.5^{0}C, latency could be maintained for 15 days and reactivation could be achieved at 37^{0}C after 4 days.

Perhaps the most realistic *in vitro* latency systems to date are those developed by Wilcox and Johnson (1988, 1990). Here NGF-dependent latency was established in rat sympathetic and rat, monkey and human dorsal root ganglia. The cells were maintained at 37^0C in the presence of acyclovir for the first 7 days and after this time the virus remained silent in the presence of NGF. The virus could be reactivated by removal of the NGF for just 1 h.. A species specific antibody which prevents NGF binding and acting on its receptor caused reactivation in a homotypic animal, and hydroxydopamine and colchicine, which interfere with steps in the NGF signal, also caused reactivation. All of these observations suggested that NGF was actively maintaining the latent state. It was also observed that if an RNA or protein synthesis inhibitor was added to the latent cultures for 1 h. reactivation followed. These results suggested that a labile factor was maintaining latency.

b) *In Vitro* Models Using Permissive Cell Types

However the use of *in vivo* models and primary neurons are limited as only small numbers of cells are accessible. The ability to grow up large numbers of cells for biochemical analyses can be very useful. Many *in vitro* systems have therefore been established in an effort to understand the molecular mechanisms involved in latency. A large number of these have used cells that are normally permissive to HSV but have employed supra-optimal temperatures, inhibitors of viral DNA replication or other factors such as cycloheximide or IFN in order to artificially hold the virus at bay. Even though these systems do not represent true neuronal latency, they have yielded much

information which in some cases has proved relevant.

For example, Cytosine Arabinoside (ara C) was used by O'Neill *et al* (1972) to treat human embryonic lung cells. After removal of the drug from infected cells, the originally silent HSV-2 reappeared and cultures were destroyed within 5-6 days. Marcon and Kucera (1976) infected human embryonic fibroblasts at 42^{0}C and HSV-2 specific DNA synthesis was totally blocked. At 40^{0}C the block was not complete, although there was a marked decrease in viral DNA replication and virus production, compared with that at 37^{0}C. Latent HSV-2 could also be maintained in human fibroblast cells at 39.5^{0}C in the presence of ara C (O'Neill *et al*, 1977), as can HSV-1 in human embryo lung cells at 40.5^{0}C (Wigdahl *et al*, 1981). HSV-1 has also been maintained in a latent state in human fibroblasts by treating the cells with BVDU and human IFN-α. Although cell survival was minimally reduced by the drug treatment, a minimum of 1-3 % of the surviving cells harboured a reactivatable genome. Reactivation could be achieved by Human Cytomegalovirus (HCMV) super-infection or by reducing the temperature of incubation (Wigdahl *et al*, 1982b). Cycloheximide has also been used to treat human embryo lung cells in which latency was established at 40.5^{0}C (Shiraki *et al*, 1986).

Russell and Preston (1986) have also established an HSV-2 *in vitro* latency system in human foetal lung cells at 42^{0}C. When switched to 37^{0}C, infectious virus was undetectable for at least 6 days. At 38.5^{0}C HSV-2 was reactivated by intertypic infection, with ts HSV-1 mutants or with HCMV, but not with Adenovirus or irradiated tsK or sub-culture (Russell and Preston, 1988). The mutant in1814 has also been used in this system (Harris and Preston, 1991). This mutant expresses no Vmw65, and the latent infections were reactivated by

ICP0 expressed in an Adenovirus vector or by shifting the temperature back to 37^{0}C.

Viruses deleted in the gene encoding ICP4 (tsK or in1411) will establish and reactivate latency in this model system. The mutant virus dl1403 (Deleted in the genes encoding ICP0 and LAT) will also establish latency, although it will not reactivate a latent HSV-2 infection (Stow and Stow, 1986, Russell *et al*, 1987). The ICP0 protein, when expressed in an Adenovirus expression vector, will reactivate (Harris *et al*, 1989) suggesting that this is a crucial protein. When regarding the mechanism by which the ICP0 gene reactivates, it is interesting to note that a virus lacking the ICP0 introns will reactivate with normal efficiency in an explant co-cultivation assay (Natarajan *et al*, 1991). An HSV-2 mutant that is unable to replicate has also been used to reactivate a latent HSV-1 infection (Wigdahl et al, 1982a).

c) Intrinsically Non-Permissive Cell Lines

Some cells grown *in vitro*, even though they are not of neural origin, are intrinsically non-permissive to HSV. As some of these are readily available to grow in culture, they have been used to study HSV latency, although it is accepted that they may not represent true neuronal latency.

These cells include Dog Kidney cells (Aurelian and Roizman, 1964). Although viral antigen, interferon and small amounts of DNA are produced, these cells are impaired in their capacity to produce infectious virions. The mechanism underlying this restriction is unknown. Rat XC cells are also intrinsically non-permissive to HSV

(Machuca *et al*, 1987), although this is specific to strain F and due to a failure to enter the cell. The ability to enter can be conferred on the F strain by the *Bam* H1 B fragment of the MP strain, but this function is not due to either Glycoprotein C or a cell fusion function which both map to the *Bam* H1 B fragment. In some cases, cells are non-permissive to one type of HSV, but permissive to the heterotypic strain. For example, hamster HDC-22 cells are permissive for HSV-1, but not to HSV-2 (Docherty *et al*, 1972). In these cells during an HSV-2 infection, there is a great reduction in antigen production and the amount of HSV-2 DNA produced; cellular DNA synthesis is not repressed. There have also been reports of cells transformed with HSV being non-permissive to superinfection, for example human embryonic lung cells (Darai and Munk, 1973) and hamster embryo fibroblasts (Doller, 1977). In the latter case, HSV-2 transformed fibroblasts could not be re-infected with either type of HSV. Furthermore, an extract of the transformed cells could inhibit viral infections of non-transformed cells. Cells of the immune system are also relatively non-permissive. These are cells such as the mononuclear phagocyte line U937 (Tenney and Marahan, 1987) which increase their permissivity on treatment with Phorbol Myristate Acetate (PMA) and also peritoneal macrophage Kupffer cells (Goodman and Filice, personal communication) which are intrinsically blocked to HSV infection and also display extrinsic antiviral activity. In mice with different susceptibilities to HSV infection, it has also been shown that the permissivities of the oligodendrocytes differ accordingly (Thomas *et al*, 1991) and that the block in the non-permissive lines appears to be at the level of adsorption and processing.

d) Neuronal Cell Lines

Some cell lines of neuronal origin have been used in an effort to study latency. In some cases, however, manipulation of the environment still seems necessary. The B103 rat neuroma line has been studied (Levine *et al*, 1980) and was found to be non-permissive dependent on the temperature of incubation and multiplicity of infection (MOI). Thus, antigens associated with the lytic cycle were not detected at low MOIs and the cells were not susceptible to cell-mediated cytolysis with αHSV-1 antiserum. PC12 cells have also been studied, particularly as they can be differentiated *in vitro* (Block *et al*, personal communication). These cells are relatively non-permissive and NGF-dependent latency has been established in differentiated PC12 cells with expression of the LATs. However, these cells are of sympathetic origin and although latency can be established in the autonomic nervous system, the sensory nervous system is the major site of HSV latency. The C1300 neuroblastoma cell line has also been extensively used as it displays properties of differentiated neurons *in vitro* (Vahlne and Lycke, 1977, 1978, Vahlne *et al*, 1981, Nilheden *et al*, 1985a, 1985b, Ash, 1986, Kemp and Latchman, 1989, Kemp *et al* 1990) These cells are of central nervous system (CNS) origin.

e) A New Model System

Recently novel hybrid cell lines were constructed by fusing the azaguanine resistant C1300 mouse neuroblastoma cell, clone N18Tg2, with rat neonatal primary sensory neurons (Wood *et al*, 1990). HAT medium was used to select for hybrids. The resultant cells, the ND

cells display many properties of the parent neuron in that they have similar electrophysiological properties, they are depolarised in response to bradykinin or capsaicin, they express molecules of the lactoseries and globoseries, phosphatidyl-inositol anchored molecules of the immunoglobulin superfamily, N-CAM, F-3 and Thy-1, and also express many neuropeptides such as substance P, CGRP and somatostatin. Moreover, they maintain the capacity to divide, but can also be switched to a non-dividing state in which processes are extended on alteration of the culture medium.

Such cells represent the true site of neuronal latency and may therefore be of immense value for *in vitro* studies studies pertaining to HSV latency. Furthermore they may also be useful to study neuronal function and differentiation.

1.viii. *In Vitro* Models of Neuronal Cell Function

Cell lines of neuronal origin have been used extensively to study neuronal function *in vitro*. For example, the mouse C1300 neuroblastoma cell line (Augusti-Tocco and Sato, 1969) has been differentiated *in vitro* by decreasing the serum concentration (Seeds *et al*, 1970), by using glass, collagen or commercially-treated tissue-culture plastic as the substratum (Schubert *et al*, 1969), by treatment with dibutyryl cAMP (Furmanski *et al*, 1971), by treatment with 5-bromo deoxyuridine (Seeds *et al*, 1971) or by treatement with dimethyl sulphoxide (DMSO) (Kimhi *et al*, 1976). The C1300 cells have also been shown to develop excitable membranes as they phenotypically mature into neuron-like cells (Nelson *et al*, 1969). The

capacity of these cells to regulate the synthesis of neurotransmitters has also been assessed. Acetylcholinesterase activity increased when cell division was arrested (Blume *et al*, 1970). However adrenegic, cholinergic or clones expressing neither acetylcholine nor catechols were derived from the C1300 tumour (Amano *et al*, 1972). Interestingly, a clone denoted N18 (from which the parent of the ND cells N18Tg2 was derived) was identified as inactive in this work. In another mouse neuroblastoma cell line, N103, tumour necrosis factor (TNFα) and IFN caused the arrest of cell growth and DNA synthesis which was accompanied by morphological differentiation (Munoz-Fernandez *et al*, 1991).

Other neuroblastoma cell lines have also been studied, including some of human origin (Prasad, 1974). Human neuroblastomas have been differentiated using all-*trans* retinoic acid (RA) (Tonini *et al*, 1991) when both protein kinase C and *c-myc* mRNA expression decreased. These observations were thought to be associated with reversion of the malignant phenotype, as differentiation of neuroblastomas in general is thought to be (Prasad, 1974). Increased expression of the HOX genes (mammalian genes containing a hom eobox) have also been observed on RA-induced differentiation of human neuroblastomas (Peverali *et al*, 1990).

Surprisingly perhaps, treatment of neuroblastoma cells with actinomycin D or cycloheximide to inhibit mRNA or protein synthesis respectively, does not inhibit the differentiation process (Seeds *et al*, 1970). In fact the introduction of such inhibitors can induce differentiation (Minana *et al*, 1991).

Attempts have also been made to immortalise cells of the mammalian

central nervous system using the temperature-sensitive (ts) products of oncogenes (Frederiksen *et al*, 1988, McKay *et al*, 1988). Such lines may be useful in studying the interaction of different cell types in the developing brain.

Neuroblastomas have also been fused with a variety of cells of the nervous system to produce hybrid cell lines. For example, neuroblastoma X glioma (Daniels and Lamprecht, 1974), neuroblastoma X sympathetic neuron (Greene et al, 1975) and neuroblastoma X dorsal root ganglion (DRG) neuron (Platika *et al*, 1985, Francel *et al*, 1987, 1988, Wood *et al*, 1990) hybrids have been developed.

By far the most studied neuron type in the mammalian nervous system is the sensory DRG neuron as, relatively, it is the easiest to remove from the animal. However the numbers produced still do not fulfil the requirements for many biochemical and molecular assays and the populations produced are highly heterogeneous. The need for large homogeneous populations of neuronal cell types to study events at the molecular level has therefore led to the development of the types of immortal cell lines outlined above. Of particular use are the neuroblastoma X DRG cell lines, as function of the primary cultures of the easily dissectable DRG neurons can be correlated with that of function assessed in immortal lines.

The availability of a large homogeneous population of cells with sensory neuronal characteristics, as the ND cells provide (Wood *et al*, 1990), is invaluable as a model to study aspects of neuronal cell function. These include neurite extension, cell:cell communication, synapse formation, intracellular transport of neuron-specific molecules and synthesis of neurotransmitters.

1.ix Aims

The aim of this work was essentially to characterise the novel ND cell lines. Preliminary characterisation had been carried out with respect to the maintenance of neuronal characteristics in these cell lines (Wood *et al*, 1990).

However, as the ND cells are of sensory neuronal origin, it seemed highly likely that they would present a model system of the natural host cell of HSV latency, where the phenomenon could be studied *in vitro*. Primarily therefore, the permissivity to HSV ofthe ND cells has been studied (**Chapter 3**).

Data and models are also presented which may explain the low permissivity to HSV seen in neuronal cells (**Chapter 4**), and also the mechanisms by which the low permissivity can be overcome by either increasing the multiplicity of infection (**Chapter 4**) or by treatment with low levels of cAMP (**Chapter 5**).. This treatment may represent a molecular mechanism of reactivation from the latent state (**Chapter 5**).

Secondly, the ND cells have been characterised with respect to their differentiated state. A prerequisite of an *in vitro* latency system is a non-dividing cell. High levels of cAMP can effect arrest of the cell cycle and differentiation and this in fact also results in a marked decrease in the permissivity to HSV (**Chapter 5**). Low serum levels can also cause differentiation (**Chapters 5** and **6**). Whilst the ND cells may in fact be already mature in terms of gene expression in the undifferentiated state, data is presented which suggests that pathways of secretion and exocytosis at the neurite tips only mature on morphological differentiation (**Chapter 6**).

The characterisation of ND cells has therefore been greatly extended. This has been accomplished with respect to the capacity of the ND cells to support non-permissive and possibly true latent HSV infections. In addition, the changes in gene expression and protein redistribution on morphological differentiation have been closely monitored.

Chapter 2 Materials and Methods

2.1. Materials

2.1.i. *E. coli* Strain

JM101 *supEthi* Δ(*lac-proAB*)F'(*traD* 36*proAB* +*lacI* ^{q}*lacZ* ΔM15), allows growth of vectors with amber mutations.

2.1.ii. Cell Lines

ND cells (Wood *et al*, 1990) were obtained from Dr. J. N. Wood, Sandoz Institute for Medical Research, London, U.K..
BHK-21 Clone 13 (McPherson and Stoker, 1962), Green Monkey Kidney (Vero) cells and NIH3T3 cells were obtained from the Cell Production Unit, Imperial Cancer Research Laboratories, London, U.K..
HB 8083 secreting the 58S antibody against the HSV IE protein ICP4 (Showalter *et al*, 1981) was obtained from the American Type Culture Collection.

2.1.iii. Virus Strain

HSV-1 strain F (Ejercito *et al,* 1968) was for all viral infections and was obtained from Drs. N. B. La Thangue and W. L. Chan, University College, London, U. K..

pRSVCAT (Gorman *et al*, 1984) and pSV2CAT (Gorman *et al*, 1982) were obtained from Dr. C. Gorman, Imperial College of Science and Technology, London, U.K..

IECAT (Stow *et al*, 1986) was obtained from Dr. C. Preston, Institute of Virology, Glasgow, Scotland.

pIG65 (Gelman and Silverstein, 1987) was obtained from Dr. S. Silverstein, Columbia University, New York, U.S.A..

WLATA-F (Zwaagstra *et al*, 1989) were obtained from Dr. S. Weschler, UCLA, U.S.A..

p111, p63, p175, N* and Sal t were obtained from Dr. R. Everett, MRC Virology Unit, Glasgow, Scotland.

pMC151 (Croen *et al*, 1987) was obtained from Dr. E. Wagner, University of California at Irvine, U.S.A..

p123 (Kemp *et al*, 1986) was obtained from Dr. L. Kemp, University College, London, U.K..

pF (Wheatley *et al*, 1991) and pF/6 were obtained from Dr. C. Dent, UCMSM, London, U.K..

pO (Latchman *et al*, 1989) was obtained from J. Partridge, NIMR, London, U.K..

pUC1813 (Kay and McPherson, 1987) was obtained from Dr. R. Kay, Washington State University, U.S.A..

DNA molecular weight markers (λ/*Hin* d III and 1 Kb DNA ladder) were purchased from Gibco/BRL, Middlesex, U.K..

Herring Sperm DNA was purchased from the Sigma Chemical Company, Dorset, U.K..

2.1.v. Enzymes

All Restriction Enzymes were purchased from Gibco/BRL, Middlesex, U.K., New England Biolabs, U.S.A. or Boehringer Corporation Ltd., Sussex, U.K..

Ribonuclease A^, Lysozyme, proteinase K, RNA-free DNAse, RNA polymerases and chloramphenicol acetyl-transferase (CAT) were purchased from the Sigma Chemical Company, Dorset, U.K..

2.1.vi. Radiochemicals

(Dichloroacetyl-1,2-^{14}C) chloramphenicol (60 mCi/mMol) and (γ-^{32}P)CTP (500 Ci/mMol) were purchased from New England Nuclear Inc., Boston, U.S.A..

2.1.vii. Other Chemicals

RNAse Inhibitor, vanadyl ribonucleoside complex (VRC), protease inhibitors, fructose phosphate, dithiothreitol, β-mercaptoethanol, tween 80, dibutyryl cAMP, PMA, IBMX and all-*trans*-retinoic acid were purchased from the Sigma Chemical Company, Dorset, U.K..

NGF was a gift from Dr. J. Winter, Sandoz Institute for Medical Research, London, U.K..

Bacto-tryptone and Yeast Extract were purchased from Difco Labs., Surrey, U.K..

^Before use, RNAse A was prepared by dissolving the solid in 10 mM tris-Cl, pH 7.5/ 15 mM NaCl at a concentration of 10 mg/ml and by boiling for 10 mins. The enzyme was stored at -20^{0}C.

B.C.A. Protein Assay Kit was purchased from Pierce Warriner (U.K.) Ltd, Cheshire, U.K..

Nucleotide triphosphates were purchased from Pharmacia, Buckinghamshire, U.K..

tRNA was purchased from the Sigma Chemical Company, Dorset, U.K..

Conjugated anti-mouse antibodies were purchased from Dako Ltd, Buckinghamshire, U.K..

Acetyl Coenzyme A was purchased from the Boerhinger Corporation Ltd., Sussex, U.K..

Tissue-culture media, sera and plasticware were purchased from Gibco/BRL, Middlesex, U.K..

2.1.viii. Miscellaneous

Nitrocellulose membrane (Hybond C) were purchased from Amersham International, Buckinghamshire, U.K..

Thin Layer Chromatography (TLC) plates were purchased from Camlabs, Cambridgeshire, U.K..

X-ray film (X-omat AR) was purchased from Kodak Ltd, Hertfordshire, U.K..

Polaroid 667 film was purchased from Polaroid U.K. Ltd, Hertfordshire, U.K..

General laboratory plasticware was purchased from Greiner Labortechnik Ltd, Gloucestershire, U.K.

2.1.ix. Buffers, Solutions and Growth Media

All of the following were sterilised by autoclaving for 20 mins. at

15 lb/sq.in. on a liquid cycle, except when stated:

L-Broth: (L^{-1}) 10 g tryptone, 5 g yeast extract and 10 g NaCl. pH was adjusted to 7.0 using 10 M NaOH.

L-Agar: agar was added to LB at 1.5% (w/v).

Minimal Medium: (L^{-1}) 1X M9 salts, 1 mM $MgSO_4$, 0.2 % glucose, 100 µM $CaCl_2$, 1 mM thymine.

Ampicillin was dissolved in water at a concentration of 50 mg/ml to make a stock solution and was diluted 1:1000 in culture media to select for plasmid-encoded resistance. This solution was filtered by passing through a 0.2 µm acrodisc (Gelman Sciences Northamptonshire, U.K..)

PBS (phosphate-buffered saline): (L^{-1}) 6.1 g NaCl, 0.135 g KCl, 0.96 g $Na_2HPO_4.2H_2O$, 0.17 g KH_2PO_4. pH was adjusted to 7.4 using HCl.

TBE (tris-borate-EDTA): (L^{-1}) 10.8 g trizma base, 5.4 g boric acid, 8 mls 250 mM EDTA, pH 8.0.

TAE (tris-acetate-EDTA): (L-1) 40 mM tris-Cl, 20 mM Na acetate, 2 mM EDTA. pH was adjusted to 8.3 using NaOH.

250 mM EDTA: (L^{-1}) 93.06 g was added to 700 mls H_2O. 10 M NaOH was addded until the pH was 8.0. The volume was then made up to 1 L with H_2O.

SSC (saline sodium citrate): (L-1) 8.76 g NaCl and 4.41 g Na citrate. pH was adjusted to 7.0 with NaOH.

Tris-Cl: 1 M tris (121.14 g L^{-1}) was prepared and the pH was adjusted appropriately with HCl.

TE (Tris-Cl/EDTA): 10 mM tris-Cl, pH 7.5, 1 mM EDTA.

Denhardt's Solution (PM): (100X stock, L^{-1}) 20 g ficoll, 20 g polyvinyl pyrrolidone, 20 g BSA).

Phenol Equilibration: approximately 200 mls. solid phenol was melted

at 65^{0}C and 0.1 g/100 ml. 8-hydroxyquinoline was added as a reducing agent. The phenol was then washed with 0.5 M tris-Cl, pH 8.0 (3X) and with 0.1 M tris-Cl, pH 8.0/0.2% β-mercaptoethanol (2X). Washing was carried out by shaking the buffer with the phenol, allowing the layers to separate and then discarding the upper aqueous layer. The last layer of buffer was left to prevent oxidisation.

Phenol/chloroform was prepared by mixing equal quantities of equilibrated phenol and chloroform. Phenol and phenol/chloroform were stored at +4^{0}C.

Herring Sperm DNA (HS DNA): 0.5 g HS DNA was dissolved in 50 mls H_2O and after sonication, the solution was autoclaved to sterilise and to further break the DNA into small fragments.

All other solutions are listed in the appropriate methods section.

2.2. Methods: DNA

2.2.i. Large-Scale Plasmid DNA Preparation (PEG Precipitation Method)

A 400 mls culture was grown overnight with aeration and bacterial cells were isolated by centrifugation at 7K for 10 mins. at +4^{0}C in a Beckman centrifuge, model no. J2 21. The cell pellet was resuspended in 4 mls of sucrose buffer (25 % sucrose, 50 mM tris-Cl, pH 8.0), lysozyme was added at 1 mg/ml and the mixture was incubated on ice for 15 mins.. EDTA was then added to a final concentration of 10 mM, and again the mixture was incubated on ice for 15 mins.. Half of the total volume was then added of Triton buffer (L^{-1}, 30 mls Triton-X-100, 150 mM tris-Cl, pH 8.0, 750 mM EDTA), the mixture was gently mixed and then further incubated on ice for 30 mins.. The lysate was then cleared by centrifugation at 18K for 1 h. at +4^{0}C in a Beckman centrifuge, model no. J2 21. The lysate was then decanted, NaCl was added to a final concentration of 0.5 mM, and it was extracted with phenol/chloroform (1X) and chloroform (1X). PEG 6000 was then added to the extracted lysate at a concentration of 10 % (w/v) and it was dissolved by incubation at 37^{0}C for 30 mins. The mixture was then left at +4^{0}C for a minimum of 1h. or overnight. The PEG/DNA precipitate was collected by centrifugation at 12K for 20 mins. at +4^{0}C in a Beckman centrifuge, model no. J2 21. The pellet was redissolved in 500 µl 0.1 M tris-Cl, pH 8.0, RNAse A was added to a final concentration of 200 µg/ml, and the mixture was incubated at 37^{0}C for 30 mins. An equal volume of PEG buffer (10 mM tris-Cl, pH 8.0, 1 mM EDTA, 1 M NaCl, 20% PEG 6000) was then added and again

the mixture was left at +4^{0}C for a minimum of 1 h. or overnight. The PEG/DNA precipitate was collected by centrifugation at high speed in a microfuge for 10 mins. The pellet was then resuspended in 400 µl of a solution of 0.5 M NaCl and 10 mM tris-Cl, pH 8.0 and extracted with phenol/chloroform (1X) and chloroform (1X). RNAse A was then added to a final concentration of 200 µg/ml and the mixture was again incubated at 37^{0}C for 30 mins. The DNA was then precipitated with 250 mM NaCl and 2.5X volume of ethanol.
All plasmid DNA preparations were checked by Restriction Enzyme digestion.

2.2.ii. Restriction Enzyme Digestion

Digests were carried out in commercially-bought buffers, each enzyme being optimally active in the particular buffer bought with that enzyme. 500 ng-1µg of plasmid DNA was digested with 1 unit of enzyme for 1 h. in a 10 µl volume for analytical purposes.

2.2.iii. Agarose Gel Electrophoresis

All digests were analysed on 0.7% agarose TBE gels. Ethidium bromide was added to each gel at a concentration of 1 µg/ml, in order to view the DNA using the UV transilluminator which emits light at a wavelength of 254 nm. Electrophoresis was carried out at 10 V/cm. Bromophenol blue was added to the digest prior to loading in order to track the DNA. This dye migrates with double-stranded DNA of approximately 300 bp.

2.2.iv. Preparation of Competent Cells and Transformation

A culture of *E. coli* of an appropriate strain was grown to saturation in L-Broth at 37^{0}C overnight with aeration. A 1:100 dilution of this culture was made and grown to an O.D. of 550 nm (for approximately 2 h.) The cells were then collected by centrifugation at 3K for 10 mins. at +4^{0}C in an IEC Centra-4R benchtop centrifuge. The cell pellet was resuspended in half of the original volume of ice-cold 50 mM $CaCl_2$ and then incubated on ice for 20 mins. the cells were then spun as before and resuspended again in one tenth of the original volume of 50 mM $CaCl_2$. The cells were then left on ice 40 mins.- 2 h..

To transform, 200 µl of competent cells were added to the DNA in a volume of 5-20 µl. Approximately 1 ng uncut supercoiled DNA was used. This mixture was then incubated on ice for 40 mins.-2 h.. After this incubation, the mixture was heat-shocked at 42^{0}C for 3 mins., 200-300 µl L-Broth was added and it was then further incubated at 37^{0}C for 30 mins..

The transformed *E. coli* were plated out on an agar plate containing an appropriate antibiotic.

2.3. Methods: RNA

2.3.i. Nuclear Run-On Assay of Transcription (see Mason *et al*, 1986)

All buffers used in RNA work were treated with Diethylpyrocarbonate (DEPC), by adding this compound at 0.1 % to buffers, leaving overnight

at 37^{0}C and then autoclaving. This compound is a strong inhibitor of RNAses and is thought to be a carcinogen.

Preparation of Nuclei

To prepare nuclei, cells were plated at 5 X 10^6 - 10^7 on a 90 mm plate (see section 1.5.v. on cell culture). The growth media was aspirated off and the cells rinsed with ice-cold PBS. The cells were then harvested into 1 ml ice-cold PBS and put into an eppendorf. The cells were then pelleted by centrifugation at low speed for 3 mins at +4^{0}C. The pellet was resuspended in 500 µl ice-cold Lysis Buffer (10 mM Hepes, pH 7.9,10 mM NaCl, 3 mM $MgCl_2$, 0.05 % NP40) and incubated on ice for 5-10 mins. The resuspended pellet was then underlaid with 500 µl Sucrose Buffer (Lysis Buffer with 30 % sucrose) and the cells were spun again under the same conditions. The nuclei from the lysed cells collect at the bottom of the tube or at the interface between the two buffers. The supernatant was discarded whilst taking care not to lose any nuclei. This procedure of lysis and spinning through sucrose was repeated twice more. Finally the nuclei were resuspended in 100 µl Freezing Buffer (50 mM Hepes, pH 7.9, 40 % glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) at approximately 5 X 10^6 nuclei/100 µl in a vial. The vials were placed immediately on dry ice and stored at -70^{0}C.

Nuclear Run-On reactions were carried out as described (Mason *et al*, 1986). After the radio-labelelled RNA had been precipitated overnight, it was collected by centrifugation at high speed, dried and resuspended in 100 µl H_2O.

Approximately 5 μg each of unlabelled probe DNAs were denatured and cross-linked to a nitrocellulose membrane. RNA probes (see below) were attached to membrane as described below. The membrane was pre-hybridised at 42^{0}C for a minimum of 24 h. in 5 mls of a buffer containing 4XSSC, 50 mM PB, 1XPM, 0.2 % SDS, 50 % de-ionised formamide and 250 μg/ml total RNA.
Hybridisation was carried out in the same buffer at 42^{0}C for 72 h..

The membranes were washed in 2XSSC/0.1 % SDS at 65^{0}C for 60 mins followed by rinsing in 2XSSC (2X) and incubating the filters with 20 μg/ml RNAse A in 2XSSC at 18^{0}C for 30-60 mins. If non-specific binding of radio-label still persisted, the membrane was again washed in 2XSSC/0.1 % SDS at 65^{0}C for 60 mins.. The membrane was then exposed to X-Ray film.

2.3.ii. Synthesis of Single-Stranded RNA Probes

RNA probes were synthesised from either DNA strand after linearisation of the plasmid template at the 3' end of the coding region (antisense probe) or at the 5' end of the coding region (sense probe). RNA polymerases used were T7 or T3. The reaction contained 5X transcription buffer (200 mM tris-Cl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 100 mM dithiothreitol (DTT), 50 u RNAse Inhibitor, 2.5 mM each of ATP, GTP and UTP, 100 μM CTP, 1 μg DNA template, 50 μCi (γ-^{32}P)CTP, 10 u RNA polymerase., in a total volume of 20 μl.
The reaction was incubated at 37^{0}C for 60 mins. afterwhich time

RNAse-free DNAse was added to a final concentration of 2 u / μg of DNA and incubation was continued for 15 mins.. The volume of the reaction was then increased to 100 μl and was extracted with phenol and chloroform. 20 μg tRNA was added and the reaction was precipitated with 20 μl 10 M ammonium acetate.

2.2.iii. RNA Slot Blots

5 μg RNA probe of known volume was added to three volumes of Northern Denaturation Buffer (50 % formamide, 18 % formaldehyde, 11 % Northern Buffer [200 mM MOPS, 50 mM Na Acetate, 10 mM EDTA, pH 7.0]). The sample was incubated at 65^0C for 10 mins. and 20X SSC was added to a final volume of 1X. The RNA was slotted onto the membrane and cross-linked using a Stratagene Stratalinker.

2.4. Methods: Protein

2.4.i. Determination of Protein Concentration

Protein concentration was determined using the BCA Kit (Pierce Laboratories). For extracts of transfected cells, assays were carried out in 0.23 M tris-Cl, pH 7.8.

2.4.ii. Chloramphenicol Acetyl Transferase (CAT) Assay
See Gorman, 1985.

All extracts were kept on ice. After equalising for protein content of

each extract, the volume of extract required was diluted with 0.25 M tris-Cl, pH 7.8 to a final volume 90 µl. To this diluted extract, 35 µl H_2O, 20 µl Acetyl Coenzyme A (ACoA) and 1 µl ^{14}C Chloramphenicol (^{14}C Chl.) were added. Control assays were set up by adding 1 µl (6.25 u) of commercially bought CAT enzyme to 90 µl 0.25 M tris-Cl, pH 7.8 (positive control) or by adding no enzyme (negative control). After incubation at 37^0C for 30 mins., the reaction mixture was extracted with 1 ml ethyl acetate. The solvent containing the ^{14}C Chl. was then dried in a freeze-drier for approximately 1 h., afterwhich time the 14C Chl. was resuspended in 40 µl ethyl acetate and spotted onto a silica gel Thin Layer Chromatography (TLC) plate (Camlabs, Cambridgeshire, U.K.). Chromatography of the samples was allowed to progress for 1 h. in a chromatography tank (BDH Ltd., Essex, U.K.) using a mixture of chloroform and methanol in a ratio of 95:5 as the solvent. The TLC plate was then air-dried and exposed to X-ray film.

The CAT activity in each sample is able to convert the ^{14}C Chl. to the acetylated forms (mono- and di- acetylated). Acetylated chloramphenicol dissolves more readily in the solvent than does the non-acetylated form and it therefore migrates further in the TLC plate. The percentages of the radioactivity in the unconverted fraction and in the converted fraction were then calculated.

After a suitable exposure had been obtained, the positions on the TLC plate containing radioactive material were cut out and the level of radioactivity was determined by liquid scintillation counting. The data could then be presented as % conversion (acetylated chloramphenicol compared with total chloramphenicol, acetylated and non-acetylated).

2.4.iii. Enzyme-Linked Immunadsorbent Assay (ELISA)

ELISA assays were carried out on duplicate fixed cell monolayers in 96-well plates (Nunc from Gibco/BRL). Infections were carried out as described in section 2.5.iii and fixed using 10 % formaldehyde followed by ice-cold methanol.

During the staining procedure care was taken to prevent the monolayers from drying out by keeping the plates in a box lined with damp paper.

The fixed monolayers were first incubated with 50 µl coating buffer (1.59 % Na_2CO_3, 2.93 % $NaHC0_3$, 0.2 % NaN_3) for 30 mins. at 37^0C. After washing (3X) with 1X PBS/% NP40, the primary antibody was then added (50 µl/well) and incubation was continued for 1 h. at 37^0C. After removal of the primary antibody and washing as described, the secondary peroxidase-conjugated antibody (50 µl/well) was added and incubation was continued as described. After washing again as described, 50 µl of the substrate solution (37.5 mls 1X citrate/phosphate buffer, 1 X 15g tablet of O-phenylenediamine dihydrochloride (O-PD) [Sigma no. P4664], 20 ml H_2O_2) was added. When the colour had developed (5-30 mins), the reaction was arrested by adding 25 µl 2.5 M sulphuric acid. The optical density was then read in an ELISA reader at 490 nm.

2.4.iv. Immunofluorescent Antibody Test (IFAT)

Cells were grown on glass coverslips (10 mm diameter) and after infection (see section 2.5.v.) were fixed in absolute methanol. The

cells were stored covered by a solution of 1X PBS/0.1 % sodium azide/1 % newborn calf serum. Each coverslip was first covered with a 35 µl drop of the primary antibody and incubation was carried out for 1 h. at 18^{0}C. The coverslips were then drained on tissue and washed with the PBS/azide/NCS solution (3X). A 35 µl drop of the PBS/azide/NCS solution was then placed on the coverslip for 10 mins.. This was then drained and the procedure was repeated for the secondary fluoroscein-conjugated antibody.

The coverslips were then mounted cells-down on slide using a solution of 90 % glycerol/10 % PBS with 0.1 % sodium azide and 2.5 % 1,4-diazabicyclo [2,2,2] octane (DABCO) as a mounting fluid. The cells were then observed under UV light.

2.5. Methods: Cell Culture

2.5.i. Cell Culture

The following media and supplements were used to grow cells used in this work:

Cell Line	Medium*	Serum* %	35 %w/v Glucose mls/200	7.5 % Na Bic* mls/200	200 mM Glut * mls/200
ND7	L15	10	1.7	9.8	0.2
ND15	L15	10	1.7	9.8	0.2
ND3	DMEM>	10	2	___	___
N18TG2	RPMI	10	___	5.2	___
BHK	DMEM>	10	2	___	___
NIH3T3	DMEM>	10	2	___	___
Vero	RPMI	10	___	5.2	___

*Purchased from Gibco/BRL; Foetal Calf serum (FCS) used for neuronal cell lines and Newborn Calf Serum (NCS) used for all other cell lines.
> No. 041-01885

Cells were routinely passaged at 37^0C with 5 % CO_2 in a humidified atmosphere in Nunc 80 cm^2 tissue culture flasks (Gibco no.) to sub-confluency and then 'split' (by hitting the flask hard (ND7s and N18Tg2s) or by trypsinisation^ (all other cell lines)) to dilutions of 1 in 10 or 1 in 20 and then grown up again for 3-4 d.. Cells were frozen in aliquots of approximately 5 X 10^5 in 1 ml medium supplemented with 10 % FCS and 10 % DMSO. Cells were stored in liquid nitrogen. To thaw the cells, the vial was warmed to 37^0C and the cells were added to 5 mls medium in a 15 mls sterile tube. The cells were pelleted by centrifugation at 1K for 10 mins at $+4^0C$ in an IEC Contra-4R benchtop centrifuge. The cell pellet was then resuspended in 6 mls fresh medium and the cells were seeded into a small flask. Alternatively doubling dilutions were carried out in a 24-well dish. Cells were counted using a haemocytometer.

2.5.ii. Differentiation of Neuronal Cells

Cells could be differentiated using three different types of medium. Care was taken to remove all traces of serum from cells. Cells were plated at a maximum density of 2 X 10^6/90 mm plate (approximately 70 cells/mm^2) and were allowed to differentiate for 72-144 h. (3-6 d.), with a medium change after 72 h.. The table below shows the constituents of the differentiation media.

^Trypsinisation was carried out by washing the cells in PBS and then adding 2 mls of a solution of 10 % trypsin in versene (Gibco/BRL). The flask was incubated at 37^0C for 2 mins. and then 2 mls medium was added to inhibit the trypsin.

Constituent	Diff/cAMP	Diff/F14	Diff/F12
Medium	100 % L15	50 % DMEM (Gibco no. 041-01966) /50 % Ham's F14	50 % DMEM (Gibco no. 041-01966) /50% Ham's F12
Serum	1 %	___	___
cAMP	1 mM	___	___
NGF	200 ng/ml	___	___
Bovine Insulin	___	250 ng/ml	5 µg/ml
Human Transferrin	___	5 µg/ml	5 µg/ml
Sodium Selenite	___	30 mM	30 mM
Putrescine	___	100 µM	___
β-oestradiol	___	1 pM	___
Progesterone	___	20 nM	___

More extensive differentiation resulted when serum-free medium was used, compared with medium containing 1 % serum. The addition of cAMP to the medium caused an increase in the size of the cell bodies and in the thickness of the neurites produced. Interestingly some cells did not survive the differentiation event, possibly due to programmed cell death or 'apoptosis'. The inclusion of cAMP to the differentiation medium was able to prevent a proportion of this death.

When grown for staining in the Histochemistry Department, RPMS, Hammersmith Hospital, the cells were plated on either teflon-coated slides (Flow Laboratories) or 8-well compartmentalised slides (Nunc from Gibco/BRL,).

For fixation, cells were washed in PBS/1 % bovine serum albumin (BSA)

(3X) and then fixed in Bouin's Fixative (74 % formaldehyde/25 % picric acid/1 % glacial acetic acid) for 30 mins.. Cells were then washed (3X) in PBS and stored at $+4^0C$ in PBS/0.1 % sodium azide.

Staining was carried at by Dr. A. Suburo using various primary antibodies. Horse Radish Peroxidase-conjugated secondary antibodies were utilised to detect all primary antibodies and the DAB (di-amino-benzidine) / $NiCl_2$ (nickel chloride) / H_2O_2 (hydrogen peroxide) reaction was used to visualise the seondary antibody.

2.5.iii. Preparation of HSV Stocks
(see Latchman and Kemp, 1991)

A 'Master Stock' of HSV strain F was obtained from Drs. N. B. La Thangue and W. L. Chan. This was a plaque-pure preparation and from it a 'Sub-Master' stock was produced. This was subsequently used to produce a working stock from which all viral infections described in this work were carried out. Following passage of HSV outside the human host, it has been observed that spontaneous deletions occur, and it is therefore necessary to limit the passage number of isolated viral strains to four to maintain the integrity of the virus.

HSV can be propagated on Baby Hamster Kidney (BHK) cells. Cells were plated on 30 X 90 mm plates (Nunc from Gibco/BRL) at sub-confluency (2 X 10^6/plate). One day later, cells were infected at a MOI of 0.001 plaque forming units (pfu)/cell.

Infecting virus was added in 1 ml/plate serum-free medium onto PBS-washed monolayers. Virus was allowed to adsorb for 30 mins., afterwhich time the cells were fed with 10 mls/plate full growth

medium. Cells were checked over the next 48-72 h. for evidence of cytopathic effect (cpe). When observed in most cells, the infected monolayers were harvested in their spent medium using a rubber policeman into 50 mls sterile 'Falcon Tubes'. Cells were pelleted at 1K for 10 mins. at +4^{0}C in an IEC Centra-4R benchtop centrifuge and the pellets were washed with PBS. The cell pellets were then pooled and resuspended in 10-20 mls serum-free medium. The infected cell suspension was freeze-thawed (3X) by transferring the tube from liquid nitrogen to 37^{0}C. The cell debris was pelleted by centrifugation at 3K for
10 mins. at +4^{0}C in an IEC Centra-4R benchtop centrifuge. The supernatant containing virus was filtered through a 0.45 μm acrodisc (Gelman Sciences, Northamptonshire, U.K.), frozen in aliquots and stored at -70^{0}C. The virus was titred on Vero cells as described in the following section.

2.5.iv. Titration of HSV

Ten-fold dilutions of the newly-made virus stock were plated onto 10^6 Vero cells in 6-well dishes in 500 μl of serum-free medium. After adsorption for 30 mins. at 37^{0}C followed by aspiration off of the infecting medium, RPMI medium supplemented with 1 % NCS, 0.15 % sodium bicarbonate and 30 % Carboxy Methyl Cellulose (CMC; 1 %) was overlain on the infected monolayers. The cells were left for 48-72 h., until plaques had formed. At this stage the monolayers were washed with PBS (3X) and the monolayer was fixed with a mixture of Glacial Acetic Acid and Methanol in a ratio of 3:1 by covering the monolayer in the fixative and then removing it. A

solution of Crystal Violet (0.7 % in Methanol/H_2O at a ratio of 1:2) was then used to stain the monolayers, again by covering the monolayer with the solution and then washing it off with H_2O. The plaques were then counted and the titre calculated.

2.5.v. HSV Infection for ELISA, IFAT, Nuclear Run-On and Virus-Growth

Cell were plated out the day before infection. For the ELISA, cells were plated on 96-well plates at a density of 5 X 10^4 cells/well, for the IFAT on 6-well plates at 5 X 10^5 cell/well and for Nuclear Run-On or Virus Growth at 5 X 10^6/90 mm plate. Cells were infected at an appropriate MOI (Mock (M), 1, 5, 10 or 20) in serum-free medium and the virus was again allowed to adsorb for 30 mins. afterwhich time the infecting medium was removed and fresh growth medium was added. The infection was allowed to proceed for 6 h. (ELISA and IFAT), 4 h. (Nuclear Run-On) and 18 h. (Virus Growth). For the ELISA, cells were fixed in 4 % paraformaldehyde in PBS and for the IFAT, in ice-cold methanol. Fixation was carried out by aspirating off the medium and then covering the cells in the appropriate fixative. The fixative was then removed and cells were stored in a solution of 1 X PBS containing 1 % serum and 0.1 % sodium azide at +4^{0}C. For the nuclear Run-On, cells were treated as described in section 2.3.i . Cells infected to assay Virus Growth were treated as in the next section.

2.5.vi. Virus Growth

Cells were harvested in their spent medium into a universal and pelleted by centrifugation at 1 K for 10 mins. at +4^{0}C in an IEC Centra-4R benchtop centrifuge. The pellet was washed with PBS and resuspended in 500 µl serum-free medium. The cells were then freeze-thawed (3X) as described in section 1.5.iii., and the cell debris pelleted by centrifugation at high speed for 10 mins. at +4^{0}C in a microfuge. The supernatant was then passed through a 0.45 µm acrodisc (Gelman Sciences Ltd., Northamptonshire, U. K..) and titred on Vero cells as described in section 1.5.iv..

2.5.vii. The Calcium Phosphate Method of Transient Transfection of Mammalian Cells (see Gorman, 1985)

Cells were plated at 10^5/well on a 6-well plate for transfection followed by infection, and at 5 X 10^5-10^6/90 mm plate for all other transfections. For the transfections on 6-well plates, appropriate reductions were made in all solutions used. A standard amount of 5 µg DNA/90mm plate was transfected except where stated and carrier Herring Sperm DNA was used to either equalise the total amount of DNA transfected onto each plate within one experiment, or to increase the bulk of DNA to optimise precipitate formation. During transfection, care was taken to maintain the CO_2 concentration in the incubator at 5 %, the cells were fed with 5 mls DMEM at least1 h. before addition of theDNA precipitate and the cells were kept out of the incubator for as little time as possible to maintain the pH of the medium. Cells were always transfected the day after being plated

out, except when cells were treated or differentiated for longer periods of time. The calcium phosphate precipitate was kept on the cell monolayer for 4 h. and the cells were then left for 48 h. to allow for the gene to be expressed. No glycerol shock or any other method of enhancement of DNA uptake was used. Cells were harvested into 1 ml ice-cold PBS and pelleted by centrifugation at low speed for 3 mins. at $+4^0$C in a microfuge. The cell pellet was resuspended in 100 µl 0.25 M tris-Cl, pH 7.8 and then freeze-thawed (3X) by transferring from liquid nitrogen to 37^0C. The cell debris was then spun down by centrifugation at high speed for 10 mins. at $+4^0$C in a microfuge. The supernatant was then collected and assayed for protein concentration as in section 2.4.i., and Chloramphenicol Acetyl Transferase (CAT) activity, see section 2.4.ii.

2.5.viii. Primary Dorsal Root Ganglion Neuronal Cell Culture

Dorsal root ganglia were dissected from neonatal rats. The animals were first decapitated and the dorsal side was then washed with alcohol before being cut from neck to tail along the dorsal side. The spinal cord was then removed and any excess fat was also removed from around the neck. The dorsal root ganglia were then clearly exposed and could be removed with fine forceps. When removed from the animal's body, the ganglia were placed in a drop of medium on a scalpel blade. When the dissection was complete (usually 4 animals) the ganglia were put into a 15 ml tube in 2 ml medium. The ganglia were then incubated with 2.5 mg/ml trypsin (in serum-free medium), 12 mg/ml collagenase and 0.4 mg/ml DNAse. After all incubations the ganglia were triturated with a fine pipette

to dissociate the neurons. A sample of neurons was counted using a haemocytometer and their bright appearance under phase-contrast microscopy was checked.

In order to reduce the number of non-neuronal cells in the cultures, pre-plating was carried out. This involved plating the dissected neurons on a 90 mm tissue-culture plate treated with 10 μg/ml poly-D-ornithine and 5 μg/ml laminin. The non-neuronal cells stick down much more strongly than do the neurons. After 24 h. therefore the neurons could be gently washed from the surface of the plate using the bathing medium and a pasteur pipette.

The neurons could then be plated onto pre-treated (poly-D-ornithine and laminin) glass coverslips. The neurons were maintained in Ham's F14 medium supplemented with 200 μM glutamine, 100 u/ml penicillin and streptomycin, 0.15 % sodium bicarbonate and Ultroser G (a synthetic serum substitute) (Gibco/BRL). Cytosine arabinoside (Sigma Chemical Co. Ltd., Dorset, U. K..) was added at 10 μM to kill mitotic (glial) cells. The medium was also supplemented with 200 ng/ml NGF as neonatal sensory neurons need NGF to survive.

The ganglion neurons could then be infected as described in section 2.5.v.

Chapter 3 Characterisation of the Non-Permissive Nature of the ND Cells

3.i. Introduction

Many cell lines grown in culture have been used as a model to study Herpes Simplex Virus Latency. However, as already described in the introduction, manipulations of the environment often have to be effected in order to block the lytic cycle and allow the latent state to arise. Ideally latency should not require manipulation of the environment to keep the virus quiescent.

Fewer alterations, if any at all, have to be made when a cell of neuronal origin is used as the host, as these cells almost certainly contain neuron-specific factors essential for the mechanisms of HSV latency to be brought about.

The C1300 cell line, for example, has been used in studies of virus:cell interactions and has been shown to have an extremely low permissivity on infection with HSV at low multiplicities of infection (MOI) (Ash, 1986, Vahlne and Lycke, 1987, Kemp and Latchman, 1989, Kemp *et al*, 1990a). A block to the lytic cycle which characterises the non-permissivity of the C1300 system is a prerequisite to study latency. The ND cells may prove to be a better model of the virus:cell interaction as their origin is, in part, from sensory ganglia.

In this chapter, a panel of ND cells has initially been characterised

with respect to their capacity to express the viral IE gene ICP4. The cells under study were ND3, ND5, ND20 and ND26, and also the C1300 and NIH3T3 lines as controls (**Table 3.1**, section 3.ii). All classes of viral genes, and also the gene encoding the LATs, have subsequently been analysed at the transcriptional level in one of these lines (ND3) in comparison to the permissive line NIH3T3 (**Table 3.2**, section 3.iii).

However, an ND cell line not included in the initial study has been characterised most fully in this work. The ND7 line is capable of differentiation when placed in defined medium, and this property is essential for establishing a latent type of infection without dilution of the viral genome. This cell line therefore represents more readily the natural host cell of HSV latency, not least due to its non-dividing state.

The results in this chapter characterise the non-permissive state of the ND cells, in particular of the ND7 cell line.

3.ii. Results 1: Screening of ND Cell Lines for their Capacity to Produce the IE Protein ICP4

The ability of a panel of ND cells to express the HSV IE protein ICP4 after infection with HSV-1 strain F has been assessed (**Table 3.1**). Cells were infected at various multiplicities of infection (MOIs) overnight and then fixed. The cells were then stained with the 58S antibody to ICP4 (Showalter *et al*, 1981). A wide variety of

Table 3.1

Immunofluorescent Staining of Cell Lines with Antibody to the HSV-1 Immediate-Early Protein ICP4

Multiplicity of Infection	Dorsal root ganglion cell line					
	C1300	ND3	ND5	ND20	ND26	3T3
1	0.78 %	1.5 %	45 %	19.4 %	4.4 %	48 %
5	12.8 %	17 %	94 %	53 %	36 %	87 %
10	15.4 %	89 %	89 %	50 %	50 %	nd

The figures represent the percentages of cells staining with the 58S antibody to ICP4 (Showalter *et al*, 1981) after overnight infection with HSV-1 strain F. nd, not determined.

permissivities in different ND lines is apparent.

3.iii. Results 2: IE Gene Transcription is Impaired in ND Cells

Nuclear run-on analysis of viral IE gene transcription in ND3 and NIH3T3 cells has been carried out (**Table 3.2**). The experiment shows that the low level of IE protein detected in ND3 cells is paralleled by a low rate of transcription. It can also be seen that the LATs are expressed when HSV infects ND3 cells, but are expressed poorly in NIH3T3 cells.

3.iv. Results 3: Production of Virions is Impaired in ND Cells

A virus growth experiment was carried out on ND7 cells and (Baby Hamster Kidney) BHK cells (**Table 3.3**). This experiment was carried out to ascertain the potential of a cell to produce infectious virions. Both cell types were infected at at multiplicity of infection (MOI) of 1, harvested after 24 h., and the number of virions produced were assayed by titration on Vero cells. The ND cells produce fewer infectious progeny than a permissive cell. A 15-fold difference is seen in the number of virions produced between the two cell types at 24 h. post infection (p. i.).

Table 3.2

Transcription Rates of the Immediate-Early Genes in Uninfected and HSV-Infected ND3 and NIH3T3 Cells

	ND3		3T3	
IE Gene	UI	I	UI	I
1	2 (1)	5 (4)	6 (-)	65(8)
2	6 (2)	7 (1)	7 (1)	195(15)
3	4 (-)	6 (3)	9 (3)	65(9)
4	4 (-)	8 (2)	5 (-)	109(6)
5	6 (1)	4 (-)	5 (1)	121(12)
LAT	3 (2)	47 (11)	3 (1)	15 (2)
RNA LAT	4 (-)	85 (7)	2 (-)	25 (3)
RNA IE1	4 (2)	5 (1)	4 (2)	95 (8)
Control	184 (11)	190 (16)	173 (12)	185 (17)

Figures (average of two determinations whose range is shown in parenthesis) are counts per minute binding to clones derived from the indicated genes following hybridisation under run-on conditions. The control clone is derived from a cellular mRNA whose abundance does not change in HSV-1 infection (Kemp *et al*, 1986). Strand-specific probes were synthesised from the plasmid pMC151 and were used to distinguish between LAT and IE-1 mRNAs (Stevens *et al*, 1987) and are designated RNA LAT and RNA IE1 respectively. (From Wheatley *et al*, 1990).

Table 3.3

Titre of Infectious Progeny after Infection of Cell Lines with HSV-1 Strain F

Cell Line	Time post-infection (p.i.) 5 h. p.i.	24 h. p.i.
BHK	500	1.5×10^6
ND7	70	1×10^5

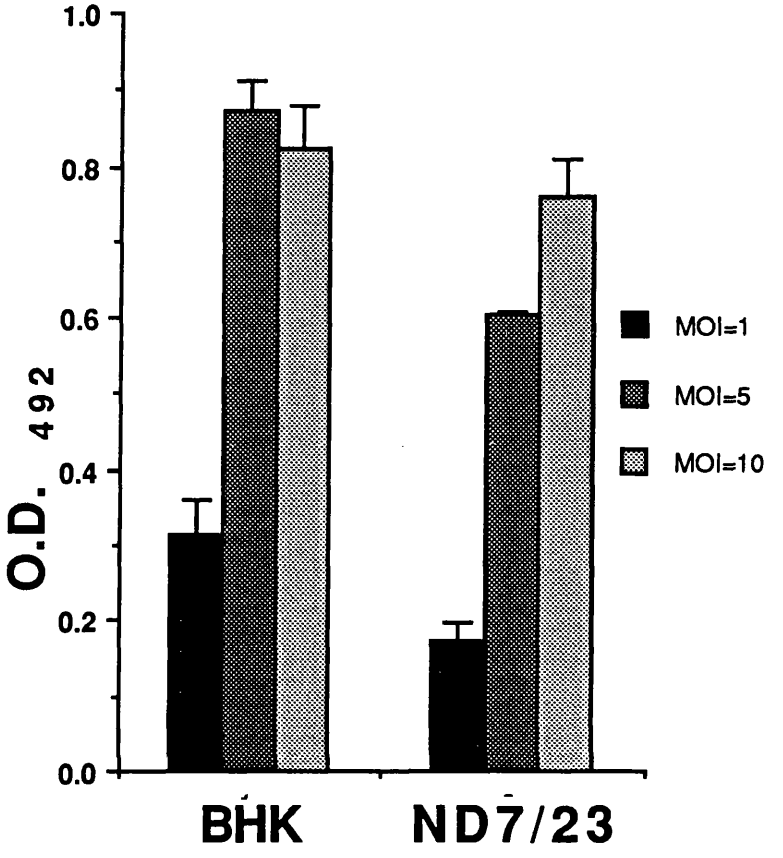
Figures show the values obtained after titration of the progeny produced on each of the cell lines after infection at a multiplicity of 1 for the indicated number of hours.

3.v. Results 3: Production of ICP4 is Impaired in ND Cells

An Enzyme-Linked Immunadsorbent Assay (ELISA) was carried out on BHK and ND7 cells (**Figure 3.1**). In this experiment, total cell populations of the ND7 and BHK cells were compared in their capacity to express the IE protein ICP4. The 58S antibody used as the primary antibody is a mouse monoclonal antibody (Showalter *et al*, 1981) and was raised against the IE protein ICP4. The difference between the two cell types is seen to be greatest at the lowest MOI. As the MOI is increased, the difference in the level of expression of ICP4 between the two cell types is reduced.

Whilst the data in **Figure 3.1** show the total amount of protein produced by each cell population, it was clear that the number of cells in each population expressing the protein should be calculated. It was important to know whether the same numbers of cells were expressing the protein, but the ND7 cell population expressing less per cell or whether fewer ND7 cells were expressing the same amount of protein per cell. An immunofluorescent antibody test (IFAT) was therefore performed and the numbers of ND7 or BHK cells which expressed the IE protein ICP4 (using the 58S antibody) were counted. The percentage of cells detectably expressing ICP4 was then denoted as a proportion of the whole population (**Table 3.4**). The decrease in the difference in the level of expression of ICP4 between the two cell types as the MOI is increased is also seen in this assay. Photographs of ND7 and BHK cells expressing ICP4 when infected at a MOI of 5 are shown in figure **Figure 3.2**.

Figure 3.1



Enzyme-Linked Immunadsorbent Assay Demonstrating the Levels of the HSV IE Protein ICP4 in BHK and ND7 Cell Populations

Bars show the average value of two replicate wells of each cell type infected at the indicated multiplicities of infection. Primary antibody was detected using an alkaline phosphatase-conjugated secondary antibody. The optical density of the product was read at 490 nm.

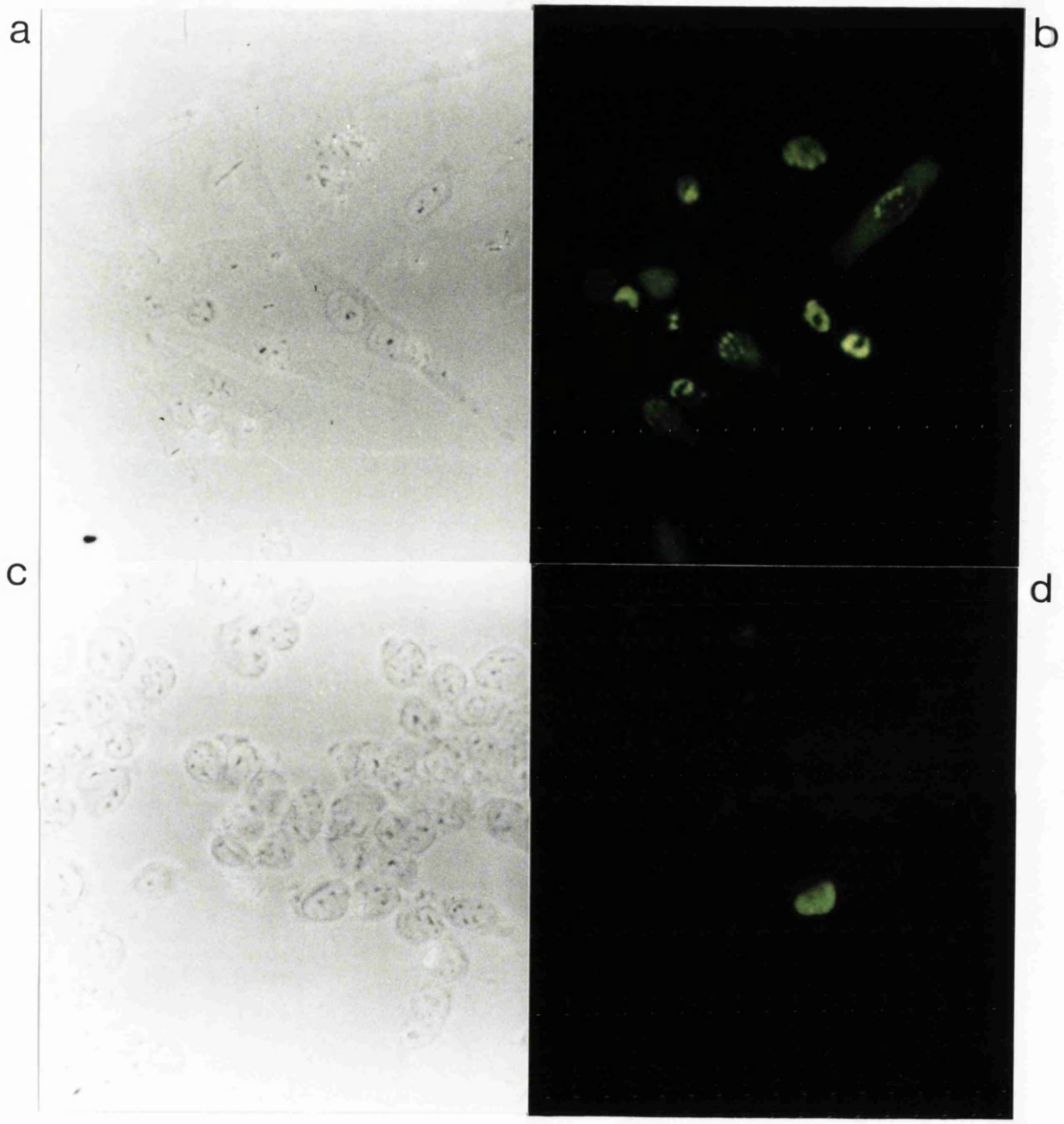
Table 3.4

Immunofluorescent Staining of Cell Lines with Antibody to the HSV-1 Immediate-Early Protein ICP4

Multiplicity of Infection	Cell Line BHK	ND7
1	30 %	1 %
5	80 %	12 %
10	80 %	26 %

The figures represent the percentages of cells staining with the 58S antibody to ICP4 (Showalter *et al*, 1981) after infection for 6 h. with HSV-1 strain F.

Figure 3.2



scale 1 cm = 62.5 μm

Immunofluorescent Staining of BHK and ND7 with the 58S Antibody Against ICP4

BHK cells and (a and b) and ND7 cells (c and d) viewed by phase contrast (a and c) and by immunofluorescent staining (b and d) with the 58S antibody to the HSV-1 immediate-early protein ICP4 (Showalter *et al*, 1981).

There is a great increase in the number of BHK cells staining over the number of ND7 cells. The BHK cells also show both nuclear and cytoplasmic staining. There have recently been reports of staining with antibodies against ICP4 in the plasma membrane of HEp 2 cells (Yao and Courtney, 1991). There also speckles of staining in the BHK cell nuclei. Such patterns of staining are not observed in the ND7 cells. The ND7 cells show a quite homogeneous type of staining, sometimes nuclear, but often not distiguishable as such as the nucleus is often as large as the cytoplasm.

3.vi. Results 4: Production of ICP4 is Impaired in Primary Sensory Neurons

To parallel the low level of ICP4 detectable in the ND cells, an IFAT was carried out on primary neonatal dorsal root ganglion (DRG) cells. These cells support latent HSV infections in *vivo*. Again the number of cells expressing ICP4 (using the 58S antibody) was calculated as a percentage of the total cell population (**Table 3.5**). The values represent the number of cells that express the IE protein ICP4 after infection with HSV-1 strain F at MOIs of 1 and 5. These data show that DRG cells are non-permissive at a MOI of 1 but that at a MOI of 5 the number of cells that stain with the 58S antibody increases to a greater degree than if the increase were directly proportional to the MOI.

Table 3.5

Immunofluorescent Staining of Dorsal Root Ganglion Neurons with Antibody to the HSV-1 Immediate-Early Protein ICP4

MOI	Cells Staining
1	4.25 %
5	48.5 %

Figures represent the percentages of cells staining with the 58S antibody to ICP4 (Showalter *et al*, 1981) after infection with HSV-1 strain F for 6 h..

3.vii. Discussion

The permissivities of a panel of ND cells has been ascertained by counting the number of cells that stain with the 58S antibody directed against the major viral *trans*-activator ICP4, the product of an IE gene and one of the earliest expressed proteins (Roizman and Sears, 1990). Latently infected cells do not express this protein (Stevens *et al*, 1987).

There is in fact a large range of permissivities within the ND cell lines. Some are highly permissive, but this was to be expected as neurons themselves display variations in their permissivities to HSV (Speck and Simmons, 1991).

ND3 cells do not express high levels of ICP4 at low MOIs. At a MOI of 1, 1.5 % ND3 cells express ICP4 wheres 48 % 3T3 cells express the protein. The nuclear run-on data show that this block in the expression of this IE protein is due to a reduction in the transcription rate of the IE-3 RNA. This is in contrast to the situation in HSV-infected U937 cells where the expression of the IE proteins is blocked due to the instability of the IE RNAs after transcription (Kemp *et al*, 1990b).

This experiment also showed that the LATs are expressed in ND3 cells. This observation has important implications as it suggests firstly that the viral genome is present in the cell. Secondly, it highlights the suitability of the ND cells for use as a model of a non-permissive virus:neuronal cell interaction.

Most of the experiments in this chapter were carried out on the ND7 cell line. This cell line is capable of differentiation after modulation

of the culture medium, see **Chapters 5** and **6**.

ND7 cells have a reduced capacity to produce infectious virions compared to BHK cells. In correlation with this, ND7 cells express low levels of IE proteins and BHK cells express high levels of IE proteins at low MOIs (1 % ND7 cells and 30 % of BHK cells express ICP4 at a MOI of 1). The capacity of each cell type to express the IE genes may therefore account for the differences between the two cell types to support a full productive infection.

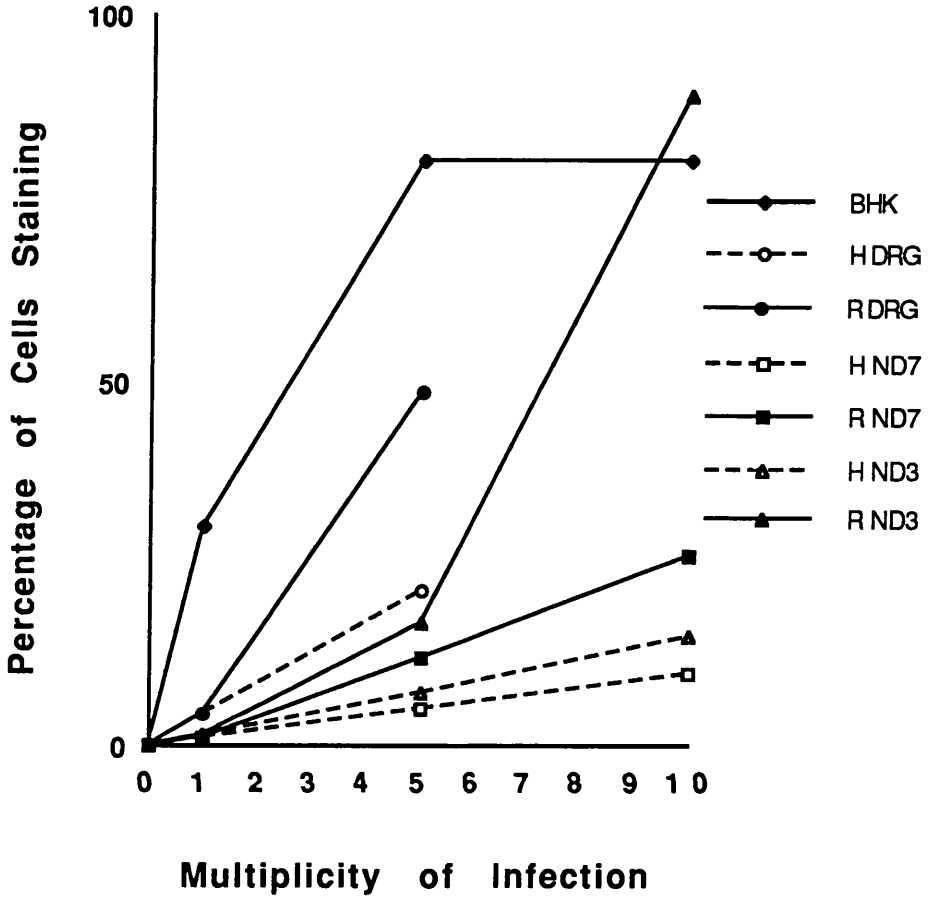
Observations have been made on the capacity of C1300 cells to produce infectious virions and the reduction seen is 100-fold at a MOI of 1 (Vahlne and Lycke, 1978), when compared with Vero cells using the strain F virus. Furthermore, in **Table 3.1**, the difference between the C1300 cells and the 3T3 cells in their capacities to express ICP4 is approximately 60-fold at a MOI of 1, which is not too dissimilar from the difference seen between the capacities of the C1300 cells and the Vero cells to produce infectious progeny. The Vero cells, BHK cells and 3T3 cells are not greatly different in their permissivities, at least in experiments carried out in the course of this work. The ND7 cells, therefore, although more permissive than the C1300 cells, are still less permissive than BHK cells (15-fold in virion-producing capacity).

The ELISA shows that a reduced quantity of protein is produced in the ND7 cells compared with an equivalent number of BHK cells. However this result does not distinguish between two possibilities. The first of these is that the amount of ICP4 produced in the positive ND7 cells is at the same level as that in each positive BHK cell. Alternatively, a reduced amount of ICP4 may be produced in every ND7 cell which has

a viral genome. Some ICP4 may in fact be produced but be undetectable. The results shown in **Table 3.4** and **Figure 3.2** show that only a few ND7 cells are producing the IE protein. The staining patterns between the two cell types also appear substantially different, possibly suggesting that the few positive cells are also expressing a reduced amount of protein when compared with the BHK cells. Alternatively, the overall nuclear staining of the ND cells may be obscuring a similar pattern as seen in the BHK cells.
The difference between the BHK and ND7 cells in their capacities to produce virions is 15-fold and in their capacities to produce ICP4 is 30-fold. Whilst an ND cell, once destined to go through a lytic cycle may theoretically double its virion-producing capacity, it is possible that the 1 % calculated to be the percentage of ND cells expressing ICP4 is subject to experimental error. Therefore, if the value had been calculated to be 2 %, no increase would have to be accounted for in the number of ND cells expressing ICP4 and the titre of the progeny virions produced in ND cells. This would result in the difference between BHK and ND cells in their capacities to produce ICP4 being nil. Thus, once started on a lytic cycle, an ND cell probably has the same capacity as a BHK cell to produce infectious progeny.

An interesting phenomenon seen with the non-permissive infections shown in this chapter is a decrease in the difference of permissivity between the two cell types as the MOI increases. That is, as the MOI increases a greater than expected number of cells produce the ICP4 protein than would be expected than if the increase were were proportional to the MOI, which is what happens in a permissive cell, see **Figure 3.3**. The data in **Table 3.5** shows that this

Figure 3.3



Graph Showing the Changes in Permissivities of BHK, ND and DRG Cells on Increasing the MOI

Percentages of cells staining after infection at increasing MOIs (full lines) and hypothetical values if the increase were linear (dotted lines). It can be seen that for both the ND cells and the DRG neurons that the actual increase is greater than if the increase were linear. This phenomenon may be accounted for by the theory of 'Multiplicity Activation', see section 3.v.

phenomenon also exists for DRG cells, and has been documented for primary sensory neurons elsewhere (Wilcox and Johnson, 1988). This phenomenon of 'Multiplicity Activation', where each cell has to be infected with more than one copy of the virus in order for a productive infection to ensue, has previously been observed in C1300s (Vahlne *et al*, 1981). In these experiments, an increase greater than if it were proportional to the MOI, is seen in the production of virions as the MOI increases. Experiments were also carried out to show that extracts of C1300 cells could reduce the capacity of permissive cells to produce infectious virions. Irradiation of the C1300 cells resulted in an increase in the capacity of the cells to produce virions and this suggested that a non-IFN like inhibitor of HSV gene expression was present. This idea was enhanced by the observation that IFN could not render permissive cells non-permissive (Vahlne and Lycke, 1978). A mutant virus (ts B5, mutant in a late protein) was able to rescue blocked infections. The explanation given was that the putative inhibitor of HSV gene expression could be bound by the mutant genomes, leaving the wild-type virus free to be expressed.

Evidence that an inhibitor of HSV IE gene expression is present in ND cells and DRG neurons has been accumulated, and this is presented in **Chapter 4**.

It is feasible that the lack of expression of the IE gene(s) seen in ND cells is due to an impairment of the virus binding or processing to the nucleus. This has in fact been described for C1300s (Vahlne and Lycke, 1978), but dismissed as only a part reason for the lack of

virus production seen in these studies. However, whilst the increase in MOI could cause an increase in uptake of virus and therefore explain the 'Multiplicity Activation' phenomenon seen in both the C1300 cells and the ND7 cells, there is still the observation that the LATs are produced during the putative latent infection seen in ND3 cells. Therefore the viral genome must be present in the ND cells during the aborted infection.

It therefore seems likely that the virus enters the cell but transcription of the IE genes do not reach high levels due to an inhibitor of viral gene expression. The hypothesis that neuronal latency may be established in the ND cells is therefore corroborated. The data shown here support the theory that the viral lytic cycle is blocked at the stage of IE gene expression in ND cells. This is the stage at which lytic infections are blocked in sensory ganglia (Stevens *et al*, 1987). Furthermore, expression of the LATs is seen in ND3 cells, further enhancing the suitability of the ND cells for studies of HSV latent-like infections. The LAT promoter is also capable of activity in ND7 cells, see **Chapter 4**.

Chapter 4 The Low Level of the HSV Immediate-Early Gene Products in ND Cells is due to a Transcriptional Inhibitor

4.i. Introduction

In the previous chapter it was shown that the transcription rates of the IE genes in ND cells are at very reduced levels when compared with those in BHK cells. This subsequently prevents high levels of the IE proteins, demonstrably ICP4, from accumulating and the lytic cycle is aborted.

Previous experiments carried out in C1300 cells *in vitro* have attempted to identify the *cis*-acting sequences and *trans*-acting factors responsible for the reduction in this transcription rate. These have included the introduction into C1300 cells of increasing quantities of an IE-CAT construct {where the promoter of the IE-3 from -330 - +33 with respect to the start point of transcription drives the expression of the bacterial gene encoding the readily assayable enzyme Chloramphenicol Acetyl Transferase [CAT] (Stow *et al*, 1986)}. In this experiment, the increase in dosage of the IE-CAT construct resulted in a sudden increase in the expression of CAT at a certain point in the the concentration series (Kemp *et al*, 1990a). However, the level of expression never reached that observed in a similar experiment in BHK cells. Further experiments (Kemp *et al*,

1990c) showed that when this promoter was truncated to -108 from the start point of transcription the difference observed between the two cell types in their capacity to express the IE-3 promoter was reduced, although a difference was still observed. This type of phenomenon is also seen when using the IE-2 promoter (Kemp *et al*, 1990c), that from the gene encoding ICP27, the only other IE protein shown to be essential for lytic infection, at least in tissue culture.

Two conclusions may be drawn from these results. The first is that an inhibitory protein is present in the neuronal cells which binds to upstream promoter elements. This theory stems from the observation that the full promoter is concentration-dependent for its expression in C1300 cells. At a crucial point in the concentration series the previously low level of expression of the full-length IE promoter significantly increases. A putative inhibitory protein that is also limiting could be bound to low levels of target promoter rendering them inactive, but at higher levels of target promoter there may be promoters free to be actively transcribed as the inhibitor has 'run out'. When the promoter is truncated however, the inhibitor cannot prevent transcriptional activation.

Secondly, it also appears that positive transcription factors which bind nearer to the TATA box and therefore still bind the truncated promoter, are absent from the neuronal cells, resulting in a lower level of expression of the truncated promoter in C1300 cells than in BHK cells. The lack of a concentration dependence of the expression of the truncated IE promoter in C1300 cells argues for an absence of a positive transcription factor acting near to the TATA box.

As described in the introduction, high-level transcriptional activation

of the IE genes is mediated by a potent viral *trans*-activator, VP16 which combines with the DNA-binding protein oct-1 and other cellular transcription factors (Kristie and Roizman, 1987). The sequence motif to which the oct-1 POU-homoeo domain binds, TAATGARATTC, acts in an enhancer-like manner while sequences around the TATA box act as a minimal promoter (Gelman and Silverstein, 1987).
Experiments have shown that in the C1300 system (Kemp *et al*, 1990a), an inhibitor of IE gene expression binds to the TAATGARATTC motif and also to the related octamer element found in many cellular promoters (ATGCAAATNA).

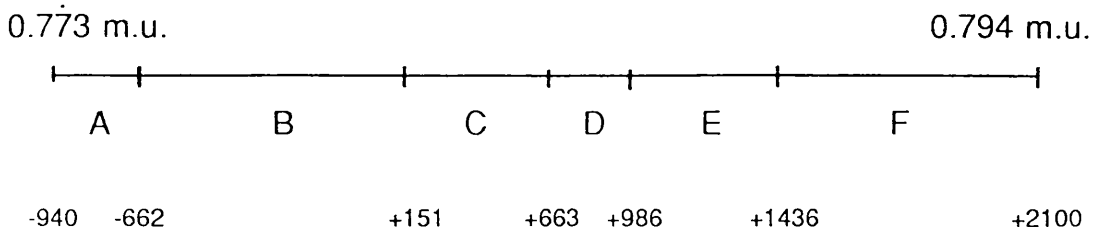
This chapter demonstrates the low activity of the IE promoters in the ND cell system which parallel the low transcription rate, and provides evidence that the reduction in the transcription rate of the IE genes is due to an inhibitory octamer-binding protein which binds to the TAATGARATTC motif.

In contrast, the LATs are expressed in the ND cells and at far higher levels than in BHK cells. This is a great advantage for using these cells as a model for latency, as only the LATs are expressed in latently infected sensory ganglia. The ND cell system is therefore more relevant for studies on latency than is the C1300 system.
Although originally identified as transcripts of approximately 2 Kb, a larger, unstable 8.3 Kb transcript has since been detected. Coupled with the fact that no promoter consensus elements have been detected near the 5' end of the 2 Kb transcript, it was suggested that the larger LAT transcript is in fact an unstable precursor from which the smaller LATs are spliced. Consensus promoter sequences were

therefore sought further upstream and a TATA box was discovered at -687 - -682 from the start point of transcription of the 2 Kb LAT (Weschler *et al*, 1988). A CAAT box and Sp 1 sites were also discovered at -817, -887, -853 and -589 upstream from the 5' end of the 2 Kb LAT (Weschler et al, 1988). To prove that these sequences were in fact functional, constructs A - F (see **Figure 4.1**) were made by Zwaagstra *et al* (1989), where fragments covering all of the coding region of the 2 Kb LAT and up to -940 upstream of the start point of transcription of the 2 Kb LAT were linked to the CAT gene. The results of experiments where these constructs were transfected into Vero cells showed that fragments A and F had promoter activity. Fragment A covers a region of the LAT promoter from -940 - -662 upstream of the start point of transcription of the 2 Kb LAT and encompasses a TATA box at -687 - -682 relative to the start point of transcription of the 2 Kb LAT. These results indeed corroborated the idea that the promoter of the LAT is a great distance further upstream from the startpoint of transcription of the 2 Kb LAT than originally thought and that the region between this TATA box and the start of the 2 Kb LAT is rapidly spliced after transcription. The fragment covering the region upstream of the 5' end of the 2 Kb LAT (fragment B) had no promoter activity, further indicating that its 5' region is an intron.

Furthermore two constructs (A+ and A++, see **Figure 4.1**) were made where a region -940 - -2592 upstream of the startpoint of transcription of the 2 Kb LAT could be assessed by their capacity to drive expression of the CAT gene in a promoter assay.

Results in this chapter therefore also show that using these constructs (A++, A+ and A - F) a promoter for the larger LAT precursor



Fragments A-F represent a portion of the HSV genome which spans the coding region of the originally identified 2 Kb LAT and a region upstream to it. Values are relative to the 5' end of the 2 Kb LAT. Fragments A+ and A++ (not shown) were derived by extending fragment A to 1271 and 2592 nucleotides respectively upstream from the 5' end of the 2 KB LAT. The A fragment contains a TATA box, a CAAT box and two Sp1 sites (taken from Zwaagstra et al, 1989). The section of the genome shown in this figure is also shown in Figure 1.2.

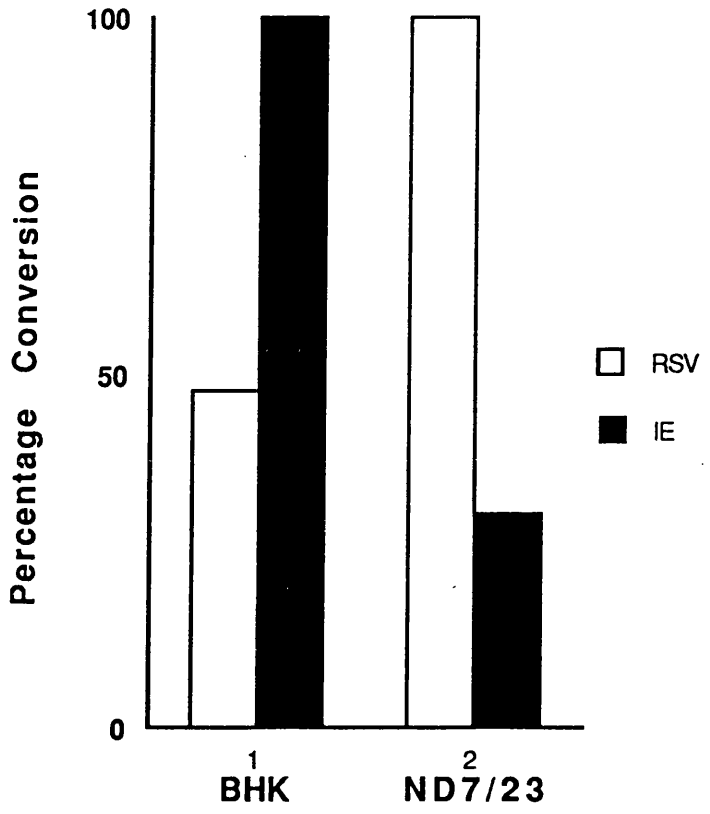
Fragments were cloned upstream of the CAT gene in the plasmid pSV0CAT (Zwaagstra et al, 1989).

has been delineated and shown to be active in ND cells. Evidence is also put forward for a neuronal cell-specific LAT promoter, which is inactive in permissive cell-types *in vitro.*

4.ii. Results 1 : Low Activity of the IE-3 Gene Promoter in ND7 Cells

To parallel the low levels of the IE RNAs in ND3 cells and of at least the protein encoded by the IE-3 gene, ICP4, after HSV infection,the activity of the IE-3 gene promoter was assessed in ND7 cells and compared with that in BHK cells. Thus, BHK and ND7 cells were transfected with the CAT gene linked to the IE-3 gene promoter (IE-CAT) (Stow et al, 1986) and the percentage conversion of chloramphenicol in a CAT assay was calculated (**Figure 4.2**). The Rous Sarcoma Virus (RSV) Long Terminal Repeat (LTR) linked to CAT (Gorman *et al*, 1984) was used as a control to show that the transfection efficiencies of the two cell types do not account for the difference seen in the expression of the IE promoter. RSV-CAT is actually expressed at a higher level in ND cells than in BHK cells, proving that the ND cells are not deficient in their ability to take up exogenous DNA.

Figure 4.2



Percentage Conversion of Chloramphenicol in a CAT Assay After Transfection of BHK and ND7 Cells with RSV-CAT and IE-CAT

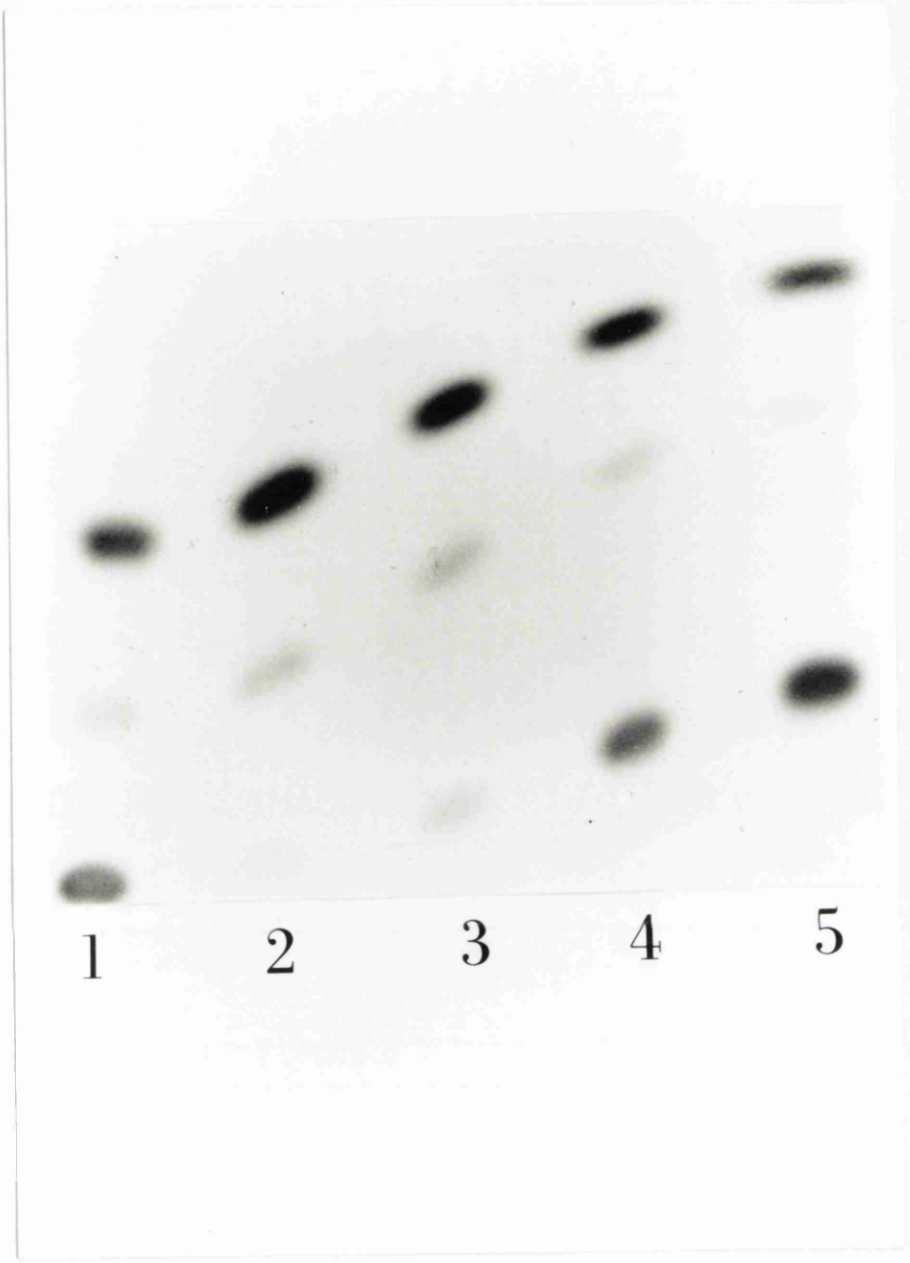
The RSV-CAT construct (Gorman, 1984) and the IE-CAT construct (Stow *et al*, 1986) were transfected into BHK and ND7 cells. CAT assays were then performed on cell extracts and the percentage conversion of chloramphenicol was ascertained. Bars represent the conversion of chloramphenicol as a percentage of the highest level of conversion in each cell type. There is clearly a difference between the two cell types in the capacities to express each promoter.

4.iii. Results 2: Elevation of IE-3 Gene Promoter Activity by Co-Transfection of TAATGARATTC or Related Elements

To lend weight to the theory that the ND cells contain an inhibitor of HSV IE gene expression, experiments were carried out where the effect of titrating out the inhibitor could be assessed. This was done by introducing extra copies of either the TAATGARATTC sequence or the octamer element and then assaying the capacity of the cell to express the IE-3 promoter. Co-transfections were therefore carried out with IE-CAT and firstly the pUC1813 plasmid (Kay and McPherson, 1987) containing six copies of the TAATGARATTC sequence (pF/6) (**Figure 4.3**). Increasing amounts of the pF/6 plasmid were introduced into ND7 cells and the amount of plasmid was always equalised with pUC1813 with no TAATGARATTC sequences. The results of this experiment show that on introduction of 5 μg of pF/6, the level of expression of IE-CAT is increased, but at higher levels than this (10-20 μg), the high level of IE-CAT expression begins to drop off. This effect may also be seen when the counts in the converted chloramphenicol are measured and expressed as a percentage of the total chloramphenicol (**Figure 4.4**).

It therefore seems that a putative neuronal cell-specific inhibitor binds to the introduced copies of the TAATGARATTC motif and leaves the sequences in the IE promoter free for oct-1 to bind. This theory is consistent with the presence of an inhibitory molecule and not the absence of a positively-acting factor. Such an effect on addition of the TAATGARATTC sequences would not be observed in

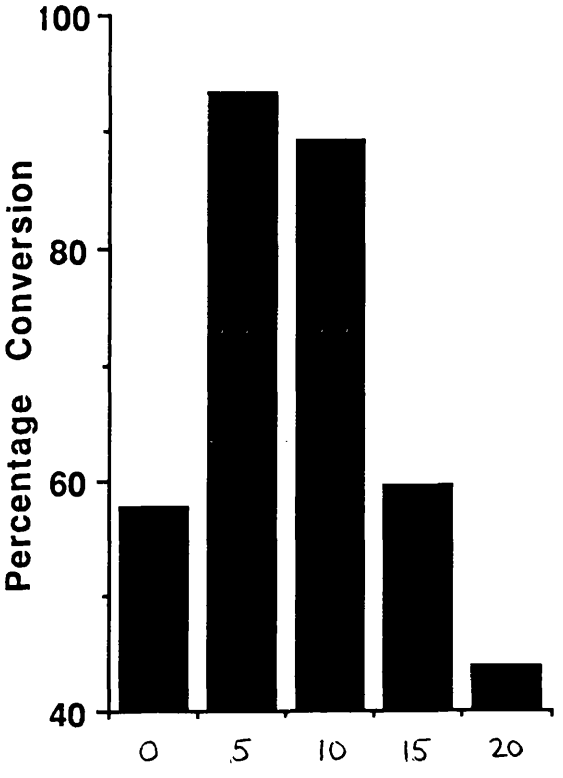
Figure 4.3



Cat Assay of ND7 Cell extracts After Co-Transfection of IE-CAT with TAATGARATTC Elements

ND7 cells were co-transfected with the IE-CAT construct (Stow *et al*, 1986) and the plasmid pUC1813 (Kay and McPherson, 1987) (track 1) and with increasing amounts of pUC1813 containing six copies of a consensus TAATGARATTC sequence cloned into the *Bam* H1site (track 2, 5 µg; track 3, 10 µg; track 4, 15 µg; track 5, 20 µg). The total amount of plasmid introduced was equalised with pUC1813. As the TAATGARATTC sequence is introduced, there is clearly an increase in IE-CAT expression (track 2). However, as greater amounts are introduced, the level of expression of IE-CAT decreases (tracks 3-5).

Figure 4.4



Percentage Conversion of Chloramphenicol in a CAT Assay After Co-Transfection of ND7 Cells with IE-CAT and TAATGARATTC Elements

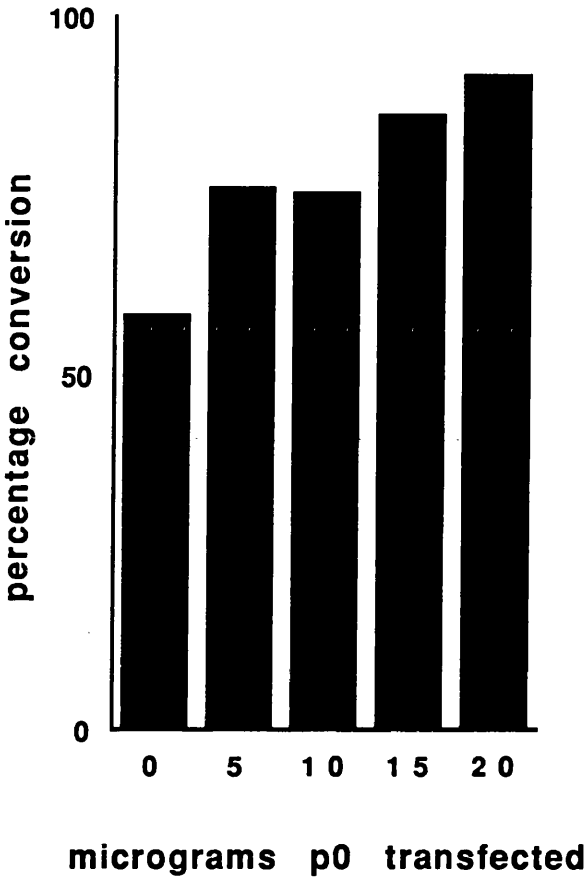
The values represented in this bar-graph have been taken from the CAT assay presented in **Figure 4.3**.

ND cells if the low expression of the IE promoters were due to the lack of a positive transcription factor. However after the initial increase the decline in the activity of IE-CAT may be due to binding of positive transcription factors, including oct-1, to the introduced TAATGARATTC sequence and removing them from the cellular pool that is available for transcription of the IE promoters.

A similar experiment was also carried out where the IE-CAT construct was transfected into ND cells with increasing quantities of a plasmid pO (Latchman *et al*, 1989), (**Figure 4.5**). This plasmid is pUC1813 containing one copy of the octamer element from the H2B gene promoter which is a perfect octamer concensus (ATGCAAATNA). The total amount of plasmid introduced was equalised with pUC1813. Again the level of CAT activity rises as the level of pO increases, but the decrease in activity is not observed. This is presumably because as the octamer sequence is present in only one copy in this construct, it has not reached high enough levels in the cell for the decrease in activity to occur. It is worth recalling that in contrast the TAATGARATTC sequence was present in six copies in the pF/6 construct.

The pO construct was again used in such an experiment, except that the cell line used was one assessed to be permissive with respect to Herpes Simplex Virus (**Tables 3.1** and **3.2**, **Chapter 3**). Thus NIH3T3 cells were co-transfected with IE-CAT and increasing quantities of pO, the difference in the amount of pO introduced being offset by pUC1813. The percentage conversion of chloramphenicol in a CAT assay was calculated (**Figure 4.6**). As these cells are highly

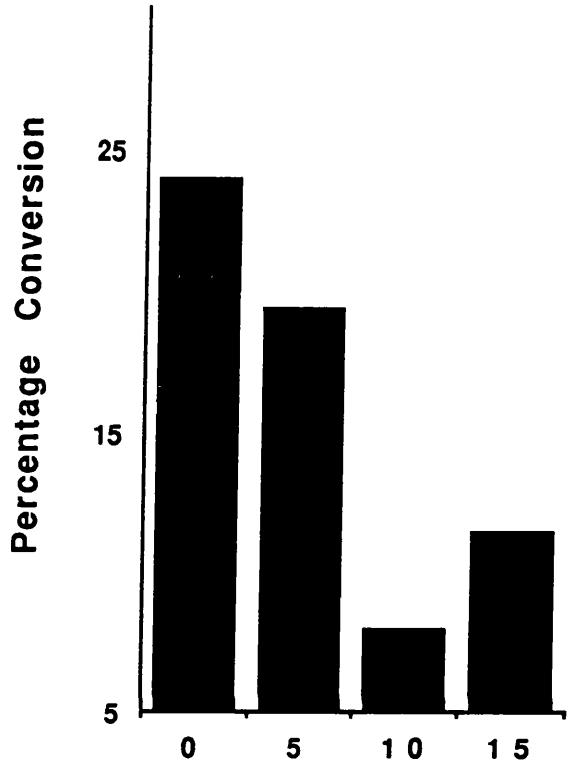
Figure 4.5



Percentage Conversion of Chloramphenicol In a CAT Assay After Co-Transfection of IE-CAT and Octamer Elements

ND7 cells were co-transfected with IE-CAT and the plasmid pUC1813 (Kay and McPherson, 1987) (track 0) or increasing quantities of pUC1813 containing one copy of the octamer element from the H2B gene promoter (pO) (tracks 5 - 20, 5 - 20 µg pO). The total amount of transfected plasmid was equalised by adding pUC1813. As the level of pO is increased, the level of expression of IE-CAT also increases.

Figure 4.6



Percentage Conversion of Chloramphenicol in a CAT Assay After Co-Transfection of NIH3T3 Cells with IE-CAT and Octamer Elements

NIH3T3 cells were co-transfected with IE-CAT and the plasmid pUC1813 (track 0) or increasing amounts of pUC1813 containing one copy of the octamer element from the H2B gene promoter (pO) (tracks 5 -15, 5 - 15 μg). The total amount of plasmid introduced was equalised using pUC1813. On introduction of the pO plasmid, the level of expression of IE-CAT drops immediately.

permissive to HSV, it may be assumed that no inhibitor is present and a productive lytic infection will proceed on introduction of HSV.
In this experiment, a decrease in the CAT activity was immediately seen on introduction of pO, probably reflecting the binding of positive transcription factors, including oct-1, to the octamer element and removal of them from the cellular pool. Totally different patterns of IE-3 promoter activity were therefore seen in NIH3T3 cells and in ND7 cells.

4.iv. Results 3 : Elevation of ICP4 Production on Infection with HSV after Transfection of TAATGARATTC or Related Elements

To parallel the increase in CAT activity seen in ND cells as increasing quantities of either the TAATGARATTC sequence or the octamer element were introduced, experiments were carried out to test the level of expression of the IE protein ICP4 in ND cells after introduction of these elements into ND cells and infection with HSV. Therefore the number of cells staining with the 58S antibody, raised against ICP4, after transfection of ND cells with a plasmid pF (containing one copy of the TAATGARATTC element in pUC1813) and infection 24 h. later was counted (**Table 4.1**). The staining on the cells was carried out 6 h. post-infection (p. i.). The results show that the level of ICP4 increases and then decreases as did the CAT activity after transfection with the pF/6 construct.

The pO construct was also used to transfect both ND cells and BHK

Table 4.1

Immunofluorescent Staining of ND3 Cells with Antibody to the Immediate-Early Protein ICP4 after Transfection of the pF Plasmid and Infection with HSV-1 Strain F.

pF Transfected/μg	% Staining
0	28.6
2	46
4	42
6	26

The figures represent the percentages of cells staining with the 58S antibody (Showalter *et al*, 1981) after transfection and infection with HSV-1 strain F at a multiplicity of infection of 5 and at 6 h. p. i..

cells to assess the number of cells which express ICP4 after infection at a MOI of 5 (**Table 4.2**). The results of these experiments are also shown graphically (**Figure 4.7**). In the ND cells, although a slight depression is seen at the beginning of the dose-response curve, there is a clear increase in the number of cells expressing the ICP4 protein. This increase subsequently decreases at high levels of pO. In contrast, the BHK cells show only a reduction in their capacity to express the ICP4 protein as the number of ICP4-positive cells decreases. It is again likely that where the graph takes a downward turn, the introduced sequences are binding oct-1 and other positive transcription factors and thus prevent expression from the ICP4 gene.

4.v. Results 4 : LAT Promoter Activity

The constructs shown in the introduction to this chapter (**Figure 4.1**) were used to transfect BHK and ND7 cells. These constructs contain the CAT gene with fragments of the 2 Kb LAT and regions upstream of its start point to -2952. A functional TATA box had previously been mapped to -687 - -682 upstream of the start of the 2 Kb LAT and a theory of a large 5' intron between the putative real startpoint of transcription just downstream of this TATA box and the 5' end of the 2 Kb LAT had been proposed. After transfection of the constructs, the percentage conversion of chloramphenicol to the acetylated form was calculated (**Figure 4.8** and **Table 4.3**).

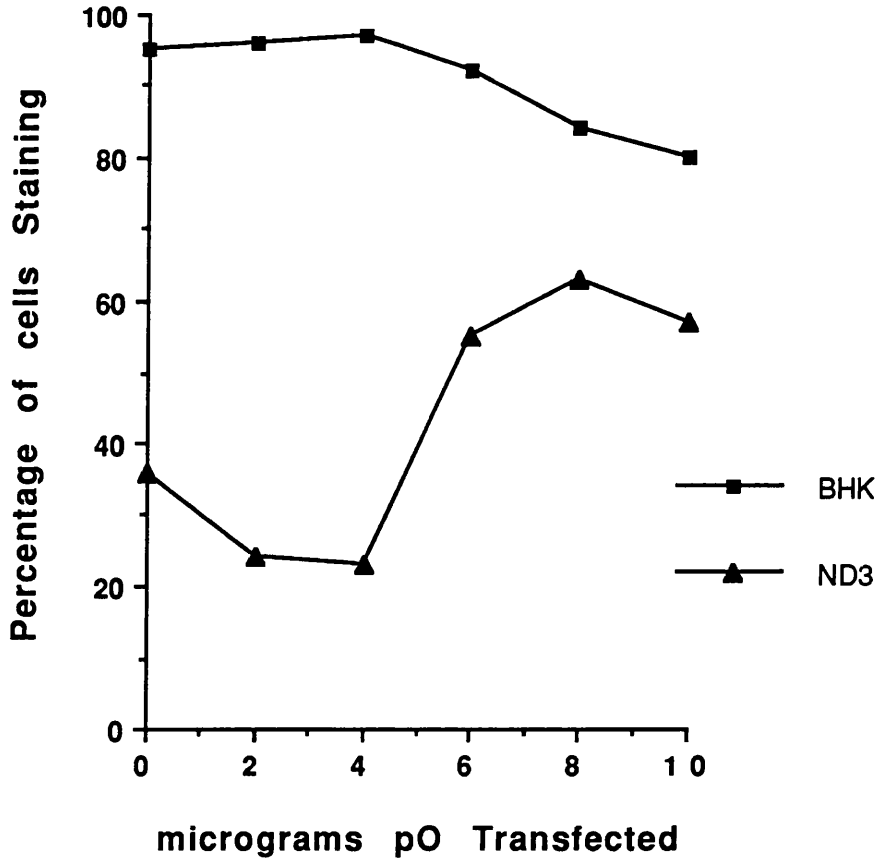
Firstly these results show that promoter activity exists for the larger, unstable LAT precursor. The TATA box identified by Weschler

Table 4.2

Immunofluorescent Staining of BHK and ND3 Cellswith Antibody to the Immediate-Early Protein ICP4 After Transfection of the pO Plasmid and Infection with HSV-1 Strain F

pO Transfected/μg	% Staining	
	ND3	BHK
0	36	95
2	24	96
4	23	97
6	55	92
8	63	84
10	57	80

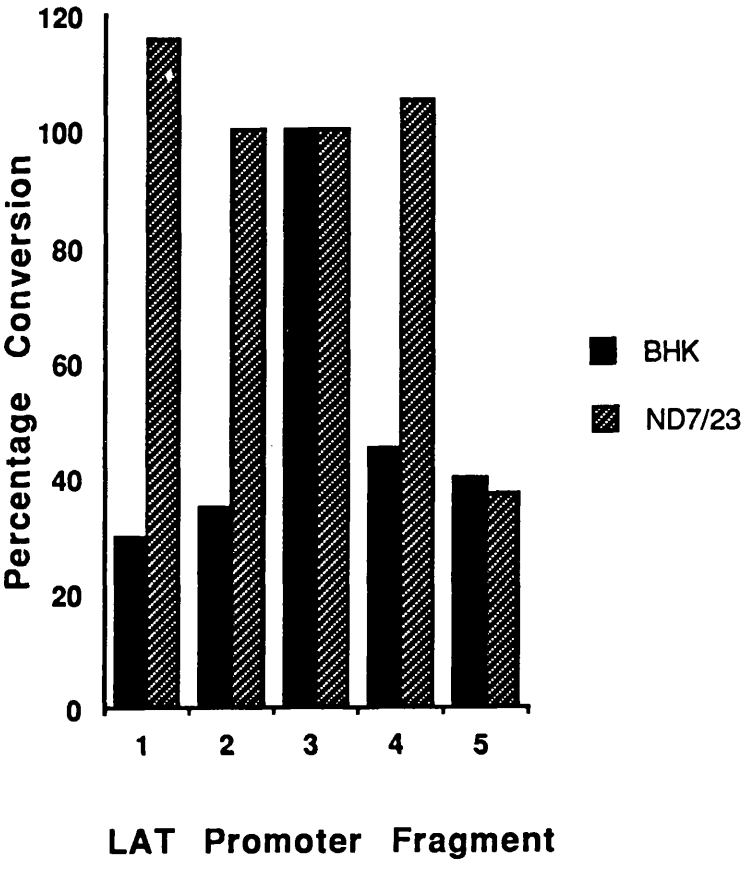
The figures represent the percentages of cells staining with the 58S antibody (Showalter *et al*, 1981) after transfection with the pO plasmid containing the octamer sequence (ATGCAAATAA) and infection with HSV-1 strain F at a multiplicity of infection of 5 and at 6 h. p. i..



Graph to Show the Percentage of a Cell Population which Expresses ICP4 in an Immunofluorescent Antibody Test After Transfection with Octamer Elements and Infection with HSV-1 Strain F

BHK and ND3 cells were transfected with pUC1813 (Kay and McPherson, 1987) and with increasing quantities of pUC1813 containing the octamer element from the H2B gene promoter (pO). The total amount of transfected DNA was equalised with pUC1813. After 24 h., the cells were infected at a MOI of 5 with HSV-1 strain F for 6 h.. The cells were then fixed and stained with the 58S antibody to ICP4 (Showalter *et al*, 1981). The ND3 cells show an initial decrease (2 μg transfected pO)in the number of cells staining followed by a clear increase in the number of cells expressing ICP4 (4-8 μg pO). At 8 μg transfected pO, the number of cells expressing ICP4 drops. However,the number of BHK cells expressing ICP4 is initially high and a gradual decrease in the number of cells expressing ICP4 is seen with increasing quantities of pO

Figure 4.8



Percentage Conversion of Chloramphenicol in a CAT Assay After Transfection of ND7 Cells with LAT Gene Fragments Linked to the CAT

ND7 cells were transfected with constructs derived by fusing the CAT gene to fragments of the gene encoding the Latency-Associated Transcripts and to sequences 5' to it (see **Figure 4.1**). CAT assays were performed on cell extracts and the conversion of chloramphenicol is expressed as a percentage of the greatest percentage conversion for each cell type. Track 1, A++; track 2, A+; track 3, A; track 4, F; track 5, SV2.

Table 4.3

Relative Percentage Conversion to Acetylated Chloramphenicol in a CAT Assay After Transfection of ND7 cells with the CAT Gene Under the Control of Various LAT Fragments.

LAT Fragment/ Promoter	Percentage Conversion	
	BHK	ND7
1 (A++)	30	116
2 (A+)	35	100
3 (A)	100	100
4 (F)	45	105
5 (SV2)	40	37

The percentage conversion by the A fragment was standardised to 100 % and the results for the other fragments have been standardised relative to A. The A fragment actually gave a conversion rate of approximately 10 % in both cell lines. All the LAT fragments were from the *Bam* H1 B fragment of HSV-1 strain F and were cloned upstream of the pSVOCAT plasmid. A++, A+ and A all have the same 3' end. The 5' ends are all relative to the 5' end of the originally described 2Kb LAT transcript. A++ ends at -2592, A+ at -1271 and A at -940, see **Figure 4.8**.

et al (1988) indeed signals binding of the RNA polymerase complex, and the putative true promoter therefore starts over 600 bp upstream from the 5' end of the 2 Kb LAT.

Fragment A++ represents the putative full-length promoter and A+ and A represent deletions of this promoter. A+ is truncated to -1271 and A is truncated to -940 upstream of the start point of transcription of the 2 Kb transcript. Fragments B-E show no promoter activity at all and in fact probably represent an intron that is removed rapidly.

The F fragment has some promoter activity although no recognisable sequence motifs have been detected. It is active in either orientation. No promoter activity therefore exists for region the region just 5' of the start of the 2 Kb LAT, further substantiating the evidence that the 2 Kb LAT is generated by splicing of the region immediately upstream of its 5' end.

These results secondly show that the LAT promoter is capable of higher activity in ND cells than in BHK cells. This correlates well with the transcription of the LAT gene seen in **Figure 3.2** in the previous chapter.

The full-length LAT promoter fragment used here (A++) is active to a far greater level in ND cells than it is in BHK cells. The same is true of the A+ promoter. These results have been standardised with respect to the A fragment as promoter activity of this fragment is similar in both cell types. These observations suggest that a silencing region exists in the LAT promoter that is only used in permissive BHK cells.

The promoter from the early region of Simian Virus 40 linked to CAT (pSV2CAT), (Gorman 1982) was used as a control. The expression of this construct was similar in both ND and BHK cells.

4.vi. Discussion

Evidence has been presented showing that the promoter from the IE-3 gene has low activity in ND cells. The low level of IE gene transcription results in low levels of IE proteins being produced and appears to be due to a transcriptional inhibitor. In comparison the LAT gene is expressed well in ND cells.

Different methods of analysis of infection have ben used by others and in this work, for example, analysis of IE transcripts, of IE proteins and of virion production. **Chapter 3** showed that it is indeed relevant to consider only the expression of the IE genes as a guide to the capacity of the cell to produce infectious virions. In latency *in vivo* it is notable that sensory ganglia are impaired in their capacity to produce IE proteins and this accounts for most if not all of the obstruction to proceed through the lytic cycle.

In the immortalised sensory neuron lines, the ND lines, it has been shown that the inhibition of IE gene transcription can be overcome by introducing extra copies of the TAATGARATTC sequence, the sequence to which the oct-1/VP16 complex binds in a natural infection. The kinetics of either IE-CAT expression in the co-transfection experiments (**Figures 4.3 - 4.5**) or ICP4 expression in the transfection and infection experiments (**Figure 4.7** and **Tables 4.1** and **4.2**) prove that it is an inhibitor that is responsible for the low levels of IE gene expression in ND cells. That is low level expression from the IE promoter is boosted on introduction of certain promoter elements. These observations are not consistent, for example, with an absence of a positive transcription factor in ND cells, as in that

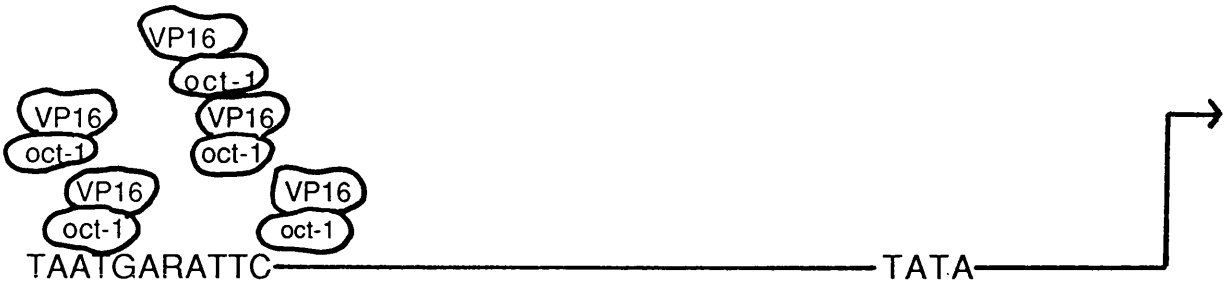
case promoter activity would not increase on introduction of the upstream promoter elements. Furthermore, the idea of viral sequences binding out a negative regulatory factor is not new, as Nilheden *et al* (1985) observed an increase in virus production in C1300 cells after co-infection of a mutant virus mutant (ts B5, mutant in a late protein) with the wild type.

In addition, **Chapter 3** describes the phenomenon of 'Multiplicity Activation', where an increase in the multiplicity of infection results in a greater than linear increase in the level of IE protein production (see **Figures 3.1 and 3.2 and Table 3.4**) or virus production (Vahlne and Lycke, 1978) in neuronal cells. This phenomenon also occurs in primary neurons (see **Table 3.5** and Wilcox and Johnson, 1988). These observations are consistent with the theory of an inhibitor of IE gene transcription present in neuronal cells. As increasing numbers of viral genomes are introduced into one cell, the putative inhibitor binds to a proportion of the genomes, but still leaves some free to be bound by positive transcription factors. This theory implies that the inhibitor has a higher affinity for TAATGARATTC than does oct-1 and also that it is present in limiting amounts. A model for this theory is presented in **Figure 4.9**.

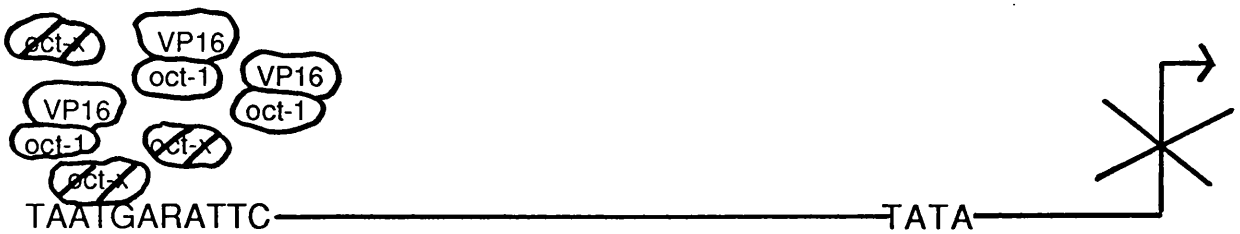
The data here show that the TAATGARATTC element or the octamer concensus can bind the putative inhibitor of viral IE gene transcription in neuronal cells. This implies that the inhibitor is an octamer-binding protein and in fact an extra octamer-binding protein has been detected by others in ND cells using the gel-retardation assay (C. Dent, personal communication), see **Figure 4.10**. This protein is therefore a candidate for the HSV IE gene transcriptional inhibitor, and it has been further characterised elsewhere (Lillycrop

Model of the Proposed Role of Introduced Multiple TAATGARATTC Sequences in Cells of Neuronal Origin after HSV Infection

a) In an infected permissive cell, oct-1 complexed with VP16 is present in abundance and binds to the TAATGARATTC element in the IE gene promoters. High level transcription of the IE genes therefore occurs.



b) In a neuronal cell, the putative inhibitor of IE gene transcription, oct-x, binds to the TAATGARATTC element with higher affinity than does the oct-1/VP16 complex. High level transcription mediated by oct-1/VP16 is therefore inhibited.



c) If copies of the TAATGARATTC sequence are introduced into a neuronal cell, they are able to bind oct-x and leave the TAATGARATTC elements in the IE promoters free to bind the oct-1/VP16 complex. Activated transcription can therefore occur.

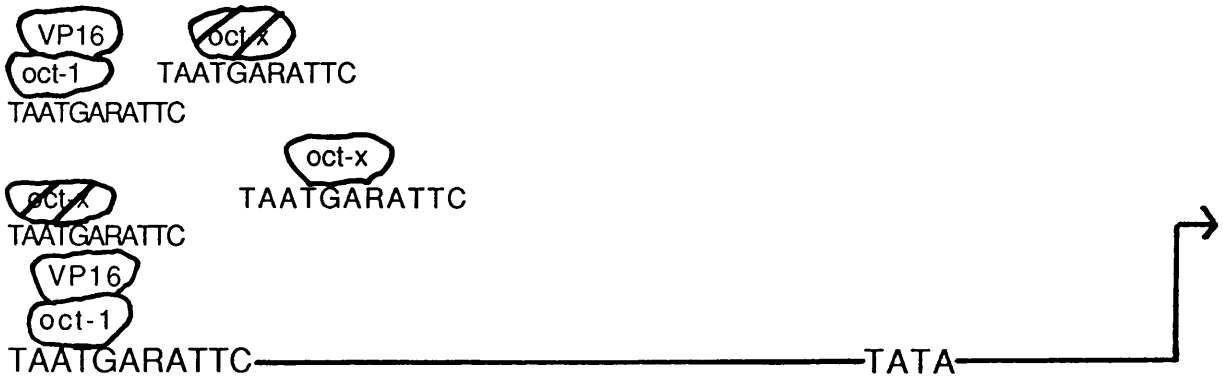
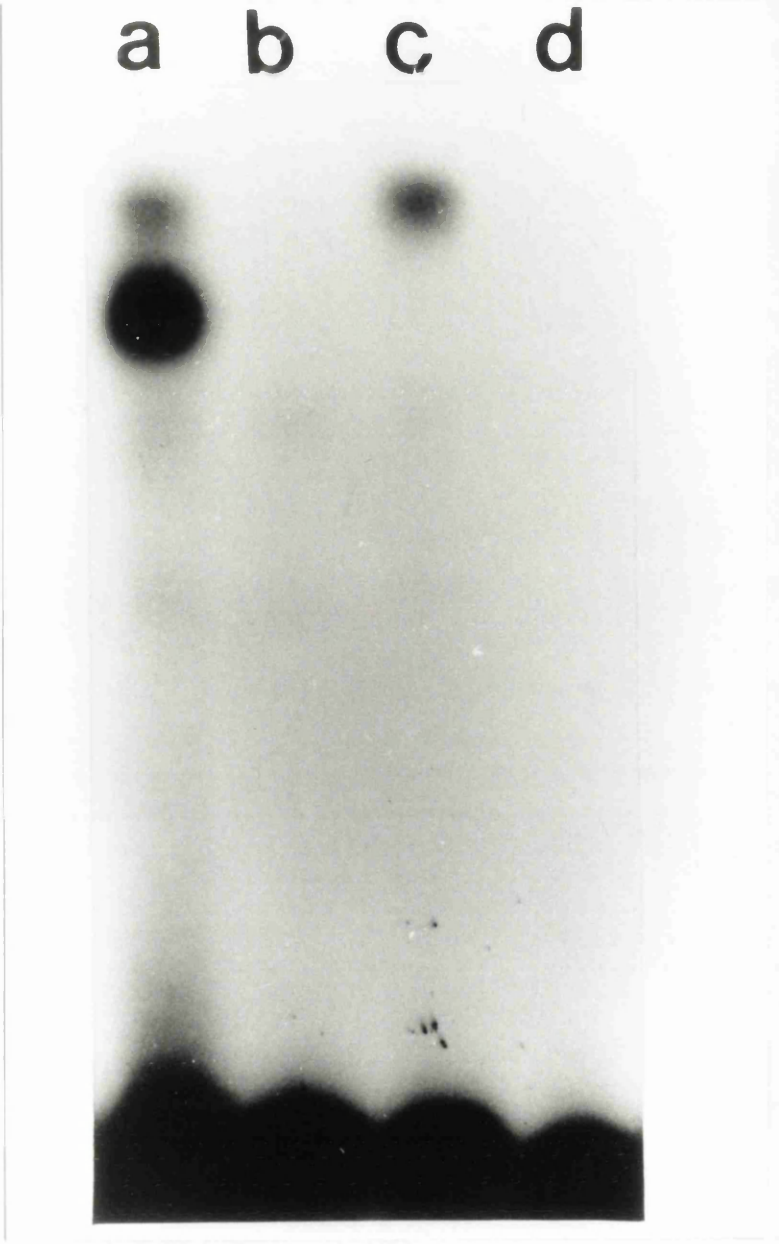


Figure 4.10



Gel retardation assay showing extracts from ND7 cells (tracks a and b) and BHK cells (tracks c and d) shifted on a probe containing the consensus IE TAATGARATTC sequence. Tracks a and c are uncompeted whereas tracks b and d are competed with cold oligonucleotide, showing that the bands seen are specific. It can clearly be seen that an extra band exists in ND7 cells (track a) which migrates faster than the upper band seen in tracks a and c.

et al, 1991).

LAT expression, in contrast, is high in ND cells and expression of these transcripts occurs to low levels in BHK cells. The functional capacity of the 8.3 Kb LAT promoter has now been corroborated, although this transcript has only been detected as a minor species (Mitchell *et al*, 1990, Zwaagstra *et al*, 1990). The promoter of this unstable transcript begins at -662 from the start point of transcription of the 2 Kb LAT and the region between the start point of the 8.3 Kb transcript and that of the 2 Kb transcript is rapidly spliced.

The LAT promoter has also been shown to contain a neuronal cell-specific region between -2592 and -940 upstream of the start point of transcription of the 2 Kb LAT. This region corresponds to -1930 - -378 upstream of the putative start point of transcription of the 8.3 Kb transcript. This region results in high levels of transcription in ND cells and low levels in BHK cells.

As described in the introduction, the LAT gene is expressed to much higher levels in sensory ganglia than in permissive cells in tissue culture (Spivack and Fraser, 1987). This experiment with truncated LAT promoter fragments provides evidence that this is due to a *cis*-acting sequence which results in neuronal cell-specific transcription. The increase seen in the expression of the LAT promoter in BHK cells when it is truncated argues for a negative regulatory mechanism at work which is absent from ND cells. Titration experiments may substantiate this theory.

Further analysis of this promoter will delineate more specifically the region involved and the mechanism of action of factors at this site.

The role of the LATs or latency-related protein remains elusive.

The ND cells have therefore been demonstrated to support only low levels of activity of the IE-3 promoter. This is consistent with findings in sensory neurons *in vivo* in which no IE transcripts have been detected (Stevens *et al*, 1987). Such cells could support IE gene transcription and the transcripts could be rapidly degraded, as occurs in the U937 cell line which is non-permissive for HSV (Kemp and Latchman, 1990b). However, a transcriptional inhibition of IE gene expression has here been demonstrated in ND cells which may argue for such a mechanism to exist in sensory neurons *in vivo*.

Chapter 5 Cyclic AMP Modulates HSV Immediate-Early Gene Expression

5.i Introduction

Cyclic adenosine monophosphate (cAMP) is an ubiquitous signal transduction molecule the intracellular levels of which are regulated by receptor-mediated activation or inhibition of the enzyme adenylate cyclase (Smigel *et al*, 1984). Cyclic AMP activates protein kinase A and the resulting phosphorylation events may affect cell metabolism or gene expression. Genes regulated by cAMP are expressed in tissues responsive to hormones, neurotransmitters or growth factors and it has been shown that an active kinase catalytic subunit is a necessary intermediate for induction of cellular gene transcription by cAMP (Grove *et al*, 1987).

Cyclic AMP has been shown to affect the rate of transcription of many genes. Deletion and mutation studies on the promoters of these genes has shown that they contain cAMP-responsive elements (CREs) which act as enhancers, or in some cases as basal promoter elements.
The first gene found to be cAMP-inducible was that for the enzyme phosphoenolpyruvate carboxykinase (Cimbala *et al*, 1982). The CRE in this gene is located at -109 - -68 upstream from the start point of transcription. The human α-chorionic gonadotrophin (αHCG) gene also contains a CRE at -146 - -111 upstream of the startpoint of transcription (Silver *et al*, 1987). Other genes containing such

sequences are those encoding the hormones somatostatin (Montminy and Bilezikjian, 1987), proenkephalin (Comb *et al*, 1986), vasoactive intestinal peptide (Tsukada *et al*, 1987), the cellular homologue of the retroviral gene from the FBJ murine osteosarcoma virus, c-fos (Van Beveren *et al*, 1983) and the neurotransmitter-synthesising enzyme tyrosine hydroxylase (Lewis *et al*, 1987). All of these genes contain a highly conserved core CRE consensus sequence, T G/T A C G T C A. The regions flanking the concensus CREs may have roles in synergistic effects with that sequence, although they show no homology between different CRE-containing genes. The level of expression induced from the isolated CRE from the phosphoenolpyruvate carboxykinase gene did not reach that from the same CRE when maintained in context with its native flanking sequences (Yoo-Warren *et al*, 1983). In that case, a CAAT box also existed in close proximity, perhaps resulting in protein:protein interactions to further induce transcription.

Cyclic AMP-dependent protein kinase (protein kinase A) affects the transcriptional efficacy of CREB whilst having no DNA-binding influence. The CRE binds a 43 KDa CRE binding protein (CREB), which has been isolated from rat brain (Yamamoto *et al*, 1988) and rat pheochromocytoma cells (PC12 cells) (Montminy and Bilezikjian, 1987). This protein binds the palindromic concensus CRE sequence as a dimer, a process which is partly regulated by calcium-dependent protein kinase (protein kinase C) (Yamamoto *et al*, 1988), although this protein kinase does not affect transcriptional activation effected by CREB. Protein kinase C can also be activated by the phosphatidyl-inositol secondary messenger pathway (Krause *et al*, 1978).

CREB has also been identified as Activating Transcription Factor (ATF) 47 (Lin and Green, 1988) and its ability to dimerise with ATF 43 has been demonstrated (Hurst *et al*, 1990). The discrepancy in the sizing is probably accounted for by the fact that heterodimerisation can occur on the CRE.

CREB and the ATFs belong to a family of factors called the bZIP proteins (Vinson *et al*, 1989). These contain a leucine zipper preceded by a similar highly basic domain which mediates the dimerisation of members of the family. Also included in the family are the so-called cellular immediate-early genes which include *c-jun* and *c-fos*. *C-jun* can also heterodimerise with ATF to activate transcription of CRE-containing genes.

The transcriptional activity of CREB is altered after treatment with cAMP via a phosphorylation mechanism mediated by protein kinase A (Gonzalez *et al*, 1989). Tryptic peptide mapping of CREB has elucidated one protein kinase A phosphorylation site, two protein kinase C phosphorylation sites and one casein kinase II phosphorylation site (Gonzalez *et al*, 1989). These findings argue for a series of phosphorylation events, brought about by different protein kinases, that regulate events near the TATA box. Protein kinase A itself causes an allosteric change in the CREB protein which may result in either a change in binding of RNA polymerase II to the promoter or in protein:protein interactions that affect the transcription initiation complex.

A second protein has also been identified that in some cases mediates the induction of gene transcription by cAMP. Activating Protein (AP)

2 is an approximately 50 KDa cell-specific protein which will also respond to phorbol myristate acetate (PMA) to induce gene expression (Imagawa *et al*, 1987). The sequence which mediates this induction has the consensus C C C C A N G C G and is present in the human metallothionein gene promoter and that of the proenkephalin gene. The binding affinity of this protein to DNA is not altered by treatment with cAMP (Imagawa *et al*, 1987), as also noted above for CREB.

A role for cAMP in the induction of reactivation from latency and by necessity of HSV IE gene expression, has evolved after observations that a number of cAMP analogues and compounds which stimulate intracellular cAMP levels can cause reactivation of latent HSV from sensory neurons *in vitro* (Wilcox, personal communication).
Adrenergic hormones such as epinephrine have also been shown to reactivate latent HSV in animal models (Trousdale *et al*, 1991). Such hormones result in an intracellular increase in cAMP, again substantiating cAMP as a stimulus for reactivation from latency.
During tissue damage (including nerve trauma) prostaglandins accumulate at the affected site (Horrobin, 1978). Prostaglandins cause an increase in the levels of intracellular cAMP (Horrobin, 1978). As nerve trauma is a well-documented stimulus for reactivation (Baringer, 1978, Stevens, 1978), prostaglandins may be the means by which the reactivation is accomplished in the cell, at least in some instances.

In addition, it has also been observed that Nerve Growth Factor (NGF) has a role in the maintenance of the latent state, thus a drop in the supply of NGF to latently infected sensory neurons resulted in

reactivation of latent HSV *in vitro* (Wilcox and Johnson,1988). NGF-dependent HSV latency has also been established in PC12 cells (Block *et al*, personal communication).

The idea that NGF maintains latency has been borne out after studying patients who have been treated for trigeminal neuralgia by dissection of the trigeminal nerve root, or who have had another sensory nerve section (axotomy or rhizotomy). In such patients, reactivation of HSV occurred and productive infection resulted at the periphery after the operation (see section 1.v.a.). In these cases, the reactivation of latent HSV may have been induced by the cessation of retrograde transport of NGF to the ganglionic cell body. A reproducible animal model of reactivation of latent HSV after nerve root section has also been established (Walz *et al*, 1974).

It has also been reported that the secondary messenger pathways of NGF and cAMP are linked. Evidence has suggested that the NGF receptor is associated with adenylate cyclase and therefore binding of the ligand NGF results in activation of the enzyme and an increase of intracellular cAMP (Skaper *et al*, 1979). However, this seems to present a paradox as evidence suggests that NGF maintains latency whereas cAMP has the ability to reactivate latent virus. Data from experiments in this chapter may shed some light on this apparently irreconcilable enigma.

Extensive studies on the major IE enhancer of human cytomegalovirus (HCMV), a related herpesvirus, have shown that it can be induced in a cell-type specific manner by cAMP (Stamminger *et al*, 1990). Thus in lymphoid lines and HeLa cells the HCMV IE enhancer is induced via a

18-19 bp *cis*-acting sequence which contains a consensus CRE.

Therefore it seemed important to investigate the effect of cAMP on HSV IE gene expression in the sensory neuron-derived cell lines, the ND cell lines. This chapter shows data indicating that the non-permissive virus:cell interaction seen with the ND cells and HSV may be at least partially overcome by treatment with a compound that raises intracellular cAMP, and which suggest that a latent HSV infection might be reactivated by cAMP. The data therefore substantiate the theory of cAMP as a reactivation agent.

5.ii Results 1 : The Block to IE Gene Expression can be at least Partially Overcome by Low Levels of cAMP

Isobutyl methylxanthine (IBMX) is a compound that can increase the intracellular levels of cAMP. It inhibits the cAMP phosphodiesterase to maintain cAMP levels high, whilst cAMP is still synthesised. The effect of this compound on ND7/23 cells has been ascertained (**Table 5.1**). ND7/23 cells were treated with 10 μM IBMX and the capacity of the HSV-infected cells to produce infectious virions was measured (**Table 5.2**). The capacity of ND7/23 cells was markedly improved after treatment with this compound.

On treatment with the same concentration of IBMX the number of cells expressing the HSV immediate-early protein ICP4 increased after infection with HSV-1 strain F at different multiplicities of infection (MOI) (**Table 5.3**). This increase in the number of cells expressing ICP4 presumably accounts for the increase in virus production

Table 5.1

Effect of 500 μM IBMX on the Level of cAMP in ND7/23 Cells in Culture

Untreated Cells	IBMX-Treated Cells
cAMP Level/coverslip	
0.99 pmol	7.55 pmol
S.E.M. 0.27	S.E.M. 5
n = 3	n = 5

Cells were treated with 500 μM IBMX for 1 h. at 37°C. Experiment carried out by J. Wood.

Table 5.2

Effect of 10 μM IBMX on the Capacity of ND7/23 Cells to produce Infectious Virions

	Untreated Cells	IBMX-Treated Cells
	Virus Titre	
Experiment 1	2.5×10^2	1.1×10^3
Experiment 2	3.75×10^2	1.3×10^3

Figures indicate the values obtained by titrating the virus produced by ND7/23 cells untreated or treated with 10 μM IBMX, harvested at 4 h. p. i. following infection at a multiplicity of infection of 1.

Table 5.3

Immunofluorescent Staining of Untreated and IBMX-Treated ND7/23 Cells with Antibody to ICP4 after Infection with HSV-1 Strain F

MOI	Untreated		IBMX-Treated	
	A	B	A	B
1	0.0	4.0	1.0	8.0
5	1.5	14.4	14.4	23.0
10	13.9	24.7	39.8	27.2

Figures indicate the percentages of cells staining after treatment with 10 μM IBMX in two separate experiments (A for 72 h. and B for 24 h.) Cells were stained with the 58S antibody to ICP4 (Showalter *et al*, 1981) after infection for 6 h..

observed in the total cell population (**Table 5.2**). ND7/23 cells were also treated with dimethyl sulphoxide (DMSO) and methanol in this assay as this is the solvent for IBMX. However no effect was detected in the number of cells expressing ICP4 (data not shown).

5.iii Results 2 : The ICP0 Gene Promoter is Induced by cAMP via a Cyclic AMP Responsive Element

Following these results, and with the knowledge of the mechanism of action of cAMP on eukaryotic gene expression, the promoters of the HSV IE viral trans-activators were investigated for CREs and AP2 sites. The search showed that a CRE is present in the promoter of the gene encoding ICP0 at -70 - -62 relative to the start point of transcription (**Figure 5.1**). This site binds a protein of the same molecular weight as that which binds an ATF site in a band shift assay (see **Figure 5.5**, discussion, this chapter). No CRE was found in either the promoter of the gene encoding ICP27 or that encoding ICP4, although two possible AP2 sites were identified in the promoter of the gene encoding ICP4 at -64 - -73 and -38 - -47 relative to the start point of transcription (**Figure 5.1**). However, the match of these two AP2 sites with the consensus (**Figure 5.1**) was not good, implying that transcripional activation of the IE-3 gene promoter by AP2 may not actually occur.

The promoters of the IE genes encoding ICP0 and ICP4 linked to the CAT gene were therefore studied to discover whether they could be activated by 10 μM IBMX in a transient transfection assay. The ICP0-CAT construct used was pIG 65 (Gelman and Silverstein, 1987).

Figure 5.1

Comparison of the Cyclic AMP-Responsive Element (CRE) and Activating Protein (AP) 2 Site Consensus Sequences with those found in the HSV IE Gene Promoters.

a) CRE		Position
Consensus :	T G/T A C G T A/C A/G	
αHCG :	T G A C G T T T	
HSV IE-1 :	A A T C G T T A	-62 - -70

b) AP2 site		
Consensus :	C C C C A N G C G	
H MTIIA :	C C G C C C G C G	
HSV IE-3 (A) :	G C C G G C G G C	-64 - -73
HSV IE-3 (B) :	C C G C C G G C C	-38 - -47

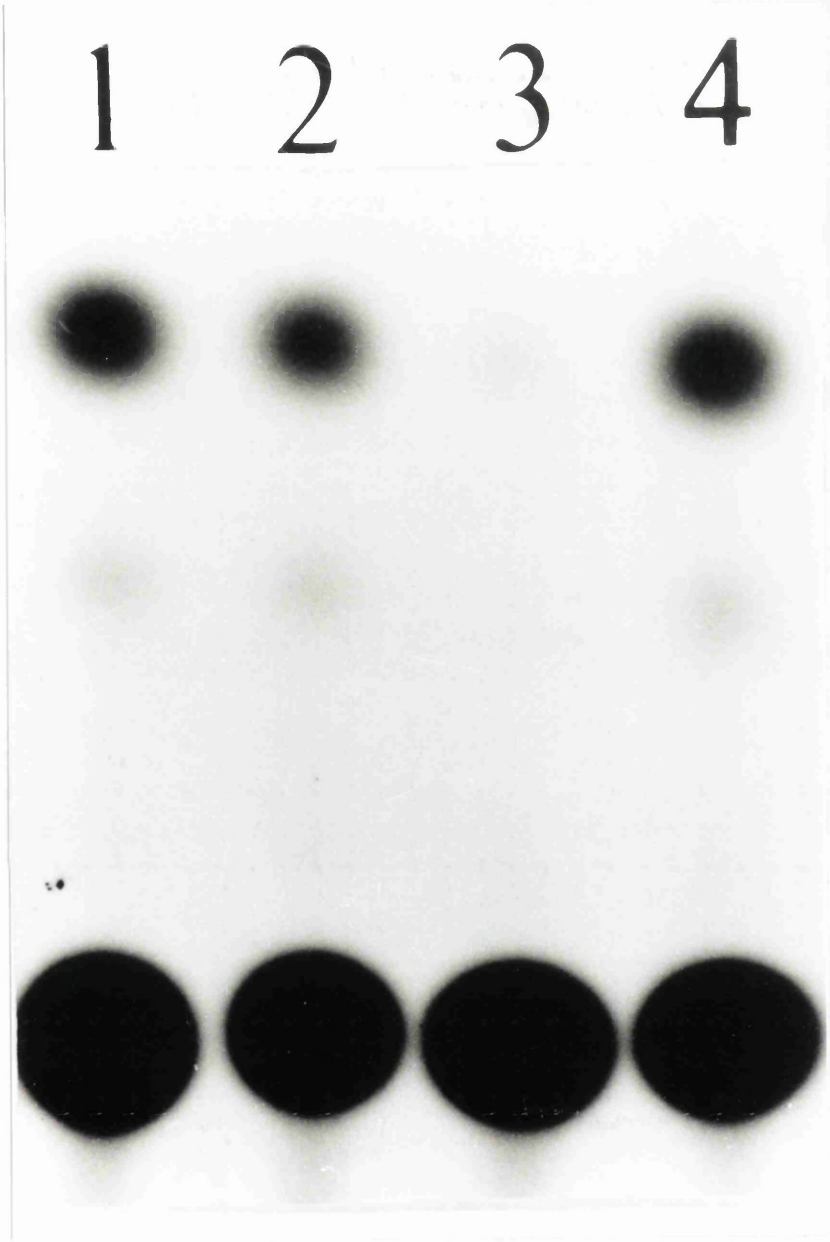
The full-length promoter from the gene encoding ICP0 was linked to CAT in this construct. The ICP4 gene promoter linked to CAT (IE-CAT) has been previously described in this work (**Chapter 4**). These studies revealed that the ICP0 gene promoter could be induced by cAMP (**Figure 5.2**). However, the ICP4 gene promoter was not induced. The induction of the ICP0 gene promoter was expected as it contains a CRE throughwhich induction by cAMP and CREB could occur. The experiment was controlled by using the RSV-CAT construct (**Chapter 4**) and the expression of this construct was unaffected by cAMP.

5.iv Results 3 : High Levels of cAMP can Inhibit IE Protein Production

It has therefore been shown that 10 μM IBMX can cause the induction of the ICP0 gene promoter, an increase in ICP4 protein production in a virus infection and increase in virion-producing capacity of ND7/23 cells. In stark contrast to this, it was also observed that at a 10-50-fold increase of IBMX to 100-500 μM, a sharp reduction occurred in the number of cells expressing ICP4 after infection with HSV-1 strain F (**Table 5.4**). This occurred to the extent that in one experiment using 500 μM IBMX no ICP4-positive cells were seen, even at a multiplicity of infection (MOI) of 10.

This observation correlates to some degree with the decrease in the expression of ICP4 in ND cells differentiated with medium containing 1 mM dibutyryl cAMP (**Table 5.5**). In this experiment cells were differentiated for 3 d. in L15 medium containing 0.5 % foetal calf

Figure 5.2



CAT Assay of Untreated and ND7/23 Cells Treated with 10 μM IBMX After Transfection of RSV-CAT and ICP0-CAT

Untreated (track 1) and treated (track 2) cells were transfected with RSV-CAT (Gorman *et al*, 1984) and little difference in the level of expression of this construct is observed. However, the expression of the ICP0-CAT construct (Gelman and Silverstein, 1987b) is greatly enhanced by treatment of ND7/23 cells with 10 μM IBMX (track 3, untreated cells; track 4, treated cells).

Table 5.4

Immunofluorescent Staining of Untreated and IBMX-Treated ND7/23 Cells with Antibody to ICP4 after Infection with HSV-1 Strain F

MOI	Untreated		IBMX-Treated	
	A	B	A	B
1	20.2	4.0	1.65	0.8
5	76.4	14.4	31.0	1.8
10	nd	24.7	34.0	nd

Figures indicate the percentages of cells staining after treatment with 100 μM IBMX in two separate experiments (A for 72 h. and B for 24 h.). Cells were stained with the 58S antibody to ICP4 (Showalter *et al*, 1981).

Table 5.5

Immunofluorescent Staining of Undifferentiated and Differentiated ND7/23 Cells with Antibody to ICP4 after infection with HSV-1 Strain F.

MOI	Undifferentiated	Differentiated
1	1.0	1.0
5	12.0	8.0
10	26.0	17.0

Figures show the percentages of cells staining after differentiation for 72 h. in medium containing 1 mM dibutyryl cAMP (Bu_2cAMP), 200 ng/ml Nerve Growth Factor (NGF) and 0.5 % foetal calf serum. Cells were stained with the 58S antibody to ICP4 (Showalter *et al*, 1981) after infection for 6 h..

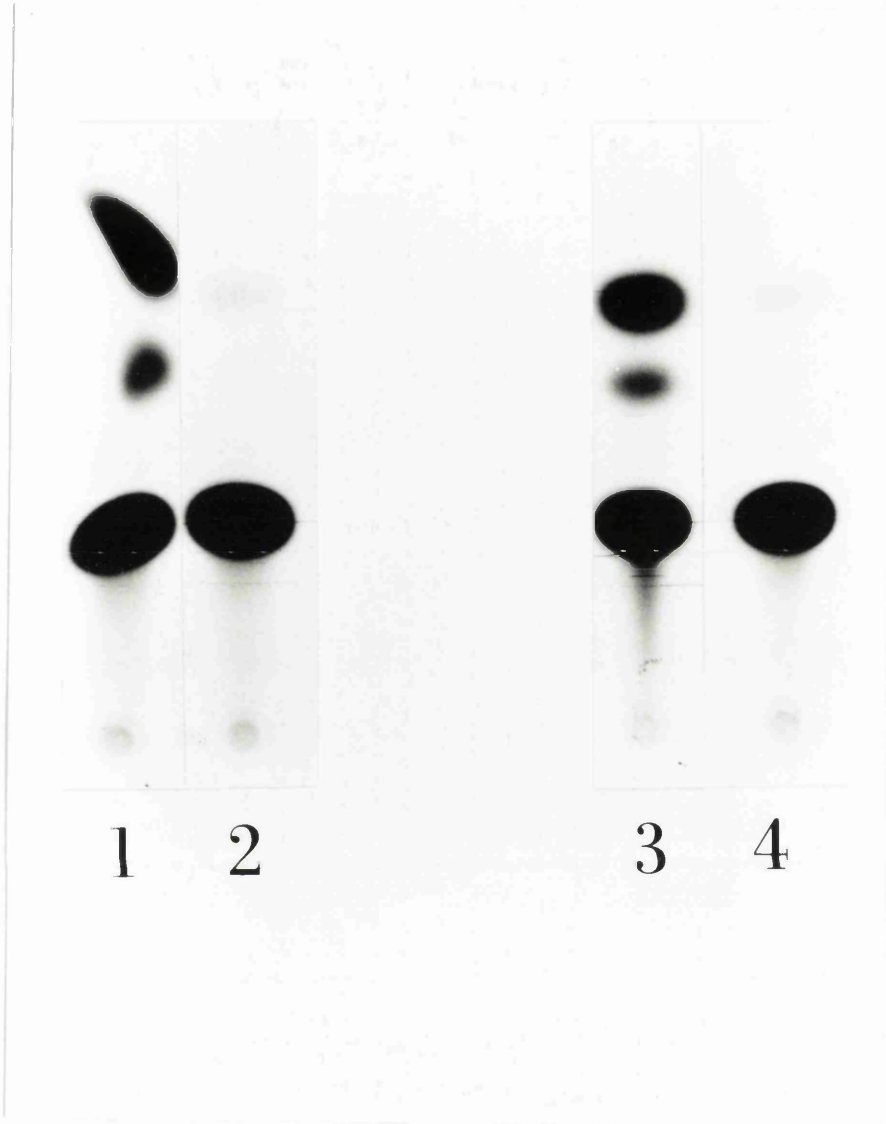
serum, 1 mM dibutyryl cAMP (Bu_2cAMP) and 200 ng/ml NGF (see **Chapter 2**, Materials ans Methods). Treatment with 500 μM IBMX also resulted in a very low yield of virions from ND cells (data not shown), paralleling the lack of expression of IE genes.

5.v. Results 4 : NGF and Serum do not Affect IE Gene Transcription

As mentioned in the introduction, it has been suggested that the secondary messenger pathways of cAMP and NGF may be linked. It has also been suggested that NGF may alter the synthesis of a labile protein that is involved in IE gene expression (Wilcox and Johnson, 1990). The RSV-CAT construct (Gorman, 1984) and the ICP0-CAT construct (Gelman and Silverstein, 1987) were transfected into ND15 cells which had been untreated or treated with 200 ng/ml NGF (**Figure 5.3**). There was no change in the expression of ICP0-CAT on treatment with NGF. The ICP0-CAT construct was also transfected into ND cells which had been treated with a concentration range of NGF (2 ng - 2 μg). However no effect of any concentration of NGF on IE gene expression was observed (data not shown).

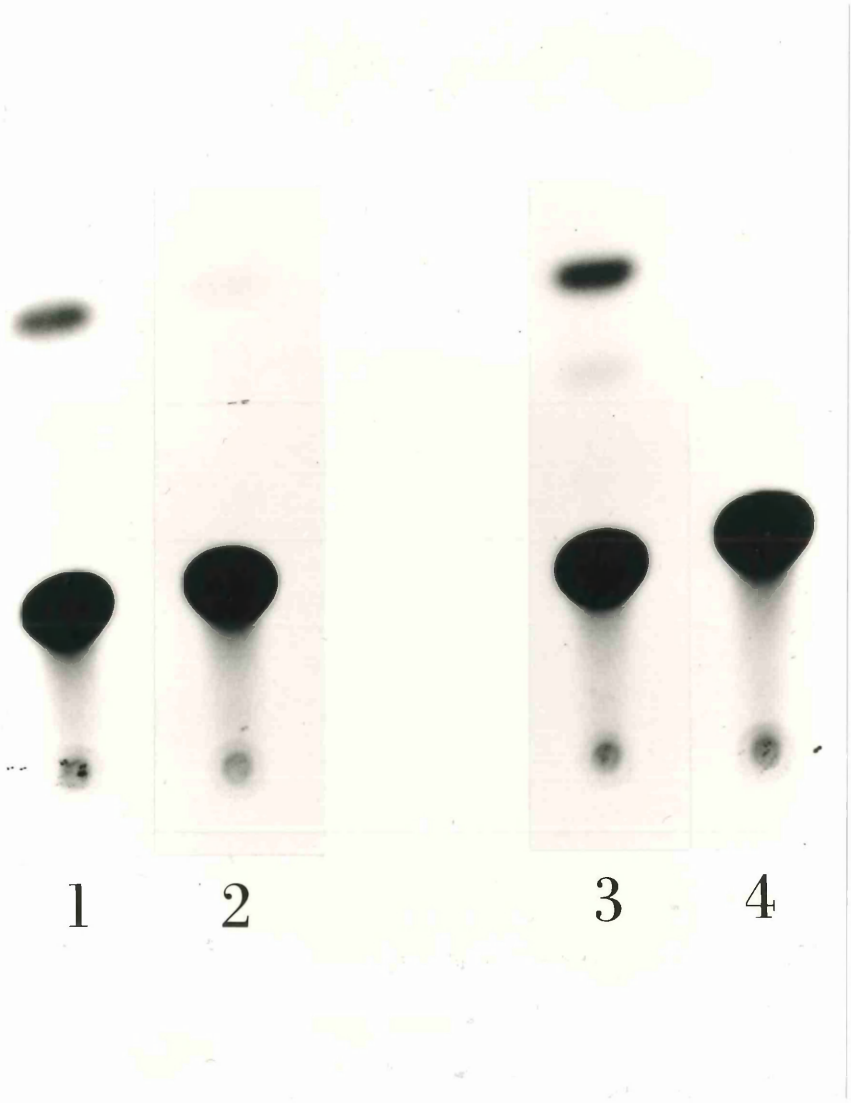
The effect of serum on IE promoter activity was also investigated. A reduction in serum concentration or indeed removal of serum results in morphological differentiation of ND cells. RSV-CAT and IE-CAT were also transfected into ND cells with 10 % serum (the concentration in full growth medium) or in serum-free medium (**Figure 5.4**). Again no effect on the IE promoter activity was seen.

Figure 5.3



CAT Assay of Untreated and ND15 Cells Treated with NGF After Transfection with RSV-CAT and ICP0-CAT

Untreated (track 1) and ND15 cells treated with 200 ng/ml NGF (track 3) were transfected with RSV-CAT (Gorman *et al*, 1984). Little difference is seen in the level of expression of RSV-CAT on treatment with NGF. The cells were also transfected with ICP0-CAT (Gelman and Silverstein, 1987b), both untreated (track 2) and Treated (track 4). Again little difference is observed in the capacity of ND15 cells to express IE-CAT on treatment with NGF.



CAT Assay After Transfection of ND7/23 Cells Grown in Full Growth medium and Serum-Free Medium with RSV-CAT and IE-CAT

ND7/23 cells grown in 10 % serum (track 1) and in 0.5 % (track 3) serum were transfected with RSV-CAT (Gorman *et al*, 1984). No difference is observed in the capacity of the cells grown in different concentrations of serum on the expression of RSV-CAT was observed. The cells grown in 10 % serum (track 2) and 0.5 % serum (track 4) were also transfected with IE-CAT. However, again no dfference was observed in the capacity ND7/23 cells to express IE-CAT when serum levels are reduced.

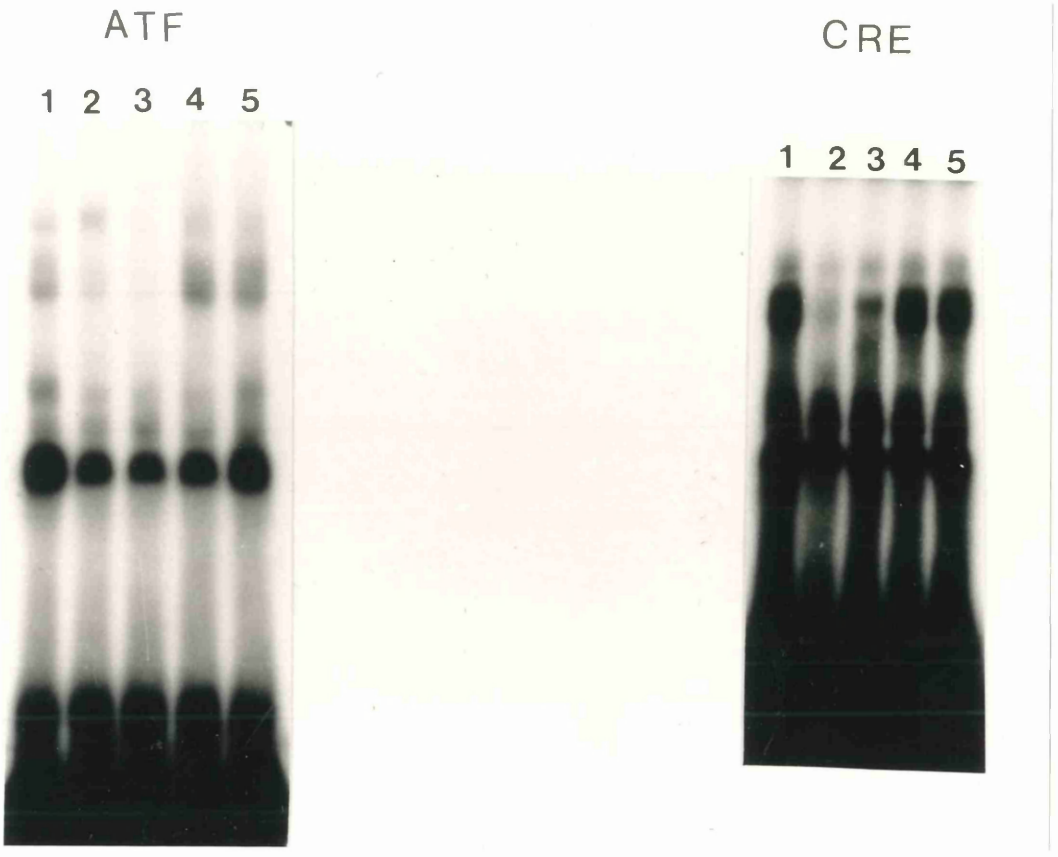
5.vi Discussion

The results in this chapter have shown that cAMP at low levels can cause an increase in the transcription of the IE gene encoding ICP0, an increase in the production of the IE protein ICP4 and also an increase in the capacity of ND cells to produce infectious virions.

The functional capacity of the ICP0 CRE has been further characterised by others in a gel-retardation assay (C. Dent, personal communication) (**Figure 5.5**). In this experiment the sequence of the CRE from the ICP0 gene was synthesised as part of an oligonucleotide, as was the CRE from the αHCG gene (a consensus CRE). These two oligonucleotides were used in gel-retardation assays and it was shown that they both bind a protein of a similar size and that each can compete for the protein from the other, although to a greater or lesser extent.

It is easy to envisage how the increase in transcription of the gene encoding ICP0 can come about when the mechanism of action of CREB is considered. It is likely that, whilst the oct-x protein binds to TAATGARAT and prevents oct-1 from binding thus preventing transcription via this *cis*-acting site, (see previous chapter), the CREB protein binds to the CRE that is further downstream in the gene promoter and is activated by protein kinase A. This may at least partly overcome the transcriptional block which is caused by the oct-x protein. A model to explain this idea more fully is presented in **Figure 5.6**.

Figure 5.5

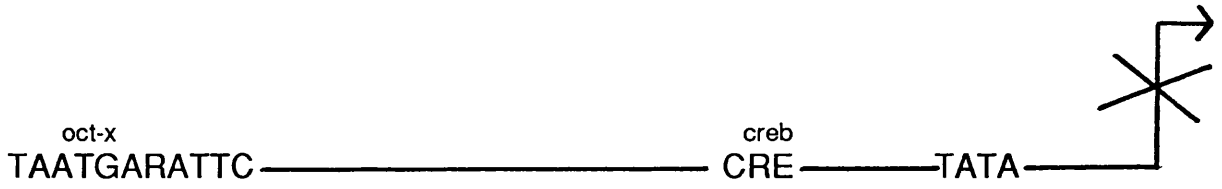


Gel retardation assay of ND7 cell extracts on probes containing the ATF site from the human chorionic gonadotrophin gene (αHCG) and the cyclic AMP responsive element (CRE) from the HSV IE-1 gene promoter. Track 1, uncompeted; track 2, competed with the IE-1 CRE; track 3, competed with the αHCG ATF site; track 4, competed with a stimulatory protein 1 (SP1) binding site; track 5, competed with an oligonucleotide containing a mutant octamer motif. It can be seen that competition occurs in tracks 2 and 3, suggesting that the bands competed are specific and that the αHCG ATF site and the IE-1 CRE are shifted by proteins that have similar binding properties.
Figure provided by C. Dent.

Figure 5.6

Model showing the Possible Mechanism of Action of cAMP in the Transcriptional Induction of ICP0 in ND cells.

a) In cells of neuronal origin, activated transcription is blocked by an octamer-binding inhibitory protein



b) When treated with cAMP, creb is activated and can override the transcriptional repression observed in untreated cells.

The induction of IE gene expression brought about by 10 μM IBMX does not seem to be due to a change in the levels of octamer-binding proteins. Thus in a gel-retardation assay no change in the abundance of octamer-binding proteins was seen on treatment of ND cells with 10 μM IBMX (N. Lakin, personal communication).

There does however appear to be a paradox as the ICP4 promoter is not activated by cAMP via its two AP2 sites. As mentioned in the introduction the AP2 sites can be activated by either cAMP or PMA, but in fact in the ND cells the ICP4 promoter cannot be activated by PMA (data not shown). AP2 is in fact cell-type specific (Imagawa *et al*, 1987) and therefore not present in certain cell types. It may therefore not be present in ND cells, thus explaining the lack of induction of the ICP4 promoter by cAMP. Alternativey the sequences present in the ICP4 promoter may not adequately match the concensus AP2 binding site required for PMA/cAMP activation.

It is seen therefore that the ICP0 promoter can be induced directly by cAMP, presumably resulting in the production of the ICP0 protein. However, whilst the ICP4 protein is induced, there is no direct effect of cAMP on the ICP4 promoter. It is useful here to recall that the ICP0 protein alone is able to cause reactivation of latent HSV in *in vitro* culture systems (Harris *et al*, 1989). By some mechanism the ICP0 protein induces ICP4 and ICP27 protein production and starts off a lytic cycle. ICP0 can activate the ICP4 promoter in a co-transfection assay (Gelman and Silverstein, 1987b). Perhaps the mechanism by which ICP0 can induce IE protein production in a latently infected cell upon a reactivation stimulus is analogous to the

mechanism in ND cells by which ICP0 can induce a lytic infection in these experiments.

Low levels of cAMP have previously been used to induce gene expression in many systems. For example, 10 μM Bu_2cAMP has previously been shown to transcriptionally induce the urokinase-type plasminogen activator gene 10-fold (Nakagawa *et al*, 1988). Furthermore 10^{-8} M cAMP will activate protein kinase A (Stryer, 1988). therefore it is not a new concept that low cAMP levels can induce metabolic events and gene expression.

Interestingly, the LAT promoter has also been shown to contain a CRE (Leib *et al*, 1991). This CRE upregulated the LAT promoter in PC12 cells after treatment with Bu_2cAMP, NGF, forskolin or PMA. Reactivation of wild-type HSV was also enhanced when intracellular levels of cAMP were increased. Reactivation of a virus containing a mutant CRE in the LAT promoter was not enhanced by cAMP. These observations imply that both the LATs and cAMP have roles in reactivation, although the mechanisms remain obscure.

In contrast high levels of cAMP can drastically decrease the level of expression of the IE genes. Thus levels of cAMP produced by 100 - 500 μM IBMX cause a great decrease in the number of cells that express ICP4 after infection with HSV. This effect was so drastic that in one experiment using 500 μM IBMX there were no ICP4-positive cells, even at a MOI of 10. Such levels of cAMP can also drastically decrease the capacity of ND cells to produce infectious virions (data not shown).

Cyclic AMP at high levels can in fact cause differentiation of

neuroblastomas (Prasad, 1974). The levels used to differentiate neuroblastomas, 1 mM dibutyryl cAMP, also results in the differentiation of ND cells, although the effect is more marked when the differentiation is carried out in serum-free medium. It may in fact be that the levels of cAMP produced by 100 μM IBMX are in excess of the levels available to the cell when bathed in medium containing 1 mM dibutyryl cAMP. If this is the case, it may explain the differences on gene expression when cells are exposed to 1 mM Bu_2cAMP compared with exposure to 100 μM IBMX.

It has also been recently demonstrated that an alternative protein to CREB will bind to the CRE. This protein, cAMP-responsive element modulator (CREM) (Foulkes *et al*, 1991a) is expressed in a cell-type specific manner as differentially-spliced isoforms. CREM is also a bZIP protein and can heterodimerise with CREB to prevent transcriptional activation (Foulkes *et al*, 1991b). Future gel-retardation experiments on the CRE using untreated and ND cell extracts treated with high levels of cAMP may show another distinct protein that binds to the CRE which may down-regulate IE gene transcription.

The lack of response of the IE promoters to NGF may be due to the unresponsiveness of the ND cells themselves to NGF. Neuroblastomas, although possessing NGF receptors, often do not respond to NGF (Prasad, 1974). In fact many assays have been carried out to test the responsiveness of the ND cells to NGF and to date no response has been observed. Immunocytochemistry using an antibody to the protein encoded by the *trk* oncogene (Hempstead *et al*, 1991) may prove the

existence of high-affinity NGF receptors on ND cells. Alternatively, NGF may not be active on adenylate cyclase.

It is difficult to reconcile the effects of NGF with changes in cAMP levels, particularly as no effects of NGF have been demonstrated which can be correlated with one of the observed effects of cAMP. There is clearly evidence that NGF can maintain latency *in vivo* and *in vitro* in some systems. Perhaps binding of NGF causes a large increase in the levels of intracellular cAMP such that the IE genes remain inhibited, possibly by the action of CREM, or by some other mechanism. A drop in the cAMP levels brought about by NGF removal may therefore result in CREB binding in place of CREM, or in a change in the levels of octamer-binding proteins, therefore resulting in an increase in IE gene expression. NGF increases oct-x in DRG cells (K. Lillycrop, personal communication). Although the theory cannot be tested in ND cells (as it is likely that ND cells are simply unresponsive to NGF), it has not been experimentally disputed in DRG or ND cells.

A change in chromatin structure correlating with gene expression has been reported to be due to changes in cAMP levels (Becker *et al*, 1987), and it has also been suggested that type II cAMP-dependent protein kinase can act as a topoisomerase (Constantinou *et al*, 1985). The cAMP response may in fact be totally separate from that of NGF in terms of secondary messenger systems.

The ND cells were also differentiated using serum-free medium to investigate the effect of serum on the activity of the IE-3 gene promoter. No change was observed in the activity of this IE gene

promoter on differentiation.

This suggests that no major changes have occured in cellular gene expression that at least could have accounted for a change in the expression of the IE-3 gene promoter. This experiment implies that the ND cells, in the undifferentiated form, may be mature in terms of gene expression. and that the differentiation seen may simply be morphological. This idea is borne out by studies where neuroblastomas were differentiated in the presence of cycloheximide (Seeds *et al*, 1970, see **Chapter 6**). In these experiments neurites were extended even in the absence of protein synthesis, implying that a redistribution of proteins was occurring rather than the synthesis of new classes. Furthermore this experiment gives evidence that the drop in serum levels (from 10 % to 0.5 %) in the experiment from which the data is shown in **Table 5.5** is probably not responsible for the decrease in IE gene expression. The following chapter shows that there is also very little detectable change in protein synthesis on differentiation of ND cells.

No change was observed in the level of expression of the IE protein ICP4 after differentiation of ND cells with serum-free medium and infection with HSV-1 strain F (K. Howard, personal communication), paralleling the lack of change in the activity of the IE-3 promoter.

It has therefore been demonstrated that the low, but induced, intracellular levels of cAMP produced by 10 μM IBMX can have a profound effect on HSV IE gene expression. The implications of the experiments presented here are that this molecule can have acute effects on the fate of the cell, as it may be able to switch the viral genome from a non-productive latent state to a productive,

replicating state.

Alternatively, high levels of cAMP induced by 100 - 500 μM IBMX, can repress IE gene expression. Although the mechanism of repression of IE gene expression by high cAMP levels is unknown, the idea of two effects of the same molecule at different concentrations is well documented, for example, for PMA.

Cyclic AMP therefore has two effects pertaining to HSV latency in cells of sensory neuronal origin. It seems likely that regulatory elements binding to the CRE in the ICP0 gene promoter control events at the molecular level.

Chapter 6 Morphological Differentiation of ND Cells

6.i. Introduction

As outlined in the introduction, the ND cells provide a homogeneous population of sensory neuronal cell-types, the large numbers of which allow neuronal functions to be studied by biochemical and molecular analysis. However the ND cells proliferate, necessitating a switch to a non-dividing differentiated state for the study of some functions. Conditions have therefore been defined under which the ND cells cease proliferating and extend neurites, see section 6.ii., this chapter.
Whilst relatively easy to dissect, the primary cultures produced from DRG are highly heterogenoeus, as separate or overlapping groups express the phosphorylated 200 KDa subunit of neurofilaments (Winter, 1987), substance P (Price, 1985), somatostatin (Price, 1985), tyrosine hydroxylase (Price and Mudge, 1983), fibrillary associated protein and various stage specific embryonic antigens (SSEA-1, SSEA-3 and SSEA-4) (Jessell and Dodd, 1984) and other neuron-specific proteins. Cell lines derived from DRG that homogeneously express a particular antigen throughout the population would therefore be useful.

Some of the novel ND clones could be differentiated *in vitro*. It therefore seemed important to test whether the neuropeptides

detected in the ND cell clones altered either quantitatively or qualitatively on differentiation. To this end, screening of undifferentiated and differentiated ND cells was carried out using a panel of antibodies directed against neuron-specific proteins. This section of the work was carried out in collaboration with Dr. Angela Suburo and Prof. Julia Polak in the Histochemistry Department at the Royal Postgraduate Medical School, Hammersmith Hospital, London, U.K..

In fact only a small degree of quantitative change was detected (see Results 1 and **Table 6.1**). However some very interesting changes in the distribution of some of the proteins under study were seen, and these were changes in the distribution of proteins carried in exocytotic vesicles.

Exocytosis is carried out in neurons by either constitutive or regulated pathways. The constitutive pathway exists in every cell and is the means by which membrane proteins and proteins destined for storage migrate to the cell periphery (De Camilli and Jahn, 1990). It is characterised by a constant flow of small vesicles from the *trans* Golgi network with no intermediate packaging and storage (Burgess and Kelly, 1987). Conversely there are two regulated pathways of exocytosis which are also present in other specialised secretory cells, for example in the neuroendocrine system (De Camilli and Jahn, 1990).

The two pathways of regulated exocytosis are characterised by Small Synaptic Vesicles (SSVs) and Large Dense-Core Vesicles (LDCVs). SSVs constitute a homogeneous population of organelles and contain

the classical neurotransmitters acetylcholine, the catecholamines, glutamate and gamma-aminobutyric acid (GABA) as well as Synapsin I and II, Synaptophysin and Synaptobrevin (De Camilli and Jahn, 1990). LDCVs are more heterogeneous and contain the chromogranin family, regulatory peptides, processing enzymes and precursor fragments. SSVs migrate to pre-synaptic nerve endings of the axon, the active zone, and the coexistence of SSVs and LDCVs is likely to be a feature of all nerve endings (De Camilli and Jahn, 1990). Regulated exocytosis occurs in response to a stimulus and both types of vesicle may be induced to exocytose in response to calcium, although different concentrations will activate the different types of vesicle and fluxes in calcium concentration may be highly localised (Sher *et al*, 1989, De Camilli and Jahn, 1990). It is also interesting that in the human neuroblastoma line IMR-32, the cells only acquire their secretory properties on drug-induced differentiation (Sher *et al*, 1989).

The proteins showing interesting redistribution patterns and under study in this work are synaptopysin, a 38 KDa integral membrane protein carried in SSVs (Jahn, 1985) and which may have a role in the mechanism of exocytosis of the SSVs (De Camilli and Jahn, 1990). This protein is located in the Golgi Apparatus in hypothalamic neurons of 16 day-old rat foetuses but it appears in mature synaptic boutons and varicosities of synaptic vesicles after 12-13 days in culture (Tixier-Vidal *et al*, 1988). The chromogranins, neuropeptide Y and its C-flanking peptide C-PON, which are carried in LDCVs, were also under study. Chromogranins A and B were originally identified as acidic protein components of bovine adrenal chromaffin granules, but

have since been detected in the nervous system (Fischer-Colbrie *et al*, 1985). GAWK is the name of a peptide homologous to a region of chromogranin B, the first four peptides being glycine (G), alanine (A), tryptophan (W) and lysine (K) (Benjannet *et al*, 1985). The function of these proteins remains unknown. However a high level of chromogranin A and ratio of chromogranin A to synaptophysin is detected in patients with Alzheimer's and Pick's diseases (Weiler *et al*, 1990). Chromogranin C, also called Secretogranin II, shares acidic properties with the other chromogranins but is immunologically unrelated (Fischer-Colbrie *et al*, 1986). Synaptophysin and the chromogranins have been previously used to study the trafficking of the SSVs and LDCVs respectivly in the PC12 cell line respectively (Cutler and Cramer, 1990).

Neuropeptide Y has previously been shown to be present in DRG and spinal cord (SC) neurons (Gibson *et al*, 1984). Its C-flanking peptide C-PON is generally co-localised with neuropeptide Y in the LDCVs (Gulbenkian *et al*, 1985). The precursor molecule of neuropeptide Y and C-PON, pre-pro-neuropeptide Y has been detected in the striatum, cortex, hippocampus and hypothalamus (Higuchi et al, 1988) and the expression of this molecule may be induced by cAMP, phorbol myristate acetate (PMA) or synergistically by both compounds.

This chapter demonstrates that the ND cells can be morphologically differentiated *in vitro* and that little change in protein synthesis accompanies this morphological change. Moreover, data are also presented which show that the pathways of regulated exocytosis mature as the differentiation process proceeds.

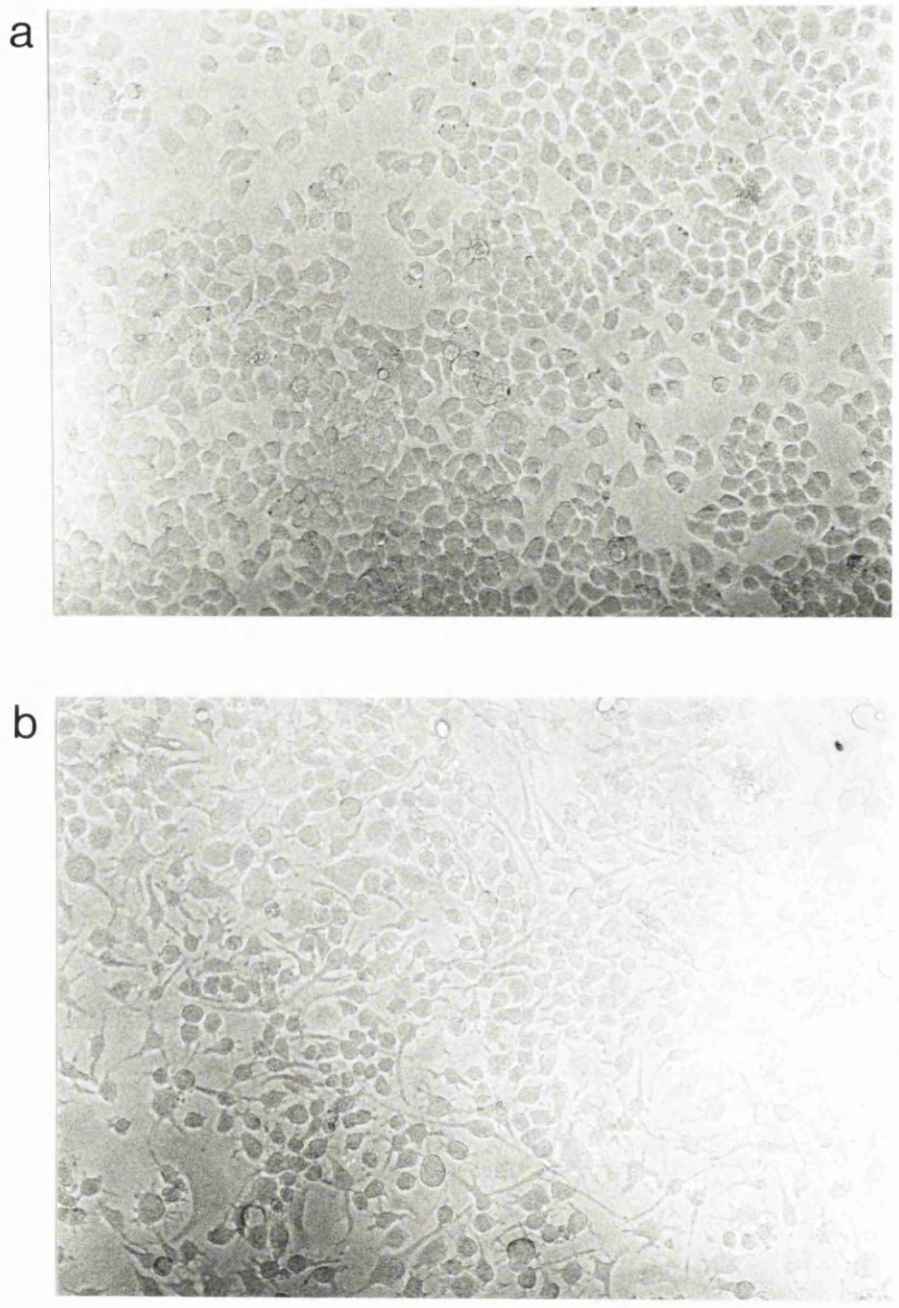
6.ii. Results 1: Neuropeptide Constitution of Undifferentiated and Differentiated ND7 Cells

ND cells were differentiated as described in section 2.5.ii. in **Chapter 2.** Briefly, cells were plated on teflon-coated slides which had 8 wells marked out on them. Cells were plated at 70/mm^2 in serum-free differentiation medium (F12 or F14). No difference was observed in gene expression when using these two types of differentiation medium. Cells were routinely differentiated for 72 h. or for different lengths of time in particular experiments. Cells were washed (3X) in PBS supplemented with 1 % bovine serum albumin (BSA) and fixed in Bouin's Fixative (see section 2.5.ii.) for 30 mins.. The cells were then washed again in PBS and stored in PBS with 0.1 % sodium azide until staining was carried out.

All staining was carried out at the Hammersmith Hospital by Dr. A. Suburo. Secondary antibodies were peroxidase-conjugated and were visualised by the diaminobenzidine (DAB) / nickel chloride ($NiCl_{2)}$ / hydrogen peroxide ($H_2O_{2)}$ reaction. The specificity of the staining reaction was tested by adsorbing the primary antibody with its antigen in solution before using the antibody to stain the cells. The specificity of the staining was tested by adsorbing the relevant peptide with the corresponding antibody to check if staining was abolished.

The morphology of ND cells, both undifferentiated and differentiated, is shown in **Figure 6.1**. This figure shows cells differentiated in serum-free medium in the presence of 1 mM dibutyryl cAMP (Bu_2cAMP). This treatment increases the survival of differentiated ND cells.

Figure 6.1



Phase-Contrast Photographs of Undifferentiated and Differentiated ND7/23 Cells

Cells were photographed grown in full growth medium (a) or in serum-free medium supplemented with 1 mM Bu_2cAMP (b). Note the long and thick processes (b) and swelled cell bodies (b).

The presence or absence in ND cells of a range of neuron-specific proteins (including hormones, enzymes, structural proteins and proteins to which no function has been assigned) is shown in **Table 6.1**. This table shows that on differentiation very few qualitative changes occur in protein expression. For example no changes in expression of the transcription factors c-fos or c-jun were detected. Few hormones were detected with this method of analysis, even though substance P had previously been detected in ND cells by radioimmunoassay (Wood *et al*, 1990). **Figure 6.2** shows ND cells stained for protein gene product (pgp) 9.5 (a ubiquitin carboxyl terminal hydrolase [Day *et al*, 1990]) and tyrosine hydroxylase (a marker of adrenergic neurons). These two proteins stain both undifferentiated and differentiated ND cells.

6.iii. Results 2: Redistribution of Proteins Associated with Exocytotic Vesicles on Morphological Differentiation of ND Cells

Whilst most of the proteins studied in **Table 6.1** do not change in abundance on morphological differentiation of ND cells, some of them change in their distribution patterns, see **Table 6.2**. There are also some exceptions to the observations of qualitative changes on differentiation, and these are the chromogranins, see **Table 6.2**. All three are expressed in differentiated ND cells, whereas no chromogranin B and little of chromogranins A and C are expressed in undifferentiated ND cells. As discussed in the introduction to this chapter, the chromogranins are found in the secretory granules of

Table 6.1

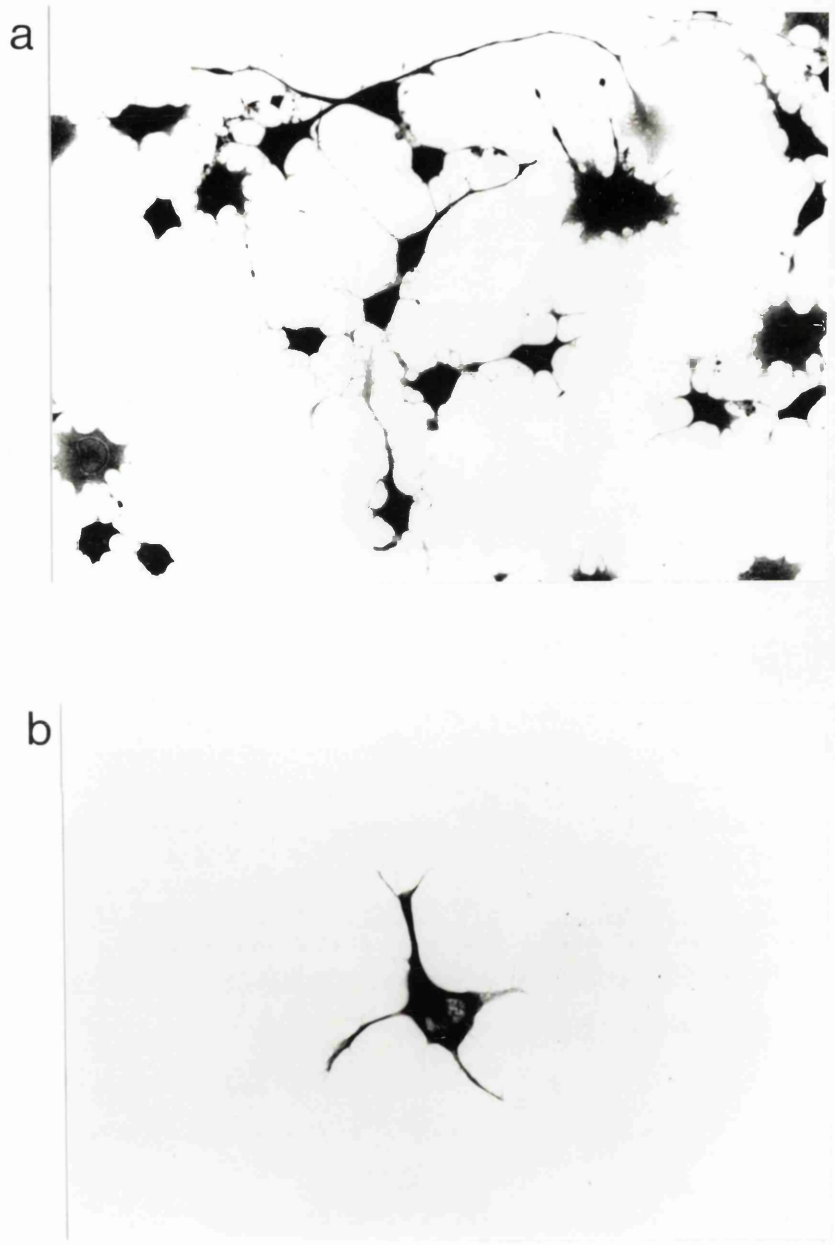
Presence or Absence of Neuronal Proteins in Undifferentiated and Differentiated ND7/23 Cells.

Protein	Undifferentiated	Differentiated
Chromogranin A	+	+
Chromogranin B	-	+/-
Chromogranin C	+/-	+
GAWK	+/-	+/-
Neuropeptide Y	+/-	+/-
C-PON	+/-	+/-
Synaptophysin	+	+
Protein Gene Product 9.5	+	+
Neuronal Enolase	+	+
Nitric Oxide Synthase	+	+
Glial Fibrillary Acidic Protein (GFAP)	-	-
200 KDa phosphorylated subunit of neurofilaments	+	+
Laminin	+	+
NGF Receptor (low affinity)	+/-	+/-
Tyrosine Hydroxylase	+	+
Dopamine-β-Hydroxylase	-	-
Carbonic Anhydrase 1	+	+
c-fos	+	+
c-jun	+	+
Peripherin	+	+
Serotonin	+/-	+/-
Substance P	-	-
Somatostatin	-	-

Enkephalin	-	-
Calcitonin Gene Related Peptide	-	-
Vasoactive Intestinal Peptide	-	-
Galanin	-	-
Neurotensin	-	-

+: present in most cells, +/-: present in some cells, -: not present.

Figure 6.2



ND7/23 cells stained with (a) pgp 9.5, a protein expressed in axons and dendrites *in vivo* (Thompson and Day, 1988) and (b) tyrosine hydroxylase, a marker of adrenergic neurons.

Table 6.2

Distribution of Selected Proteins on Differentiation of ND7/23 Cells

	Undifferentiated	Differentiated
Chromogranin A	Staining in most of the cell.	Staining in most of the cell. Concentration in the neurite tips.
Chromogranin B	Staining in tips of small processes when present.	Staining in tips of processes.
Chromogranin C	Staining in the cell periphery and in small processes when present.	Staining in the tips of processes.
GAWK	Golgi-like staining.	Staining in Golgi Apparatus and in the tips of processes.
Neuropepide Y	Staining in cell periphery, Golgi Apparatus and small processes when present.	Staining in tips of processes.
C-PON	Staining in cytoplasm Staining in tips of and Golgi Apparatus.	
Synaptophysin	Staining in the Golgi Apparatus .	Staining in the Golgi Apparatus and also in few tips of processes.

Cells were differentiated for 72 h. in differentiation medium containing 0.5 % or no serum (see Materials and Methods).

neuronal cells, the LDCV; their final destination, the tips of neurites, are therefore not present in undifferentiated cells. The expression of chromogranin B at least may therefore be delayed until neurites have been extended.

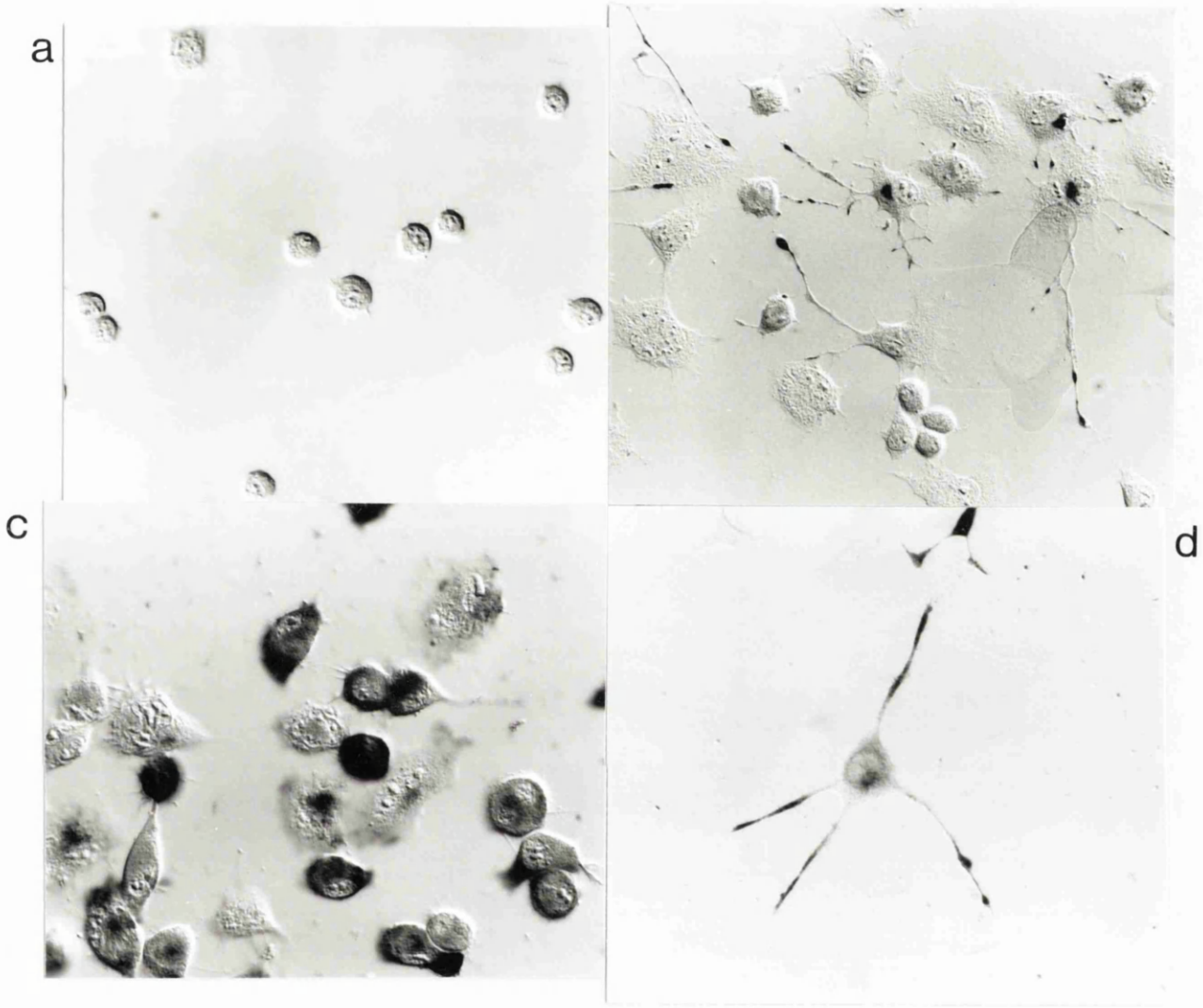
A similar type of redistribution is seen with neuropeptide Y and its C-flanking peptide C-PON. These proteins are also found in the, the LDCVs, although these proteins are present in undifferentiated ND cells in the Golgi Apparatus. Migration of the LDCVs to the tips of neurites is therefore accompanied by immunostaining for the chromogranins, neuropeptide Y ansd C-PON at the neurite tips.

A protein associated with SSVs also migrates to the tips of neurites in differentiated ND cells, although to a far limited extent than do the proteins associated with LDCVs. Thus immunostaining for synaptophysin is detected in a very small proportion of the tips of neurites in differentiated ND cells after 72 h. (see **Table 6.4**, section 6.iv.), whereas most of the differentiated cells are stained in the Golgi Apparatus. Synaptophysin is associated with the active zone of the synapse and may play a role in release of neurotransmitters to the extracellular space. It therefore appears that most of the SSVs have not yet migrated to the tips of the neurites at this time point. Photographs of cells stained for chromogranins A and B, C-PON and synaptophysin in undifferentiated ND cells and cells differentiated for 72 h. are shown in **Figure 6.3**.

At this time point therefore, one of the regulated pathways of exocytosis has therefore matured, whilst one is still immature. The LDCVs are present at the termini of neurites but the great proportion of SSVs are immature in their location.

Table 6.3 shows that some of the proteins under study (the

Figure 6.3



ND7/23 cells stained for (a) chromogranin B (undifferentiated, no staining); (b) chromogranin B (differentiated, staining in neurite tips); (c) C-PON (undifferentiated, staining in the Golgi Complex); (d) C-PON (differentiated, staining in the Golgi Complex and in the neurite tips); (e) synaptophysin (undifferentiated, staining in the Golgi Complex); (f) synaptophysin (differentiated, staining in the Golgi Complex); (g) synaptophysin (differentiated, staining in the Golgi Complex and in the neurite tips); (h) synaptophysin (differentiated , staining in the neurite tips).

Figure 6.3/contd.

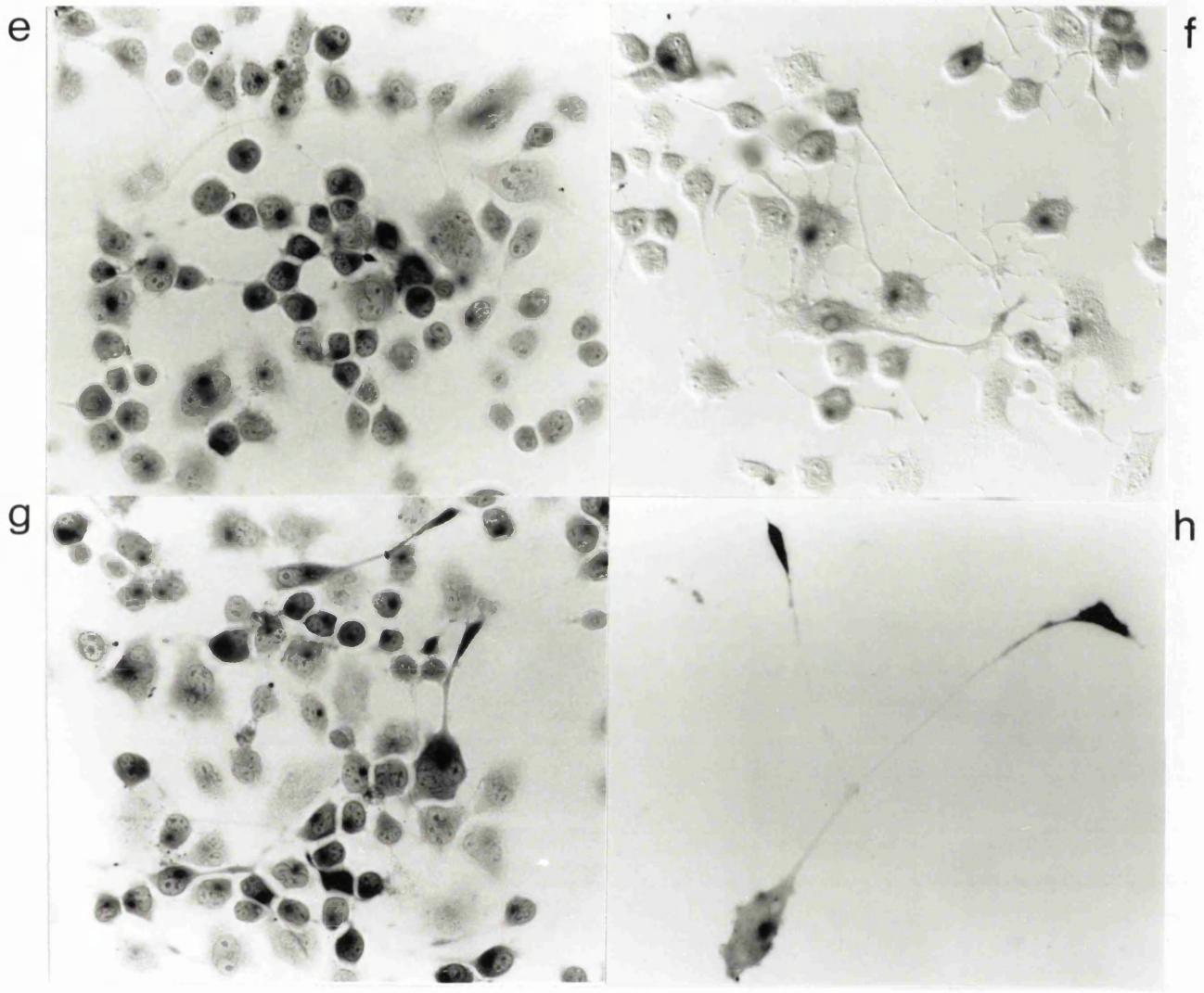


Table 6.3

Presence and Absence of Proteins in ND7/23 Cells and in Murine C1300 Neuroblastoma Cells (clone N18Tg2).

	ND7/23	N18Tg2
Chromogranin A	+	-
Chromogranin B	+	-
Neuropeptide Y	+	+
C-PON	+	+
Synaptophysin	+	-
Pgp 9.5	+	+

chromogranins and synaptophysin) are only present in the ND cell system and not in the parent neuroblastoma cell line N18Tg2. ND cells are therefore a more suitable model for studying regulated exoc ytosis in neurons than is the N18Tg2 neuroblastoma line. **Figure 6.4** shows N18Tg2 cells immunostained for pgp 9.5 and C-PON, but not for synaptophysin.

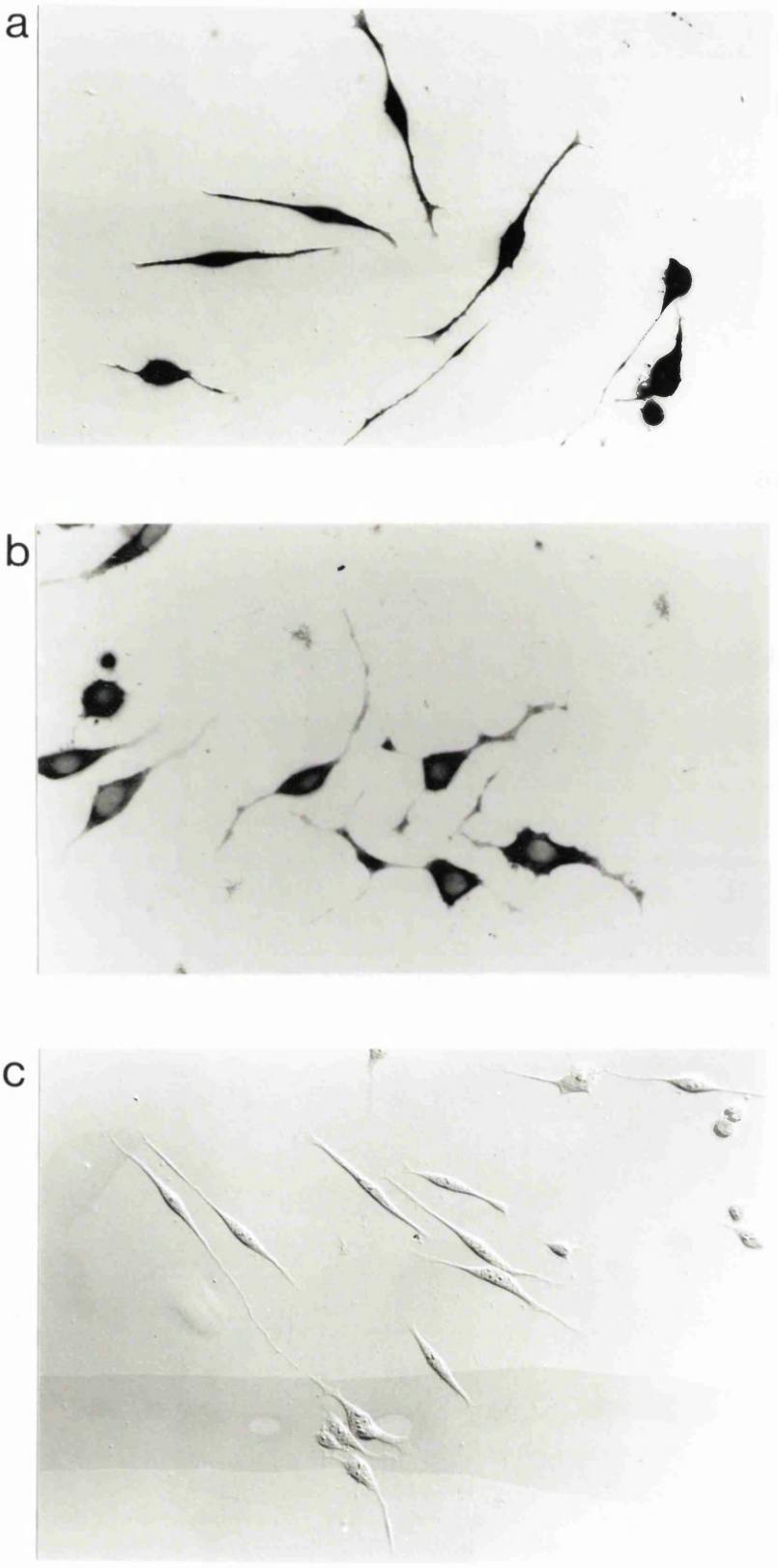
6.iv. Results 3: The ND Cells May be Used to Study the Maturation of the Pathways of Regulated Exocytosis

The results in section 6.iii. show that there is a difference in the spatial expression of proteins associated with LDCVs and SSVs at a time point of 72 h. of differentiation. The difference in the maturation times of these two pathways therefore warranted further investigation.

As very few neurite termini stained for synaptophysin at 72 h., a time course was carried out where ND cells were differentiated for defined periods and the staining for synaptophysin and C-PON was studied. The results of this study showed that as the time course (36, 72 and 144 h.) progressed, proportionally more neurite termini stained for synaptopysin (see **Table 6.4**), that is at 36 h., 4.3 % of cells were immunostained for synaptophysin, at 72 h., 8.1 % whereas at 144 h., 22.4 % were stained. However the percentages of the processes staining for C-PON altered only slightly during the time course (46.7 % - 66.7 %).

To further quantitate these observations, the lengths of processes

Figure 6.4



N18Tg2 cells stained for (a) pgp 9.5; (b) C-PON; (c) synaptophysin (no staining).

Table 6.4

Staining of Processes on ND7/23 Cells with Antisera to C-PON and Synaptophysin after Various Periods in Differentiation Medium

Antibody	C-PON			Synaptophysin		
Time/h.	36	72	144	36	72	144
No. Stained	213	314	307	27	49	126
No. Unstained	243	152	153	603	558	437
% Stained	46.7	67.4	66.7	4.3	8.1	22.4
P Value	-0.001	0.89	-	0.008	0.001	-

The P value (Pearson's Coefficient of Contingiency) represents the probability in a chi-square test that each value of the percentage of stained processes is not significantly different from the value at the previous time point.

which stained for either C-PON or synaptophysin were measured and the results of this experiment are shown in **Figure 6.5**. This graph shows that the processes which immunostain for synaptophysin are considerably longer than those that immunostain for C-PON (119.3 μM - 189.3 μM compared with 25.1 μM - 32.6 μM). It is very likely therefore that as the differentiation process progresses, more cells acquire processes that are greater than a threshold length, resulting in migration of mature SSVs to the active zone at the neurite tips.

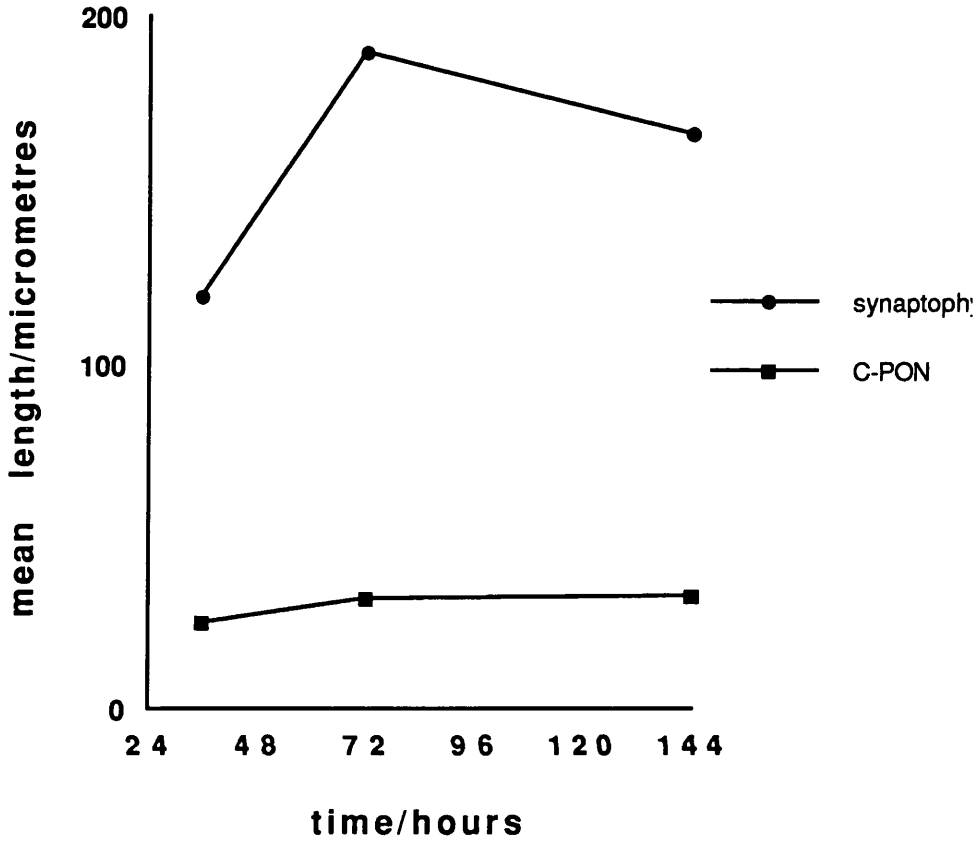
6.v. Results 4: Effect of NGF on Redistribution

It has been demonstrated that NGF can cause an increase in the number of SSVs in PC12 cells (Cutler and Cramer, 1990). The effect of a concentration series of NGF was also studied in ND cells differentiated in serum-free medium. No effect of NGF on growth, neurite extension or gene expression in ND cells had previously been observed. The range of concentrations used was 0.2 ng - 2 μg/ml for 72 h.. However no modulation of the distribution pattern of any proteins associated with exocytotic vesicles was observed.

6.vi. Discussion

ND cells can be differentiated *in vitro* by reducing or removing serum. Differentiation is enhanced by adding cAMP to the culture medium. NGF appears to have no effect on the differentiation process.

Figure 6.5



Graph to Show the Lengths of Processes Staining for Synaptophysin and C-PON at Different Time-Points

The lengths of processes staining for synaptopysin are longer than those staining for C-PON at each time-point. As the time-course progresses, the lengths of processes in the cell population staining for synaptophysin increases.

On differentiation it appears that the structural changes seen are in fact morphological changes and not akin to the differentiation of a precursor cell to a terminally differentiated phenotype. This type of change would normally be accompanied by a large change in the repertoire of genes expressed, and that is clearly not the case in ND cell differentiation, at least for the proteins studied in this work.

The mature nature of the ND cells is not in fact surprising. The C1300 mouse neuroblastoma, and indeed many other neuroblastomas, represent mature neuronal types (Prasad, 1975) and the primary DRG neuron, although neonatal and dependent on NGF is already morphologically mature. In effect the ND cells represent an immortalised mature neuron.

The treatment of neuroblastomas with RNA or protein synthesis inhibitors throws light on this subject. When treated with cycloheximide, C1300 cells extend neurites almost immediately and continue to do so for a few hours until neurite extension is impeded by the inhibition of protein synthesis. Protein synthesis is therefore not required for the first few hours of neurite extension suggesting that the subunits of the neurofilaments are already synthesised. Treatment of differentiating C1300 cells with colchicine or vinblastine arrests the differentiation process (Seeds *et al*, 1970), further substantiating the idea that that differentiation involves the polymerisation of existing subunits. Similar experiments on ND cells have not yet been attempted.

In avian embryonic DRG cells neuropeptide Y and tyrosine hydroxylase could not be detected in non-dividing precursor cells but appeared after 3 days of culture *in vitro* (Xue *et al*, 1987). These two markers

are present in both undifferentiated and differentiated ND cells. Pgp 9.5 has mainly been detected in the axons and dendrites of brain neurons (Thompson and Day, 1988), but is present in undifferentiated ND cells. These observations argue for a mature undifferentiated ND cell.

It has also been demonstrated that proteins associated with the two types of regulated exocytotic vesicles, the SSVs and the LDCVs, migrate to the tips of neurites on differentiation. The two types move in a time-dependent manner, related to the lengths of cell processes. The experimental data therefore shows that the ND cell system indeed may used to study the pathways of regulated exocytosis existing in neurons.

The unresponsiveness of ND cells to NGF may be due an absence of functional NGF receptors. This is in fact the case for many neuroblastoma cell lines (Prasad, 1975), although it is conceivable that the NGF-dependent primary neuron could have conferred NGF responsiveness on the hybrid. Analysis of the expression of the *trk* oncogene which confers high affinity properties onto the low affinity NGF receptor (Hempstead *et al*, 1991) may elucidate this mystery.
Future work in this area using the ND cell system may concentrate on studying the secretion of the redistributed proteins after appropriate stimuli.

Chapter 7 Conclusions

The ND cells represent a model sensory neuron system. Such a system is invaluable to correlate functions studied *in vivo* and on primary sensory neurons *in vitro* with those assessed by biochemical and molecular methods on cell lines *in vitro*.

The non-permissive nature of the interaction of HSV with the ND cells is reminiscent of that of HSV with sensory neurons *in vivo*. For example, low IE gene expression, reduced virus production, abortion of the lytic cycle, exhibition of the 'Multiplicity Activation' phenomenon, expression of at least one extra octamer-binding protein (the putative inhibitor of HSV IE gene transcription) in ND cells and DRG neurons and induction of IE gene expression by cAMP in ND cells and the observation that cAMP can reactivate latent HSV infections in sensory neurons *in vivo*.

Further functional similarity between ND cells and primary sensory neurons has been demonstrated in the capacity of the ND cells to develop regulated pathways of exocytosis on morphological differentiation. Such pathways only mature *in vivo* late on in the development of the nervous system.

However the ND cell system may not be used to study the differentiation of neuroblasts to mature neurons as occurs in the nervous system *in vivo*. The ND cells are in fact a mature cell type in the undifferentiated state and already express proteins characteristic of mature neurons. Functions expressed in mature

neurons only may be studied in the ND cells. However the morphology of the mature neuron can be reproduced by modulation of the culture medium so that functions associated specifically with neurites can be analysed.

While characterising the ND cell system a problem has been highlighted in the study of NGF responses. No responses, for example growth responses, neurite extension, alterations in gene expression, were observed when NGF was administered to the bathing culture medium. This observation was puzzling, as neonatal sensory neurons are dependent on NGF for survival and adult sensory neuron function and gene expression are modulated by NGF. Whilst it is possible that the neonatal DRG neuron could have been rescued by the neuroblastoma after the hybridisation event to render it independent of NGF and to therefore be unaffected by it, it is also possible that the ND cells, as many neuroblastomas, do not carry functional high-affinity NGF receptors. NGF effects may therefore not be transduced by ND cells.

Although HSV can be reactivated by many factors both *in vivo* and *in vitro* the unresponsiveness of the ND cells to NGF prevents testing a well-characterised mechanism of reactivation on latent infections in these cells.

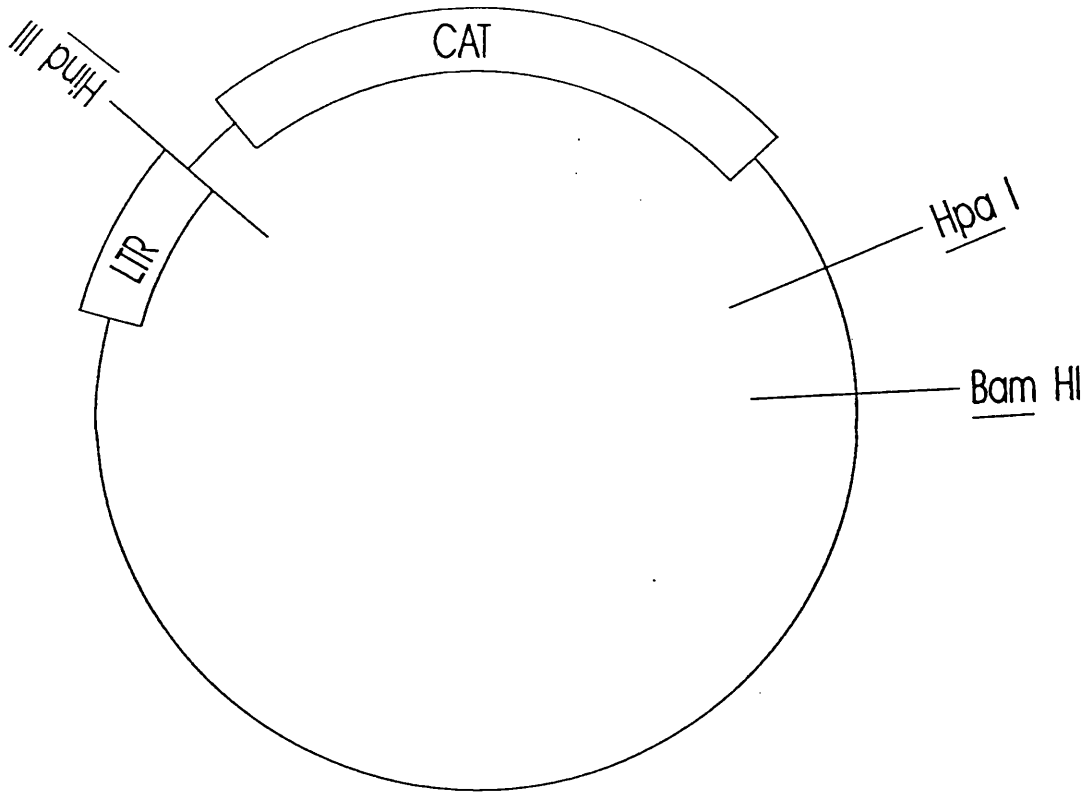
Despite the lack of NGF responsivness, the ND cells exhibit an array of sensory neuronal characteristics. Although not usable for studying differentiation as occurs during development *in vivo* some functional systems mature on morphological differentiation, for example, secretory pathways and possibly synapse formation.

The phenomena studied in this work in the ND cell system are therefore likely to be representative of those in sensory neurons *in vivo*.

Appendix: Plasmid Maps

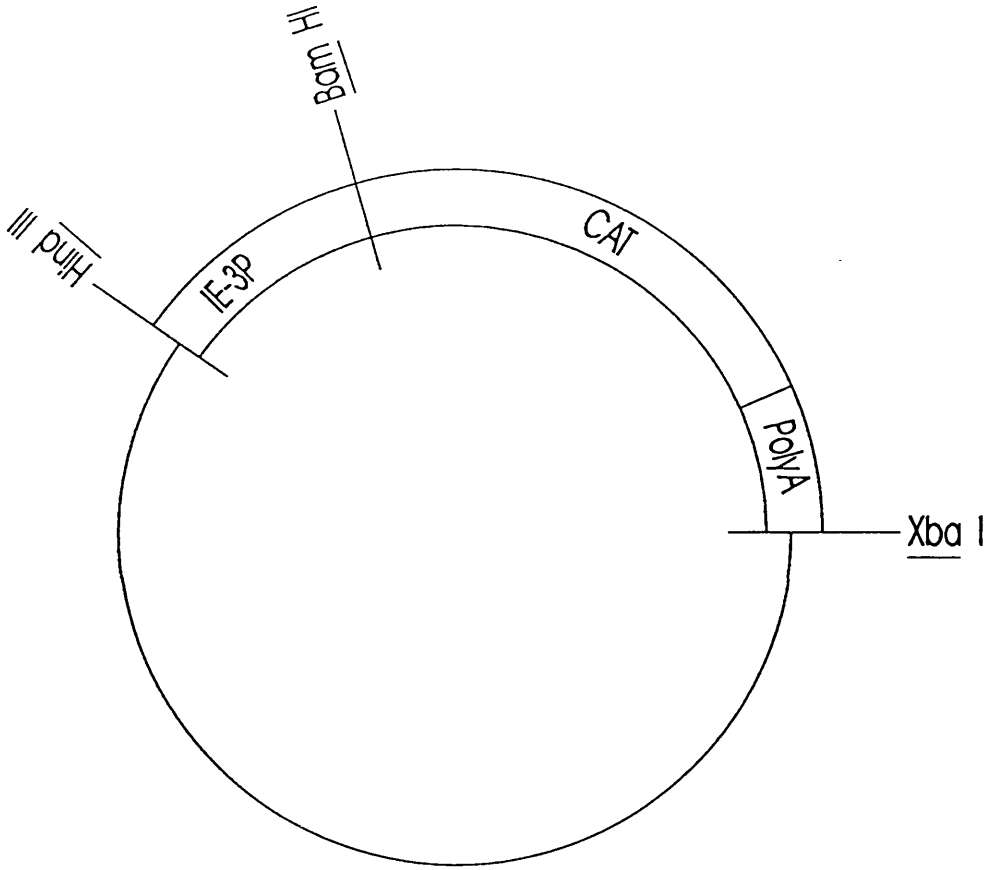
pRSVCAT

Gorman *et al*, 1984.



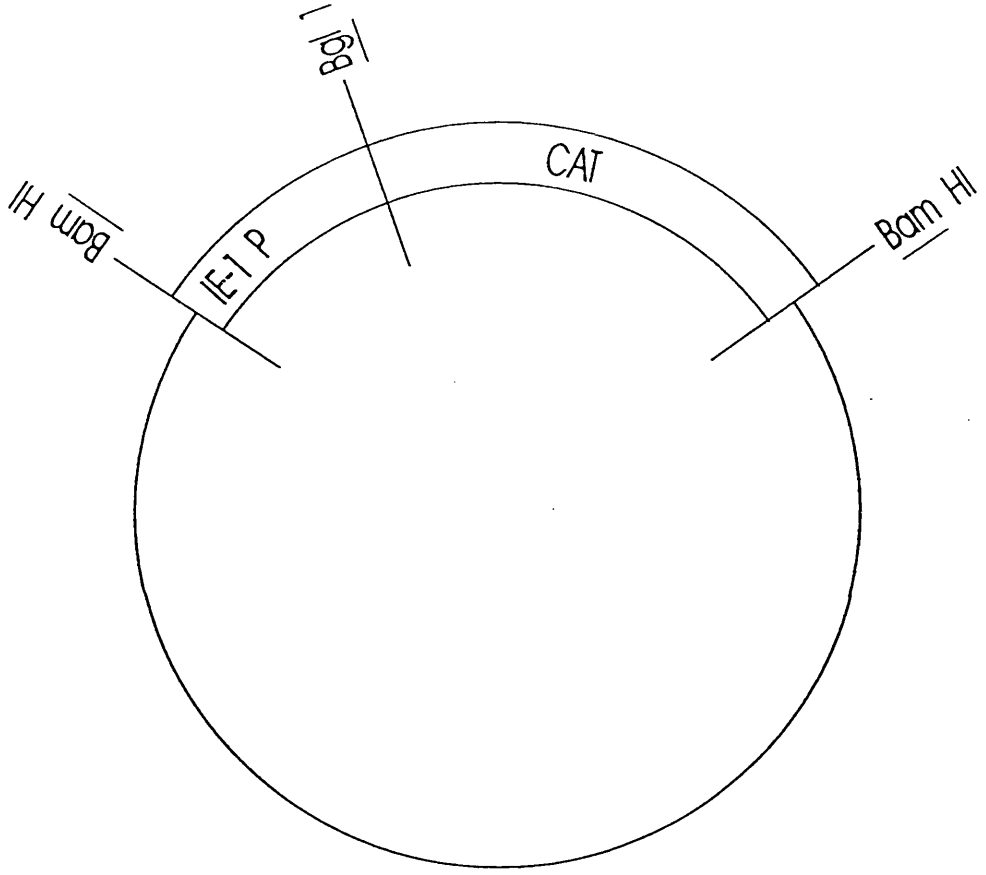
IECAT

Stow *et al*, 1986.



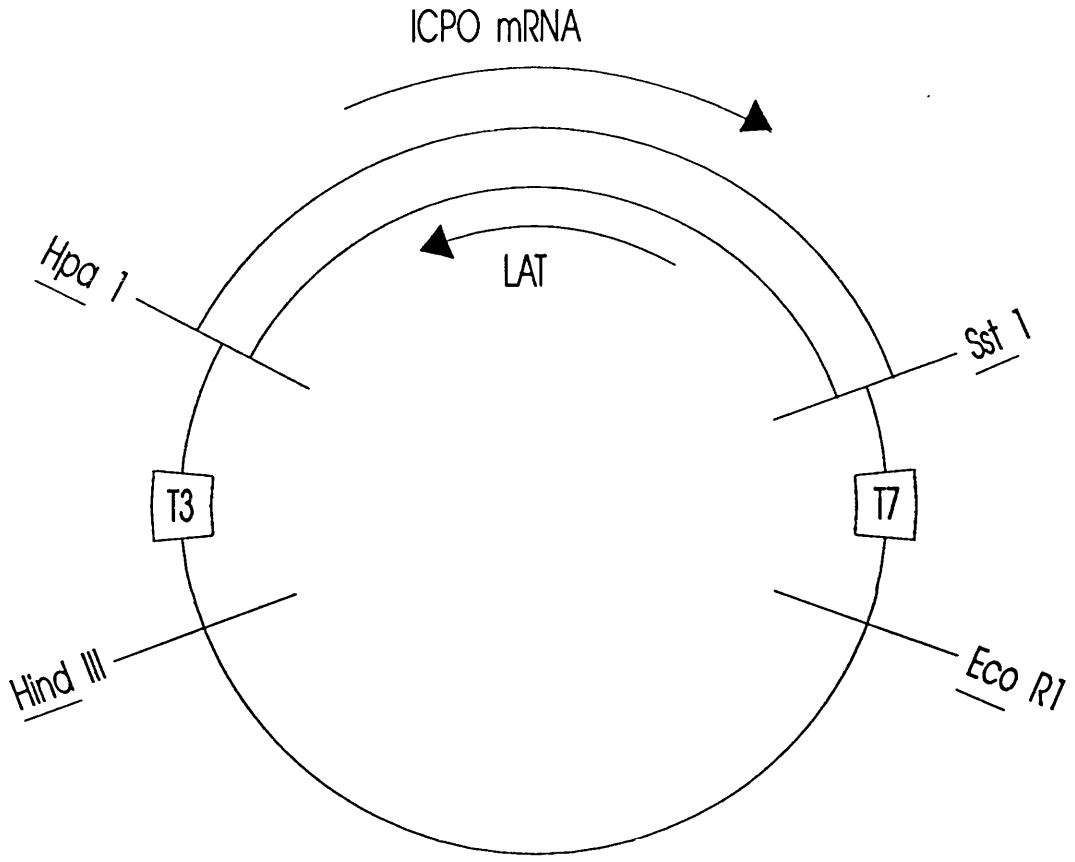
pIG65

Gelman and Silverstein,1987b.



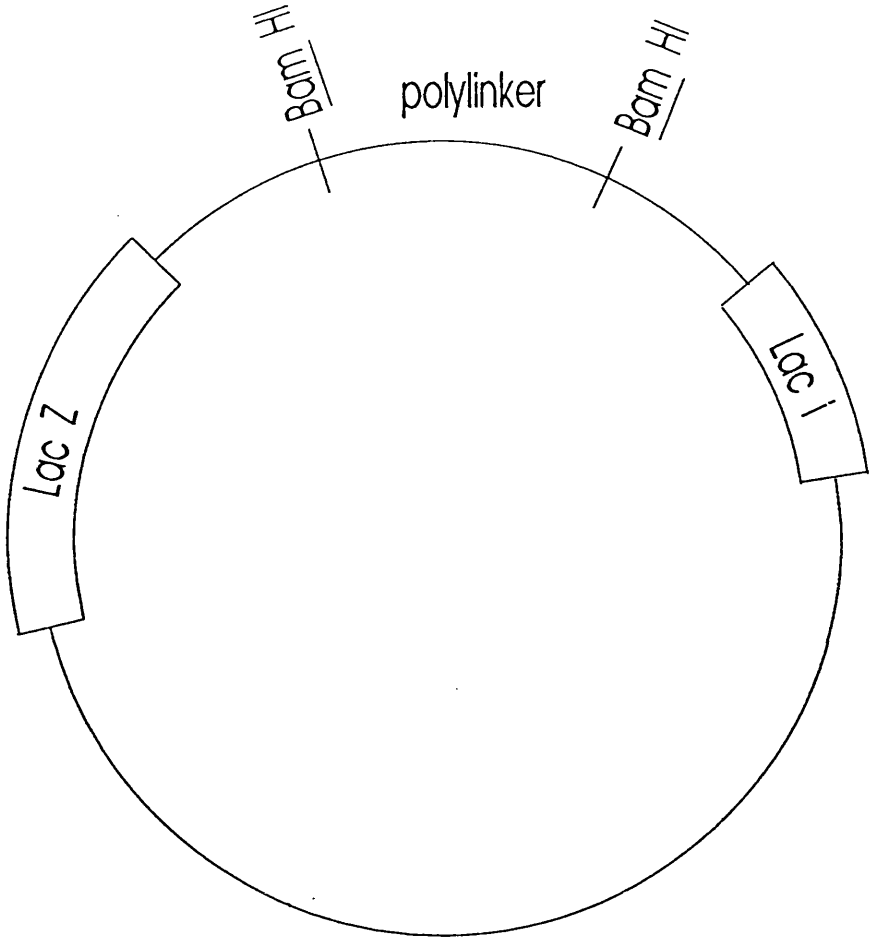
pMC151

Stevens *et al*, 1987



pUC1813

Kay and McPherson, 1987.



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