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expression in cells of neural origin**

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DEGREE

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**Cytokine regulation of human immunodeficiency virus type 1 gene
expression in cells of neural origin**

**A thesis presented for the degree of PhD in Biological Sciences by
Simon Swingler, BSc (Hons) [Reading]**

WARWICK UNIVERSITY

Submitted March 1992

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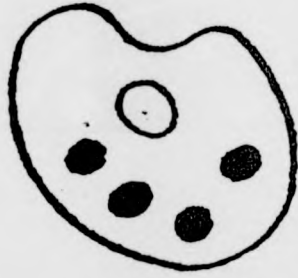


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Declaration

All work reported in this thesis was performed by the author in the laboratory of Dr. Alan Morris in the Department of Biological Sciences, University of Warwick, any exceptions are noted in the text. Work described in this thesis has not been submitted for a degree at any other institution.

List of publications and presentations

Control of HIV-1 transcription in neural cells: Meeting abstract and poster presentation at the 1990 AIDS directed programme meeting, 23-26th September, University of Exeter.

Swingler, S. Easton, A. Morris, A (1992): Cytokine augmentation of HIV-1 LTR-driven gene expression in neural cells. AIDS Research and Human Retroviruses; Vol 8, No 4 (April). pages 487-493.

Abbreviations

aa	amino acid	GFAP	glial fibrillary acidic protein
ADC	AIDS dementia complex	G-CSF	granulocyte-colony stimulating factor
AIDS	acquired immune deficiency syndrome	GM-CSF	granulocyte macrophage-colony stimulating factor
AMV	avian myeloblastosis virus	GR	glucocorticoid receptor
ARC	AIDS-related complex	GVHD	graft versus host disease
ATP	adenosine triphosphate	GTP	guanosine triphosphate
bp	base pair	HBS	Hepes buffered saline
β -gal	β -galactosidase	HBV	hepatitis B virus
BSE	bovine spongiform encephalopathy	Hepes	<i>N</i> -2-hydroxyethyl-piperazine- <i>N</i> -2-ethanesulfonic acid
CAT	chloramphenicol acetyl transferase	HHV-6	human herpes virus type 6
CAP	Catabolite activator protein	HIV-1	human immunodeficiency virus type 1
CIAP	calf intestinal alkaline phosphatase	HSV-1	herpes simplex virus type 1
CJD	Cru zfeld-Jacob disease	HTLV-I	human T lymphotropic virus type 1
CMV	cytomegalovirus	I-CAM1	intracellular adhesion molecule 1
CPE	cytopathic effect	ICP-O	infected cell protein
CNS	central nervous system	IE	immediate early protein
CNS	<i>cis</i> -acting repressive sequence	IFN	interferon
CSF	cerebro-spinal fluid	Ig	immunoglobulin
CT	computed tomography	IL	interleukin
CTP	cytidine triphosphate	IPTG	isopropyl-1-thio- β -D-galacto-pyranoside
DEAE	diethyl amino ethyl	K	killer
DMEM	Dulbecco's modification of Eagle's medium	KIMSV	Kirsten murine sarcoma virus
DMS	dimethyl sulphate	LB	luria broth
DMSO	dimethyl sulfoxide	LPS	lipopolysaccharide
DNA	deoxyribonucleic acid	LTR	long terminal repeat
DNAse	Deoxyribonuclease	MHC	major histocompatibility complex
dNTP	deoxynucleotide triphosphate	M _r	Molecular weight
ds	double stranded	MRC	Medical Research Council
DTT	dithiothreitol	MRI	magnetic resonance imaging
EDTA	ethylenediamino-tetra-acetic acid	Nef	negative factor
EGTA	ethyleneglycol-bis (2'-amino-ethylether) tetra-acetic acid	NK	natural killer
eIF-2	elongation initiation factor 2	Nonidet-P40	Nonidet-P40
Env	envelope	NRE	negative regulatory element
FBS	foetal bovine serum	OD	optical density
FITC	fluorescein isothiocyanate	ONPG	<i>o</i> -nitrophenyl- β -D-galactopyranoside
Gag	group associated antigen		
GalC	galactoside-ceramide		

PBS	phosphate buffered saline	VP1	virus protein 1
PCR	polymerase chain reaction	Vpr	virus protein R
PDL	progressive diffuse leukoencephalopathy	Vpu	virus protein U
PE	phycoerythrin	x-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside
PHA	phytohaemagglutinin		
PIPES	piperazine- <i>N,N'</i> -bis(2-ethane-sulfonic acid; 1,4-piperazine diethanesulfonic acid		
PKA	protein kinase A		
PKC	protein kinase C		
PMA	phorbol 12-myristate 13-acetate		
PMSF	phenyl methyl sulphonyl fluoride		
Poly (dIdC)- (dIdC)	poly deoxyinosine-deoxycytidine double stranded co-polymer		
Pol	polymerase		
UTP	uridine triphosphate		
UV	ultraviolet		
Rev	regulator of virion expression		
RPMI	Roswell park memorial institute		
RNA	ribonucleic acid		
RNAse	Ribonuclease		
RNAse	RNAse inhibitor		
RRE	Rev-responsive element		
RSV	Rous sarcoma virus		
SDS	sodium dodecyl sulphate		
ss	single stranded		
SV40	simian virus 40		
TAE	Tris-acetate electrophoresis buffer		
TAR	transactivation response element		
Tat	transactivator of transcription		
TBE	Tris-borate electrophoresis buffer		
TE	Tris-EDTA buffer		
TEMED	<i>N,N,N',N'</i> , tetramethyl-ethylene-diamine		
TGFβ	transforming growth factor β		
TNF	tumour necrosis factor		
tRNA	transfer RNA		
Vβ	variable β chain		
Vif	virion infectivity factor		
VIP	vasoactive intestinal peptide		

Summary

In response to the growing body of evidence suggesting the direct infection of neural cells may contribute to the pathogenesis of the neurological syndrome associated with HIV infection, the AIDS dementia complex, the regulation of HIV-1 gene expression by cytokines was investigated in cells of central nervous system origin.

Expression from a reporter gene under the control of the HIV-1 LTR was determined by transient transfection assays in a collection of cells representative of the major neural components of the central nervous system. These were human neuroblastoma, astrocytoma, glioblastoma cell lines, primary murine astrocyte cultures and a murine oligodendrogloma cell line. Cellular stimulation with a range of cytokines, TNF α , IL-1 β , IL-6, IFN α and IFN γ , individually and in pairs revealed a number of these capable of significantly augmenting expression from the LTR. TNF α was found to stimulate LTR-driven gene expression in all neural cells as did IL-1 β in astrocytoma, glioblastoma and astrocyte cultures. IL-6 enhanced expression only in astrocyte cultures. The interferons generally suppressed LTR-driven gene expression except IFN γ which consistently augmented expression in murine astrocyte and oligodendrogloma cells and IFN α augmenting reporter gene expression in one neuroblastoma cell line.

The HIV-1 *tat* gene product was found to be functional in all cell types with varying degrees of efficiency and in one cell line the combination of an activating cytokine or phorbol ester and Tat resulted in an enhancement above that obtained by co-transfection with Tat alone. In most the level of expression did not significantly change.

Analysis of the interaction of sequence-specific DNA-binding proteins with the HIV-1 LTR demonstrated that in both neuroblastoma and astrocytoma cells the augmentation of LTR-driven gene expression by TNF α or IL-1 β correlated with the induction of factors recognizing the NF κ B motifs of the HIV enhancer. These proteins were rapidly induced and no other DNA-binding activities recognizing the LTR were found to be regulated by cytokines. Many constitutive DNA-binding factors were observed to interact with the LTR, such as LBP-1, Sp1, TATA-, Site A- and Site B-like binding activities previously noted in lymphocytes and HeLa cells. In addition two neural specific factors were discovered which recognize octamer- and GTI-like binding motifs in the LTR.

The results obtained demonstrate that the cytokines can regulate cellular mechanisms that can lead to augmented transcription from the HIV-1 LTR in neural cells and suggest that a latent infection of neural cells by HIV-1 will be activated by TNF α and IL-1 β in the central nervous system of patients with the AIDS dementia complex.

CHAPTER 1

Chapter 1: Introduction

1.1. Initial considerations

The acquired immune deficiency syndrome and its etiological agent, the human immunodeficiency virus, are evolving into a pandemic of great proportions across many countries of the world and the number of those seropositive for the virus increases yearly. Ultimately infection produces fatal disease through the suppression of host immunity gradually debilitating the immune reactivity until it fails to control often otherwise innocuous pathogens. A number of opportunistic infections are prevalent amongst AIDS sufferers and immunosuppression also increases the occurrence of certain neoplasms. In addition, HIV-1 infection will manifest neurological abnormalities in many infected individuals producing a progressive and lethal dementia in the absence of, or in conjunction with symptoms of AIDS. The syndrome of neurological disease is known as the AIDS dementia complex (ADC) and is a significant factor in the morbidity and mortality of HIV-1 infection.

The broad spectrum of disease that HIV-1 can induce and the insidious nature with which it begins this process, usually causing no undue harm to the host for several years, indicate it possesses the means to finely control its life-cycle. The ability of the virus to evade destruction by host immunity through the establishment of latent infection is being gradually understood through the analysis of processes that control virus expression and transcription in the infected cell. Work in this area is central to the pathogenesis of HIV-1 infection and provides insights into how HIV-1 subverts normal cellular control mechanisms. The furtherance of this research to include the AIDS dementia complex, is similarly important and examination of the regulatory mechanisms operating in neural cells is likely to be equally enlightening and may also contribute to the understanding of the pathogenesis of ADC.

1.2. The human immunodeficiency virus type 1 and AIDS

Infection with the human immunodeficiency virus has the capacity to begin a slow and progressive disease of the immune system which ultimately leads to a state of profound immunodeficiency known as the acquired immune deficiency syndrome, AIDS. This state is characterized by the establishment of life-threatening opportunistic infections and neoplasms that invariably lead to the death of the infected individual (Fauci, 1988). The mean time between infection and the onset of immunodeficiency is variable but is likely to exceed five years and may be as long as 15 years (Haseltine *et al.*, 1990). HIV-1 ultimately follows a varied clinical course due to involvement of other infectious agents and cancers in the pathogenesis of AIDS but infection progresses through several distinctive stages.

1.2.1. Asymptomatic Infection

Initially HIV-1 may replicate efficiently in cells of the lymphoid system and produce an acute infection with mononucleosis-like symptoms and readily detectable plasma viremia. During this period, which may occur from three weeks to six months post-infection, the virus can infect more than 1% of circulating CD4+ T lymphocytes and becomes further disseminated to other cells of the body (Haseltine *et al.*, 1990; Garcia-Blanco and Cullen, 1991). HIV-1 has the propensity to infect many cell types of the lymphoid system, including bone marrow progenitor cells (Folks *et al.*, 1988; Zucker-Franklin and Cao, 1989) and cells of the monocyte/macrophage lineage which serve as one reservoir of virus in tissues throughout the body (Gendelman *et al.*, 1989). Moreover, HIV-1 may enter the central nervous system at this time (Haseltine, 1989). Subsequently cells productively infected with HIV-1 are subject to destruction and clearance by an effective humoral immune response and the amount of circulating virus declines as the levels of antiviral antibody increase, resulting in the loss of detectable free virus and a drop in the numbers of infected CD4+ lymphocytes (Garcia-Blanco and Cullen, 1991). The level of circulating CD4+ cells, which decline during acute infection as a result of the prolific and cytopathic replication of the

virus, return to normal and the seropositive individual enters the asymptomatic period of infection (Redfield and Burke, 1988).

A completely asymptomatic period may continue for around one year and is commonly followed by symptoms of mild to severe lymphadenopathy for a variable period of typically three to five years (Redfield and Burke, 1988). During this time the patient's CD4+ T cell count steadily declines as far more CD4+ T lymphocytes are lost than are infected with HIV-1 and they often reach a low or undetectable level within two or three years, whilst the number of HIV-1 infected cells in the circulation gradually increases (Koenig and Fauci, 1990).

1.2.2. CD4+ lymphoid cell depletion

The circulatory CD4+ cells mostly represent the T helper cell (T_H) population which are fundamental in the initiation of immune reactivity to T cell dependent antigens (Roitt *et al.*, 1985) and there is evidence for a number of mechanisms by which HIV-1 mediates their selective loss. The prolonged latent period before the development of frank immune deficiency supports the view that direct cytopathic effects induced by viral replication are not responsible for the loss of significant numbers of CD4+ T cells and even the envelope glycoprotein-induced cell fusion of CD4+ cells observed *in vivo* in thymus and brain tissue is not likely to be a major cause of the additional depletion of uninfected T_H cells characteristic of HIV-1 infection (Habeshaw and Dalgleish, unpublished manuscript; Ho *et al.*, 1987). Free envelope glycoprotein, gp120, can sensitize other uninfected CD4+ cells to cell killing by antibody dependent cellular cytotoxicity and the actions of NK and K cells (Tyler *et al.*, 1989). Autoantibody production is common in HIV-1 seropositive individuals and persistently produced lymphocytotoxic antibodies to an unidentified surface antigen will cause the death of CD4+ T cells (Stricker *et al.*, 1987). Further mechanisms based on the induction of autoimmunity have been suggested due to gp120 bound to CD4 mimicking the physiological interaction of major histocompatibility (MHC) class II antigens and CD4. This may produce a cross-reactive immune response to MHC class II-bearing cells, as well as an antidiotypic response to CD4+ cells, leading to

their elimination by mechanisms equivalent to graft versus host disease [GVHD] (Fauci, 1988; Koenig and Fauci, 1990). Recent evidence also indicates that HIV-1 may encode a 'super-antigen', examples of which cause the selective elimination of T cells expressing particular V β -antigen receptors independent of their antigen specificity and this could also lead to T_H cell impairment and depletion (Imberti *et al.*, 1991).

Other immunosuppressive properties of HIV-1 infection can further contribute to the state of immune dysfunction caused by the loss of T_H cells and are a consequence of individual viral gene products. The envelope glycoprotein gp120 and specific peptide sequences from transmembrane protein gp41, as well as the transactivator protein, Tat, are all capable of inhibiting mitogen or antigen-induced proliferation of lymphocytes *in vitro* (Habeshaw and Dalgleish, unpublished manuscript; Ruegg *et al.*, 1989; Viscidi *et al.*, 1989).

1.2.3. AIDS-related complex

The gradual decline in CD4+ T cell numbers correlates with the disease progression to the AIDS-related complex (ARC) in which symptoms of local immune suppression begin to become apparent and lymphadenopathy may become severe (Gurley and Groopman, 1990). Furthermore the replicative potential of HIV-1 isolated from the host correlates inversely with the fate of circulating CD4+ lymphoid cells such that during the asymptomatic stage of infection virus isolates tend to grow slowly and to low titres *in vitro*, whereas during ARC or AIDS, virus isolates grow more rapidly and to high titres which can continuously replicate in CD4+ cell lines and often induce syncytia (Cheng-Mayer *et al.*, 1988). This situation equates with the high mutation rate that exists amongst RNA viruses (Dougherty and Temin, 1988) and is suggestive of HIV-1 evolution *in vivo*, a hypothesis further supported by the significant variation between the RNA sequence of circulating and cell-associated virus and integrated proviral DNA (Simmonds *et al.*, 1991). The accumulation of mutations within the viral genome appears related to the gradual increase in viral cytopathology. The emergence of viral 'species' that escape host immune defences has been shown to relate directly to mutations within the neutralization domains of the envelope glycoprotein gp120 (LaRosa *et al.*, 1990). Therefore mutation in virus epitopes

provides one mechanism for the persistence of HIV-1 after acute infection and would also be necessary for the 'super-antigen' mediated elimination of CD4+ T_H cells (Imberti *et al.*, 1991).

1.2.4. Acquired immune deficiency syndrome

Progression to AIDS is a clinical definition based on the appearance of certain diseases or neoplasms that are at least moderately predictive of defective cell-mediated immunity by comparison with the spectrum of disease known to occur in an immuno-compromised host. These conditions are characterized by serious opportunistic infection with normally innocuous pathogens and neoplasms such as malignant lymphoma and Kaposi's sarcoma (Gurley and Groopman, 1990).

1.3. The AIDS dementia complex

1.3.1. Central nervous system involvement in HIV-1 infection

It has become clear that infection with HIV-1 is capable of causing a progressive syndrome of neurological disease that is not strictly dependent upon the state of immunosuppression in the infected individual (Price *et al.*, 1988). Although the central nervous system is often the target for opportunistic infections in the later stages of ARC or in AIDS, which may potentiate general CNS pathology, the AIDS dementia complex (ADC) is by far the most common cause of neurological dysfunction (Elder and Sever, 1988) and may occur at any stage of the disease (Fischer and Enzensberger, 1988).

Neuropsychiatric abnormalities are often the prevailing symptom of the later stages of HIV-1 infection and occur in 40 to 50% of adults and as many as 70 to 80% of children with clinically defined AIDS (Petito, 1988). Other reports suggest that around two thirds of HIV-1 seropositive patients will suffer significant manifestations of the AIDS dementia complex before the terminal phases of their disease (Price and Brew, 1988). Rarely, the sole indication of HIV-1 infection is the involvement of the central nervous system (Navia

and Price, 1987; Wiley and Nelson, 1990). Furthermore at post mortem the majority of AIDS sufferers have signs of central nervous system damage (Petito *et al.*, 1986).

The neurological syndrome associated with HIV-1 infection portrays a slow, progressive degeneration of cognitive and motor functions that does not remit (Moller *et al.*, 1988). Frequently it begins with mild symptoms of impaired concentration and motor impairment that gradually increase in severity leading to the loss of major intellectual capacity and concomitant motor disability. Finally patients enter a nearly vegetative state that is terminal (Price and Brew, 1988). The time course of progression through the stages of ADC is variable and probably dependent on unknown host or viral factors (Wiley and Nelson, 1990) but significant deterioration can occur in the course of two months to more than one year (Moller *et al.*, 1988).

1.3.2. Invasion of the central nervous system

The time at which HIV-1 gains entry in to the central nervous system is unclear. Several reports document aseptic meningitis as the dominant symptom of HIV-1 infection at the time of seroconversion (Wiley and Nelson, 1990) and further evidence of intrathecal HIV-1-specific antibody synthesis (Gallo *et al.*, 1988) and the regular recovery of virus from cerebro-spinal fluid [CSF] (Brew *et al.*, 1988) suggest an early viral entry. However, as noted from studies on herpes simplex virus, viral meningitis seldom progresses to encephalitis and other factors must influence this progression (Wiley and Nelson, 1990). By analogy to another lentivirus, Maedi Visna, free virus could cross the blood brain barrier following replication in cells of the choroid plexus (Wigdahl, 1989). Alternatively a more widely accepted view is that HIV-1 may be carried across a compromised blood brain barrier by infected macrophages, possibly after damage caused by the early infection of brain capillary endothelial cells (Wigdahl, 1989; Wiley and Nelson, 1990).

1.3.3. Neuropathological changes

The major histopathological changes in the central nervous system associated with ADC fall into three groups of focal, diffuse or overlapping lesions (Budka, 1989).

Focal changes are initially found in the cerebral white matter and less frequently in the grey matter, where prominent microgranulomatous foci of multinucleate giant cells and reactive astrocytes occur (Rostad *et al.*, 1987; Budka, 1989). There is mild infiltration by lipid-laden macrophages and lymphocytes and as the disease progresses these abnormalities are found more often in the grey matter (Petito, 1988). The formation of multinucleate giant cells with accompanying cell infiltration is often incorrectly referred to as sub-acute encephalitis.

Vacuolar myelopathy is found frequently during pathological examination of the CNS and resembles the spongiform changes seen in unconventional encephalopathies such as CJD and BSE (Rostad *et al.*, 1987). The earliest lesions consist of intramyelin swelling causing vacuolation, whilst severe cases show extensive vacuolation and evidence of gross demyelination and axonal loss with atypical reactive gliosis (Petito *et al.*, 1986; Wiley and Nelson, 1990). Vacuolar myelopathy is most regularly observed in tissues of the spinal cord where it is often intense and the severity often correlates with the symptoms of spinal cord disease, such as spastic paraparesis. Additionally, the regions of the brain affected by vacuolar myelopathy, and the extent of damage also relate frequently to the symptoms of dementia (Petito, 1988; Wiley and Nelson, 1990). These lesions are further notable by the usual minimal inflammatory response in which only lipid-laden macrophages are observed occasionally around sites of vacuolation, with the cellular infiltrate containing mainly macrophage elements otherwise restricted to the perivascular regions (Wiley and Nelson, 1990). The occurrence of vacuolar myelopathy can not be predicted by the presence of multinucleate giant cell encephalitis and the two may occur independently or concurrently, although multinucleate giant cell formation is found more frequently in patients with severe vacuolar myelopathy (Petito *et al.*, 1986).

Radiological methods of examining CNS damage detect some gross changes during the course of the AIDS dementia complex. Computed tomography (CT) shows a rise in brain atrophy and ventricular enlargement with an increased signal from white matter as the disease progresses (Price *et al.*, 1988) and magnetic resonance imaging (MRI) studies show more frequent abnormalities in additional regions of the CNS not clinically

implicated by the symptoms and gives evidence of inflammatory changes (Wiley and Nelson, 1990). Furthermore the degree of cerebral atrophy as judged from MRI correlates with the symptoms and progression of ADC (Levin *et al.*, 1990).

Diffuse damage is evident in the cerebral and cerebellar white matter and shows three characteristic traits of reactive gliosis, demyelination and disseminated perivascular infiltration by cells of the macrophage lineage often accompanied by multinucleate giant cells which also stain for macrophage-specific markers (Budka, 1989). This is most accurately described as a progressive diffuse leukoencephalopathy (PDL) as it shows evidence of an intensifying, generalized degeneration of the white matter without gross inflammation (Rostad *et al.*, 1987; Budka, 1989).

1.3.4. Cellular localization of HIV-1 in the CNS

i). Identification of HIV-1 within cells of the central nervous system

Resident cell types of the central nervous system can be divided into two categories of neuronal and glial cells. Neuronal cells are obviously involved in the transmission of nervous signals whilst the glia can be further classified into astrocytes and oligodendrocytes. Astrocytes perform many homeostatic and immunological functions within the CNS (see Section 1.6) and the primary role of oligodendrocytes is to form the myelin sheaths around the axons of neuronal cells. A third class of cell within the CNS, the microglia perform a role as the macrophage of the brain (see Section 1.3.4. ii).

Following the close association between neurological dysfunction and HIV-1 infection many studies were undertaken to determine whether direct infection of the central nervous system was the underlying cause of the AIDS dementia complex. Shaw *et al.*, (1985) were the first to report that integrated and non-integrated HIV-1 sequences were present in the brains of patients suffering from ADC and the abundance of viral nucleic acids often exceeded that recovered from lymphoid tissues, such as lymph nodes and spleen.

Ultrastructural examination of brain tissue sections from three patients with ADC demonstrated virus particles within multinucleate giant cells and astrocytes (Epstein *et al.*,

1984/5; Clague *et al.*, 1986), and, although none were detected in neurons or oligodendrocytes, Gyorkey *et al.* (1987) described HIV-1 virions in oligodendrocytes and again in astrocytes. Further analysis by *in situ* hybridization confirmed the presence of HIV-1-specific nucleic acid sequences in up to 15% of multinucleate giant cells, and immunocytochemical studies indicated that these cells were composed of macrophage-like cells. No neural cell markers were identified on giant cells by staining with specific antisera (Koenig *et al.*, 1986). The major cell type supporting HIV-1 replication was, by ultrastructural (Meyenhofer *et al.*, 1987) and immunocytochemical (Vazeux *et al.*, 1987) criteria, macrophage in origin (encompassing microglia and infiltrating macrophages) and HIV-1 RNA and virus particles were frequently detected in these cells around regions of demyelination (Koenig *et al.*, 1986). Another report confirmed that HIV-1 infection was predominantly localized to infiltrating mononuclear cells and multinucleate giant cells. In this study Wiley *et al.* (1986) also showed HIV-1 infection occurred significantly in capillary endothelial cells by immunocytochemistry and *in situ* hybridization techniques. In brain tissue from the most severe case of HIV-1 encephalopathy neurons and astrocytes also contained HIV-1 antigens (Wiley *et al.*, 1986). Other investigations have confirmed this less frequent infection of neural cells and shown virus-specific antigens in astrocytes, neurons and oligodendrocytes (Stoler *et al.*, 1986). *In situ* hybridization also demonstrated HIV-1 specific RNA and DNA in macrophage-like cells and to a lesser extent in glial cells and occasional neurons (Gosztonyi *et al.*, 1988).

Macrophage-derived cells are most frequently found to harbour HIV-1 which, from the presence of virus particles, viral DNA and RNA support active replication in the brain (Wiley *et al.*, 1986) and neural cells apparently form a site of less frequent HIV-1 infection. Whether the infrequent detection of neural cell infection noted by some investigators represents limitations in the sensitivities of the techniques employed or a true low level of infection remains to be determined (personal communication, Dr. C. A. Wiley, Department of Pathology, University of California, San Diego, La Jolla). A latent infection at low copy number may well be below the threshold of detection of the methods employed especially with the sampling techniques available for human tissues (Wiley and Nelson, 1990).

ii). Macrophage and microglia

The origin of the macrophage-like cells infected with HIV-1 in the CNS of AIDS encephalopathy is unclear and complicated further by the long-held controversy over the existence of a distinct lineage of microglia. The most widely held view is that microglia, the brain-specific macrophage, are derived from monocytes entering the CNS during the late embryonic and early post-natal periods (Perry and Gordon, 1989). There is no convincing evidence to demonstrate the existence of a population of cells in the CNS that have the morphology of microglia but are derived from the neural ectoderm. Monocytes and microglia share many antigens and there appears to be no definitive immunocytochemical distinctions between them (Perry and Gordon, 1989). Vazeux *et al.* (1987) identified the majority of HIV-1 infected mononuclear cells as microglia on the basis of lacking CD14 and CD4 surface antigens, whilst other infected cells were CD14⁺ and CD4⁺ and categorized as macrophages of haematogeneous origin. Experimental animal systems have shown that expression of the CD4 antigen on microglial cells is not constitutive and is absent unless induced by inflammation or raised levels of CSF protein (Perry and Gordon, 1989). *In vitro* studies on HIV-1 replication in microglial cells isolated from normal human brain demonstrated these cells to be CD4⁺ which served also as a receptor for virus infection (Jordan *et al.*, 1991). However, these cultures of microglial-like cells may equally be contaminating blood-derived macrophages on the basis of CD4 expression. Other *in vitro* studies demonstrated that CD4⁺, CD14⁺, CD68⁺ and Ki-M8⁺ microglial cells (monocytes were positive for all four antigens) could not be infected with either monocytotropic or lymphotropic HIV-1 (Peudenier *et al.*, 1991). Further work is needed to define the factors which induce CD4 expression on microglial cells and whether amongst these AIDS encephalopathy can render them targets for the viral infection.

1.3.5. Infection of neural cells by HIV-1 *in vitro*

i). Cellular tropism of HIV-1

HIV-1 becomes adapted *in vitro* to the two main lymphoid cell types that it infects such that virus isolated from T lymphocytes replicates poorly in monocytes, and virus isolated from monocytes, although capable of replicating in T lymphocytes quickly loses the ability to re-infect monocytes after passage in lymphocytes (Gendelman *et al.*, 1989; Meltzer *et al.*, 1990a). The characteristics of HIV-1 replication also differ; in lymphocytes the majority of virus strains replicate to high titre and cause cytopathic effect (CPE) dependent on the interaction of CD4 and gp120, whereas in monocytes only a few strains follow this course and HIV-1 replication is usually limited to transient CPE followed by low levels of replication with little release of infectious particles or CPE (Cheng-Mayer and Levy, 1990). Macrophage-adapted strains are substantially less cytopathic in these cells and infected macrophages are likely to form a reservoir of HIV-1, increasing persistence and virus spread (Gendelman *et al.*, 1989; Meltzer *et al.*, 1990a). A similar phenomenon is observed with brain-derived isolates which show distinct macrophage-tropism with reduced cytopathogenicity (Cheng-Mayer *et al.*, 1989).

Many isolates of HIV-1 will productively infect glioma (Dewhurst *et al.*, 1987; Weber *et al.*, 1989; Keys *et al.*, 1991), astrocytoma (Cheng-Mayer *et al.*, 1987; Harouse *et al.*, 1989) and neuronal cell lines (Li *et al.*, 1990; Shapshak *et al.*, 1991) and the virus usually exhibits low levels of replication and limited or absent cytopathic effects. Most studies have used HIV-1 propagated in T cells, which on occasion required high multiplicity of infection to establish viral replication in neural cell types (Weber *et al.*, 1989). Macrophage-tropic isolates showed similar persistent non-cytopathic replication in neural cell lines (Keys *et al.*, 1991). In some glial cells lines HIV-1 replication is more abundant and analogous to the chronic infections observed in lymphocyte and macrophage cell lines (Cheng-Mayer *et al.*, 1987; Dewhurst *et al.*, 1988).

Primary human glial cell cultures infected with HIV-1 initially produce high levels of antigen and release considerable amounts of infectious virus until both progressively subside and the infection enters a persistent, latent phase where viral antigens are often

undetectable (Christofinis *et al.*, 1987; Tornatore *et al.*, 1991). During the productive stages of replication HIV-1 antigens have been localized to astrocyte cells (glial fibrillary acidic protein [GFAP] positive), and enriched populations of primary human GFAP+ cells also support HIV-1 replication with very little cytopathic effect (Rytik *et al.*, 1991).

ii). Neural cell specific receptors for HIV-1

The expression of CD4 was apparent at low levels on some glial (Dewhurst *et al.*, 1987) and neuronal cell lines (Shapshak *et al.*, 1991) where it was required for infection. But many glial and one neuronal cell line did not express detectable levels of CD4 antigen or mRNA (Harouse *et al.*, 1989; Weber *et al.*, 1989; Li *et al.*, 1990) and their infection was not impeded by monoclonal antibody to CD4 (Harouse *et al.*, 1989; Weber *et al.*, 1989) or soluble recombinant CD4 (Li *et al.*, 1990). Furthermore, transfection of a CD4 expression vector in to a susceptible glial cell line did not augment virus infection (Chesebro *et al.*, 1990). Entry of HIV-1 in to these cells is not dependent on expression of the CD4 antigen and in some examples where expression of CD4 has been demonstrated infection appears to occur by other means (Weber *et al.*, 1989). HIV-1 infection of primary human glial cells may also operate via alternative mechanisms as Christofinis *et al.* (1987) failed to detect surface expression of CD4 by immunocytochemistry.

For cells of lymphoid origin the CD4 antigen is the receptor by which HIV gains entry into the cell (Haseltine, 1990). However, many neural cells capable of infection lack CD4 and this implies there are other receptors for HIV. The use of alternative receptors is not restricted to cells of neural origin. HIV-1 will infect CD4⁻ muscle (Clapham *et al.*, 1989) and fibroblastoid cell lines (Tateno *et al.*, 1989) where infection is unaffected by monoclonal antibody to CD4 or soluble recombinant CD4. Although virus entry could be non-specific and result from endocytosis or direct fusion of the plasma membrane perhaps due to potential fusogenic regions in the envelope protein gp41 (Gonzalez-Scarano *et al.*, 1987) other candidate receptors have been suggested.

Studies on whole brain sections show structures immunologically similar to CD4 in many locations which can interact with gp120 and microscopically were observed over

neurons and glial cells (Pert *et al.*, 1988a). CD4 mRNA was also present in brain homogenates from the same areas that express CD4 antigen (Maddon *et al.*, 1986). Therefore *in vivo* CD4 may permit HIV-1 infection of the CNS, in agreement with the CD4-dependent infection of some neural cells. Nervous tissue also contains receptors for vasoactive intestinal peptide, VIP, which shares homology with a discrete region of gp120, that in the form of a synthetic peptide [peptide T] is biologically active (Pert *et al.*, 1988a) and can interact with CD4 on lymphoid cells (Brenneman *et al.*, 1988). The distribution of VIP receptors in brain is remarkably similar to CD4 (Pert *et al.*, 1988a) and VIP is a potent inhibitor of peptide T binding to CD4 (Pert *et al.* 1988b). VIP competes with the interaction of gp120 and neuronal cells in an analogous way to the competition by anti-CD4 monoclonal antibodies (Brenneman *et al.*, 1988). The VIP receptor may therefore act as a secondary receptor for HIV in neural cells.

The existence of a distinct neural cell-specific receptor for HIV-1 is indicated by two CD4⁺ neural cell lines, of neuronal and glial origin, in which infection can be inhibited by the antisera to galactoside-ceramide (GalC). This is a membrane-bound glycolipid identified originally on the surface of oligodendrocytes and Schwann cells where it could also serve as a receptor for HIV-1 (Harouse *et al.*, 1991).

1.3.6. Pathogenesis of the AIDS dementia complex

i). Mechanisms of nervous system dysfunction

There is a degree of correlation between the locations of pathological lesions noted in HIV-1 encephalopathy and the detection of HIV-1 infected cells (Koenig *et al.*, 1986; Wiley *et al.*, 1986) and a relationship exists between the affected structures of the brain and the symptoms of AIDS dementia, especially in patients who suffer a more progressive and severe clinical course (Price *et al.*, 1988). But there is believed to be insufficient detectable virus in the CNS to account for the extent of neurological dysfunction (Wiley and Nelson, 1990) and no direct replication-associated pathology within neuronal or glial cells (Wiley *et al.*, 1986; Koenig *et al.*, 1986). Therefore the body of evidence points to an indirect

mechanism of neuronal dysfunction (Wiley *et al.*, 1986; Price *et al.*, 1988; Elder and Sever, 1988).

Such mechanisms of indirect neuronal damage may result from the actions of several viral gene products, particularly the envelope glycoprotein gp120. The binding of free gp120 to neurons in culture causes their death via an interaction with receptors that also bind the neurotrophic factor, VIP which is implicated in neuronal survival (Brenneman *et al.*, 1988). Astrocytic cells also possess receptors for VIP where gp120 may also exert deleterious effects (Wilkin and Cholewinski, 1988). A further region of gp120 shows homology with another neurotrophic factor, neuroleukin, which promotes the growth of motor neurons *in vitro* (Gurney *et al.*, 1986). However, the suggestion of a similar interaction with neuroleukin receptors contributing towards the pathogenesis of ADC was questioned following the discovery that neuroleukin is probably an enzyme, phosphohexose isomerase (Chaput *et al.*, 1988). Intracerebral inoculation of gp120 reduces cerebral glucose metabolism globally in experimental animals (Kimes *et al.*, 1991) and parallels the clinical findings of altered glucose metabolism particularly in the sub-cortical regions of the brain that are targets of HIV-1-induced neuropathology (Price *et al.*, 1988). Furthermore, the reduction in cerebral glucose metabolism by gp120 was greatest in regions of the brain known to be rich in receptors for VIP (Raymon *et al.*, 1989). Current evidence indicates the neurotoxic potential of gp120 may operate via the mis-regulated increase in intracellular calcium in neurons (Dreyer *et al.*, 1990).

The Tat protein of HIV-1 is toxic to neurons, glioblastoma and neuroblastoma cell lines *in vitro* and intracerebral injection of Tat is lethal to mice. It is suggested that the cationic properties of Tat protein are responsible for alterations in cell membrane permeability which mediate toxicity (Sabatier *et al.*, 1991). Other studies have shown that Tat is secreted from expressing cells (Helland *et al.*, 1991) and therefore the close proximity of cells actively replicating HIV-1 could promote neuronal damage.

By analogy with the proposed means of T_H cell depletion in systemic HIV-1 infection, powerful immunological mechanisms may operate in brain tissue causing the elimination of uninfected neural cells. Autoantibodies reactive to brain antigens are

present in some AIDS patients and patients suffering ADC show a much greater frequency in their production (Kumar *et al.*, 1989). Astrocytes share the immunodominant epitope of the transmembrane protein gp41 and astrocyte-reactive antibodies were found in the CSF of some AIDS patients with neurological complications (Yamada *et al.*, 1991). It has also been noted that monoclonal antibodies directed against HIV-1 p17^{gag} recognize uninfected astrocytes (Parravicini *et al.*, 1988). The perturbation of astrocyte function could profoundly influence neuronal cells and the integrity of the blood brain barrier (Yamada *et al.*, 1991). Infection of capillary brain endothelial cells (Wiley *et al.*, 1986) and astrocyte proliferation [reactive gliosis], particularly the hypertrophy of astrocyte foot processes may account for structural changes noted in the capillary walls and may compromise the blood brain barrier (Taruscio *et al.*, 1991). This would be expected to lead to vasogenic oedema and consequent neurological dysfunction (Wiley and Nelson, 1990).

Immunologically activated and HIV-1 infected monocytes will produce cytokines within the CNS, particularly TNF α , IL-1 and IFN α (Macé *et al.*, 1989; Meltzer *et al.*, 1990a). TNF α is cytotoxic to oligodendrocytes and, alone or with IL-1, leads to their degeneration and demyelination (Robbins *et al.*, 1987; Hofman, 1989). IFN α will induce MHC class I antigen expression on astrocytes in culture (Borgeson *et al.*, 1989) and the release of IFN γ from activated lymphocytes will further amplify immunological reactions through the induction of both class I and II MHC antigens (Hofman, 1989) [see also Section 1.6.1]. IL-1 is also the cause of astrocytic proliferation (Gullian and Lachman, 1985) and with IFN γ promotes the release of a TNF-like factor from astrocytes (Chung and Benveniste, 1990) [see also Section 1.6.2]. Therefore the actions of cytokines within the central nervous system may account for several of the pathological changes observed in the CNS occurring in ADC.

HIV-1 infection of neural cell lines, and more prominently of primary glial cell cultures, would suggest that the general failure to detect HIV-1 nucleic acids or antigens in resident neural cells in AIDS encephalopathy (see Section 1.3.4) is a consequence of the latent or low level of replication observed *in vivo*. Indeed polymerase chain reaction (PCR) was required to detect HIV-1 infection in foetal CNS tissue from some seropositive

mothers (Lyman *et al.*, 1990). Therefore, latently infected neural cells and particularly GFAP+ cells may form another important reservoir of the virus (Tornatore *et al.*, 1991). Moreover, infection of glial and neuronal cells by HIV-1 could contribute to the pathogenesis of the AIDS dementia complex due to direct antagonism of cell function (Price *et al.*, 1988; Li *et al.*, 1990; Wiley and Nelson 1990; Cheng-Mayer and Levy, 1990).

1.4. The human immunodeficiency virus type 1

1.4.1. Virus and genetic structure

i). The virion

HIV-1 is a retrovirus of the sub-family lentivirinae. Morphologically viruses of this class have an elongated and tapered cylindrical nucleocapsid surrounded by a protein matrix internalized within a lipid bilayer containing transmembrane and surface viral glycoproteins (see Figure 1.1, p19). The nucleocapsid contains two copies of the viral genome, consisting of 9.2 Kb of single-stranded RNA, in association with the reverse transcriptase, protease and integrase proteins common to all retroviruses (Gallo and Montagnier, 1988), and an additional virus-specific protein, Vpr (Cohen *et al.*, 1990a).

ii). *Gag, pol and env*

In common with "classical" retroviruses the RNA genome of HIV-1 contains the typical *gag*, *pol* and *env* open reading frames that encode the virus structural proteins, the polymerase, protease and integrase functions and the membrane glycoproteins, respectively. However, the genetic structure of HIV-1 is more complex with an additional six genes located in different overlapping reading frames that produce non-structural regulatory proteins. A genetic map of the HIV-1 proviral DNA genome is represented in Figure 1.2, p28.

The primary translation product of the *gag* gene, p55, is proteolytically cleaved by the viral protease to produce four polypeptides, p24, p17, p7 and p9 which form the structural proteins of the capsid and matrix in the mature virus particle (di Marzo Veronese

et al., 1988; Haseltine *et al.*, 1990). The major structural component of the nucleocapsid is p24. The matrix protein, p17, forms the outer surface of the matrix and possibly interacts with the lipid bilayer through myristylation at its NH₂ terminus. The *gag* proteins p7 and p9, by analogy to other retroviruses, are thought to bind the viral RNA [see Figure 1.1] (Haseltine *et al.*, 1990). The reverse transcriptase, protease and integrase functions are encoded by the *pol* gene. These are produced from a *gag-pol* fusion protein which arises by a translational frame-shift and is subsequently proteolytically processed to generate distinct polypeptide species. In addition, the reverse transcriptase protein possesses a ribonuclease H-like function also essential for the conversion of the viral genome into the DNA proviral form (Haseltine *et al.*, 1990).

The envelope mRNA is translated to form the precursor molecule, gp160 which is post-translationally modified to produce the external, gp120, and transmembrane, gp41, glycoproteins of the virion (see Figure 1.1). Fully processed gp120 is heavily glycosylated and has more than 25 potential sites for N-linked glycosylation in the external region of the protein. The transmembrane protein, gp41 is also glycosylated though to a lesser extent (Haseltine *et al.*, 1990).

The gp120 protein binds to the CD4 antigen with high affinity and this molecule serves as a cellular receptor for HIV-1 (Matthews *et al.*, 1987). The CD4 antigen is expressed abundantly on the surface of T lymphoid cells of the helper subclass (T_H) and to a lesser extent on monocytes, macrophages and Langerhans cells. The interaction of gp120 with CD4 is the major determinant of the tissue tropism for HIV-1 (Haseltine *et al.*, 1990), although CD4 is not the sole receptor for HIV-1 (see Section 1.3.5. ii.).

iii). Accessory proteins

The genome of HIV-1 contains numerous open reading frames encoding non-structural proteins which perform a variety of functions that influence or control the complex life cycle of the virus. Several proteins, Tat, Rev and Nef regulate viral gene expression and are discussed in detail in Section 1.5.2, whilst Vif, Vpr and Vpu influence later stages in the replication of HIV-1.

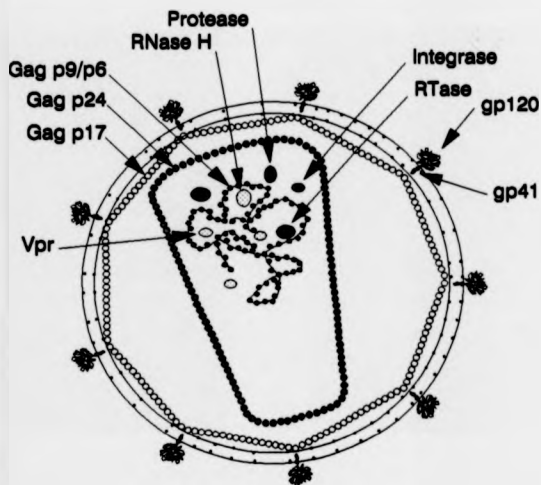


Figure 1.1. Structure of the human immunodeficiency virus type 1

Experiments on the *in vitro* infection of CD4+ cells with HIV-1 suggest that expression of the Vif protein, which remains in the cytoplasm of the infected cell, enhances the infectivity of the progeny virus particles (Haseltine *et al.*, 1990). Vif reportedly has a cysteine protease activity and modifies the processing and conformation of the *env* protein (Guy *et al.*, 1991).

Disruption of the *vpu* open reading frame results in the accumulation of cell surface-associated viral proteins and a decline in the release of progeny virus from infected cells, indicating that Vpu would appear to influence steps in the maturation of HIV-1 (Klimkait *et al.*, 1990). Recently Vpu has been shown to enhance the processing of the envelope precursor gp160 in CD4+ cells, where in the absence of Vpu the formation of intracellular complexes between CD4 and gp160 severely disrupts the production of gp120 (Willey *et al.*, 1992).

1.5. Control of HIV-1 replication

1.5.1. Latency

During the long asymptomatic period before the onset of life-threatening disease, HIV-1 clearly pursues strategies that avoid immune elimination and lead to the establishment of a persistent infection within susceptible cells (Fauci, 1988). From the analysis of infected T_H cells *in vivo*, it was found that around ten-fold more harboured latent proviral DNA than expressed detectable viral mRNA or protein (Garcia-Blanco and Cullen, 1991). Moreover, even in cells expressing viral antigens, virus replication appears to be controlled tightly, with the release of few infectious particles and little cytopathology (Haseltine *et al.*, 1990). In tissue culture HIV-1 has been found to establish a latent infection by two distinct pathways which are believed to function *in vivo*.

The infection of resting T_H cells is non-permissive for replication unless the cells are stimulated to leave the G₀ phase of the cell cycle (Rosenberg and Fauci, 1989). However, quiescent cells are still targets for virus binding and entry but reverse transcription is inefficient and there is a block on proviral integration (Garcia-Blanco and Cullen, 1991).

Yet, from *in vitro* experiments it appears that the extrachromosomal HIV-1 genomes remain viable for periods of days to weeks and may provide a reservoir of viral sequences (Cullen and Greene, 1989). This hypothesis was borne out by the direct examination of lymphocytes from asymptomatic individuals where full length HIV-1 DNA was found to remain episomal in a large proportion of resting lymphocytes and these genomes retained the ability to integrate upon cellular activation (Bukrinsky *et al.*, 1991). In monocytes there is no similar restriction on viral replication and productive HIV-1 infection can occur independent of cellular DNA synthesis prior to mitosis (Weinberg *et al.*, 1991).

Activation of CD4+ lymphocytes is normally transient *in vivo* and when cellular proliferation ceases cells re-enter G₀ and it is suggested that this may initiate a second period of latency in the form of an integrated but quiescent provirus (Fauci, 1988; Cullen and Greene, 1989). Monocytes *in vitro* will similarly maintain latent proviral DNA without productive replication or the release of virus (Meltzer *et al.*, 1990a). The maintenance of latency and re-activation of virus replication are, for the most part, controlled by a complex interplay between viral regulatory proteins and host cell factors with the long terminal repeat and other regulatory regions of the viral genome.

1.5.2. The regulatory genes of HIV-1 and viral replication

i). Viral gene expression

Primary RNA transcripts from the HIV-1 genome are subject to a complex series of splicing events that regulate the expression of structural and non-structural gene products (Felber *et al.*, 1989). During the early phases of HIV-1 replication, in the absence of significant Rev function, unspliced and singly spliced RNAs accumulate in the nucleus but only the short (2 kb) multiply-spliced mRNAs reach the cytoplasm and lead predominantly to the expression of the regulatory genes, *tat*, *rev* and *nef* (Cullen and Greene, 1989). The *gag* and *pol* structural genes of the virus are expressed only from unspliced (9.2 kb) mRNA that serve also as HIV-1 genomes, and *env* from singly-spliced (4.3 kb) mRNAs (Pavliakis *et al.*, 1991). These mRNAs are dependent on the interaction of Rev and the *cis*-acting *rev*-responsive element (RRE) located within the envelope gene for their expression and in

the early phases accumulate in the nucleus (Emerman *et al.*, 1989). In the later stages of virus replication full length mRNAs begin to appear due to the action of Rev which controls processes leading to the cytoplasmic appearance of unspliced or singly-spliced RNAs containing the RRE (Cullen and Greene, 1989). In doing so Rev coordinately represses its own synthesis and that of the other regulatory proteins Tat and Nef (Felber *et al.*, 1990). Although *tat* is also encoded by a singly-spliced mRNA containing the RRE (Pavlakis *et al.*, 1991). Thus RNA synthesis switches to the production of viral genomes and structural proteins (Cullen and Green, 1989).

ii). *rev*

The *rev* gene product is a 19 Kd phosphoprotein (Cochrane *et al.*, 1989) located primarily in the nucleolus of infected cells and is encoded by several multiply-spliced mRNAs (Pavlakis *et al.*, 1991). Rev action requires binding to the RRE which is a highly structured, stem loop RNA element of 250 nucleotides in the central region of the *env* coding sequence [see Figure 1.2, p28] (Sharp *et al.*, 1991). The function of Rev appears to depend on the relatively inefficient process of splicing affecting HIV-1 mRNAs and the interaction of splice sites with the cellular splicing machinery being sufficient to retain transcripts in the nucleus. The interaction of Rev with the RRE either regulates the formation, or promotes the dissociation of spliceosome-RNA complexes allowing unspliced precursor transcripts to be transported to the cytoplasm (Chang and Sharp, 1989). A report that a HIV-1 intron regulated by Rev is stabilized by forming a complex with U1 snRNP, a spliceosome component, would also support this hypothesis (Lu *et al.*, 1990). Furthermore, the additional interaction of cellular proteins and the RRE might suggest that Rev is interrupting a normal cellular process (Wong-Staal *et al.*, 1991).

Other regulatory sequences, the *cis*-acting repressive sequences (CRS) located in the *gag*, *pol* and *env* open reading frames serve to repress expression of the structural genes and contribute to the retention of these RNAs within the nucleus through a mechanism that can be alleviated by Rev in the context of the RRE (Rosen *et al.*, 1988; Cochrane *et al.*, 1991). These are thought to represent additional sites that promote recognition with components

of the splicing machinery (Sharp *et al.*, 1991; Maldarelli *et al.*, 1991). Indeed cellular factors have been found to interact with CRS elements and monoclonal antibodies against specific splicing components interfere with complex formation (Cochrane *et al.*, 1991). However, there is a body of evidence from the analysis of CRS elements in heterologous systems that suggests Rev also acts to improve the translational capacity of RRE-containing mRNAs (Arrigo & Chen, 1991; Cochrane *et al.*, 1991).

iii). tat

The *tat* gene is encoded by three multiply-spliced and one singly spliced transcripts. Its expression is believed to be independent of Rev action during early events and dependent on Rev in the later phases of replication (Cullen and Greene, 1989; Pavlakis *et al.*, 1991). Tat is a 14 Kd nuclear protein located predominantly in the nucleolus (Kalland *et al.*, 1991) which functions as a powerful transactivator capable of raising viral mRNA and protein expression by over 100-fold and, like rev, is required for viral replication (Sharp and Marciniak, 1989). The specificity of Tat-mediated transactivation resides in the transactivation response element, TAR, which is positioned from nucleotides -17 to +80 (relative to the start of transcription) and is located at the 5' termini of all HIV-1 transcripts. The TAR RNA sequence is orientation and positionally dependent and forms a stable base-paired stem-loop structure that specifically binds Tat at a bulge region located just below the hairpin loop (Dingwall *et al.*, 1990).

Transactivation by Tat increases the levels of steady-state RNA from the HIV-1 LTR by around 20-fold and further enhances the translational capacity of the mRNA 5-fold (Cullen, 1986; Sharp and Marciniak, 1989). However, the primary function of Tat is to stabilize the elongation of transcripts from the LTR, the rise in the rate of transcription initiation is believed to be a secondary effect (Cullen, 1990). The HIV-1 LTR is an active but relatively inefficient promoter in that the majority of nascent transcripts terminate within 50 to 100 bp of the cap site, yet in the presence of Tat the proportion of transcripts which proceed and result in a full length polyadenylated mRNAs significantly increases (Ratnasabapathy *et al.*, 1990). The initiation complexes become more processive in the

presence of Tat and raises the proportion of full length transcripts without greatly affecting the total number (Ratnasabapathy *et al.*, 1990; Sharp *et al.*, 1991). Further *in vitro* analysis of transcription from LTR-linked templates of varying length indicates that two forms of initiation/elongation complexes assemble on the HIV-1 LTR which are either more or less processive and Tat functions to increase the ratio of the more processive type by 10-fold (Marciniak and Sharp, 1991).

Cellular factors are also implicated in the control of transactivation by Tat (Newstein *et al.*, 1990) and several RNA-binding proteins have been detected that interact directly with TAR (Marciniak *et al.*, 1990; Gatignol *et al.*, 1989; Waterman *et al.*, 1991a). Interestingly, the binding of individual proteins has been demonstrated to both the hairpin loop and the Tat-binding site and cellular factors appear to bind in conjunction with, or in competition against Tat (Waterman *et al.*, 1991a; Wu *et al.*, 1991). Furthermore one protein, TRP-185, which specifically interacts with the Tat-binding bulge region will activate transcription from the LTR *in vitro* (Wu *et al.*, 1991). This, together with the data of discussed above further suggests that Tat manipulates a cellular process (of transcriptional elongation) that is part of a more widely regulated process in mammalian cells than previously anticipated (Marciniak and Sharp, 1991).

Tat may also exert an effect on the translational capacity of mRNAs through a direct interaction with TAR such that efficient translation of TAR-containing mRNA requires exposure to Tat within the nucleus (Braddock *et al.*, 1989). This finding has gone largely unexplained, and its relevance difficult to assess since experiments were performed by microinjection of *Xenopus* oocytes.

iv). Regulation of gene expression by TAR

The interferon-induced p68 kinase is induced and activated by the double stranded RNA that forms the TAR element of HIV-1 mRNAs (SenGupta and Silverman, 1989) and this in turn enhances phosphorylation of eIF-2 and represses translation (Laurence, 1990). Many RNA viruses have evolved strategies to inactivate or sequester p68 kinase to avoid its detrimental effects on viral protein expression and HIV-1 is no exception. The p68 kinase

binds TAR RNA with high affinity at sites on TAR distinct from those for Tat and TAR-binding factors and it is suggested that this mechanism may contribute to limit the expression of viral RNAs in latently infected cells and regulate the levels of regulatory proteins (Roy *et al.*, 1991). Conversely, in actively replicating cultures p68 kinase levels are decreased and the actions of Tat may account for the observed repression (Roy *et al.*, 1990). Another interferon-regulated enzyme 2'-5' oligoadenylate synthetase is also induced by TAR RNA (SenGupta and Silverman, 1989) and leads to the activation of the single strand specific RNase L (Laurence, 1990). Similarly Tat has been reported to block this activation (Schroder *et al.*, 1990). The production of short stable RNA transcripts from the HIV-1 LTR also maps to the TAR element and these may bind Tat keeping it functionally inactive by removing it from the transcription unit (Ratnasabapathy *et al.*, 1990). Therefore the HIV-1 TAR sequence may negatively regulate viral protein expression and aid the maintenance of latency.

v). *nef*

The *nef* gene encodes two closely related proteins of 25 and 27 Kd by the alternative use of a downstream internal methionine codon for the initiation of translation (Kaminchik *et al.*, 1991). The majority of Nef p27 is myristylated at its NH₂-terminus and located mainly perinuclearly in the Golgi complex and also at the nuclear membrane (Ratner *et al.*, 1991; Ovod *et al.*, 1992). Nef p25 lacks the myristylation residue and is consequently restricted to the cytoplasm (Kaminchik *et al.*, 1991). The alternative initiation codon (position 20) is not universally conserved amongst all HIV-1 isolates, yet the two forms of Nef occur in strains where it is absent (Guy *et al.*, 1990a).

Expression of Nef is from multiply-spliced mRNAs and is therefore independent of regulation by Rev (Pavliakis *et al.*, 1991). In addition to myristylation, Nef is phosphorylated, capable of autophosphorylation, and *in vitro* possess weak GTP-binding and GTPase activities which indicated a certain degree of homology to G proteins and implies a role in signal transduction (Guy *et al.*, 1987; Guy *et al.*, 1990a).

The precise function of Nef has still not been determined. Early reports indicated that Nef served to down-regulate transcription from the LTR up to 10-fold *in vitro* and the effect required a specific region of the LTR, the negative regulatory element [NRE; see Figure 1.2] (Ahmad and Venkatesan, 1988). *In vivo* RNA accumulation was similarly reduced and viral replication decreased 30- to 50-fold, although these investigators mapped the LTR sequences involved to a position over the cap site (Niederman *et al.*, 1989). Studies on the interaction of DNA-binding proteins supported the data of Ahmad and Venkatesan (1988) who later confirmed their original work (Maitra *et al.*, 1991), and indicated that Nef operated at the level of transcription, as a cellular activation associated factor that bound to the NRE was found to be down-regulated in Nef expressing cells (Guy *et al.*, 1990b). Yet, work presented by other groups disputed both the *in vivo* and *in vitro* findings and concluded that Nef had no negative function on viral transcription or replication (Kim *et al.*, 1989; Hammes *et al.*, 1989). Careful manipulation of proviral DNA of several HIV-1 strains to create chimeric proviruses and isogenic *nef*-defective versions indicates that sequences within *env* regulate the effect of *nef* on viral replication and this may be positive or negative depending on the isolate and cell line examined (Terwilliger *et al.*, 1991). Recently it has again been demonstrated that Nef suppresses HIV-1 LTR-driven expression but only through the actions of myristylated, membrane-bound p27 (Yu and Felsted, 1992). In addition, Nef will also down-regulate the levels of the CD4 antigen on the cell surface of cell lines stably expressing Nef (Guy *et al.*, 1990a; Garcia and Miller, 1991). This effect does not occur at the level of transcription and results in the cytoplasmic accumulation of CD4 (Garcia and Miller, 1991). Furthermore similar cell lines expressing Nef exhibit an altered signalling pathway via the T cell receptor that does not result in the expected expression of IL-2 mRNA after induction with PHA and PMA. Activation of the IL-2 promoter is defective yet augmentation of expression from the HIV-1 LTR is unaffected (Luria *et al.*, 1991).

Messenger RNAs encoding Nef predominate in early infection [77%] and far outweigh the level of *rev* transcripts [20%], whereas mRNAs for *tat* account for only a small minority [3%] (Robert-Guroff *et al.*, 1990). The overall conclusion from the known

functions of Nef strongly implicate it as having a central role in the establishment and maintenance of proviral latency (Guy *et al.*, 1987; Guy *et al.*, 1990a; Guy *et al.*, 1990b; Garcia and Miller, 1991; Luria *et al.*, 1991). The *nef* open reading frame may further determine viral pathogenesis, suggested by the finding that rhesus monkeys infected with the simian immunodeficiency virus SIV_{mac} containing a deletion in *nef* survive infection whilst those receiving wild type virus die of AIDS (Groopman, 1991).

vi). *vpr*

The 15 Kd Vpr protein is, like Nef, Vif and Vpu, dispensable for virus replication *in vitro* (Cohen *et al.*, 1991) and differs from the other regulatory genes discussed here in that it is expressed from a singly-spliced mRNA and is subject to regulation by Rev (Arrigo and Chen, 1991). Evidence of expression is found in all seropositive individuals (Dedera *et al.*, 1989) and would occur late in the replication cycle when structural proteins begin to accumulate (Cohen *et al.*, 1991). Vpr is a virion-associated protein and multiple copies of Vpr are found in mature virus particles, suggesting that it may be the first viral gene product to function in the infected cell (Cohen *et al.*, 1990a). The inactivation of the 96 amino acid allele of *vpr* results in reduced cytopathogenicity and delayed replication kinetics compared to wild type, isogenic virus (Ogawa *et al.*, 1989). However, unless infectious virus particles are used, derived originally from *vpr*⁺ and *vpr*⁻ proviruses, no differences are observed following infection of susceptible cells (Dedera *et al.*, 1989). This correlates with the reported inclusion of Vpr in the virion and implies that the protein creates a cellular environment more permissive for the early stages of viral replication (Cohen *et al.*, 1990a; Cohen *et al.*, 1990b). Vpr was demonstrated subsequently to encode a second viral transactivator that increased gene expression from the HIV-1 LTR 3-fold (Cohen *et al.*, 1990b). Deletion analysis of the LTR indicates that transactivation requires sequences within the NRE downstream of nucleotide position -167 [relative to the start of transcription] (Cohen *et al.*, 1990b). Vpr also transactivates the HIV-2 LTR and other viral promoters, such as those of CMV and HTLV-I to a similar degree (Cohen *et al.*, 1990b; Cohen *et al.*, 1991).

The genetic structure of the HIV-1 proviral genome

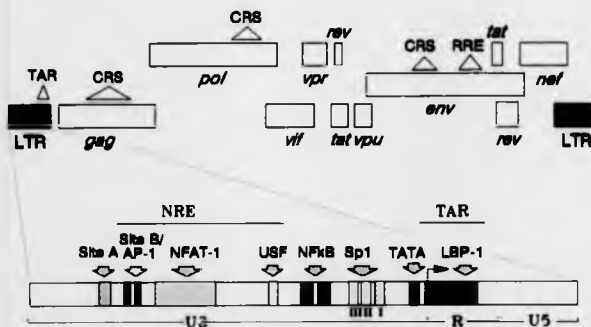


Figure 1.2. Genetic structure of the HIV-1 proviral genome and long terminal repeat (LTR). Top. The genetic map of HIV-1, indicating open reading frames (open boxes), LTRs and other cis-acting regulatory sequences: TAR; Transactivation-response element. CRS; cis-acting repression sequence. RRE: rev-responsive element. Bottom. Location of major protein binding sites within the LTR. NRE; negative regulatory element encompassing Site A, Site B (also AP-1 sites), NFAT-1 and USF. The enhancer of two NFkB sites which is upstream of three Sp1 binding sites, the TATA box and four LBP-1 sites within TAR. The arrow TAR indicates the location of the transcriptional start.

1.5.3. Activation of HIV-1 replication

It is apparent from the ultimate progression to disease that HIV-1 does not maintain an entirely quiescent state forever and the close association between the pattern of virus replication and the status of host cell indicates that specific cellular signals will re-activate latent HIV-1. Clearly the identification of cellular signals which lead to augmented HIV-1 transcription and the production of virus will be important in the understanding of latency.

i). Induction of HIV-1 gene expression: role of cytokines

After the initial cellular stimulation and proliferation required to permit the integration of the HIV-1 provirus (Garcia-Blanco and Cullen, 1991), CD4+ lymphocytes return to the G₀ phase of the cell cycle and enter a quiescent state as memory cells (Greene, 1990). Subsequent exposure of memory cells to challenge with their cognate antigen in the context of a proper MHC interaction will re-activate events leading to their proliferation and acquisition of immunological function (Crabtree, 1989). Signals emanating from the T cell receptor also initiate events leading to augmented expression from the HIV-1 LTR (Tong-Starksen *et al.*, 1989). The enhanced transcription from the LTR, probably amplified through the increased synthesis of Tat (Rosenberg and Fauci, 1990), leads to a rise in the level of Rev which alters the pattern of mRNA processing and produces a shift from regulatory to structural gene expression and productive viral replication (Cullen and Greene, 1989; Pomerantz *et al.*, 1990).

The exposure of CD4+ T lymphocytes to antigen and the accessory signal provided by the antigen-presenting cell are necessary for the activation of T cells and induction of IL-2 synthesis (Crabtree, 1989) and similarly both signals facilitate the maximal induction of expression from the HIV-1 LTR (Tong-Starksen *et al.*, 1989). In addition, HIV-1 expression will respond sub-optimally to non-mitogenic signals such as stimulation of CD2 or CD28 T cell surface antigens with antibody to mimic physiological activation independent of the T cell receptor. Also, signalling via the lymphocyte homing antigen and hyaluronin acid receptor, CD44, enhances HIV-1 LTR-driven gene expression to a lesser

extent. Moreover, CD28 stimulation synergizes with the cytokine-induced activation of protein kinase C stimulated by exposure to phorbol ester (Gruters *et al.*, 1991).

The process of T cell activation ultimately leads to the production of cytokines, in addition to IL-2, which mediate diverse immune reactions (Roitt *et al.*, 1985). Immunologically competent T lymphocytes secrete IFN α , IFN γ , IL-3 and GM-CSF, and the synergistic action of IL-2 and IFN γ lead to TNF β release. In the induction of the antibody response from B cells, IL-6 will also be released (Hamblin, 1988). Certain cytokines prompt intracellular events that lead to the activation of HIV-1 gene expression (Okamoto *et al.*, 1989) [see Section 1.5.3. ii, iii and iv] and in lymphocytes, HIV-1 replication is strongly enhanced by TNF, (Matsuyama *et al.*, 1989a; Ito *et al.*, 1989; Matsuyama *et al.* 1989b; Vyakarnam *et al.*, 1989) a cytotoxic cytokine with many functions including those central to inflammatory reactions (Hamblin, 1988).

Furthermore TNF α activates HIV-1 replication in macrophages (Poli *et al.*, 1990a; Rosenberg and Fauci, 1990) and HIV-1 replication in monocytic cells is activated by a significantly wider range of cytokines than is known for lymphocytes, with IL-3 (Koyanagi *et al.*, 1988; Schuitemaker *et al.*, 1990), IL-6 (Poli *et al.*, 1990a), M-CSF (Koyanagi *et al.*, 1988; Meltzer *et al.*, 1990a) and GM-CSF (Koyanagi *et al.*, 1988; Schuitemaker *et al.*, 1990) all increasing virus replication, an effect that does not rely upon a mitogenic response by the host cell (Koyanagi *et al.*, 1988; Poli *et al.*, 1990a). The increasing exposure of macrophages to IFN γ also primes and then activates these cells to produce IL-1, TNF α and reactive oxygen metabolites involved in immune and inflammatory reactions (Hamblin, 1988). Exposure of HIV-1 infected T cells to oxidative stress mediated by these metabolites induces HIV-1 replication and the efficient replication of HIV-1 in macrophages may also be a consequence of cellular mechanisms for the production of an effective oxidative burst (Schreck *et al.*, 1991).

In contrast, HIV-1 replication can be negatively regulated by IFN $\alpha\beta$ in both lymphocytes and macrophage-derived cell lines (Fernie *et al.*, 1991) and IFN γ may have a limited antiviral effect *in vivo* (Harshorn *et al.*, 1987). The action of IFN α , however, does

not greatly reduce virus replication as such, but rather limits the release of mature virions from chronically infected lymphoblastoma and promonocytic cells (Femie *et al.*, 1991).

Infection with a heterologous viruses has been implicated as a co-factor in the stimulation of HIV-1 replication and experiments using HIV-1 LTR-driven reporter gene constructs have identified a number of gene products from DNA viruses and another retrovirus that transactivate expression from the LTR. Cytomegalovirus early proteins IE1 and IE2 are potent inducers of LTR-driven gene expression (Barry *et al.*, 1990) and further synergize with signals that activate HIV-1 expression in lymphocytes (Paya *et al.*, 1991). In addition, the presence of CMV regulatory proteins, under the control of the CMV promoter, confers indirect inducibility to the HIV-1 LTR via another signalling pathway [PKA-dependent] (Paya *et al.*, 1991). The X protein from HBV (Siddiqui *et al.*, 1989), Tax protein from HTLV-1, ICP-O protein from HSV-1, a gene product from the polyoma virus JC (Rosenberg and Fauci, 1989) and co-infection of lymphocytes with HHV-6 (Ensoli *et al.*, 1989) or HSV-1 (Gimble *et al.*, 1988) all result in the up-regulation of HIV-1 replication.

ii). Interaction of cellular transcription factors and the HIV-1 LTR

The 630 bp long terminal repeat of the human immunodeficiency virus type-1 contains a unique array of *cis*-acting regulatory elements (see Figure 1.2) that interact with specific cellular factors to coordinate viral transcription (Greene, 1990; Orchard *et al.*, 1990; Waterman *et al.*, 1991a). These transcription factors control basal expression from the LTR and mediate the induction of expression upon activation of the host cell and are therefore likely to be the initial determinants that release the provirus from latency (Siekevitz *et al.*, 1987; Waterman *et al.*, 1991a).

iii). Inducible factors: NF κ B and NFAT-1

The physiological activation of T_H lymphocytes via the T cell receptor, in conjunction with the accessory signal simulated by PMA, triggers two signal transduction mechanisms in an overlapping manner; a rise in the intracellular calcium levels, and protein kinase C (PKC) translocation (Crabtree, 1989). Stimulation of the PKC-dependent pathway

results in the functional activation and nuclear localization of members of the NF κ B family of transcription factors (Franza *et al.*, 1987; Bohnlein *et al.*, 1988; Crabtree, 1989; Molitor *et al.*, 1990), whilst both stimuli lead to the *de novo* synthesis of the NFAT-1 transcription factor (Crabtree, 1989; Hivroz-Burghaud *et al.*, 1991). Neither of these factors are found in unstimulated T lymphocytic cell lines (Shaw *et al.*, 1988; Molitor *et al.*, 1990). NF κ B-binding factors interact with tandem recognition sites that constitute a strong enhancer in HIV-1 (Siekevitz *et al.*, 1987; Osborn *et al.*, 1989) and NFAT-1 binds to specific sequences further upstream within the same U3 region of the LTR [see Figure 1.2] (Shaw *et al.*, 1988).

HIV-1 gene expression is concomitantly induced following these stimuli and deletion analysis of the LTR demonstrates that the effect is predominantly due to the interaction of NF κ B factors with the enhancer (Siekevitz *et al.*, 1987; Tong-Starksen *et al.*, 1989; Tong-Starksen *et al.*, 1990). Although the integrity of the NFAT-1-binding domain is required for maximal induction of the LTR (Siekevitz *et al.*, 1987; Tong-Starksen *et al.*, 1989), it does not activate LTR-driven expression significantly in the absence of a functional enhancer (Tong-Starksen *et al.*, 1989).

iv). The NF κ B family of DNA-binding proteins

The induction of NF κ B also correlates with the augmentation of HIV-1 gene expression (Israël *et al.*, 1989; Osborn *et al.*, 1989) and replication in lymphocytic and monocytic cells by TNF α (Meltzer *et al.*, 1990a; Rosenberg and Fauci, 1990) and transactivation due to co-infection by HSV-1 (Gimble *et al.*, 1988), HHV-6 (Ensoli *et al.*, 1989) and the HTLV-1 *tax* gene product (Rosenberg and Fauci, 1989). Furthermore NF κ B-like factors are also induced by IL-1 in certain cell lines (Osborn *et al.*, 1989; Brasier *et al.*, 1990).

Moreover, the interest in NF κ B created by its involvement in HIV-1 expression has also highlighted the central role that NF κ B has in the intracellular events leading to the initiation of immune reactions, as many critical genes contain binding sites for this and its related transcription factors. The NF κ B motif has been described in the promoter regions of genes encoding the cytokines IL-1 (Trede *et al.*, 1991), IL-2, IL-6, GM-CSF, M-CSF,

TNF α , TNF β and IFN β (Baeuerle, 1991) and the intracellular adhesion molecule I-CAM1 (Stade *et al.*, 1990), class I MHC antigens (Baldwin and Sharp, 1988), the T cell receptor β -chain, immunoglobulin light chain, β_2 -microglobulin (Baeuerle, 1991) and IL-2 receptor α -chain (Bohnlein *et al.*, 1988).

Analysis of the inducible factors which bind to the HIV-1 enhancer reveals a family of structurally and functionally related polypeptides recognizing the NF κ B motif. These represent the NF κ B complex of *rel*-related proteins which bind DNA as heterodimers [or pairs of heterodimers, Baeuerle, 1991] of 50 Kd DNA-binding (p50 or p50B) and 65 Kd (p65) transmodulator proteins (Ghosh *et al.*, 1990; Ballard *et al.*, 1990; Bours *et al.*, 1992), and products of the proto-oncogene *c-rel* and the related gene *relB*, that also bind as heterodimers with members of NF κ B and other as yet unknown cellular proteins (Franza *et al.*, 1987; Molitor *et al.*, 1990; Lee *et al.*, 1991; Hansen *et al.*, 1992; Ryseck *et al.*, 1992).

The *c-rel* and NF κ B group of transcriptional activators share many properties that underlie their role as signal transducers. The p50 or the related p50B subunit of NF κ B contain dimerization and DNA-binding domains (Ghosh *et al.*, 1990; Bours *et al.*, 1992) and are present in the cytoplasm in a transcriptionally inactive form complexed via p65 or *relB* to an inhibitory subunit I κ B (Urban and Baeuerle, 1990; Ryseck *et al.*, 1992). Agents that stimulate the PKC-dependent signalling pathway induce phosphorylation of I κ B, which exists in two forms α and β , and permit the translocation of NF κ B to the nucleus where it activates expression of genes containing the NF κ B motif (Baeuerle and Baltimore, 1988; Baeuerle, 1991; Kerr *et al.*, 1991). I κ B β inhibition of NF κ B action is additionally responsive to PKA-dependent phosphorylation and I κ B β is probably identical to the *rel*-associated protein pp40 (Baeuerle, 1991; Kerr *et al.*, 1991). The *c-rel* proteins are also found in a cytoplasmic complex with a protein that represents the precursor of p50B or the precursor of p50 (p105), and an inhibitory subunit (Kerr *et al.*, 1991) and exhibits similar cytosol to nuclear translocation upon cellular activation (Franza *et al.*, 1987; Molitor *et al.*, 1990; Lee *et al.*, 1991). The *c-rel* gene product p85 has recently been shown to bind to the NF κ B motif as a complex with the p65 subunit of NF κ B (Hansen *et al.*, 1992) and *in vitro* forms complexes with p105 or a protein analogous to p50B (Schmid *et al.*, 1991).

NF κ B p50/p65 (Kawakami *et al.*, 1988), p50/RelB (Ryseck *et al.*, 1992), p50B/p65, p50B/RelB (Bours *et al.*, 1992) and c-Rel (Muchardt *et al.*, 1992) will transactivate expression from a promoter containing tandem NF κ B sites and the HIV-1 LTR. The NF κ B/*rel* proteins now constitute a growing family of transcriptional activators and DNA-binding proteins that can mix and form heterodimers which can specifically interact with the NF κ B motif, a situation analogous to products of the *fos* and *jun* proto-oncogenes.

HIV-1 infection also seems specifically to induce nuclear NF κ B during the course of infection and higher levels of active NF κ B are found in cells undergoing a productive infection (Bachelier *et al.*, 1991). *In vitro* the HIV-1 protease will process the precursor of the p50 DNA-binding subunit of NF κ B p105 to a 45 Kd protein capable of interacting with NF κ B p65 which can bind DNA and may contribute to the increased levels of active NF κ B (Rivi re *et al.*, 1991). This process may also contribute to the autocrine stimulation of constitutive and inducible virus replication by TNF apparent in chronically infected cells (Poli *et al.*, 1990b). This cytokine both induces NF κ B binding activity and contains NF κ B regulatory domains in its promoter region and may mobilize an increased intracellular pool of inactive NF κ B due to the actions of the HIV-1 protease. The same phenomenon may apply to IL-6 which is induced by TNF α (Kohase *et al.*, 1986) and stimulates HIV-1 replication in monocytes, predominantly via a post-transcriptional mechanism (Poli *et al.*, 1990a), and also contains an NF κ B binding site in its promoter (Hirano *et al.*, 1990). Furthermore the promoter of the gene encoding p105, the precursor of p50 NF κ B, contains multiple NF κ B motifs and expression from this promoter is up-regulated by phorbol ester and TNF α , a process that should replenish the cell with part of the NF κ B complex (Ten *et al.*, 1992).

Phorbol esters act via PKC in the induction of NF κ B yet TNF does not invoke the same pathway (Baeuerle, 1991). Indeed, TNF acts through a mechanism independent of PKC-, PKA- and calcium-regulated protein kinases and demonstrates that other uncharacterized signalling pathways also lead to the modification of I κ B (Feuillard *et al.*, 1991).

In addition, there is evidence to suggest that PKC induces another transcription factor AP-1 which plays a role in the T cell expression of IL-2 (Crabtree, 1989). The HIV-1 LTR contains two AP-1 recognition sites within the NRE (Franza *et al.*, 1988) and three sites located in the *pol* open reading frame (Van Lint *et al.*, 1991). Furthermore both sites in the HIV-1 genome bind AP-1 (Franza *et al.*, 1988; Van Lint *et al.*, 1991) but AP-1 does not appear to contribute to the inducibility of gene expression directed by the HIV-1 LTR (Siekevitz *et al.*, 1987).

v). Constitutive NF κ B expression

The p65 subunit of NF κ B regulates the subcellular localization of the active complex, such that in T cells NF κ B is only translocated to the nucleus when the cells are activated by the appropriate stimulus (Urban and Baeuerle, 1990). Constitutive NF κ B-binding factors have been identified as H2TF1 (Baldwin and Sharp, 1988), KBF1 (Yano *et al.*, 1987) and EBP-1 (Wu *et al.*, 1988) in some cell lines such as HeLa. These factors are homodimers of p50 molecules and are transcriptionally inactive (Kieran *et al.*, 1990; Baeuerle, 1991). Only heterodimers (see above) strongly transactivate gene expression (Schimtz and Baeuerle, 1991). Active heterodimeric NF κ B is constitutively present in the nucleus of only a restricted range of cells, such as mature B cells, monocytes and macrophages (Baeuerle, 1991). In these cells NF κ B expression becomes activated as these cells undergo terminal differentiation and is associated only with the mature phenotype (Griffin *et al.*, 1989; Baeuerle, 1991). The constitutive levels of active NF κ B may contribute to the different characteristic of HIV-1 infection in monocytes compared to lymphocytes where there are higher levels of viral transcripts and steady production of infectious particles that are sequestered in intracellular vacuoles (Gendelman *et al.*, 1989).

vi). Basal transcription from the HIV-1 LTR

The HIV-1 LTR contains three GC-rich sites, located downstream of the TATA box, which bind the sequence-specific transcription factor Sp1 [see Figure 1.2] (Jones *et al.*, 1986). Sp1 falls generally into the class of promoter proximal transcription factors and

binding sites are found in a wide variety of viral and cellular promoters (Mastrangelo *et al.*, 1991). A single Sp1 binding site can activate transcription from a synthetic promoter *in vitro* and the interaction of Sp1 with the HIV-1 LTR contributes to the basal level of expression (Jones *et al.*, 1986). The third Sp1 site has the highest affinity for Sp1 (Jones *et al.*, 1986) and contrary to what may be expected there is no co-operation in Sp1 binding to adjacent sites (Courey *et al.*, 1989). Site-directed mutagenesis to disrupt the individual Sp1 sites does not effect transcription from the LTR but the inactivation of all three sites will result in up to a ten-fold reduction (Jones *et al.*, 1986; Harrich *et al.*, 1989).

The functional integrity of the TATA box present in the HIV-1 LTR is necessary for full promoter activity and contributes to the basal level of expression (Jones *et al.*, 1988) through the interaction with TFIID and the other components of RNA polymerase II initiation complex (Greenblatt, 1991). Additionally the TATA box is required for the correct initiation of HIV-1 transcripts (Jones *et al.*, 1988). Downstream of the transcription initiation site also lie four sites for the leader binding protein, LBP-1 which forms another basal promoter element of the LTR (Waterman *et al.*, 1991a). Mutations in this region have a negative effect on transcription from the LTR *in vitro* and confirm that the 63 Kd LBP-1 protein stimulates transcription (Jones *et al.*, 1988). Although these binding sites are located in the TAR element, conservative mutagenesis that disrupts LBP-1 sites but not important residues of TAR indicate that these motifs function as DNA recognition sites for LBP-1 (Jones *et al.*, 1988). However, another factor, UNF 1 exhibits identical sequence-specificity but binds the same sequence in both DNA or RNA (Gaynor, 1991).

LBP-1 may also negatively regulate transcription from the LTR as an additional site overlapping the TATA box which binds LBP-1 with lower affinity was observed with purified LBP-1 (Kato *et al.*, 1991). Interaction of LBP-1 with this site competes with TFIID for binding to the TATA box *in vitro* and mutations which abolish LBP-1 binding but leave TFIID intact increased transcription from the LTR in cell lines (Kato *et al.*, 1991).

vii). *Negative regulatory element*

Deletion of the 5' portion (nucleotide positions -420 to -157) [see Figure 1.2] of the HIV-1 LTR significantly enhances gene expression directed by the LTR (Siekevitz *et al.*, 1987) and the insertion of this 263 bp fragment downstream of a heterologous promoter, such as SV40 late or HTLV-1, suppresses promoter activity (Lu *et al.*, 1991). Thus *cis*-acting sequences within this region have a negative effect upon transcription and constitute the NRE.

Interaction with one cellular factor, USF, originally characterized as a positive regulator of expression from the adenovirus major late promoter (Pognonec and Roeder, 1991), provides a considerable proportion of the repressive effect on HIV-1 LTR-driven gene expression (Lu *et al.*, 1990). An identical sequence is present in the IL-2 receptor α -chain gene promoter and this interacts with the same factor that recognizes the HIV-1 LTR and also negatively regulates gene expression (Smith and Greene, 1989). The USF region has been shown to interact with three cellular proteins of different molecular weights, 44 kd representing a USF-like protein (Smith and Greene, 1989; Giacca *et al.*, 1992) and 70 and 110 kd, which are all constitutively expressed and, although whilst USF has been demonstrated to repress gene expression from the LTR, the functions of the other two have not been clearly defined (Giacca *et al.*, 1992). The USF interaction exerts a similar negative effect on the LTR in the presence (Lu *et al.*, 1990) or absence of Tat and after cellular stimulation with phorbol ester (Giacca *et al.*, 1992).

The NRE contains a palindromic binding site designated Site B which interacts with constitutive DNA-binding proteins that are members of the steroid hormone receptor super-family (Orchard *et al.*, 1990). Site B was also demonstrated to interact with the human homologue of the chicken transcription factor COUP, a member of the steroid hormone family (Cooney *et al.*, 1991) and this may account for the binding activity observed by Orchard *et al.*, (1990). Mutation of this region to destroy one half site of the palindrome increases gene expression directed by the LTR in the presence of Tat (Orchard *et al.*, 1990) as does deletion of the entire domain in the absence of Tat transactivation (Zeichner *et al.*,

1991). Therefore the interaction of Site B-binding factors also appears to contribute towards the effect of the NRE.

Another protein-binding site within the NRE, Site A also interacts with constitutive DNA-binding factors (Orchard *et al.*, 1990) but deletion of this region does not effect gene expression directed by the LTR in the presence (Lu *et al.*, 1990) or absence (Zeichner *et al.*, 1991) of Tat. Furthermore deletion of the NFAT-1 domain which is located in the negative regulatory region does not effect the level of gene expression from the LTR in unstimulated cells (Zeichner *et al.*, 1991).

1.6. Neuroimmunology

The central nervous system may form a distinct environment for the development and pathogenesis of HIV-1 infection, and immune responses occurring within the brain are likely to be modulated within the constraints of CNS immunology. The CNS has long been referred to as an immunologically privileged site because of the lack of lymphatic drainage and the physical separation by the blood brain barrier (Frei and Fontana, 1989). The penetration of the CNS by the cellular components of the immune system may be minimal and immune surveillance low, yet the CNS retains the ability to initiate responses against neurovirulent pathogens and the effector functions of lymphocytes are intact in this environment (Borgeason *et al.*, 1989; Frei and Fontana, 1989). In order to maintain the CNS as a site of limited immune reactivity and minimize immunopathological damage to important neuronal and oligodendroglial cells evidence suggests that astrocytes, microglia, and capillary brain endothelial cells to some extent, mediate the interaction with components of the immune system and possess a number of important immunoregulatory properties (Hughes *et al.*, 1988; Frei and Fontana, 1989).

1.6.1. Antigen presentation and major histocompatibility antigen expression within the CNS

The absence or low levels of MHC antigens on resting neural cells undoubtedly contributes to the partial shielding of the brain from immune reactions (Pryce *et al.*, 1986). MHC class I antigens are not displayed on the surface of neurons or oligodendrocytes *in vivo* and their expression is limited to microglial cells, and a subset of astrocytes and brain endothelial cells (Pryce *et al.*, 1986; Mauerhoff *et al.*, 1988; Frei and Fontana, 1989). IFN γ and TNF α will induce MHC class I expression on oligodendrocytes (Suzumura *et al.*, 1986; Mauerhoff *et al.*, 1988) and both cytokines will function to increase the proportion of MHC class I+ astrocytes and endothelial cells (Pryce *et al.*, 1986) but will not stimulate MHC class I antigen expression on neurons (Mauerhoff *et al.*, 1988; Borgeson *et al.*, 1989). This a property that protects neurons but not astrocytes from class I restricted T cell-mediated cytotoxicity (Borgeson *et al.*, 1989) and may ensure the survival of latently infected neurons.

In addition, IFN γ will induce strongly MHC class II antigen expression on the surface of brain endothelial cells, astrocytes and microglial cells and I-CAM1 expression on brain endothelial cells. This corresponds with the ability of these cells to behave as antigen presenting cells [APC] (Pryce *et al.*, 1986; Fontana *et al.*, 1987; Frohman *et al.*, 1989). TNF α synergizes with IFN γ in MHC class II antigen induction on astrocytes (Frohman *et al.*, 1989) and brain endothelial cells (Hughes *et al.*, 1988) and the induction of adhesion molecules on the latter cell type may facilitate increased lymphocyte traffic into the CNS (Hughes *et al.*, 1988) conceivably allowing the entry of HIV-1 infected cells. It has been suggested that the predominant endogenous APC in the brain is the astrocyte (Fontana *et al.*, 1987) and that these cells, perhaps initially via the brain endothelium and microglia, control the development of immune reactivity within the CNS (Pryce *et al.*, 1986).

1.6.2. Generation of cytokines within the CNS

The majority of the available data on neuroimmunological processes described below has been determined using animal models and *in vitro* analysis of cultured cells.

However, studies on the pathology of multiple sclerosis *in vivo* generally supports these findings (Hofman, 1989).

Activated astrocytes and microglia are potent sources of particular cytokines within the CNS, although microglia are essentially the same as macrophages in this respect (Goetzl *et al.*, 1989). IL-1 is secreted by astrocytes and microglia after exposure to stimulants such as LPS or neurotropic virus infection (Lieberman *et al.*, 1989; Goetzl *et al.*, 1989), and is necessary for effective antigen presentation and IL-2-induced proliferation of receptive T cells (Roitt *et al.*, 1985) and plays a central role in inducing inflammatory reactions (Dinarello, 1989). Stimulated astrocytes also release IL-3, which leads to the proliferation of microglia and macrophages (Frei and Fontana, 1989), an IFN $\alpha\beta$ -like molecule (Goetzl *et al.*, 1989) that enhances MHC class I antigen expression (Borgeson *et al.*, 1989) and the multipotent stem cell factors, GM-CSF and M-CSF are also released by astrocytes which may further recruit immune cells to the CNS (Frei and Fontana, 1989).

In addition, astrocytes stimulated with LPS or neurotropic virus infection secrete TNF α (Lieberman *et al.*, 1989) which, like the IFNs, will perpetuate immune reactions through the induction of MHC antigens (Frohman *et al.*, 1989) and exert cytotoxic effects on oligodendrocytes (Robbins *et al.*, 1987). Moreover, TNF α stimulates the production of GM-CSF from astrocytes (Frei and Fontana, 1989) and other factors chemotactic for leukocytes from endothelial cells (Mantovani and Dejana, 1989). IL-6 is released from these cells under similar conditions and will have pleiotropic effects, including the enhancement of T cell proliferation, B cell differentiation and antibody production within the CNS (Hirano *et al.*, 1990). Microglial cells also release IL-1, IL-6 and TNF α after appropriate stimulation (Frei and Fontana, 1989).

Furthermore astrocytes are sources of eicosanoids, metabolites of arachidonic acid, and certain of these molecules will increase vasodilation and oedema (Murphy *et al.*, 1988). Moreover prostaglandins have neuromodulatory functions (Murphy *et al.*, 1988) and along with another potent immunosuppressive factor, analogous to TGF β , released from astrocytes provide a mechanism by which these cells can suppress immune reactions within

the CNS through interference with the proliferation of T cells (Murphy *et al.*, 1988; Goetzl *et al.*, 1989).

The interaction of the molecular mechanisms operated by cytokines and HIV-1 expression is an area of intense investigation in cells of the lymphoid system and is providing valuable insight in to cellular regulatory mechanisms that activate productive infection from latent proviruses. Following the finding of considerable neurological illness associated with HIV-1, the abundance of virus within the brain and the low-level of direct neural cell involvement, in conjunction with the latent nature of HIV-1 infection, a complementary analysis of resident neural cells was prompted. In the environment of the central nervous system HIV-1 expression may follow a similar pattern of regulation by cytokines and therefore experiments were designed to first investigate and then to determine the mechanisms by which this may occur in neuronal and glial cells.

CHAPTER 2

Chapter 2: Materials and methods

2.1. Cell lines

The cell lines in this study were established originally from human or murine tumours of neural cells. These were chosen to represent a range of the CNS cell types (see Table. 2.1), and were selected from the cell types from which tumour cell lines have been derived. No permanent or tumour-derived microglial cell lines have been described (Personal communication, Dr. David Male, Institute of Psychiatry, DeCrespigny Park, Denmark Hill, London).

Two human neuroblastoma cell lines, SK-N-SH and SK-N-MC (Spengler *et al.*, 1973), were purchased from the American Type Culture Collection (ATCC). The human astrocytoma U373MG and glioblastoma U138MG (Pontén and Macintyre, 1968) were obtained from the MRC AIDS reagent programme and were originally also from the ATCC. The murine oligodendroglioma G26-24 (Sundarraj *et al.*, 1975) and Jurkat (Osborn *et al.*, 1989), a human T lymphoblastoma cell line, were donated by Dr. Alan Morris, Department of Biological Sciences, University of Warwick. Also primary murine astrocytes were prepared as described in Section 2.6.2.

2.2. Bacterial strains

The *E.coli* K12 strain TG2 (Sambrook *et al.*, 1989) was used in all transformations and for the amplification and maintenance of plasmid vectors. It was originally derived from strain JM101 and has the genotype: $\Delta lacpro$, *thi*, *SupE*, *hsdD5*, *FtraD36*, *proAB*, *lacI^q*, *Z Δ M15* and *recA⁻*.

Table 2.1. Properties of neural cells

Cell Designation	Cell Type	Phenotypic marker	origin
U138MG	Glioblastoma	Cell morphology §	Human
U373MG	Astrocytoma	GFAP+ §	Human
-	Primary astrocytes	GFAP+	Mouse
G25-24	Oligodendroglioma	GalC+	Mouse
SK-N-SH	Neuroblastoma	Dopamine β -hydroxylase §	Human
SK-N-MC	Neuroblastoma	Dopamine β -hydroxylase §	Human

§ Also produces appropriate tumour in nude mice.

2.3. Media

2.3.1. Tissue culture media

All neural cells were cultured in DMEM and lymphoid cells in RPMI-1640, with the addition of 10% (v/v) foetal bovine serum, 2 mM L-glutamine and antibiotics; penicillin (100 IU/ml) and streptomycin (100 µg/ml) [media containing these additions is referred to as complete in the text]. Cell growth in DMEM was buffered by $\text{HCO}_3^-/\text{CO}_2$ in a 5% (v/v) CO_2 atmosphere, and in RPMI-1640, 25 mM Hepes was included. All cells were cultured at 37°C in a humidified incubator (Flow Laboratories).

2.3.2. Bacterial growth media

E. coli TG2 clones were grown in L broth supplemented with 100 µg/ml ampicillin to maintain the plasmid vector. Solid media was obtained by the addition of 1.5% (v/v) Difco bacto-agar prior to autoclaving at 121°C for 15 min. Indicator agar plates for the detection of *lac z* expression in recombinant colonies also contained 0.5 mM IPTG and 0.02% (w/v) X-gal.

LB broth: 10 g tryptone.

5 g yeast extract.

5 g NaCl

2 ml 1 M NaOH (to adjust to pH 7.2)

distilled water to 1 litre.

2.4. Oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems DNA synthesizer as single-stranded molecules. Complementary strands were annealed from equimolar mixtures by heating to 90°C for 10 min, and cooling slowly for 3-4 hrs until below the melting temperature (T_m) of each oligonucleotide. T_m was estimated from the base composition

using the following formula: $T_m = 4[G+C] + 2[T+A]$ (Sambrook *et al.*, 1989). The sequences of all oligonucleotides employed in the analysis of nuclear proteins are given in Table 2.2.

The sequence of the oligonucleotide VPR, a 42mer containing the wild type coding sequence for the repair of the BH10 *vpr* gene is given below. Novel restriction sites are indicated by underlining and those forming the cohesive termini by bold text (see also Chapter 3, Section 3.3.3).

	Eco RI-Pst I-Pvu II	Sal I	
5'--	AATTCTGCAGCAGCTGCTGTTTATCCATTAGAATTGGTG		-3'
3'--	SACGTCGTCGACGACAAATAGGTAAATCTTAACCCACAGCT		-5'

2.5. Sources of cytokines

Recombinant human and murine IFN γ , partially purified from transfected CHO cells (Morris and Ward, 1987), was obtained from Dr. Alan Morris. All other cytokines were purchased from external suppliers (see Appendix A., list of Suppliers).

Table. 2.2. Sequence composition of double-stranded oligonucleotide probes

Site A (30 mer)
[Orchard *et al.*, 1990].

AGGCTACTTCCCTGATTAGCAGAACTACAC
TCGGATGAAAGGAACTAATCGTCTTGAATGTG

NFκB wild type consensus
sequence (29mer) [Osborn *et al.*, 1989].

CAAGGACTTTCCGCTGGGACTTTCCAG
GTTCCCTGAAGGCGACCCCTAAAGGCT

Sp1 high affinity consensus
sequence (15mer) [Harrich *et al.*, 1989].

ACCGGGCGGGCTG
CGCGGGCGGGCGAC

Site B (30mer)
[Orchard *et al.*, 1990].

TCGACAGGGTCGATATCCACTGACCTTC
AGCTGTCCCCAGTCTATAGTGACTGGAAAG

BM5 Site B mutant sequence
(30mer) [Orchard *et al.*, 1990]

TCGACACACTAGCGATATCCACTGACCTTC
AGCTGTGTGATCGCTATAGTGACTGGAAAG

TFIID consensus sequence
[La Thangue and Rigby, 1988].

AGAGCATATAAGGTGAAGTAAGA
TCTGGTATATTCCACTCCATCGT

CTF/NF1 consensus
sequence [Jones *et al.*, 1985].

CCTTTGGCATGCTGCGAATATG
GGAAACGTACGACGGTTATAC

NFκB mutant sequence (29mer)
[Osborn *et al.*, 1989].

CAACTCACTTTCCGCTGCTCACTTTCCAG
GTTGAGTGAAGGCGGACGAGTGAAGGCT

159 oligo, HIV-1 LTR -249/-219
(30mer).

ACACCCGTGTAGCCCTGCATGGAAATGATGA
TGTGGGCACTCGGACGTACCTTACCTACT

5C159 oligo, HIV-1 LTR
261/-240

CACCACTTGTACACCCCTGT
GTGGTCGAACAATGTGGGACA

Notes:

- Underlined text indicates the nucleotides which constitute the protein binding site, except for Site A [Orchard *et al.*, 1990] and 5C159 (Chapter 8) which indicate the extent of protected region in DNase I footprint .
- All sequences are given 5' to 3' with the coding strand above the non-coding strand.

2.6. Tissue culture

2.6.1. Growth of tissue culture cells

Adherent cells were in grown tissue culture-grade plastic flasks in complete DMEM, with fresh medium at 2-3 day intervals. Cells were passaged by trypsinization when confluent, at a ratio of 1:8 - 1:12 depending upon cell type. All solutions were pre-warmed and cultures were rinsed briefly in PBS then incubated with trypsin-EDTA solution (5 mg/ml trypsin, 5 mM EDTA, 145 mM NaCl) at 37°C until detached (1-2 min). The cell suspension was mixed gently with 10 ml complete DMEM and the cells recovered by centrifugation at 400g for 5 min. The pellet was then resuspended in fresh medium and plated at the required dilution in a new flask.

Suspension cells were grown in upright plastic flasks and cells were maintained at a density of approximately 100,000 cells/ml by the resuspension in fresh media every 2-3 days.

2.6.2. Preparation of primary murine astrocyte cultures

The method used was based on that of Morris and Tompkins, (1989). Ten to twenty neonatal Balb/c mice were killed by decapitation with sterile dissecting scissors and the heads washed in ethanol. The skin over the skull was removed and the skull carefully cut from the base of the neck to the nose. The brain was exposed by gentle pressure with forceps on the snout and the complete brain excised into 5 ml sterile PBS. The remainder of the procedure was performed in a tissue culture hood. The meninges were stripped from each brain by carefully rolling on dry filter paper (UV-sterilised for 20 min prior to use), and the remaining material was transferred to a 60 mm Petri dish containing 5 ml PBS and a stainless steel mesh. The tissue was disaggregated by forcing through the wire mesh with a glass rod using gentle pressure, then the mesh was washed a further three times with 5 ml PBS and the washes combined. The homogenate was further disaggregated by passage through a 21 gauge needle and the cells were pelleted by centrifugation at 400g for 5 min. A

single-cell suspension was prepared by resuspension of the cell pellet in 10 ml PBS containing 0.2 mM $MgCl_2$, trypsin (1 mg/ml) and DNase I (40 μ g/ml) followed by incubation at 37°C for 30 min. Ten millilitres of complete DMEM (which contained 10% [v/v] foetal bovine serum) was added to inhibit the trypsin digestion and the cells collected by centrifugation as described previously. The medium was carefully aspirated and the cells were resuspended in complete DMEM for counting in a Neubauer chamber. Approximately 0.5×10^6 cells were obtained per brain and 1.5×10^6 were seeded into a 25 cm² tissue culture flask in 5 ml complete DMEM. Cells were cultured as previously described with media changes on every alternate day until the monolayers were confluent, by around day 8. Following this period, and when plated at this cell density, astrocytes grew out from the brain cell cultures and formed the predominant neural cell type, as assessed by indirect immunofluorescent staining for the astrocyte marker glial fibrillary acidic protein (see Figure 2.1).

2.6.3. Liquid nitrogen storage of cells

Confluent monolayers of cells were trypsinized and collected by centrifugation. The cell pellet was resuspended in approximately 1 ml of complete medium containing 20% (v/v) DMSO, per 25 cm² area of cells harvested and aliquots of 1 ml transferred to freezing vials. The vials were wrapped in paper towel, placed in a plastic beaker and stored at -70°C overnight. The next day vials were transferred to liquid nitrogen for storage. Initially a vial of each cell type was recovered in the following days to check the viability of the frozen stock.

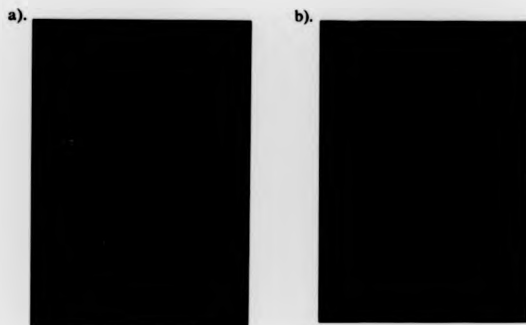


Figure 2.1. Indirect immunofluorescence staining of the astrocyte-specific marker glial fibrillary acidic protein (GFAP) and FITC-labelled second antibody in a). Primary murine astrocyte cultures at day 8 (magnification $\times 150$) and b). U373MG astrocytoma cells which were known to express GFAP and were stained to provide a positive control (magnification $\times 300$).

2.6.4. Recovery of cells from liquid nitrogen

Vials were removed from liquid nitrogen and thawed quickly in a 37°C water bath. The contents were mixed with 10 ml complete media and the cells pelleted by centrifugation to remove the DMSO. The pellet was then resuspended in 5 ml complete media and seeded into a 25 cm² tissue culture flask. The next day the cultures were either trypsinized if confluent or the medium replenished.

2.7. Indirect Immunofluorescence

Cells were grown on sterile glass coverslips until nearly confluent in a Petri dish containing tissue culture medium, prior to cell staining. Coverslips were washed once in PBS (all washes were with 10 ml of solution at room temperature) and fixed in 4% (w/v) paraformaldehyde for 10 min. After two washes in PBS supplemented with 0.05% (v/v) Tween 20, the cells were permeabilized by immersion in 0.2% (v/v) Triton X-100 for 5 min and washed twice again in 0.05% (v/v) Tween 20 in PBS. The coverslips were then incubated in PBS containing 10% (v/v) FBS for 5 min, and washed twice more in 0.05% (v/v) Tween 20 in PBS. Antibodies were diluted in 10% (v/v) FBS in PBS at 1:50 - 1:100 (monoclonal antibodies, supplied as cell culture supernatants, were used neat) and 60 µl aliquots placed onto a strip of Parafilm lying flat on wet filter paper in a plastic box. Coverslips were placed cell side down onto each of the antibody solutions and incubated for 40 min at room temperature. After which they were washed three times in 0.05% (v/v) Tween 20 in PBS and incubated with 60 µl of the relevant FITC or biotin-conjugated secondary antibody for 30 min. Cells were washed three times in 0.05% (v/v) Tween 20 in PBS and coverslips stained with FITC-labelled antibodies allowed to air dry in the dark for 20 min. Phycoerythrin conjugated to streptavidin was used to detect biotinylated antibodies. Cells were also washed three times in 0.05% (v/v) Tween 20 in PBS then incubated for 10 min with PE-streptavidin (diluted 1:50 in PBS containing 10% [v/v] FBS), and washed three times (1 min each) in 0.05% (v/v) Tween 20 in PBS. Coverslips were then treated

with stabilizer (Amersham) for 20 min and air-dried for 20 min in the dark. The coverslips were placed cell side down onto a drop of mountant (Amersham) on a microscope slide and observed under a Zeiss UV microscope. Photographs were taken with Kodak Ektachrome 160 tungsten film using an automatic exposure meter.

The following antibodies were employed for cell staining by this method, all antibodies were polyclonal unless otherwise indicated and the relevant dilution is given in parenthesis: rabbit anti-cow glial fibrillary acidic protein (1:100), goat anti-rabbit FITC conjugate (1:100), mouse IgM anti HIV-1 tat monoclonal, kindly donated by Dr. Sheila Green, Laboratory of Molecular Biology, Hills Road, Cambridge; and goat anti mouse immunoglobulin biotin conjugate (1:50).

2.8. Routine manipulation of nucleic acids

2.8.1. Phenol extraction

Phenol extractions were performed to remove contaminating protein from samples of nucleic acids. An equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1) containing 0.1% (v/v) hydroxyquinolone was added, the sample vortexed briefly, and the phases separated by centrifugation (20,000g) in a microcentrifuge for 5 min. The upper aqueous layer was removed to a fresh tube and the sample was usually back-extracted with 0.5 volumes of distilled water. This was then combined with the first extraction and the nucleic acids precipitated with alcohol (see Section 2.8.2).

2.8.2. Precipitation of DNA

All precipitations of nucleic acids, unless otherwise stated, were carried out in the following manner.

DNA solutions were adjusted to 0.3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol or 1 volume of isopropanol added. Precipitations were carried out either at -20°C overnight, -70°C for 30 min or in an ethanol/dry ice bath for 5-10 min. Nucleic

acids were recovered by centrifugation (microcentrifuge) for 5-15 min at 20,000g. The pellets were washed once in 70% ethanol (0.9 ml) and dried briefly under vacuum before resuspension in TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA) or sterile distilled water.

2.5.3. Gel electrophoresis of DNA

i). Agarose gels.

DNA was routinely analysed for purity or following restriction enzyme digestion by electrophoresis through horizontal agarose slab gels submerged in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The percentage of agarose, made up in 0.5 x TAE, was varied between 0.8 and 2.0% (w/v) depending upon the size of the DNA fragments to be resolved. Electrophoresis was performed using a Gibco-BRL Model H5 gel tank which allowed gels to be poured in a plastic tray of dimensions 11.0 x 14.0 cm (width x length). Wells were formed using 1 or 2 mm thick combs of 10 or 20 teeth depending upon the number or volume of samples to analysed. Samples were supplemented with one fifth volume of load dye (5 x TAE, 30% [v/v] glycerol, 0.25% [w/v] bromophenol blue) and electrophoresis performed at 50-150 V at room temperature with buffer containing 0.4 µg/ml ethidium bromide. Size markers were included in all gels; at least one track contained 1-4 µg of Gibco-BRL 1 Kb ladder (fragment sizes ranged from 12-0.1 Kb). DNA was visualised by placing the gel on a UV trans-illuminator and photographed using a Polaroid camera and type 55 4" x 5" land film.

ii). Extraction of DNA from agarose gels

DNA was purified from agarose gels by electro-elution (Sambrook *et al.*, 1989). A dialysis membrane was first rinsed with 0.5 x TAE, sealed at one end with a dialysis clip and filled with this buffer. The region of the gel containing the desired DNA fragment was excised with a clean scalpel and placed into the dialysis bag. Excess fluid was removed, leaving just sufficient to keep the gel slice from touching the sides of the membrane, and

the bag sealed with another clip. The bag was then submerged in electrophoresis buffer (1 x TAE) in the gel tank and a voltage (100-200 V) applied for 1-2 hrs. The electrophoretic removal of the DNA from the gel slice was checked by UV trans-illumination and the polarity was reversed for 30 s to free the DNA from the dialysis tubing. The buffer containing the DNA was removed to a microcentrifuge tube and the bag rinsed out with 0.5 volumes (approximately 200 μ l) of 0.5 x TAE. The eluted DNA was subsequently extracted with phenol/chloroform/iso-amyl alcohol (25:24:1) and ethanol precipitated before use in further reactions.

iii). Polyacrylamide gels

Native polyacrylamide gels were employed in the preparation of end-labelled DNA probes or, at low ionic strength, for the resolution of nucleo-protein complexes in gel retardation assays. Radiolabelled RNA probes were also purified by electrophoresis through denaturing 8M Urea gels. In all cases 4 or 5% (w/v) gels (20:1 acrylamide:bis-acrylamide) were formed in a 150 x 170 x 0.8 mm (width x length x thickness) apparatus and polymerized with 200 μ l (w/v) ammonium persulphate and 25 μ l TEMED for 1 hr. Electrophoresis was carried out in a Gibco-BRL model V16 system at 150 V in 1 x TBE (89 mM Tris-HCl [pH 8.3], 89 mM Boric acid, 10 mM EDTA) or 0.2 x TBE for low ionic strength gels.

iv). Extraction of DNA from polyacrylamide gels

Radiolabelled DNA fragments or oligonucleotides were recovered from polyacrylamide gels by the 'crush and soak' method (Sambrook *et al.*, 1989). The gel slice containing the DNA was placed in a microcentrifuge tube and crushed against the side with a pipette tip. One to two volumes of elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA [pH 8.0], 0.1% [w/v] SDS) was added and the sample shaken overnight at 37°C on a rotary platform to elute the DNA. The sample was then vortexed, centrifuged briefly and the supernatant removed. Another 0.5 volumes of elution

buffer was added and the procedure repeated. Both supernatants were then combined and contaminating acrylamide fragments removed by passage over siliconized glass wool. A 1 ml syringe was loosely packed to 2-3 cm height with glass wool and placed in a 15 ml Corex tube above a microcentrifuge tube. The sample was pipetted into the syringe body and forced over the glass wool by centrifugation at 500g for 1 min. The solution was collected and subsequently extracted with phenol/chloroform/iso-amyl alcohol (25:24:1), ethanol precipitated, washed with 70% ethanol, re-precipitated and washed with 70% ethanol twice more in order to remove acrylamide contaminants. After each step excess ethanol was removed from the samples by brief re-centrifugation and aspiration with a micro-pipette. Samples were then finally resuspended in TE or sterile distilled water for further use.

2.8.4. Use of DNA modification enzymes

i). Restriction enzyme digests

The DNA to be cleaved by the restriction enzyme(s) was prepared in sterile distilled water and the appropriate 10 x buffer concentrate added. Enzymes were supplied by either Gibco-BRL or Amersham and the specified manufacturer's buffers were used in all cases. The number of units of enzyme required to digest the DNA in the given time (1-24 hrs) was calculated from the activity of the enzyme with λ DNA as the substrate. This was then added to start the reaction and the tube incubated at the required temperature.

ii). Dephosphorylation of DNA

Following digestion with restriction endonucleases plasmid vector DNA was usually treated with CIAP to remove 5' terminal phosphates and prevent re-ligation. Digests were first extracted with an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1), back extracted with 0.5 volumes of distilled water and ethanol precipitated. The pellet was resuspended in 50 μ l CIAP buffer (10 mM Tris-HCl [pH 8.3], 1 mM ZnCl₂, 1 mM MgCl₂) and for protruding 5' ends, 1 unit of CIAP per 100 picomoles of DNA was added and

incubated for 30 min at 37°C. Blunt or recessed termini were incubated with 1 unit/2 picomoles for 15 min at 37°C, then with a second aliquot of CIAP for a further 45 min at 45°C (Sambrook *et al.*, 1989).

iii). Blunt end reaction

DNA molecules possessing protruding 5' termini generated by restriction enzyme digest were converted to blunt ends when required by incubation with the Klenow fragment of *E.coli* DNA polymerase I in the presence of the required nucleoside triphosphates. The restricted DNA was extracted with phenol/chloroform/iso-amyl alcohol (25:24:1), back extracted, ethanol precipitated and resuspended in buffer (0.5 M Tris-HCl [pH 7.5], 0.1 M MgSO₄, 1 mM DTT, 500 µg/ml bovine serum albumin {fraction V}.) containing the dNTPs at 1 mM final concentration. The reaction was started by the addition of 1 unit of enzyme per µg of DNA and incubated at room temperature for 30 min. This was then terminated with 1 µl 0.5 M EDTA (pH 8.0) followed by extraction with phenol/chloroform/iso-amyl alcohol (25:24:1), back extraction and ethanol precipitation.

iv). Ligation

The ligation of DNA fragments was performed as follows: one hundred microgrammes of vector DNA was mixed with an equimolar amount of insert DNA, containing compatible termini, in a final volume of 50 µl ligase buffer (50 mM Tris-HCl [pH 7.6], 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000). One Weiss unit of T4 DNA ligase was added and the reaction incubated at 16°C overnight. Cohesive and blunt-ended termini were ligated in the same way.

2.8.5. Bacterial transformation and preparation of plasmid DNA

i). Transformation of plasmid vectors

Competent cells were prepared from an overnight culture of *E.coli* TG2 by a method based on Sambrook *et al.*, (1989). One millilitre was subcultured into 50 ml fresh L broth

and incubated in an orbital incubator at 37°C until the OD₅₉₀ was approximately 0.3 (about 90 min). The cells were incubated on ice for 30 min followed by centrifugation at 4°C for 10 min at 1500g. The pellet was resuspended in 25 ml ice-cold 100 mM MgCl₂ (made fresh from solid), immediately centrifuged as before and resuspended in 2.5 ml 100 mM CaCl₂ (also ice-cold and made fresh from solid). The cells were then incubated on ice for 60 min before use.

Competent cells (200 µl) were transformed with 25 µl of ligation reaction, made up to 100 µl with 100 mM Tris-HCl (pH 7.4), by mixing in a 12 ml polypropylene Falcon tube and incubating on ice for 30 min. Control reactions contained no DNA or 0.5-1 ng of circular, untreated vector. The tubes were heated at 42°C for 2 min and incubated for a further 30 min on ice before the addition of 700 µl of L broth and incubation at 37°C (orbital shaker) for 30 min. Aliquots of cells, typically 250 µl, 50 µl, 10 µl and 1 µl were each plated out onto an LB agar plate containing ampicillin and incubated overnight at 37°C. All vectors conferred ampicillin resistance to their host; pUC and pBSII vectors also carried the β -galactosidase gene, the expression of which was inactivated by a cloned insert, and these transformants were screened on LB agar plates also containing X-gal and IPTG (see Section 2.3.2).

ii). Small-scale plasmid preparation

The 'mini-prep' procedure of Serghini *et al.*, (1989) was used to prepare plasmid DNA from bacterial colonies. The DNA was subject to further analysis by restriction enzyme digestion and agarose gel electrophoresis in order to screen for recombinant plasmids.

A single colony was inoculated into 5 ml L broth containing ampicillin and grown overnight in an orbital incubator at 37°C. An aliquot of 1.5 ml was transferred to a microcentrifuge tube and the cells pelleted by centrifugation at 20,000g for 3 min. The culture supernatant was aspirated and the pellet resuspended in 50 µl TNE buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA). An equal volume of

phenol/chloroform/iso-amyl alcohol (25:24:1) was added, vortexed vigorously for several seconds and centrifuged for 5 min at 20,000g. Fifty microlitres from the aqueous phase was transferred to a fresh tube containing 50 μ l of 4 M ammonium acetate and the DNA precipitated by the addition of 200 μ l ethanol followed by a 15 min incubation on ice. The DNA was collected by centrifugation for 10 min at 20,000g and resuspended in 15 μ l of distilled water. Aliquots of 5 μ l were then taken for restriction enzyme digestion in the presence of RNase A (50 μ g/ml).

iii). *Large-scale plasmid preparation*

Large quantities of plasmid DNA (1-3 mg) were prepared by the alkaline lysis method of Sambrook *et al.* (1989). Twenty microlitres were taken from a glycerol stock of bacteria (1 ml overnight culture plus 1 ml 80% [v/v] glycerol; stored at -20°C) and inoculated into 5 ml LB containing ampicillin. This was incubated overnight in an orbital shaker at 37°C then used to inoculate 500 ml fresh LB plus ampicillin which was incubated for a further 6-8 hrs, at which time chloramphenicol (20 μ g/ml final concentration) was added in order to amplify plasmid numbers (Sambrook *et al.*, 1989). The culture was incubated for a further 15-18 hrs overnight. The cells were harvested by centrifugation (MSE 6L, 2800 rpm for 30 min) and the pellet resuspended in 7 ml of Solution I (25 mM Tris-HCl [pH 8.0], 50 mM glucose, 10 mM EDTA) containing 5 mg/ml lysozyme. The suspension was transferred to a Beckman SW28 polyallomer tube, allowed to stand for 5 min, and the bacteria lysed by the addition of 14 ml of freshly made Solution II (0.2 M NaOH, 1% [w/v] SDS). After gentle mixing, the tube was kept on ice for 10 min and 10.5 ml of ice-cold Solution III (5 M potassium acetate; 5 M K⁺, 3 M CH₃COO⁻) was added, mixed and allowed to stand on ice for a further 10 min. The tube was loaded into a Beckman SW28 rotor and centrifuged for 20 min at 20,000 rpm to pellet the cell DNA and bacterial debris. The supernatant was then mixed with 0.6 volumes of isopropanol. This was incubated at room temperature for 15 min and the DNA recovered by centrifugation (MSE Chilspin 4500 rpm for 30 min at room temperature). The pellet was washed with 70%

ethanol and dried in a vacuum desiccator before being resuspended in 25 ml TE for purification on a caesium chloride density gradient.

iv). Caesium chloride density gradients

Caesium chloride (26.5 g) and ethidium bromide (3-4 mg) were dissolved in the DNA solution, prepared from the method described above, and loaded into a Beckman 38 ml quickseal tube. The tube was then filled with paraffin oil, heat-sealed and centrifuged for 16-20 hrs at 45,000 rpm in a Beckman Vti 50 rotor at 15°C. The closed circular plasmid DNA band was located with long wave UV light and the top of the tube removed with a hot scalpel. The solution containing the plasmid was then carefully aspirated into a syringe. The ethidium bromide was removed from the sample by extracting with an equal volume of water saturated 1-butanol followed by brief centrifugation to separate the phases. This was repeated a further three times before the CsCl was removed by dialysis against three changes of TE (pH 7.8), 5 litre volume, each for 1 hr. The plasmid DNA was subsequently recovered by ethanol precipitation and resuspended in 1 ml sterile distilled water. The yield was estimated by measuring the OD₂₆₀ (1.0 OD₂₆₀ = 50 µg/ml ds DNA) and the purity by taking the ratio of OD₂₆₀/OD₂₈₀. For pure DNA this is 1.8. If plasmid preparations contained excess protein they were extracted with an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1) then twice with water saturated diethyl ether, followed by incubation at 68°C for 5-10 min to drive off contaminating ether. The OD readings were then measured again and the procedure repeated until the OD₂₆₀/OD₂₈₀ ratio was within 0.1 of this value. Plasmid solutions were stored at -20°C.

2.9. Transfection of eukaryotic cells

2.9.1. Transfection of adherent cells

Adherent cells were transfected by a modified calcium phosphate co-precipitation technique (Gorman *et al.*, 1985; Chen and Okayama, 1987). Confluent monolayers were

trypsinized 24 hrs prior to transfection and re-plated to achieve approximately 80% confluence the next day. The number of cells was $3.5 - 6.5 \times 10^5$ per 60 mm dish depending upon the cell type. One millilitre of $\text{Ca}_3(\text{PO}_4)_2$ -DNA precipitate was prepared for each pair of 60 mm dishes in the following way: up to 20 μg of each plasmid DNA (caesium chloride gradient-purified) was mixed with 50 μl 25M CaCl_2 and the volume increased to 500 μl with tissue culture-grade sterile distilled water. This was gently mixed with an equal volume of HBS (50 mM Hepes-NaOH [pH 7.12], 280 mM NaCl, 10 mM KCl, 12 mM glucose, 3 mM Na_2HPO_4) and allowed to stand for 10-15 min, during which time a fine, translucent precipitate formed. The precipitate (0.5 ml) was added dropwise to the cell culture medium with gentle swirling and the cells returned to the 37°C incubator in an atmosphere of 3% CO_2 for 20 hrs. The next day, monolayers were treated with 20% (v/v) glycerol in PBS for 2 min, washed twice with PBS and cultured in 5 ml complete DMEM. Cells were then placed back at 37°C in a 5% CO_2 atmosphere and incubated for 22-24 hrs. When the fresh medium had equilibrated with respect to temperature and carbon dioxide, approximately 30-60 min, cells were treated with cytokines or phorbol ester if required. This was added dropwise to the medium in each dish with gentle swirling and then dishes incubated as previously described for 22-24 hrs.

2.9.2. Transfection of suspension cells

Lymphocytes were transfected by a DEAE-Dextran technique (Durand *et al.*, 1987). Cells from a growing culture were collected by centrifugation into aliquots of 12×10^6 cells and resuspended in 1 ml serum-free RPMI-1640 with 500 μg DEAE-Dextran (diluted from a 10 mg/ml stock made in Tris buffered saline; 25 mM Tris-HCl [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl_2 , 0.5 mM MgCl_2 , 0.6 mM Na_2HPO_4). This was mixed with a further 1 ml of serum-free RPMI containing 0.2 mM chloroquine and up to 20 μg of each plasmid DNA, and incubated at room temperature for 70 min to transfect the cells. The aliquots were pelleted by centrifugation, washed once in RPMI without serum and

resuspended in 15 ml complete RPMI. Cultures were then returned to the incubator at 37°C.

2.9.3. G418 selection

The production of permanent cell lines was achieved by transfection of cells with a plasmid carrying the neomycin resistance gene, aminoglycosidase 3'phosphotransferase (APH), driven by the SV40 late promoter, and subsequent selection in the presence of the neomycin analogue, G418 (Geneticin, Gibco-BRL). A retroviral vector, pMoLTRcat (Dingwall *et al.*, 1989), kindly supplied by Dr. J. Karn, Laboratory of Molecular Biology, Hills Road, Cambridge, was used to create permanent lines expressing HIV-1 *cat*. This vector also facilitated expression of the APH marker. Co-transfection of pLC2R with pWLneo (Stratagene), an APH expression vector (20:1 ratio), was used to generate cell lines expressing CAT under control of the HIV-1 LTR. Preliminary experiments were performed to ascertain the lowest concentration of G418 which killed all the cells within 10-14 days. Cells were passaged 48 hrs after calcium phosphate transfection (as Section 2.9.1) at a ratio of 1:2-1:4, and incubated for a further 24 hrs. Fresh medium was added with 500 µg/ml G418, changed every 2-3 days, and the growth of resistant colonies monitored by microscopy. Once G418 resistant colonies had become established the flasks were trypsinized and re-plated to form a bulk culture. This was either employed directly in experiments or cloned by limiting dilution. A cell suspension was diluted so as to contain 1 cell in 10 µl, and 3 µl and 30 µl (equivalent to 0.3 and 3 cells) seeded separately into 48 wells of a flat-bottomed 96 well plate. Plates were cultured in medium containing G418 and scored after 2 days for colonies consisting of, or derived from, a single cell. Fresh medium was added to such wells and when confluent the cells were transferred to 6 well plates (35 mm diameter). Alternatively cloning rings were used, these were held down with vacuum grease and although cumbersome allowed most of an individual colony to be harvested, thus avoiding re-growing from a single cell. Clones and bulk cultures were propagated in complete DMEM in the presence of 500 µg/ml G418.

2.10. Analysis of cytoplasmic extracts from transfected cells

2.10.1. Preparation of cytoplasmic extracts

Cytoplasmic extracts were prepared from transiently-transfected cells for the analysis of reporter gene expression. Duplicate 60 mm dishes of cells, 22-24 hrs after glycerol shock, were washed briefly with 5 ml of ice-cold PBS and dislodged with a cell-scraper into a fresh 5 ml aliquot of PBS. All subsequent manipulations were performed on ice or at 4°C. Residual cells were removed by washing once with 5 ml PBS and the cells harvested by centrifugation at 4°C (1200g, 5 min). Lymphocytes were harvested 40-42 hrs after DEAE-Dextran transfection by centrifugation (1200g for 5 min at 4°C) and washed once with 10 ml ice-cold PBS. The cell pellet was resuspended in 150 μ l ice-cold CAT lysis buffer (250 mM Tris-HCl [pH 7.8], 5 mM DTT, 10% (v/v) glycerol, 0.25% (v/v) Nonidet-P40) and kept on ice for 10 min. Cell lysis was achieved by three cycles of freeze-thaw; ethanol/dry ice bath for 2 min, 37°C water bath for 1 min. The cell debris was pelleted in a microcentrifuge at 20,000g for 12 min. The supernatant (cytoplasmic extract) was retained and stored on ice until use.

2.10.2. Protein assay

The protein content of all cell extracts was determined using the Bio-Rad assay kit. Reagent (200 μ l) was placed in a 1 ml plastic cuvette and the volume increased to 1 ml with distilled water plus 1-10 μ l of extract. The mixture was vortexed briefly and allowed to stand for 5 min before the colour change was measured in a spectrophotometer at a wavelength of 595 nm. The zero control was reagent (200 μ l) and water (800 μ l) only. Samples were diluted, if necessary, to produce an absorbance of 0.1-0.6 units which was in the linear (1-10 μ g) range of the microassay procedure (Bio-Rad manual).

2.10.3. β -galactosidase assay

The β -galactosidase activity present in cytoplasmic extracts was measured by monitoring *o*-nitrophenol production following enzymatic cleavage of the substrate ONPG. This produced a yellow colour in solution (Rosenthal, 1987).

The following reagents were combined in a 1 ml plastic cuvette: 3 μ l 100 x magnesium buffer (100 mM MgCl_2 , 5 M β -mercaptoethanol), 66 μ l ONPG solution (4 mg/ml in sodium phosphate buffer (100 ml of 0.1 M Na_2HPO_4 adjusted to pH 7.3 at 37°C with 0.1 M NaH PO_4) and sufficient sodium phosphate buffer to give a final volume of 300 μ l including cell extract. An equal amount of fresh extract^a was transferred from ice to each cuvette, mixed and placed at 37°C to commence the reaction. Cuvettes were incubated for 30-120 min until a yellow colour was visible, and the reaction stopped by the addition of 0.5 ml 1 M Na_2CO_3 . The absorbance was measured in a spectrophotometer at a wavelength of 410 nm with water as zero control. Occasionally incubation at 37°C would cause the precipitation of material from the extract, making the solution turbid and producing a higher OD reading than anticipated. If this occurred, the contents from all cuvettes were transferred to microcentrifuge tubes and centrifuged at 20,000g for 10 min to clear the solution before the OD₄₁₀ was measured. A positive control for the enzyme assay was run using 100 ng of purified enzyme in place of the extract.

2.10.4. Chloramphenicol acetyl transferase (CAT) assay

The direct scintillation diffusion method (Eastman, 1987) was used to assay CAT activity. The number of units of extract^a employed in each assay was normalised for

a). The amount of extract was varied for each cell type to obtain a sufficient signal; this was due to different transfection efficiencies and levels of expression from the reporter gene constructs. One unit of extract was defined as the volume of required to produce an absorbance of 1.0 in the protein assay and 1-5 units (17.5 to 87.5 μ g) were used in each assay.

transfection efficiency by the ratio of β -galactosidase activity between samples. Because the promoters driving β -gal expression are responsive to cytokines (experimental observation) it was not possible to normalise between extracts from cells treated with cytokines, only between duplicates. Fresh extract was heated to 65°C for 15 min to inactivate any endogenous acetyltransferase activity and this served to reduce background as bacterial CAT is relatively resistant to such treatment. One to five units was then mixed with the following reagents in a plastic scintillation vial: 50 μ l 5 mM chloramphenicol (aq), 25 μ l 1 M Tris-HCl (pH 7.8), 124 μ l distilled water and 0.1 M Tris-HCl (pH 7.8) to give a final volume of 300 μ l. ^3H -acetyl coenzyme A (0.1 μCi) was added to each vial to start the reaction and 4.5 ml Econofluor scintillation fluid quickly added. The vials were incubated at 37°C and the formation of labelled acetyl chloramphenicol product measured by counting in a scintillation counter for 10 s every 30 min over 2 hours. Controls were included to monitor the background diffusion of label into the scintillant and a positive control of purified CAT enzyme. Also, extracts from cells transfected with pOCAT served as a measure of the background expression from a promoter-less CAT gene. This data was used to plot graphs of product formed by the CAT enzyme (CPM of ^3H -acetyl coenzyme A) against time (min) to demonstrate the linearity of the enzyme reaction during the assay. An example of such is given in Figure 2.2.

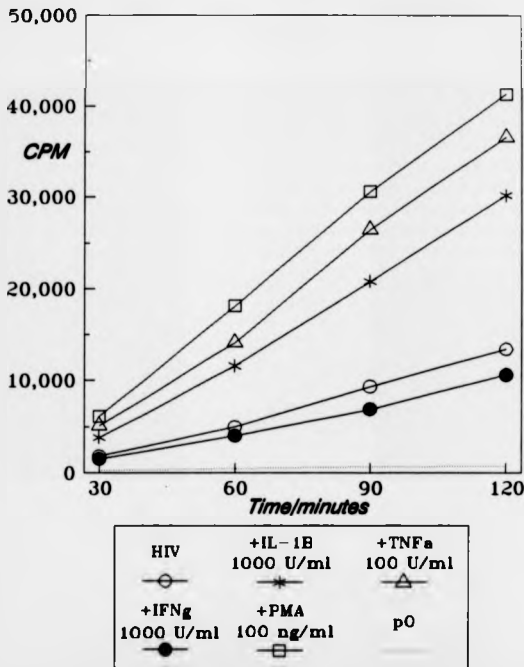


Figure 2.2. CAT activity directed by the HIV-1 LTR in extracts from U138MG glioblastoma cells. Cells were transiently transfected with pLC2R and incubated with or without cytokines for 22-24 hrs before CAT activity was determined. The line graph shows the linear relationship between product formation (CPM of ^3H -acetyl chloramphenicol) and time during the two hour incubation period of the CAT assay.

2.11. Analysis of RNA

2.11.1. Extraction of cytoplasmic RNA

This method was based on that of Sambrook *et al.* (1989). Total cytoplasmic RNA was extracted from cells transfected with pLC2R, or from one permanent cell line containing at least one integrated copy of this plasmid. RNA was prepared after 12 hrs incubation. Adherent cells were transfected as detailed in section 2.9.1, except that cells were seeded into 100 mm dishes and 70 μ g of only pLC2R DNA was used in 1 ml of calcium phosphate precipitate. The incubation period began after the cells were glycerol shocked.

Monolayers in 100 mm dishes were washed once with ice-cold PBS and stored on ice before the cells were harvested by gentle scraping into 10 ml PBS with a cell scraper. The cell suspension was centrifuged at 400g for 5 min at 4°C and the pellet washed again with 10 ml of ice-cold PBS before being finally resuspended in 200 μ l RNA extraction buffer (10 mM Tris-HCl [pH 8.6], 0.14 M NaCl, 1.5 mM MgCl₂, 0.5% (v/v) Nonidet P-40, 1 mM DTT, 1000 U/ml RNasin). This was vortexed for 15 s and stood on ice for 5 min, then the unlysed cells and nuclei were pelleted in a microcentrifuge at 12,000g for 90 s. The supernatant was transferred to a fresh tube, mixed with 200 μ l proteinase K digestion buffer (0.2 M Tris-HCl [pH 8.0], 25 mM EDTA [pH 8.0], 0.3 M NaCl, 2% [w/v] SDS) and incubated with 50 μ g/ml proteinase K for 30 min at 37°C. Following extraction with an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1) and centrifugation at 5,000g for 10 min, the aqueous phase was placed in fresh tube and precipitated with 400 μ l ice-cold isopropanol for 30 min on ice. The RNA was collected by centrifugation for 10 min at 20,000g and washed with 70% ethanol. This was carefully aspirated and the pellet allowed to air-dry before being redissolved in 200 μ l 50 mM Tris-HCl (pH 7.8), 1 mM EDTA (pH 8.0). Input DNA or contaminating DNA from the nuclei was removed by digestion with DNase I. The MgCl₂ and DTT concentrations were adjusted to 10 mM and 1 mM, respectively, and RNasin included to 1000 U/ml. Five units of RNase-free DNase I

(Promega) was added and the tube incubated at 37°C for 60 min. The enzyme was inactivated by the addition of EDTA (pH 8.0) to 10 mM and SDS to 0.2% (w/v), followed by extraction with an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1). The phases were separated by centrifugation at 12,000g for 5 min and the aqueous portion transferred to a fresh tube. The RNA was precipitated by the addition of 1/10th volume of 3 M sodium acetate (pH 7.0) and 2.5 volumes of ethanol on ice for 30 min and then collected by centrifugation at 20,000g for 5 min. The pellet was air-dried and resuspended in 100 µl TE. The yield was estimated by measuring the OD₂₆₀ assuming a solution with an OD₂₆₀ of 1.0 contained 40 µg/ml RNA. Approximately 30-100 µg of RNA was recovered from a 100 mm dish of cells. The quality of the RNA was assessed by the electrophoresis of 1 µg in loading buffer (80% [v/v] formamide, 0.1% [w/v] xylene cyanol, 0.1% [w/v] bromophenol blue, 2 mM EDTA [Solution E, RPA I kit, Ambion Inc.]) through a 2.0% agarose gel [as Section 2.8.3, i)] for 5-10 min at 150 V in 1 x TAE electrophoresis buffer containing 0.4 µg/ml ethidium bromide and visualizing the integrity of the ribosomal RNA bands under UV light.

2.11.2. Production of radiolabelled RNA probes

Antisense riboprobes specific for HIV-CAT mRNA were synthesised from the T3 promoter of plasmid pBSII-LTRF, linearized at the Bam HI site, by an *in vitro* transcription reaction in the presence of ³²P-αCTP.

The following reagents were mixed in a microcentrifuge tube: 4 µl transcription buffer (200 mM Tris-HCl [pH 7.5], 30 mM MgCl₂, 10 mM spermidine, 50 mM NaCl), 2 µl 100 mM DTT, 1 µl RNAsin (25 units), 4 µl of a solution containing 2.5 mM ATP, 2.5 mM GTP, and 2.5 mM UTP, 2.4 µl 100 µM CTP, 500 ng linearized template DNA, and 5 µl ³²P-αCTP (50 µCi) in a final volume of 19 µl. The reaction was started by the addition of 40 units of T3 RNA polymerase and incubated for 60 min at 37°C. After transcription the template DNA was destroyed by incubation with 1 U RNase-free DNase I (Promega) for a further 15 min at 37°C and the probe purified by electrophoresis through a 5% 8 M Urea

polyacrylamide gel [see Section 2.8.3, iii)]. The position of the labelled probe was located by autoradiography for 2-3 min and excised with a clean scalpel into 350 μ l elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS [Solution F, RPA I kit, Ambion Inc.]). This was placed on a rotary platform and incubated overnight at 37°C. The gel slice was removed with sterile forceps and the probe stored at -70°C until required.

2.11.3. Ribonuclease protection assay

This was performed using the RPA I kit obtained from Ambion Inc., and the protocol as detailed below.

Cytoplasmic RNA (50-100 μ g) and riboprobe (5×10^5 cpm) were co-precipitated with 2.5 volumes of ethanol, in 100 μ l final volume containing 0.5 M ammonium acetate, by storage at -70°C for 15 min. In the assay each riboprobe also required two controls consisting of an equal amount (cpm) of probe and 10 μ g yeast RNA. These were i); to check the integrity of the probe during the assay (no RNase) and ii); to confirm no positive signal was produced from the carrier RNA after digestion with RNase or due to incomplete digestion of free probe. The RNA was collected by centrifugation at 20,000g for 15 min and the ethanol carefully removed. The tube was then re-centrifuged briefly and the remaining liquid aspirated. The RNA pellet was redissolved in 20 μ l hybridization buffer (Solution A: 80% [v/v] deionized formamide, 40 mM PIPES, 400 mM ammonium acetate [pH 6.4], 1 mM EDTA), heated to 95°C for 3-4 min and incubated at 43°C for 12-16 hrs. Solution R (50 U/ml RNase A, 10,000 U/ml RNase T1) was diluted 1:100 in RNase digestion buffer (Solution B) and 200 μ l added to each experimental tube and to one control tube. The second control tube received 200 μ l of solution B only. This was mixed by brief vortexing and incubated at 37°C for 30 min. An equal volume of Solution D1 (Proteinase K/Yeast RNA) and D2 (20% [w/v] SDS) were mixed together and 20 μ l added to each tube. These were vortexed and incubated for a further 15 min at 37°C, followed by extraction with 250 μ l phenol/chloroform/iso-amyl alcohol (25:24:1). The aqueous phase was removed and the RNA precipitated with 625 μ l ethanol at -70°C for

15 min. After centrifugation for 15 min at 20,000g and removal of the ethanol as described earlier, the pellet was resuspended in 5 μ l loading buffer (Solution E) and then separated on an 8% (w/v) acrylamide 8 M urea sequencing gel [see Section 2.12.3, ii)].

2.12. Analysis of DNA binding proteins

2.12.1. Preparation of nuclear extracts

i). Cytokine treatment, harvesting and storage of cells

This method required two to four roller bottles of confluent cells depending upon the cell type. When required the necessary cytokine was added to the medium of confluent cells prior to harvesting by trypsinization. U373MG astrocytoma cells were treated with 250 U/ml of IL-1 β for 1½-2 hr and SK-N-MC and SK-N-SH neuroblastoma cells with 100 U/ml of TNF α for 2 hrs. After trypsinization the cells were washed once with 25 ml of ice-cold PBS and the cell pellet (\leq 1 ml), was resuspended in 2-3 pellet volumes of 30% (v/v) glycerol in PBS and stored at -70°C for up to 6 months.

ii). Preparation of nuclear extracts

This method was based on that of Lubon and Hennighausen (1987). Nuclear extracts were prepared from either freshly harvested cells or frozen cells thawed on ice immediately before use. All solutions were kept chilled and protease inhibitors and DTT added just prior to use. Centrifuges were also cooled to 4°C for this procedure. The cells were pelleted and washed once with 10 ml PBS and resuspended in 3 pellet volumes of 0.3 M sucrose in buffer A (10 mM Hepes-KOH [pH 7.8], 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, and 2 μ g/ml each of antipain, leupeptin and pepstatin A) which was transferred to a Dounce tissue homogenizer. Cell lysis was achieved by 10-12 strokes of a B pestle, followed by 3-4 strokes in the presence of 0.1% (v/v) Nonidet P-40. Complete lysis was confirmed by phase contrast microscopy. The nuclei were pelleted by centrifugation (1200g, 10 min) and washed twice with 3 ml buffer A without

NP-40 (the cytoplasmic supernatant was retained after the first centrifugation step and stored at -70°C). The nuclei were then resuspended in 1-2 pellet volumes of buffer B (400 mM NaCl, 10 mM Hepes-KOH [pH 7.8], 1.5 mM MgCl_2 , 0.1 mM EGTA, 0.5 mM DTT, 5% [v/v] glycerol and 0.5 mM PMSF) and thoroughly dispersed by 10 strokes (B pestle) in the tissue homogenizer. The suspension was then transferred to a 7 ml glass universal and stirred slowly at 4°C for 30 min to elute the nuclear proteins. The extracted nuclei and insoluble material were pelleted by centrifugation at 100,000g for 1 hr. The supernatant was dialysed against 100 volumes of buffer C (20 mM Hepes-KOH [pH 7.8], 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% [v/v] glycerol and 0.5 mM PMSF) for 2-3 hrs and precipitated material, mostly lipid, removed by centrifugation (25,000g, 15 min). The remaining protein solution was aliquoted into microcentrifuge tubes which were flash-frozen in liquid nitrogen before storage at -70°C .

2.12.2. Production of labelled DNA probes

DNA fragments, for gel retardation or footprinting assays, were radiolabelled at one end to produce high specific activity probes for each strand (Goodwin, 1990). One of two methods were employed, either the T4 polynucleotide kinase reaction (5' end) or end-filling with reverse transcriptase (3' end), in order to locate the labelled nucleotide(s) at the most favourable position relative to the proposed nuclear protein binding site. An overview of the procedure followed for the production of each DNA probe specifically labelled at one end is given in Table 2.3 (p74) and the region that it spans is given in Figure 2.1 (p75). Double-stranded oligonucleotides were labelled only by the T4 polynucleotide kinase reaction, on both 5' termini for gel retardation assays.

i). T4 polynucleotide kinase reaction

Fifty microgrammes of the relevant plasmid DNA, this was either pBSII-LTRF or -LTRB which contain specific restriction fragments from the 5' or 3' regions of the HIV-1 LTR (see Figure 2.3 and Chapter 3, Section 3.4), was digested with the first restriction

enzyme in a final volume of 100 μ l. An aliquot was removed and the extent of digestion checked by electrophoresis through a 1% agarose gel [see Section 2.8.3, i)] before proceeding to add 20 units of CIAP to the incubation buffer. This was incubated at 37°C for a further 30 min to remove 5' terminal phosphates from the linearized DNA. After addition of 2 μ l 0.5 M EDTA (pH 8.0) and 5 μ l 20% (w/v) SDS the solution was extracted twice with 100 μ l of phenol/chloroform/iso-amyl alcohol (25:24:1) and then once with chloroform/iso-amyl alcohol (24:1). The DNA was precipitated with two volumes of ethanol and centrifuged at 20,000g for 10 min. The pellet was washed once with 70% ethanol and the remaining ethanol removed by brief centrifugation and aspiration with a micropipette before resuspension in 20 μ l TE buffer. A portion (10 μ g) of the DNA was labelled with 32 P by mixing the following components: 4 μ l of the digested and phosphatased DNA, 5 μ l of kinase buffer (500 mM Tris-HCl [pH 7.6], 100 mM MgCl_2 , 50 mM DTT, 1 mM Spermidine, 1 mM EDTA), 12.5 μ l 32 P- γ -dATP (5000 Ci/mmol; 125 μ Ci), 27.5 μ l distilled water and 2 μ l (20 units) T4 polynucleotide kinase in a final volume of 50 μ l. This was incubated at 37°C for 45 min before EDTA and SDS were added to stop the reaction and the solution phenol extracted and ethanol precipitated (as before). The pellet was resuspended in 200 μ l TE, then re-precipitated by the addition of 20 μ l 3 M sodium acetate (pH 7.0) and 2.5 volumes of ethanol. The DNA was centrifuged and washed twice with 70% ethanol as described earlier, before being redissolved in 20 μ l distilled water and digested with the second restriction enzyme in a volume of 50 μ l for 1-2 hrs. The following were then added: 1 μ l 0.5 M EDTA (pH 8.0), 2.5 μ l 20% (w/v) SDS and 12 μ l 20% (w/v) Ficoll, and the sample loaded into two wells of a 5% native polyacrylamide gel and electrophoresed at 150 V [see Section 2.8.3, iii)]. Formamide load dye (80% [v/v] deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% [w/v] Xylene Cyanol, 0.1% [w/v] Bromophenol blue) was placed in adjacent wells to monitor the electrophoresis which was continued until the bromophenol blue reached the bottom (2½-3 hrs). The gel plates were separated from the apparatus, one glass plate removed and the gel face covered with Saran wrap. In a dark room, a film (Fuji RX) was placed over the gel and exposed for up to 4 min.

This was developed to locate the band of interest which was excised with a clean scalpel. The DNA was extracted from the gel slice by the 'crush and soak' method [see Section 2.8.3, iv)] and resuspended in 100 μ l TE to give a solution of 15,000-45,000 cpm/ μ l ($2.4 - 7.2 \times 10^6$ cpm/ μ g).

Double-stranded oligonucleotides were labelled in a similar procedure: oligonucleotide (5 pmol) was mixed with 2 μ l kinase buffer, 2.5 μ l ^{32}P - γ -ATP (5000 Ci/mmol; 25 μ Ci), 1 μ l T4 polynucleotide kinase and distilled water to give 20 μ l final volume and incubated at 37°C for 45 min. The reaction was stopped by the addition of 0.8 μ l 0.5 M EDTA (pH 8.0) and 1 μ l 20% (w/v) SDS, mixed with 5 μ l 20% (w/v) Ficoll, and loaded into one well of a 10% non-denaturing polyacrylamide gel [see Section 2.8.3, iii)]. This was electrophoresed at 150 V for approximately 1 hr (with formamide load dye in adjacent wells) and the labelled oligonucleotide located by autoradiography for 2 min as described earlier. After elution from the gel slice and removal of polyacrylamide fragments [see Section 2.8.3, iv)] the solution was extracted once with phenol/chloroform/iso-amyl alcohol (25:24:1) and ethanol precipitated in the presence of carrier tRNA (5 μ g). The oligonucleotide was recovered by centrifugation at 20,000g for 25 min, washed twice with 70% ethanol and resuspended in 100 μ l TE. This gave a solution of 50,000-100,000 cpm/ μ l ($3.5 - 7.0 \times 10^7$ cpm/ μ g).

ii). End-filling with reverse transcriptase

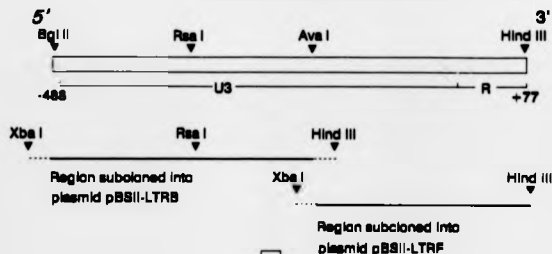
The protocol described above for the labelling of DNA fragments was followed except the end-filling reaction was performed in place of the kinase reaction. Ten microgrammes of DNA (4 μ l), digested with the first restriction enzyme, was mixed with 5 μ l RT buffer (100 mM [pH 8.3], 800 mM KCl, 100 mM MgCl_2 , 20 mM dGTP, 20 mM dTTP), 2 μ l 300 mM β -mercaptoethanol, 12.5 μ l ^{32}P - α -ATP (6000 Ci/mmol; 125 μ Ci), 12.5 μ l ^{32}P - α -CTP (6000 Ci/mmol; 125 μ Ci), 12 μ l distilled water and 2 μ l (40 units) AMV reverse transcriptase. This was incubated for 1 hr at 37 °C. The reaction was terminated and the protocol continued as previously described. Due to the more efficient

incorporation of two labelled nucleotides into each DNA molecule the exposure time for autoradiography of the gel was reduced to 5-20 seconds and the final LTR fragment resuspended in 200 μ l TE. This gave a solution of 135,000-175,000 cpm/ μ l ($4.4 - 5.7 \times 10^7$ cpm/ μ g).

Plasmid	First enzyme	Labelling protocol	Second enzyme	Probe
pBSII-LTRB	{ Xba I Hind III }	<i>Kinase</i>	Rsa I	194C
			Rsa I	159NC
pBSII-LTRB	{ Xba I Hind III }	<i>End-filling</i>	Rsa I	194NC
			Rsa I	159C
pBSII-LTRF	Hind III	<i>Kinase</i>	Xba I	FNC
pBSII-LTRF	Hind III	<i>End-filling</i>	Xba I	FC

Table 2.3. Production of radiolabelled probes spanning the HIV-1 LTR, indicating the starting plasmid, sequence of restriction enzyme digestions and labelling protocols followed in order to produce each probe end-labelled on one strand only. For additional information on the region of the LTR contained in each probe see Figure 2.3.

HIV-1 LTR



↓

Radio labelling protocol
(see Section 2.12.2 and Table 2.3)

↓

Double stranded DNA probes end-labelled on one strand:

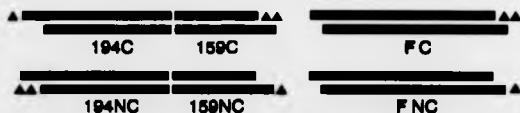


Figure 2.3. Schematic diagram showing the radiolabelled probes derived from the HIV-1 LTR (positions -488 to +77). The Bgl II - Hind III LTR fragment was subcloned in two separate fragments to produce plasmids pBSII-LTRF and pBSII-LTRB. These were employed in the radiolabelling procedures detailed in Section 2.12.2 and Table 2.3 which gave rise to six double stranded DNA probes corresponding to the entire LTR. Probes 194C, 159C and FC were labelled on the coding strand and 194NC, 159NC and FNC on the non-coding strand. ▲ indicates the position and number of labelled nucleotides in each probe.

2.12.3. Sequencing of double stranded DNA

i). Chemical cleavage method

The LTR probes were sequenced by this method (Ausubel *et al.*, 1990) to allow the pattern of fragments produced by DNase I foot-printing to be correctly orientated with respect to the sequence that they define.

Sufficient DNA (see Table 2.4) for the required chemical cleavage reactions was precipitated with isopropanol and the pellet washed twice with 70% ethanol. The DNA was dried and resuspended in sterile distilled water then distributed between the sequencing reactions. The chemical cleavage protocol was carried out as detailed in Table 2.4 and the DNA, after the final evaporation, redissolved in 5 μ l formamide load dye. The compositions of the buffers are given below.

DMS reaction buffer

50 mM sodium cacodylate (pH 8.0)

1.0 M β -mercaptoethanol

100 μ g/ml tRNA

DMS stop buffer

1.5 M sodium acetate

1 mM EDTA (pH 7.0)

Filter sterilized, then

100 μ g/ml tRNA added.

Hydrazine stop buffer

0.3 M sodium acetate (pH 7.0)

0.1 mM EDTA

Filter sterilized then 25 μ g/ml

tRNA added.

Table 2.4.
Outline of Maxam and Gilbert
chemical sequencing reactions

Component	Specificity of DNA cleavage			
	G	G+A	T+C	C
DNA, μ l	5 ^a	10 ^b	10 ^b	5 ^a
DMS Reaction buffer, μ l	200	-	-	-
Water, μ l	5	-	10	10
5 M NaCl, μ l	-	-	-	5
Base-specific modification reactions.				
DMS, μ l	1	-	-	-
Formic acid, μ l	-	25	-	-
Hydrazine, μ l	-	-	30	30
Time (min), at RT	4	10	15	8
Reaction stopped by dilution with stop buffer and -30°C ethanol and immersing in ethanol/dry ice bath for 5 min.				
DMS stop buffer, μ l	50	-	-	-
Hydrazine stop buffer, μ l	-	200	200	200
Ethanol, μ l	750	750	750	750
<i>DNA collected by centrifugation at 20,000g for 10 min, washed twice with 70% ethanol and air-dried.</i>				
Remaining reagents removed by re-precipitation (as above)				
Water, μ l	200	200	200	200
3 M Sodium acetate, μ l	20	20	20	20
Ethanol, μ l	500	500	200	200
Strand cleavage reaction at modified bases by incubation for 30 min at 80°C in 10% piperidine.				
10% (v/v) piperidine, μ l	70	70	70	70
<i>70 μl water added after incubation, vortexed and evaporated</i>				
All traces of piperidine removed by evaporation twice from water.				
Sterile water, μ l	80	80	80	80
Sterile water, μ l	50	50	50	50

^a10,000, ^b20,000 Cerenkov cpm

ii). Sequencing gels

DNAse I digested DNA from foot-printing reactions along with chemically sequenced DNA, or RNA from ribonuclease protection assays were resolved on 8% (w/v) acrylamide (19:1 acrylamide: bis-acrylamide) 8 M urea gels.

Wedge gels (0.4-1.2 mm) were poured between glass plates measuring 31.0 x 38.5 cm (width x length) and allowed to polymerize overnight by the addition of 850 μ l 10% (w/v) ammonium persulphate and 95 μ l TEMED. Gels were electrophoresed in a Gibco-BRL model S2 apparatus at 85 W (approximately 50 mA, 1500-1600 V) in 1 x TBE for 40-60 min to pre-run and warm the gel. The samples were loaded after being denatured at 90°C for 3-4 min, frozen in ethanol/dry ice and thawed on ice. The gels were run at the same settings for 2½-3 hrs to achieve the desired degree of separation. One glass plate was then removed and the gel fixed by immersion in 10% (v/v) acetic acid for 45 min. Afterwards, the gel face was covered with Saran wrap and the gel dried under vacuum at 80°C for 1½ hrs. Gels were autoradiographed at -70°C for 2-5 days with two intensifying screens using Fuji RX film.

2.12.4. Gel retardation assay

The method used was based on that of Goodwin (1990). To perform a gel retardation assay the following reagents were gently mixed in a microcentrifuge tube: 9 μ l GR buffer (40 mM Hepes-KOH [pH 7.6], 8% [w/v] Ficoll, 10 mM $MgCl_2$, 80 mM NaCl, 0.2 mM EDTA, 1 mM DTT and 0.5 mM $ZnCl_2$), 1 μ g pUC13 plasmid DNA, end-labelled DNA probe (1×10^4 cpm)^b, poly (dI:dC)-(dI:dC)^c, 10-25 μ g of crude nuclear extract and distilled water to 20 μ l. This was incubated on ice for 60 min and loaded into one well of a 4% (for oligonucleotide probes) or 5% (DNA fragment probes) non-denaturing polyacrylamide gel at low ionic strength [see Section 2.8.3, iii)]. Gels were

b). Probes were either oligonucleotides (10-20 *femt*mol, see Table 2.2) or end-labelled LTR fragments (2-5 *femt*mol, see Section 2.12.2).

c). This was 0.3-0.4 μ g for oligonucleotides or 3-5 μ g for DNA fragments.

pre-electrophoresed for 60 min and the buffer (0.2 x TBE) re-circulated prior to loading and during the electrophoresis. Formamide load dye was placed in empty wells to provide a marker and samples electrophoresed until the free probe was at the bottom of the gel, at 150 V this was approximately 5-6 hrs for DNA fragments and 1½-2 hrs for oligonucleotides. The gels were fixed for 20 min in 10% (v/v) glycerol, to retard cracking, and dried at 80°C under vacuum for 30 min, prior to autoradiography at -70°C for 1-3 days with two intensifying screens.

2.12.5. DNase I foot-printing

A limited DNase I digestion of an end-labelled LTR probe was performed *in vitro* in the presence of bound nuclear protein by the method of Goodwin (1990). Crude nuclear extract (0-225 µg) was gently mixed with 4 µg poly (dl:dC)-(dl:dC) and 2-5 femtomoles of end-labelled DNA Fragment (2×10^4 cpm) in a 100 µl final volume of 20 mM Hepes-KOH (pH 7.8), 40 mM NaCl, 2 mM CaCl₂, 5 mM MgCl₂, 1 mM DTT, 0.5 mM ZnCl₂, 10% (v/v) glycerol. This was incubated on ice for 60 min then digested with 23 ng of DNase I for 15 s on ice and 60 s at room temperature. The reaction was stopped by the rapid addition of 100 µl stop buffer (2% [v/v] SDS, 10 mM EDTA [pH 8.0], 0.1 mg/ml tRNA) followed by 200 µl phenol/chloroform/iso-amyl alcohol (25:24:1) and mixed by vortexing. The phases were separated by centrifugation (5 min, 20,000g) and the supernatant extracted again with phenol/chloroform/iso-amyl alcohol (25:24:1). The DNA was precipitated with 2.5 volumes of ethanol then pelleted at 20,000g for 10 min. The pellet was washed once with 70% ethanol and all traces removed from the sample by a brief re-spin and aspiration with a micro-pipette. It was then resuspended in 5 µl formamide load dye and heated to 90°C for 3 min, frozen rapidly in an ethanol/dry ice bath and allowed to thaw on ice before loading on to an 8% sequencing gel [see Section 2.12.3, ii)].

CHAPTER 3

CHAPTER 3

Chapter 3: Construction of plasmid vectors

Introduction

In the study of HIV-1 gene expression in mammalian cells the molecular biological techniques employed centred upon the use of plasmid vectors to express HIV-1 or reporter genes under the control of viral promoters and to manipulate HIV-1 sequences. Details of the construction and structure of plasmid vectors are given in the following chapter and summarized in Table 3.1. The numbering system for the HIV-1 sequences used was the same as that employed by the Beckman Microgenie data base and cited by the original reference for the sequence involved, except for the HIV-1 LTR which was numbered relative to the start of transcription where +1 was the first nucleotide of nascent transcripts.

3.1. Chloramphenicol acetyl transferase expression vectors

All plasmids contained the reporter gene bacterial chloramphenicol acetyl transferase (CAT), SV40 donor and acceptor splice sites and polyadenylation signals from the gene encoding small T-antigen, in addition to a bacterial origin of replication and β -lactamase gene for the propagation of recombinant *E. coli* using ampicillin selection.

The construct pLC2R (Herbomel *et al.*, 1984) expressed CAT under the direction of the HIV-1 long terminal repeat (LTR) subcloned from the LAI isolate (Wain-Hobson *et al.*, 1985; also see Wain-Hobson *et al.*, 1991) as an 826 bp Xho I to Nar I fragment spanning the entire U3, R and U5 regions. This plasmid was kindly supplied by Dr. J. Karn, MRC Laboratory of Molecular Biology, Hills Road, Cambridge. Due to the absence of a detailed map of the HIV-1 LTR CAT expression vector this plasmid was subjected to a limited restriction mapping, shown in Figure 3.1

Table 3.1. Summary of the plasmid functions

Plasmid Designation	Promoter driving expression	Gene
pLC2R	HIV-1 LTR	Chloramphenicol acetyl transferase
pSV2CAT	SV40 late promoter	
pKILTRCAT	Kirsten MSV LTR	
pOCAT	none	β -galactosidase
pCH110	SV40 late promoter	
pRSV β gal	RSV LTR	
pSVLtat	SV40 late promoter	HIV-1 (BH10) <i>tat</i>
pSVLnefS		HIV-1 (BH10) <i>nef</i>
pSVLnefII		HIV-1 (SF-2) <i>nef</i>
pSVLvpr		HIV-1 (BH10) <i>vpr</i>

Plasmids pSV2CAT and pKiLTRCAT contained the SV40 late promoter (Gorman, 1985) and Kirsten murine leukemia virus LTR (Norton *et al.*, 1984) respectively, to drive expression of the CAT gene. Plasmid pOCAT contained only the CAT reporter gene and no inserted viral or cellular promoter sequences. This was constructed from pSV2CAT by removal of the SV40 promoter as an Acc I - Hind III fragment and re-ligation of the plasmid after blunt ends had been formed. These plasmids were prepared and donated by Mr. George Ward, Department of Biological Sciences, University of Warwick.

3.2. β -galactosidase expression vectors

Plasmids pCH110 (Pharmacia) and pRSV β gal expressed the bacterial *lac z* gene (β -galactosidase) from the SV40 late promoter and Rous Sarcoma virus LTR respectively. Polyadenylation and small T-antigen donor and acceptor splice sites for the processing of transcripts were from SV40. These vectors also contained a bacterial origin of replication and β -lactamase gene.

Plasmid pRSV β gal was constructed by restriction digestion of pCH110 DNA with Hind III and Bam HI to release a 3736 bp fragment (nucleotides 1-3736) containing the *lac z* open reading frame. This was isolated by electro-elution after electrophoresis of the digest through an agarose gel and ligated into CIAP-treated, Hind III/Bgl II digested RSV-33. RSV33 is a eukaryotic expression vector, obtained from Mr. George Ward, which contains the RSV LTR, SV40 processing signals and sequences for maintenance in bacteria. The ligation mixture was used to transform *E. coli* TG2 and positive colonies selected. These were blue due to the expression of β -galactosidase from a prokaryotic promoter in the inserted sequence when recombinants were grown on LB agar plates containing ampicillin, X-gal and IPTG. Plasmid DNA was prepared by the 'mini-prep' method from overnight cultures of one white and several blue colonies and was screened by restriction digestion

followed by agarose gel electrophoresis to identify pRSV β gal. A diagram of pRSV β gal is shown in Figure 3.2.

3.3. Construction of vectors for the expression of HIV-1 genes

All plasmids were constructed using pSVL, a eukaryotic expression vector in which cloned genes inserted into a multiple cloning site were expressed under the control of the SV40 late promoter. Transcripts were spliced and polyadenylated using SV40 VP1 processing signals and the vector also contained a bacterial origin of replication and β -lactamase gene.

3.3.1. *tat*

Plasmid pSVL*tat* was constructed to express the *tat* gene of HIV-1. A molecular clone of isolate BH10, plasmid pBH10 Δ R3 (Ratner *et al.*, 1985), which contained a proviral DNA copy of an incomplete genome was obtained from the MRC AIDS reagent programme. DNA was digested with Sal I and Bam HI to release a 2686 bp fragment (nucleotides 5145-7831) which was agarose gel-purified and ligated into dephosphorylated Xho I/Bam HI restricted pSVL. The ligation mixture was then used to transform competent *E. coli* TG2 and plasmid DNA from ampicillin resistant colonies screened by restriction enzyme digestion. The recombinant plasmid (Figure 3.3.) contained both exons of *tat* and a functional protein was expressed when measured by transactivation of pLC2R in CAT assays.

3.3.2. *nef*

Vectors were constructed to express the *nef* open reading frame from two HIV-1 isolates. Plasmid pSVL*nefII* contained a functional *nef* gene derived from pARV2 Δ 7A, a molecular clone of the SF-2 proviral genome (formerly ARV-2) which lacked the 5' LTR and was permuted by pUC19 at a unique Eco RI site

(Sanchez-Pescador *et al.*, 1985). Plasmid pARV2 λ 7A was obtained from Dr. Dino Dina, Chiron Corporation, Emeryville, California, USA. Plasmid pSVLnefS contained a defective *nef* gene from pBH10 λ R3 (Ratner *et al.*, 1985) truncated by a stop codon at position 124 of 207 amino acids.

Plasmid pSVLnefII was constructed in the following way: a 961 bp fragment (nucleotides 8627-9588) was isolated from Pst I and Sst I digested pARV2 λ 7A by electrophoresis of the digested DNA through an agarose gel, followed by electro-elution of the fragment. The 961 bp fragment was then digested with Taq I. This produced two fragments of 521 bp (nucleotides 8916-9437) and 288 bp (nucleotides 8627-8915) which contained the *nef* open reading frame, designated *nef* L and *nef* S respectively, and a third 150 bp fragment (nucleotides 9438-9588) derived from the 3' LTR. *Nef* L and S were isolated separately by electro-elution following electrophoresis of the digest through an agarose gel and, after dephosphorylation of *nef* L, were combined in a ligation with Pst I/Acc I restricted pUC13 (Yanisch-Perron *et al.*, 1985). Competent bacterial cells were transformed and inoculated onto agar plates containing ampicillin, X-gal and IPTG. The agar plates were incubated overnight at 37°C. Due to the insertional inactivation of the β -galactosidase gene colonies containing inserts were white. Restriction digests of 'mini-prep' DNA from recombinant colonies were carefully analysed to determine the correct arrangement of *nef* fragments. The resulting clone contained the complete *nef* gene (nucleotides 8627-9437) and was designated pUC13nefARV2. Caesium chloride density gradient-purified pUC13nefARV2 DNA was then digested with Hind III and the termini blunt-ended with Klenow DNA polymerase I before digestion with Bam HI to release all the HIV-1 sequences. The insert was isolated from an agarose gel and used to ligate CIAP-treated Sma I/Bam HI restricted pSVL. Plasmid pSVLnefII was subsequently generated following transformation and screening of plasmid DNA from recombinant bacterial colonies by restriction enzyme digestion (see Figure 3.5).

In order to produce pSVLnefS a 1096 bp Bam HI - Sst I fragment (nucleotides 7832-8928) was isolated from a pBH10ΔR3 Bam HI/Sst I digest by agarose gel electrophoresis and subsequent electro-elution which was then ligated into CIAP-treated Bam HI/Sst I digested pUC13 DNA. The ligation mixture was used in a transformation reaction and 'mini-prep' plasmid DNA from ampicillin resistant colonies was screened by restriction enzyme digestion to obtain pUC13nef containing the *nef* fragment. Caesium chloride density gradient purified pUC13nef DNA was digested with Xba I and Sst I to release the *nef* gene and, after gel purification, was ligated into dephosphorylated, Xba I/Sst I digested pSVL. Plasmid pSVLnefS was identified following the usual transformation and screening procedure (see Figure 3.6).

3.3.3. *vpr*

Both available sources of cloned HIV-1 DNA, (from the isolates BH10 and SF-2) contained defective *vpr* genes as only the 96 amino acid allele is functional (Cohen *et al.*, 1990b). The *vpr* gene from pBH10ΔR3 was truncated at 84 aa due to a frame-shift mutation, and a three base pair insertion in the corresponding SF-2 gene encodes a reportedly inactive 97 aa protein (Cohen *et al.*, 1990b). However, the fortunate positioning of restriction sites in the BH10 *vpr* gene allowed simple repair of the mutated sequence with an oligonucleotide. (designated VPR, see Chapter 2, Section 2.4.).

Initially the gene was subcloned into plasmid pBSIIKS+ [Stratagene] (Mead *et al.*, 1985) in the following procedure. Plasmid pBH10ΔR3 was digested with Stu I and a 1424 bp fragment (nucleotides 4764-6188) containing the *vpr* gene isolated from an agarose gel by electro-elution. The fragment was then digested with Rsa I and a 490 bp Stu I - Rsa I fragment (nucleotides 4764-5254) gel-purified and ligated into Hinc II restricted and dephosphorylated pBSIIKS+. Following transformation of *E.coli* and inoculation onto ampicillin/X-gal/IPTG LB agar plates, recombinant

colonies were again white due the inactivation of β -galactosidase expression by the foreign insert. Plasmid DNA was prepared from suitable colonies by the 'mini-prep' procedure and analysed by restriction enzyme digestion and agarose gel electrophoresis in order to identify the recombinant plasmid, pBSIIvprS. Caesium chloride density gradient purified plasmid pBSIIvprS DNA was subsequently digested with Eco RI and Sal I. This released a 43 bp fragment (nucleotides 5102-5145) containing the single base pair insertion in the *vpr* gene and a 337 bp Eco RI - Eco RI fragment containing the part of the *vpr* gene (nucleotides 4764-5101) due to the presence of an Eco RI site in the multiple cloning site of pBSIIKS+. The *vpr* Eco RI fragment and the pBSIIKS+ vector band, which also possessed part of the *vpr* sequence from the Sal I site to the Rsa I/Hinc II junction (nucleotides 5146-5254), were excised from an agarose gel and extracted by electro-elution in order to remove the 43 bp fragment. A synthetic oligonucleotide, VPR, which comprised of coding sequences necessary to return the open reading frame to its wild-type, pre-mutated status was then substituted for the 43 bp fragment. The *vpr* Eco RI fragment, VPR oligonucleotide and the isolated pBSIIKS+ vector DNA were used as substrates in a ligation reaction, in which the DNA fragments were at equimolar ratios and the oligonucleotide at 100-fold molar excess (oligonucleotides would not concatenate due to the absence of 3' terminal phosphates) and subsequently used to transform competent *E.coli*. Novel Pst I and Pvu II sites were introduced into the *vpr* gene by conservative mutations in the oligonucleotide and these were used to identify recombinants carrying the repaired gene, designated pBSIIvprR.

Caesium chloride density gradient purified pBSIIvprR DNA was digested with Xho I and Hind III to release the repaired *vpr* gene, followed by a Klenow end-filling reaction to produce blunt ends. The Xho I - Hind III fragment was then purified by electro-elution from an agarose gel and used in a ligation with Sma I digested and phosphatased pSVL. Following transformation of *E.coli* and screening

of ampicillin resistant colonies, a vector containing the *vpr* gene in the correct orientation was obtained, and this was designated pSVL*vpr* (see Figure 3.4).

3.4. Subcloning of the HIV-1 LTR

The HIV-1 long terminal repeat sequences were subcloned from the CAT expression plasmid pLC2R. Plasmid DNA was linearized by digestion with *Ava* I, at site a 159 bp 5' to the junction of the U3 and R regions (see Chapter 1, Figure 1.1 and Chapter 2, Figure 2.3), and treated with Klenow DNA polymerase I to produce blunt termini. The DNA was then digested with *Bgl* II and *Hind* III to release the complete LTR in two fragments. A 236 bp fragment (*Ava* I - *Hind* III) spanning most of R and 159 bp into U3 (LTR *F*), and one of 329 bp (LTR *B*) covering the remaining 5' end of U3. These were separately isolated from an agarose gel, purified by electro-elution, and then ligated into dephosphorylated pBSIIKS+ which had been digested with enzymes to generate compatible termini. For LTR *F* the vector was digested with *Sma* I and *Hind* III, and LTR *B* *Bam* HI and *Eco* RV. The ligation was used to transform competent *E.coli* TG2 and cells were plated on LB agar plates containing ampicillin, X-gal and IPTG in order to select for colonies containing inserted DNA. Plasmids pBSII-LTR*F* and pBSII-LTR*B* (Figure 3.7 and 3.8, respectively) were identified following digestion of 'mini-prep' plasmid DNA with suitable restriction enzymes and analysis by agarose gel electrophoresis.

Figure 3.1. Plasmid pLC2R

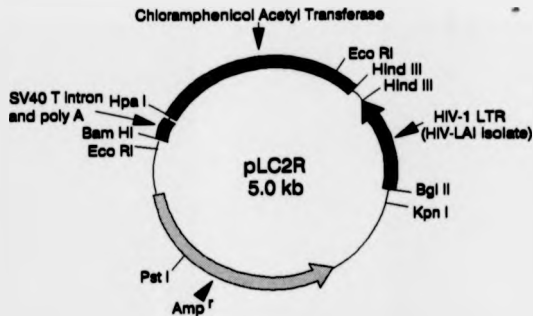


Figure 3.2. Plasmid pRSV β gal

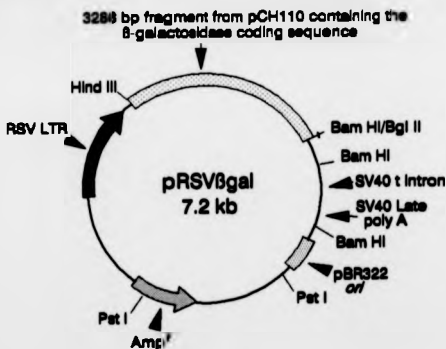


Figure 3.3. Plasmid pSVLtat

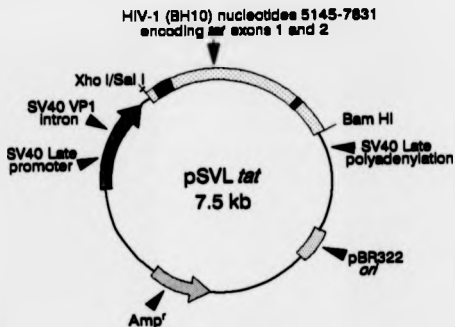
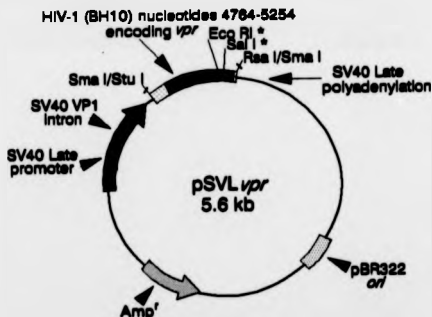


Figure 3.4. Plasmid pSVLvpr



* Inserted synthetic de oligonucleotide (VPR) from Eco RI to Sal I sites
to remove the mis-sense mutation in the BH10 coding sequence.

Figure 3.5. Plasmid pSVLnefII

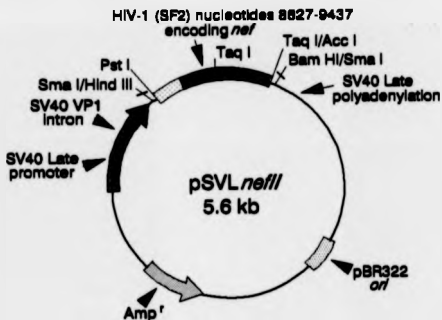
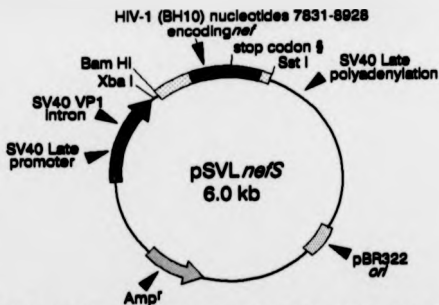


Figure 3.6. Plasmid pSVLnefS



§ UAG translational stop codon at position 124 of 207 amino acids.

Figure 3.7. Plasmid pBS-LTR F

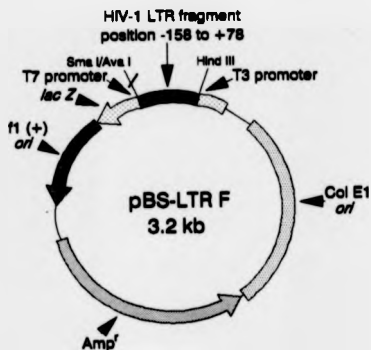
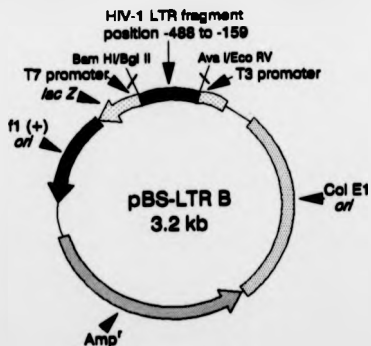


Figure 3.8. Plasmid pBS-LTR B



Discussion

All CAT expression plasmids were available from the sources given in the relevant section of the text and it was unnecessary to construct any further vectors of this type for the study.

The β -galactosidase expression plasmid, pCH110, was employed in the co-transfection of most cell types as the internal control for transfection efficiency. Expression of β -galactosidase from the SV40 late promoter (pCH110) was not detectable in Jurkat lymphoblastoma or SK-N-MC neuroblastoma cells so a similar vector expressing β -galactosidase under the control of the RSV LTR was constructed. When tested in transient co-transfection experiments with Jurkat and SK-N-MC cells pRSV β gal produced a satisfactory signal and was subsequently used in the standardisation procedure for these cell lines.

For reasons of biological containment, only cloned HIV-1 sequences that ^{derived} were from incomplete viral genomes were suitable for genetic manipulation and ^{as} were replication incompetent and unable to produce progeny virus.

Plasmid pSVL_{lat} was constructed to express both exons of the transactivator protein Tat. The large Sal I - Bam HI fragment (nucleotides 5145-7831) subcloned into pSVL also spanned the open reading frames of several other HIV-1 genes; *vpr*, *vpu*, the first exon and part of the second exon of *rev*, and most of *env* (containing gp120 and part of the gp41 coding sequences). But no other gene should result in the expression of a functional protein. The *vpr* gene of this viral isolate (BH10) has already been described in Section 3.3.3 and is non-functional due to frame-shift mutation (Cohen *et al.*, 1990b). *Rev* function would also not be present as there was insufficient coding sequences from the second exon to produce a functional protein (Hadzopoulou-Cladaras *et al.*, 1989; Kjems *et al.*, 1991). Also, it has been noted that splicing of RNA from *lat* expression vectors containing the large Sal I - Bam HI fragment, such as pSVL_{lat}, are Rev-responsive due to the possession of the RRE in

the gp120 coding sequence (Malim *et al.*, 1988). In the absence of Rev, the transcript is spliced using a splice donor site just downstream of the first exon of *tat* and a splice acceptor upstream of exon 2. Splicing of the transcript will preclude expression of *vpu* or any *env* coding sequences as both depend upon Rev for their expression (Arrigo *et al.*, 1990).

Plasmid pSVL nefS contained a truncated *nef* gene that is reported not to produce a stable Nef protein (Hammes *et al.*, 1989) and the construct was employed to assess the effect of transfection of such plasmid on HIV-1 LTR driven CAT expression. Plasmid pSVL nefII was constructed to express the entire *nef* open reading frame, although from a different isolate of HIV-1, which promised the expression of the complete functional Nef protein.

As already described, each *vpr* gene contained in isolates BH10 and SF-2 was defective, and so the BH10-derived coding sequence was returned to wild type by insertion of the VPR oligonucleotide. This repair was necessary as, like *nef*, *vpr* is often found to be inactivated by mutation in cloned isolates of HIV-1 derived from viruses isolated by tissue culture. The 96 aa protein that the 'repaired' gene should encode has an identical amino acid composition to the functional LAJ Vpr protein (Cohen *et al.*, 1990b).

The HIV-1 LTR was removed as two fragments, LTRF and LTRB, and each subcloned separately in order to produce constructs that were suitable for radiolabelling the LTR (see Chapter 2, Section 2.12.2). The convenient positioning of restriction enzyme sites within and at the borders of the HIV-1 LTR in plasmid pLC2R allowed this procedure to be performed without the disruption of any known nuclear factor binding sites contained within the HIV-1 LTR.

CHAPTER 4

Chapter 4: Transient gene expression directed by the HIV-1 LTR and the effects of the regulatory genes, tat, nef and vpr

Introduction

The assessment of promoter activity was performed by the use of reporter gene assays in which bacterial chloramphenicol acetyl transferase, linked to the HIV-1 LTR or other viral promoters, provided a measurement of the level of expression in eukaryotic cells. These studies relied upon the efficient introduction of plasmid constructs into cells where they were expressed transiently in an episomal form (Alam and Cook, 1990), and the mRNA processed and translated. The level of enzymatic function of the protein product was then determined in cytoplasmic extracts. Reporter genes, however, do not provide a direct assessment of transcription from the promoter, rather a measurement of gene expression. This is likely to be related to the steady-state RNA levels and, perhaps to the frequency of transcriptional initiation at the promoter but other factors, such as regulation at a post-transcriptional level and mRNA stability, should be taken into consideration. The efficiency of translation into functional enzyme may also effect the level of CAT activity present. These qualifications aside, reporter gene assays provide useful data on the functions of cloned regulatory elements.

In order to produce a more accurate measurement of CAT activity in transfected cells, a standardisation procedure was employed to compensate for any variation in the efficiency of transfection. A second reporter gene, β -galactosidase, was co-transfected with the CAT plasmid in question, expression of β gal was driven by either the SV40 late promoter or the RSV LTR, and its activity in cellular extracts provided an estimate of transfection efficiency, such that the CAT activity was determined from a portion of extract from different dishes of transfected cells within an experiment that expressed the same level of β -galactosidase activity.

Hence variations in transfection efficiency would do little to effect the true level of CAT expression.

The direct scintillation diffusion method was performed to measure CAT activity in extracts and allowed the detection of lower amounts of CAT enzyme than the previous TLC-based procedure (Neumann *et al.*, 1987). Indeed, no CAT reporter gene activity was detected from the HIV-1 LTR in extracts from Jurkat cells (Okamoto *et al.*, 1989) yet a sufficient signal is produced in the same cells when assayed by the direct scintillation diffusion protocol (Figure 4.1); both in the absence of HIV-1 Tat. This assay provides a linear relationship between the amount of CAT enzyme present and radiolabelled product over a wide range so long as less than 50% of the tritium label (approximately 110,000 cpm) enters the scintillation cocktail during the 2 hr incubation.

4.1. HIV-1 LTR-driven reporter gene expression in a T lymphoblastoma and cells of neural origin

4.1.1. Assessment of relative promoter strength

Preliminary experiments were performed to estimate the level of CAT expression from the HIV-1 LTR in the range of neural cell types under study (see Chapter 2, Section 2.1 and Table 2.1). To provide an estimate relative to other viral promoters, independent of the transfection efficiency, cells were also transfected with CAT expression constructs under control of the SV40 late promoter or the K₁MSV LTR.

The results showed that the HIV-1 LTR produced a detectable signal in all cell types, although it was usually weaker than the other viral promoters in directing basal gene expression in the absence of Tat (Figure 4.1), and revealed a wide variation in the activities of different promoters in directing CAT expression in the range of cell types studied. Direct comparison between cell types is difficult due to

their varying transfection efficiencies and different amounts of extract protein were required in the assays to produce either a detectable signal or one that was not above the range of the CAT assay. There was one notable similarity in the pattern of expression from the different promoter constructs amongst one pair of cells of the same lineage: the primary murine astrocyte cultures and U373MG astrocytoma cells both produced a very strong signal from the SV40 late promoter compared to much weaker expression from the other two constructs. In contrast, the two human neuroblastoma cell lines, SK-N-SH and SK-N-MC, produced opposite levels of CAT activity from the viral promoter-CAT plasmids.

The results also show the level of expression from a CAT construct lacking any defined promoter or enhancer sequences. This represented the background level above which CAT activity was considered to be due to the inserted transcriptional element. In Jurkat cells, however, an anomaly appeared to exist, pOCAT directed relatively high levels of CAT expression, especially when compared to SV40 which was almost non-functional in these cells. This result has been found in another laboratory (personal communication, Dr. Marion Major, Regional Virus laboratory, East Birmingham Hospital) and presumably results from undefined sequences present in the prokaryotic elements of the plasmid. A similar result was not seen with any neural cell tested.

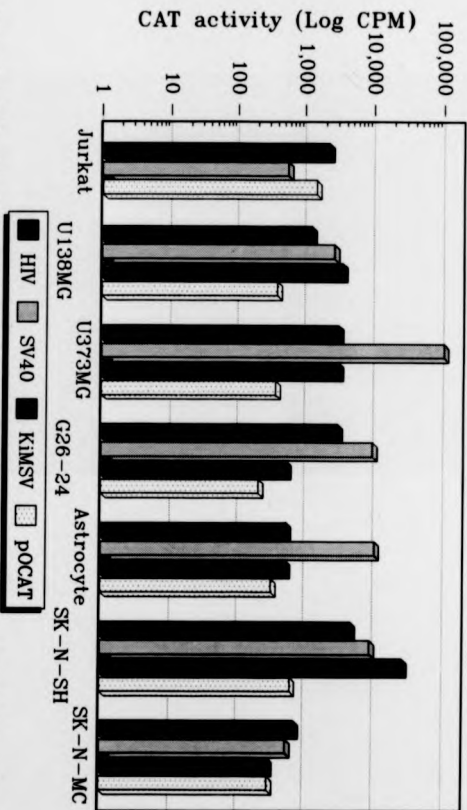


Figure 4.1. CAT activity (CPM) from the HIV-1 LTR (HIV), SV40 late promoter (SV40), K1MSV LTR (K1) and promoter-less (pO) CAT expression vectors in lymphoblastoma and neural cells 42-44 hrs after transfection. Note: absolute levels of reporter gene activity is also constrained by different transfection efficiencies between the cell types, such that the activity of different promoters may be accurately compared only within the same cell type, data is presented as such for ease of interpretation.

4.2. Modulation of HIV-1 gene expression by the regulatory genes of HIV-1

Because of the involvement of the regulatory genes of HIV-1 in the control of transcription and gene expression from the viral LTR (discussed in Chapter 1, Section 1.5.2), eukaryotic expression vectors were constructed to express the *tat*, *nef* and *vpr* genes. Each was co-transfected into cells with pLC2R in order to evaluate the individual effect of these genes on HIV-1 LTR-driven reporter gene expression.

4.2.1 Augmentation of CAT expression by the transactivator, Tat

The HIV-1 Tat protein was demonstrated to be functional in all neural cells and able to transactivate expression from the LTR to varying degrees of efficiency when cells were co-transfected with the Tat expression vector, pSVLtat, and pLC2R (Table 4.1). The precise levels of transactivation were found to be less than that achieved in the T lymphocyte cell line, Jurkat, especially in the murine G26-24 oligodendrogloma and primary astrocyte cells where a high level of Tat-mediated transactivation was not demonstrated. Murine cells are reported to lack at least one cellular protein co-factor that is required for the full activity of Tat in human cells (Newstein *et al.*, 1990).

The optimum amount of pSVLtat required for maximal transactivation was determined in preliminary experiments. It was noted that the different cell types varied in their sensitivity to increasing microgramme amounts of the Tat vector in the transfection buffer (20 μ g of pLC2R was always used). CAT activity from Jurkat and primary astrocyte cultures was transactivated to the same level over a range of pSVLtat concentrations (5-20 μ g and 2-8 μ g, respectively). However, with the other neural cell types there was an optimum amount of pSVLtat required to give maximum transactivation and excess resulted in reduced augmentation (except also U138MG in which 5 or 10 μ g of pSVLtat produced the same level of

transactivation). Hence quite precise amounts were often needed. For example, in one such cell line, SK-N-MC, optimum transactivation of HIV-CAT occurred with 2.5 μg of pSVLtat, and there was less CAT activity in extracts from cells transfected with 5 μg of pSVLtat than from cells transfected with pLC2R only. G26-24 cells were also as sensitive to the amount of pSVLtat, here 2 μg produced the highest level of transactivation and 5 μg reduced the amount of CAT expression to below that achieved with pLC2R alone. In other cell lines CAT expression was reduced to a lesser extent by co-transfection with amounts of pSVLtat above the optimum, such as U373MG cells where the highest transactivation was seen with 5 μg of pSVLtat and 10 μg of pSVLtat produced the same level of transactivation as 2 μg , some 25% less than the transactivation achieved with 5 μg .

4.2.2. The effect of the *nef* gene product on CAT expression

Initially the truncated *nef* gene, subcloned from HIV-1 isolate BH10, was used in transfection experiments to determine its effect on HIV-1 LTR-driven gene expression in Jurkat T lymphoblastoma cells. Co-transfection of pSVLnefS with pLC2R produced a dose-dependent reduction of CAT activity (Figure 4.2.A) sufficient to reduce expression to less than background levels when equal amounts of pLC2R and pSVLnefS were used. With lower amounts of pSVLnefS relative to pLC2R in the transfection there was still a significant reduction in CAT expression to around 45% of basal activity driven by the HIV-1 LTR.

The *nef* expression vector, pSVLnefII contained the full length *nef* gene from the SF-2 isolate of HIV-1. In co-transfection experiments with U138MG glioblastoma cells a decrease in CAT activity driven by the HIV-1 LTR was also found (Figure 4.2. B), although when a smaller amount of pSVLnefII was transfected with the same amount of pLC2R there was a reduction in CAT expression to a slightly lesser extent, compared to pSVLnefS in Figure 4.2. A. The suppression of CAT activity was similar for the two constructs when 10 μg of *nef* expression vector

was transfected, whereas at 5 μ g, pSVLnefS was slightly more effective, albeit in different cell types. U138MG glioblastoma cells were chosen to evaluate pSVLnefII due to their relative high efficiency of transfection compared to the other neural cells and Jurkat. U138MG therefore produced higher levels of CAT expression above background, indicated by the level of CAT expression from the 'promoter-less' CAT plasmid, p0CAT.

4.2.3. The effect of the vpr gene product on CAT expression

The expression vector for *vpr*, pSVLvpr, was constructed to contain an intact open reading frame which should be expressed and translated to produce the functional 96 aa form of the protein. However, co-transfection of the vector with pLC2R into U138MG glioblastoma cells resulted in a reduction of CAT expression that was not directly proportional to the amount of pSVLvpr contained in the transfection buffers (Figure 4.2. C). This was contrary to the reported function of Vpr which transactivates expression from the HIV-1 LTR (Cohen *et al.*, 1990b). In the absence of a direct assay for the expression of the Vpr protein this result may not accurately reflect the function of Vpr in this cell line. Although, as in all experiments duplicate dishes of cells were transfected and there was only the usual slight variation in 3 H-acetyl chloramphenicol production between duplicates.

Cell type	§ Fold transactivation
U138MG glioblastoma	18.6 ± 4.7
U373MG astrocytoma	10.4 ± 2.6
G26-24 oligodendroglioma ∞	2.2 ± 0.5
SK-N-MC neuroblastoma	12.3 ± 2.7
SK-N-SH neuroblastoma	30.1 ± 1.6
Primary astrocytes ∞	4.0 ± 0.8
Jurkat T-lymphoblastoma	41.4 ± 1.2

Table 4.1. Transactivation of the HIV-1 LTR by the Tat protein of HIV-1 in neural cells and a T lymphocyte cell line. § Figure represents the mean transactivation ± the standard deviation from two CAT assays, 42-44 hours post transfection, calculated as the ratio of ³H-chloramphenicol in extracts from cells transiently transfected with an optimum amount of pSVLtat, versus cells transfected with pLC2R alone. ∞ murine cells.

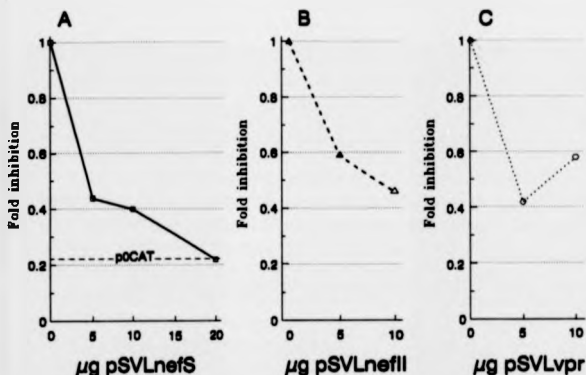


Figure 4.2. Effects of the regulatory genes, *nef* and *vpr* on HIV-1 LTR-driven gene expression in lymphoblastoma or glioblastoma cells. A. Inhibition of CAT expression from the HIV-1 LTR in extracts from Jurkat T lymphoblastoma cells after co-transfection with pSVLnefS (5-20 µg); the level of expression from pOCAT is indicated by a dashed line.

B. Inhibition of CAT expression from the HIV-1 LTR in extracts from U138MG glioblastoma cells after co-transfection with pSVLnefl (5-10 µg).

C. CAT activity driven by the HIV-1 LTR in extracts from U138MG glioblastoma cells after co-transfection with pSVLvpr (5-10 µg).

Note. Parts B and C: the level of CAT expression from pOCAT is not indicated due to the high level of CAT expression obtained with U138MG. This corresponds to a fold inhibition of 0.013 units.

Discussion

Reporter gene assays

The investigation of reporter gene expression directed by the different viral promoters served to establish the transfection and CAT assay techniques as suitable for the analysis of HIV-1 LTR-driven gene expression. Other reporter genes were considered, such as luciferase and secreted alkaline phosphatase (Alam and Cook, 1990), but neither one can be measured at more than one time point during the assay. In the direct-diffusion CAT assay the enzyme reaction was monitored over four time points during the two hour incubation which permitted the linear relationship of the enzyme/substrate reaction to be established and ensured a greater degree of accuracy in the results. Although, the numbers of assays done in this project regrettably preclude the use of line graphs an example is given in Chapter 2 (see Figure 2.2).

Experimental technique

In order to maintain the highest levels of transfection, buffers were prepared fresh monthly and stored at -20°C as deterioration in transfection efficiency had been noted with buffers stored for longer periods, and especially when at room temperature. Plasmid DNA for transfection purposes was always purified by caesium chloride density gradient centrifugation and samples were checked by agarose gel electrophoresis to ensure greater than 50% of the DNA was present in the closed circular form. This was estimated by visualization of an ethidium bromide stained agarose gel under UV light. Plasmid DNA that is linear or contains excessive single-strand 'nicks' does not transfect cells well and may fail to be expressed to the same extent (Gorman, 1985). These precautions resulted in transfection experiments that were always successful. Experience with commercial transfection 'kits' had shown that great care was needed with the quality of reagents.

The measurement of CAT activity by the method chosen and the incorporation of the β -galactosidase control for transfection efficiency allowed reproducible and precise results to be obtained. The variation in CAT activity between duplicate dishes of transfected cells was found to be extremely low. From a random selection of twenty-one pairs of measurements of the CAT activity from duplicates the standard deviation from the mean was determined for each pair and used to estimate the mean variation between duplicates as a percentage, this was found to be $4.9\% \pm 3.4$ (representing the sample mean \pm the standard error).

Tat transactivation

Expression of the Tat protein of HIV-1 in neural cells confirmed its role as a strong transactivator of gene expression from the HIV-1 LTR. From the clear enhancement of reporter gene expression seen it was evident that pSVLtat was able to express a functional protein and it was unnecessary to confirm the presence of Tat using the specific antibodies that were available. The highest level of Tat-mediated transactivation in each cell type was reported in Table 4.1, however, in repeat experiments with the same optimum amount of pSVLtat there was often an unusually large variation in the transactivation observed, even when the same preparations of plasmid DNAs were used. This suggests that slight differences in transfection efficiency occurring between different experiments were sufficient to alter the amount of Tat that is produced in the cells.

The variation in the levels of transactivation noted between the different neural cells may also have explanations which were not addressed by the simple co-transfection of pSVLtat and pLC2R. Apart from being related to the different nuclear environments of each cell type, the response to increasing amounts of the Tat expression vector in transfections indicates that different cell types require variable amounts of Tat protein to achieve maximal transactivation in this assay.

Although it is probable that different transfection efficiencies contribute, this may not account for the sensitivity of the transactivation response to higher doses of the Tat vector. It is likely that by employing amounts of pSVLtat above that needed for the optimum transactivation, Tat was over-expressed and the deleterious effect seen on gene expression due to a cytotoxic property of Tat which varied between the cell types. To highlight one example, SK-N-MC cells appeared from previous experiments to transfect at the lowest efficiency of all the neural cells examined, yet the transactivation response was one of the most sensitive to increases of only 2.5 μ g in the amount of pSVLtat co-transfected. Thus, whilst transfection efficiency may effect the absolute amount of expression vector required in the transfection these cells possess a sensitivity to Tat. This may relate to the neurotoxic potential of Tat which exerts significant toxicity towards neuroblastoma and glioblastoma cells in culture (Sabatier *et al.*, 1991).

The effect of Nef

The function of the Nef protein of HIV-1 in human cells has not been completely determined (see Chapter 1. Section 1.5.2. v). Early reports suggested that Nef was able to down-regulate both virus production (Kim *et al.*, 1989) and LTR-driven reporter gene expression and thus function as a transcriptional repressor of HIV-1 (Niederman *et al.*, 1989). However, further work suggested that the LTR sequences at the 3' end of the *nef* gene when present in an expression vector were responsible for the reduction of reporter gene expression from the LTR (Hammes *et al.*, 1989). The *nef* open reading frame is located at the 3' end of the viral genome in a region that also includes most of the U3 region of the LTR, the final codon of *nef* in HIV-1 isolate BH10 (Ratner *et al.*, 1985) and SF-2 (Sanchez-Pescador *et al.*, 1985) is 20 nucleotides upstream of the enhancer (see also Chapter 1, Figure 1.2). Therefore, two expression vectors for Nef were constructed: one, pSVLnef11, to encode a full length open reading frame (from SF-2) located in a

fragment that did not contain any unnecessary LTR sequences (the *Taq* I site at the 3' end *nef* is 3 nucleotides past the stop codon); and two, pSVL*nef*S, which contained the truncated *nef* gene from BH10 which does not give rise to a stable protein (Hammes *et al.*, 1989), located in fragment from the viral genome that extended past the enhancer into the R region of the LTR. Thus, containing most of the nuclear protein binding sites in the HIV-1 LTR. The two constructs were used to distinguish any function of Nef on LTR-driven reporter gene expression from transcriptional interference. The 3' LTR sequences that span the regulatory elements of the LTR are thought to sequester nuclear factors that are in limiting concentrations and in the absence of the Nef/LTR vector would direct increased gene expression from the LTR-CAT construct (Hammes *et al.*, 1989). Ideally, these plasmid constructs should be derived from the same allele of *nef* and attempts were made to obtain a *nef* gene derived from BH10 in which the stop codon at position 124 had been removed by site-directed mutagenesis. But this work had been performed by Glaxo Inc. (Geneva, Switzerland) who were not willing to release the vector (personal communication, Dr. Mark Harris, Department of Veterinary Pathology, University of Glasgow).

The results obtained from the co-transfection of the Nef expression vectors and pLC2R neither confirmed nor convincingly disproved an artificial role for the 3' LTR sequences in reducing LTR-driven gene expression as both constructs caused a decrease in CAT expression. However, contrary to results reported here, other workers (Hammes *et al.*, 1989) report that removal of LTR sequences to the *Taq* I restriction site described above results in no repression of CAT activity directed from the HIV-1 LTR. This finding was not reproduced here and may suggest a real suppression of LTR-driven gene expression by Nef, although further experimentation would be required. Hammes *et al.* (1989) transfected COS, Jurkat and U937 cells (a myelomonocytic cell line) with HIV-1 LTR-CAT, Nef and Tat expression vectors, presumably to allow sufficient CAT activity to be detected by the TLC-based method, and the effect of Nef alone on HIV-LTR-CAT expression was

not addressed. Whether co-transfection of Tat as well as Nef expression vectors would mask any effect of Nef remains to be determined. In the absence of an assay for the production of Nef from the pSVL_{Nef} vectors constructed here it is difficult to attribute directly the reductions seen in LTR-driven CAT expression to the Nef protein, particularly as no consensus exists in the literature for the function(s) of Nef. Antibody to Nef was not available from the MRC AIDS reagent programme nor was there a commercial source at the time. Also, it cannot be ruled out that the slight differences in the effects of the Nef vectors on the expression of CAT from the HIV-1 LTR may be due to different cell types employed.

The effect of Vpr

The effect of co-transfection of the Vpr expression vector was inconclusive in establishing a role for Vpr in HIV-1 LTR-driven gene expression. The aberrant result may be due to errors in the coding sequence introduced in the cloning protocol as ideally the 'repaired' gene should be sequenced. In addition, perhaps cells are very sensitive to the amount of vector transfected, similar to that noted with pSVL_{Tat}, and too large an amount was employed. Although, a recent report from Cohen *et al.*, (1991) showed a 3-fold transactivation of LTR-driven reporter gene expression with a ratio of Vpr to LTR-CAT vectors of 1:4, the same as tested here. The expression of antigenic Vpr in the transfected cells could also not be examined as no specific antiserum was available commercially nor even serum from HIV-1 positive individuals through the MRC AIDS reagent programme. As defining the precise function(s) of Vpr, like Nef, would require great deal of experimentation it was decided not to be pursued any further. For these reasons further work concentrated on the investigation of the function of Tat in the regulation of HIV-1 gene expression. However, the experiments done with *nef* and *vpr* were useful in that they validate the experimental system used.

CHAPTER 5

Chapter 5: Regulation of HIV-1 LTR-Driven gene expression by cytokines and phorbol ester

Introduction

Cytokines

The response of gene expression directed by the HIV-1 LTR to cytokines and phorbol ester was also investigated by the use of the chloramphenicol acetyl transferase (CAT) reporter gene. As described previously, cells were transiently transfected with the HIV-1 LTR-CAT construct (pLC2R), and a β -galactosidase expression vector, and subsequently exposed to cytokine(s) or PMA for 22-24 hrs. CAT activity was then determined in cell extracts to assess the effect of each cytokine on expression from the LTR relative to that in unstimulated cells. The range of cytokines was chosen on the basis of those which are already known to be important in the activation and regulation of immune responses to foreign antigens and further qualified by those that are also produced by resident cells of the central nervous system (see Chapter 1, Section 1.3.3 and 1.4). Although there may be other molecules that would regulate gene expression from the HIV-1 LTR other considerations, such as cost and the multiplication of the numbers of reporter gene assays required, served to confine investigations to five cytokines; TNF α , IL-1 β , IL-6, IFN $\alpha\beta$ and IFN γ and the known activator of HIV expression, PMA (Kaufman *et al.*, 1987). Human recombinant TNF α , IL-1 β and IL-6 were employed for both murine and human neural cells as these molecules are fully active in murine systems (manufacturer's data). Only the interferons from this group of cytokines are species-specific, therefore natural human IFN α and recombinant human IFN γ , and natural murine IFN $\alpha\beta$ and recombinant murine IFN γ were used.

Experimental technique

The β -galactosidase control plasmid was included in the transfections in order to standardize for transfection efficiency as described in Chapter 4, but because the promoters driving β -galactosidase expression are responsive to cytokines and PMA (unpublished observations) it was not possible to normalize between extracts from untreated cells and those treated with different agents. Therefore, the β -galactosidase activity in an equal amount of extract protein was determined for all samples, and each pair of duplicates, treated with the same or no cytokine/PMA, normalized so that CAT activity was measured in an aliquot containing the same amount of β -galactosidase activity.

To reduce further the variation in transfection efficiency between samples the calcium phosphate-DNA precipitate used to transfect cells was prepared in bulk, as the same plasmids were used to transfect many experimental samples, and an equal aliquot added to each dish of cells. The difference in transfection efficiency between duplicates, estimated from the β -galactosidase assay, was observed to be typically less than 10%. Indeed, from a random selection of 21 pairs of β -galactosidase assays taken from duplicates of cytokine-treated cells the mean variation was $6.2\% \pm 5.0$ (sample mean \pm standard error).

In initial experiments the effects of cytokines and PMA were determined on cells transfected with the 'promoter-less' control plasmid, p0CAT, in order to confirm that no sequences in the CAT gene itself or in the transcript processing signals resulted in increased background expression of CAT following exposure of cells to cytokines. As expected no cytokine, or PMA, could enhance the background level of expression from p0CAT, and in subsequent experiments it was only necessary to include unstimulated cells transfected with p0CAT as a control.

Co-transfection with the Tat expression vector

When cells were co-transfected with the Tat expression vector, pSVLtat, and pLC2R then exposed to cytokines or phorbol ester, duplicates treated with each agent and those also expressing Tat were normalized together as expression of Tat did not effect the expression of β -galactosidase from either the SV40 late promoter or RSV LTR (results not shown). For the measurement of CAT expression from the HIV-1 LTR in permanent cell lines expressing Tat from integrated retroviral vector (pMoLTRtat, see Chapter 2, Section 2.9.3 and Dingwall *et al.*, 1989) extracts were also standardized with respect to β -galactosidase activity between duplicates of untreated cells or those treated with the same agents.

5.1. Control of HIV-1 gene expression by cytokines and phorbol ester

5.1.1. Tumour necrosis factor- α

i). Augmentation by TNF α

The exposure of neural cells to TNF α resulted in a large augmentation of CAT activity directed by the HIV-1 LTR (Figure 5.1). TNF α was typically able to activate expression by two- to three-fold in all cells, with the highest values of 2.8- and 3.3-fold seen for U138MG glioblastoma and U373MG astrocytoma cells, respectively. For the other cell types TNF α produced approximately a two-fold enhancement of CAT activity.

The data presented in Figure 5.1 shows the highest level of augmentation of CAT expression by TNF α seen at its optimum concentration for each cell type, from the duplicate samples of one experiment. The optimum concentration was determined only for the neural cell lines and the concentration tested on the primary astrocyte cultures was estimated from these results. However, each cell type was tested between two and three times in separate experiments and TNF α was found to

always augment CAT expression. The concentration of TNF α required for maximum induction of gene expression from the HIV-1 LTR was in the region of 100 to 250 U/ml for most cell types. The one exception was the astrocytoma line U373MG, where a ten-fold higher concentration at 2500 U/ml was optimum.

Interestingly, no cytopathic effects of TNF α on any cell type were noted when added to the culture medium for up to 24 hrs (exposure for longer periods of time was not examined). TNF α is extremely cytotoxic for certain cell lines, such as L cells and COS (Okamoto *et al.*, 1989) but neural cells appeared to tolerate exposure well and in one case, U373MG, even proliferate in response to high concentrations of TNF α ($\leq 10,000$ U/ml; Lachman *et al.*, 1987) which probably accounts for the higher doses of TNF α employed for this cell line.

ii). The response of HIV-1 LTR driven CAT expression to increasing concentrations of TNF α in neural cell lines

Dose response curves for all the neural tumour cell lines were constructed from the augmentation of CAT expression in cells treated with TNF α at different concentrations in order to determine the nature of the dose-response and the cytokine concentration at which optimum stimulation of expression occurred. The results are shown in Figures 5.2 and 5.3.

From the data in Figure 5.2 it can be seen that significant augmentation of HIV-1 LTR-driven CAT expression occurred at low concentrations of TNF α in the three glial cell lines, U138MG glioblastoma, U373MG astrocytoma and G26-24 oligodendroglioma. At 1 U/ml of TNF α , U138MG and G26-24 produced over a 1.5-fold stimulation of CAT expression and in U373MG at 10 U/ml this was over 2-fold. In U138MG the level of augmentation then increased steeply reaching a peak at 10 U/ml, falling slightly at 100 U/ml, and producing maximum augmentation at 250 U/ml of TNF α . Although, there was not a great difference in the augmentation when TNF α was present at 10-250 U/ml. The graph for U373MG is very similar.

Augmentation rises sharply to a peak at 100 U/ml, falls again slightly at 1000 U/ml but reaches the optimum at 2500 U/ml where U373MG cells produced the highest level of HIV-1 LTR-driven CAT expression in response to TNF α . There is also little difference in the amount of augmentation between 100-2500 U/ml of TNF α . In G26-24 the initial rise in the level of augmentation is not as steep and develops more progressively than in U138MG or U373MG cells. The increase in augmentation begins to fall off at higher concentrations and forms a reasonable curve with a small difference in the enhancement of CAT expression between 100 and 250 U/ml. The optimum was also at 250 U/ml TNF α . The graphs in Figure 5.2 indicate that the response of HIV-1 LTR-driven reporter gene expression to TNF α in the glial cell lines appears to become saturated at the higher cytokine concentrations, and substantial responses occur at relatively low concentrations of TNF α .

In the neuroblastoma cell lines there was significant augmentation of LTR-driven CAT expression at 10 U/ml of TNF α and this reached an optimum at 100 U/ml in both cell lines. However at 1000 U/ml of TNF α the level of augmentation fell quite sharply to around the same as that produced by 10 U/ml in SK-N-MC and to less than this amount in SK-N-SH cells (Figure 5.3). The optimum enhancement by TNF α in neuroblastoma cells was defined by a sharp peak within the range of cytokine concentrations tested. The extreme sensitivity of the response to higher TNF α concentrations in the neuroblastoma cells may reflect the degree to which neuronal cells are susceptible to the cytotoxic effects of TNF α .

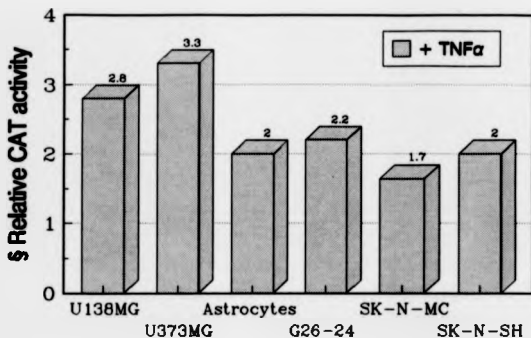


Figure 5.1. Augmentation of HIV-1 LTR-CAT expression by TNF α in neural cells. \$ Relative CAT activity was calculated from the mean cpm of ^3H -acetyl chloramphenicol product from extracts of cells transfected with pLC2R after TNF α treatment for 22-24 hrs divided by mean cpm without. Cytokine concentrations (U/ml) were U138MG 250; U373MG 2500; astrocytes 100; G26-24 250; SK-N-MC and SK-N-SH 100.

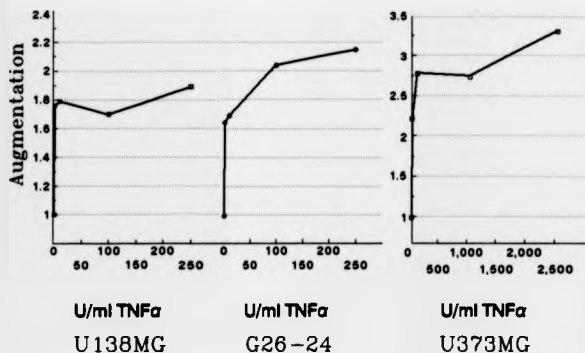


Figure 5.2. Augmentation of HIV-1 LTR-driven CAT expression in U138MG glioblastoma, U373MG astrocytoma and G26-24 oligodendrogloma cells by TNF α over a range of concentrations. Cells were transiently transfected with pLC2R and treated with TNF α for 22-24 hrs before CAT activity was determined in cytoplasmic extracts.

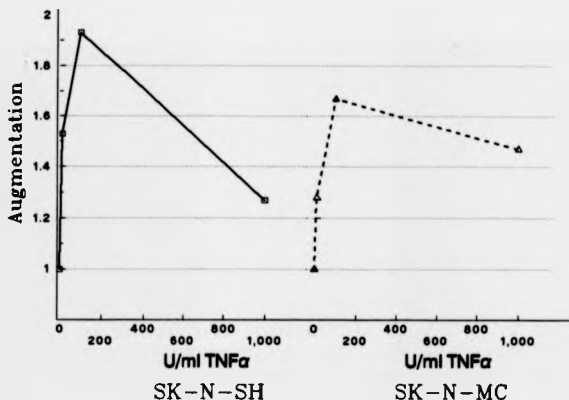


Figure 5.3. Augmentation of HIV-1 LTR-driven CAT expression in SK-N-SH and SK-N-MC neuroblastoma cells by TNF α over a range of concentrations. Cells were transiently transfected with pLC2R and treated with TNF α for 22-24 hrs before CAT activity was determined in cytoplasmic extracts.

5.1.2. Interleukin 1- β

i). Augmentation by IL-1 β

IL-1 β was demonstrated also to be a strong activator of HIV-1 LTR-driven gene expression in neural cells (Figure 5.4). It was able to augment CAT activity over two-fold in three of the four cell types tested. These were U138MG glioblastoma, U373MG astrocytoma and primary astrocyte cultures with a 2.5-, 2.5- and 2.1-fold enhancement of CAT expression, respectively. The oligodendrogloma cell line G26-24 did not respond to IL-1 β , even when exposed to high doses of 1000 or 2500 U/ml. The neuroblastoma cell lines SK-N-SH and SK-N-MC were not tested with this cytokine, this was primarily so as not to increase the number of reporter gene assays any further.

The results in Figure 5.4 demonstrate the highest level of augmentation seen at the optimum concentration of IL-1 β for U138MG and U373MG and at 1000 U/ml for primary astrocytes (the optimum concentration for astrocytes was not determined). The response of HIV-1 gene expression to IL-1 β was tested with each cell type between two and four times. The concentration of IL-1 β necessary for optimum induction of CAT expression varied between cell lines, this was at 500 U/ml for U138MG and 100 U/ml for U373MG, with a 1000 U/ml producing a comparable level of induction in primary astrocytes. U373MG cells also proliferate in response to high concentrations of IL-1 β ($\leq 20,000$ U/ml; Lachman *et al.*, 1987), but maximum induction of LTR-driven gene expression was at the relatively low concentration of 100 U/ml. Indeed, proliferation in response to IL-1 is a property of astrocyte cells in general (Giulian and Lachman, 1985), which may also explain why at concentrations of IL-1 β up to 2500 U/ml no cytopathic effects were noted.

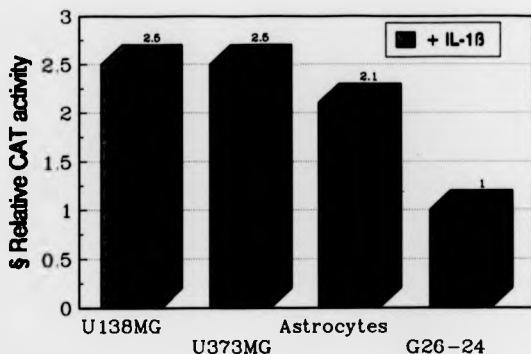


Figure 5.4. Augmentation of HIV-1 LTR-CAT expression by IL-1 β in glial cells. \$ Relative CAT activity was calculated from the mean cpm of ^3H -chloramphenicol product from extracts of cells transfected with pLC2R after IL-1 β treatment for 22-24 hrs divided by mean cpm without. Cytokine concentrations (U/ml) were U138MG 500; U373MG 100; astrocytes and G26-24 1000.

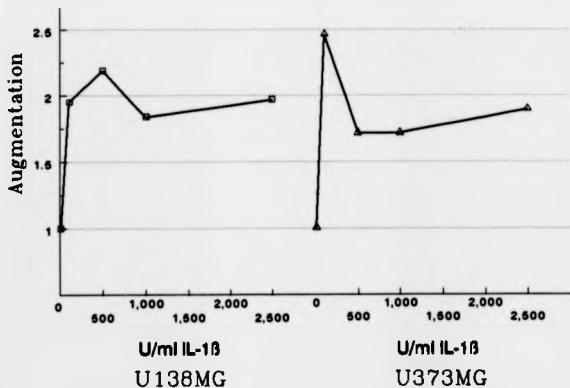


Figure 5.5. Augmentation of HIV-1 LTR-driven CAT expression in U138MG glioblastoma and U373MG astrocytoma cells by IL-1 β over a range of concentrations. Cells were transiently transfected with pLC2R and treated with IL-1 β for 22-24 hrs before CAT activity was determined in cytoplasmic extracts.

ii). The response of HIV-1 LTR driven CAT expression to increasing concentrations of IL-1 β in glial cell lines

Graphs displaying the response of HIV-1 LTR-driven gene expression to increasing IL-1 β concentrations in U138MG and U373MG cells are shown in Figure 5.5. Following information from the manufacturer on the range of IL-1 β concentrations that promote biological responses and that of Lachman *et al.* (1987), IL-1 β was tested in a higher range of concentrations at 100, 500, 1000, and 2500 U/ml.

In U138MG and U373MG cells the response to IL-1 β reached a maximum at the lower end of the range of concentrations tested, at 500 and 100 U/ml, respectively and in both cell lines the level of augmentation then appeared to stabilize. In U138MG there was only a small difference in the level of augmentation between 1000 and 2500 U/ml. In U373MG the greatest enhancement of CAT expression was found at the lowest concentration (100 U/ml) of IL-1 β so the experiment may not have defined the true optimum if the response were to have reached a peak below or indeed above this concentration of cytokine. At higher concentrations the response was not as strong and essentially unaltered from 500 to 2500 U/ml of IL-1 β .

5.1.3. Interleukin-6

The effect of IL-6 on HIV-1 LTR-driven gene expression was examined again in four cell types, U373MG astrocytoma, U138MG glioblastoma, G26-24 oligodendroglioma and primary astrocytes (Figure 5.6). Only in the primary astrocytes was expression from the HIV-1 LTR augmented significantly following treatment with IL-6, here there was a 2.3-fold increase, the highest level of augmentation obtained with any cytokine in these cells. The other cell types showed a very modest increase in CAT expression with IL-6 to around 1.2-fold which, although small was always reproducible in repeat experiments.

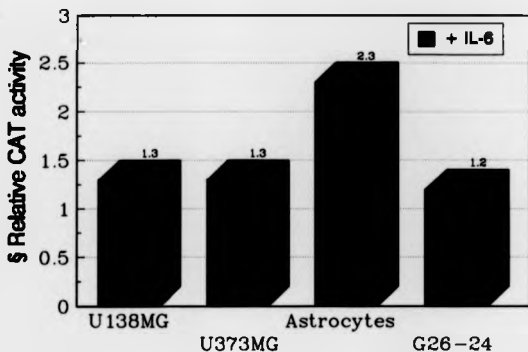


Figure 5.6. The effect IL-6 on HIV-1 LTR-CAT expression in glial cells. \$ Relative CAT activity was calculated from the mean cpm of ^3H -acetyl chloramphenicol product from extracts of cells transfected with pLC2R after IL-6 treatment for 22-24 hrs divided by mean cpm without. Cytokine concentration for all cells was 400 U/ml.

Cells were treated with IL-6 at a concentration of 400 U/ml, which would appear from the large augmentation in primary astrocytes to be in the correct range. No other concentrations of IL-6 were tested, and it is of course possible that at a higher concentration augmentation of HIV LTR-driven gene expression could occur.

5.1.4. Interferon $\alpha\beta$

i). The effect of interferon $\alpha\beta$

Treatment of the cell lines, U138MG and SK-N-SH with human IFN α and primary astrocytes and G26-24 oligodendrogloma with murine IFN $\alpha\beta$, always resulted in a slight reduction in the level of CAT expression directed by the HIV-1 LTR, to around 80% of the uninduced value (Figure 5.7). Yet, in SK-N-MC neuroblastoma cells IFN α reproducibly stimulated expression by up to 1.7-fold, which is comparable with that seen by TNF α and PMA in these cells. All assays were repeated between two and four times with IFN and produced similar results.

All cells were treated with interferon at 1000 U/ml except SK-N-MC in which IFN α at 100 U/ml appeared to be the optimum. These doses of interferon did not appear to cause any undue harmful effects to the cells when monitored by phase contrast microscopy during the experiments.

ii). The response of HIV-1 LTR driven CAT expression to increasing concentrations of IFN α in SK-N-MC neuroblastoma cells

In SK-N-MC the level of augmentation of CAT expression by IFN α increased rapidly with increasing cytokine concentration to a defined maximum at 100 U/ml (Figure 5.8), and at 1000 U/ml declined to approximately that produced by 10 U/ml. Interestingly, this response was almost identical to that observed with TNF α in this cell line and hence it may also suggest a similar sensitivity of SK-N-MC cells to IFN α .

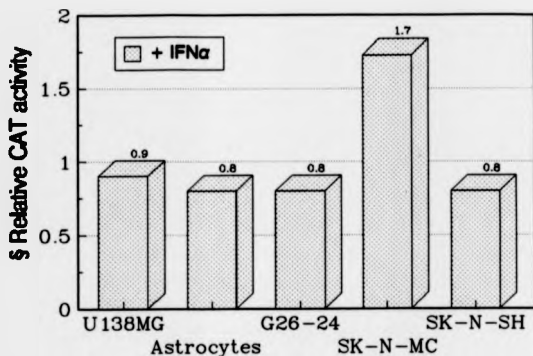
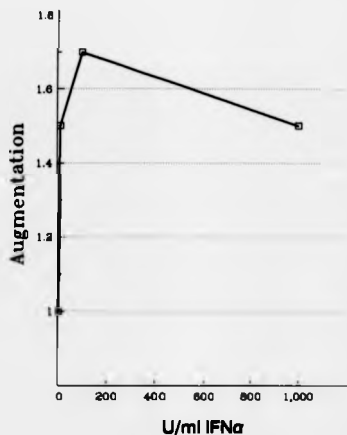


Figure 5.7. Modulation of HIV-1 LTR-CAT expression by IFN α in neural cells. § Relative CAT activity was calculated from the mean cpm of ^3H -acetyl chloramphenicol product from extracts from cells transfected with pLC2R after IFN treatment for 22-24 hrs divided by mean cpm without. Cytokine concentration was 1000 U/ml for all cells, except SK-N-MC 100 U/ml. IFN α was used for all human cells and IFN α for murine cells.



SK-N-MC

Figure 5.8. Augmentation of HIV-1 LTR-driven CAT expression in SK-N-MC neuroblastoma cells by IFN α over a range of cytokine concentrations. Cells were transiently transfected with pLC2R and treated with IFN α for 22-24 hrs before CAT activity was determined in cytoplasmic extracts.

5.1.5. Interferon γ

i). *The effect of interferon γ*

Interferon γ was also quite effective in suppressing expression from the HIV-1 LTR in the four human cell lines, U138MG glioblastoma, U373MG astrocytoma and the two neuroblastomas, but with the murine primary astrocyte and G26-24 oligodendrogloma cells there was significant augmentation of CAT expression to 1.5- and 2.3-fold, respectively (Figure 5.9). In SK-N-SH neuroblastoma cells IFN γ showed the strongest inhibition of expression down to 30% of the basal level, compared to 80% observed in the other cell lines. All cells were treated with IFN γ at 1000 U/ml. Repeat experiments further confirmed that gene expression from the HIV-1 LTR in G26-24 and primary astrocyte cells was indeed augmented by IFN γ .

ii). *The response of HIV-1 LTR driven CAT expression to increasing concentrations of IFN γ in G26-24 oligodendrogloma cells*

In G26-24 cells the dose-response curve constructed for the stimulation of LTR-driven CAT activity by IFN γ shows that augmentation does occur at low concentrations (10 U/ml), although to a lesser extent than with TNF α . The level of augmentation then increases more progressively with higher concentrations and reaches an optimum at 1000 U/ml (Figure 5.10). At the highest concentration of IFN γ (2500 U/ml) the graph shows that the response begins to decline.

iii). *Neutralizing antiserum to TNF α does not abolish the up-regulation by IFN γ*

In order to confirm that the augmentation of HIV-1 LTR-driven CAT expression by IFN γ in G26-24 oligodendrogloma cells was not due to the induction of TNF secretion and subsequent autocrine stimulation (See Discussion) cells were stimulated with IFN γ at the optimum concentration of 1000 U/ml just after the addition of a neutralizing antiserum to murine TNF α (Genzyme). The results show

that the antibody to TNF α did not effect either basal or IFN γ augmented expression from the HIV-1 LTR in G26-24 cells (Figure 5.11). An enhancement of around 1.5-fold was observed with or without the addition of anti-TNF α antibody to the culture medium indicating that TNF α was not responsible for the effect.

5.1.6. Phorbol ester

i). Augmentation by PMA

As expected, the protein kinase C activator, phorbol 12-myristate 13-acetate or PMA, was a strong activator of HIV-1 LTR-driven gene expression in all cells tested (Figure 5.12). Primary astrocytes were not treated with PMA but in the other cell lines there was a three- to four-fold enhancement of CAT expression, except for SK-N-MC in which there was 1.8-fold rise. Comparing with the data for the cytokines it was noted that PMA was capable of activating expression from the LTR in all cells to a level in excess of that produced by any cytokine. The optimum concentrations of PMA determined for U138MG, U373MG and G26-24 cells were at 10, 250 and 250 ng/ml, respectively (dose-response data is given below), the neuroblastoma cell lines were tested at two concentrations only, 50 and 100 ng/ml, and the highest level of augmentation recorded and given in Figure 5.12. These experiments were also repeated twice for each cell line to verify that PMA does indeed function as an activator of HIV-1 LTR-driven gene expression in neural cells similar to that seen in lymphocytic and monocytic cells (Rosenberg and Fauci, 1990).

ii). The response of HIV-1 LTR-driven CAT expression to increasing concentrations of PMA in glial cell lines

The level of augmentation of HIV-1 LTR-driven CAT expression was further examined in the three glial cell lines U138MG, U373MG and G26-24 at four different PMA concentrations, 1, 50, 100, and 250 ng/ml (see Figure 5.13).

In U373MG and G26-24 cells the response to increasing concentrations of PMA is very similar, the stimulation of CAT expression rises steeply at lower concentrations and then begins to fall slightly at the higher end of the range of PMA concentrations. In both cell lines maximum augmentation of CAT expression was at 250 ng/ml. The dose-response curve produced from U138MG suggests that maximum stimulation of LTR-driven CAT activity in these cells occurred at, or below the lowest concentration (10 ng/ml) of PMA tested. Higher concentrations of PMA produced a slightly erratic variation in the augmentation but clearly the effect was essentially close to maximum at concentrations of PMA above 10 ng/ml.

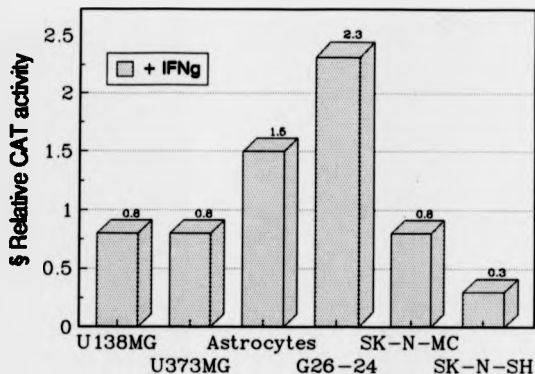


Figure 5.9. The effect of IFN γ on HIV-1 LTR-CAT expression in neural cells. \$ Relative CAT activity was calculated from the mean cpm of ^3H -acetyl chloramphenicol product from extracts from cells transfected with pLC2R after IFN treatment for 22-24 hrs divided by mean cpm without. The IFN γ was used at 1000 U/ml for all cells.

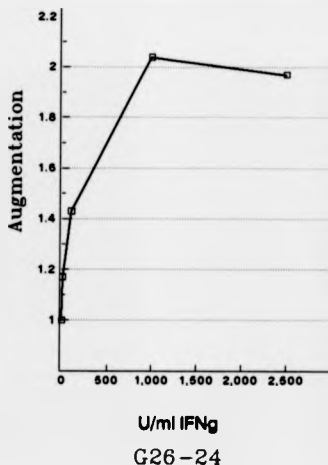


Figure 5.10. Augmentation of HIV-1 LTR-driven CAT expression in oligodendrogloma cells by IFN γ over a range of cytokine concentrations. Cells were transiently transfected with pLC2R and treated with IFN γ for 22-24 hrs before CAT activity was determined in cytoplasmic extracts.

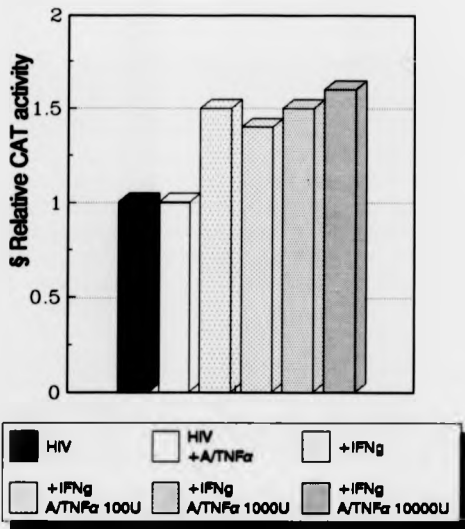


Figure 5.11. Augmentation of HIV-1 LTR-driven CAT expression by IFN γ in G26-24 oligodendrogloma cells in the presence of neutralizing antiserum to murine TNF α (A/TNF α). Polyclonal antibody (Genzyme) sufficient to neutralize the indicated number of U/ml of TNF α , was added to the culture medium just prior to the addition of IFN γ to 1000 U/ml and CAT activity determined in extracts prepared after 22-24 hrs. § Relative CAT activity was calculated from the mean cpm 3 H-acetyl chloramphenicol in the presence of IFN γ (\pm A/TNF α) divided by the mean cpm without.

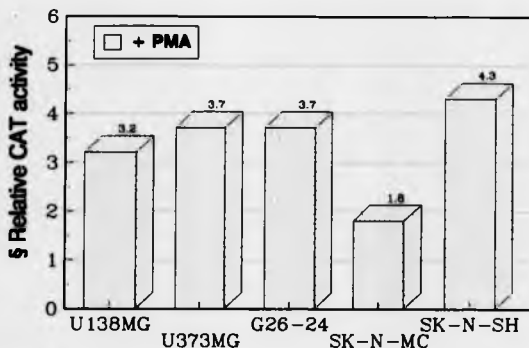


Figure 5.12. Augmentation of HIV-1 LTR-CAT expression by PMA in neural cells. \$ Relative CAT activity was calculated from the mean cpm of ^3H -acetyl chloramphenicol product from extracts of cells transfected with pLC2R after PMA treatment for 22-24 hrs divided by mean cpm without. Phorbol ester concentrations (ng/ml) were U138MG 100; U373MG 250; G26-24 250; SK-N-MC 50 and SK-N-SH 100.

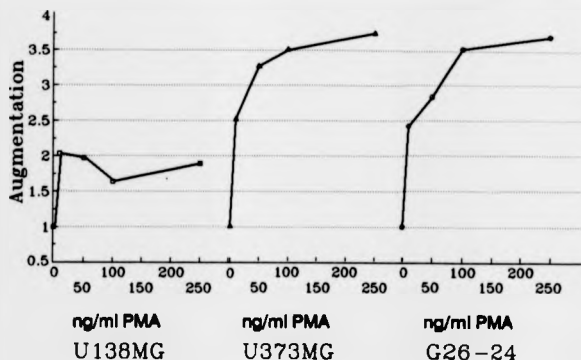


Figure 5.13. Augmentation of HIV-1 LTR-driven CAT expression in U138MG glioblastoma, U373MG astrocytoma and G26-24 oligodendroglioma cells by PMA over a range of concentrations. Cells were transiently transfected with pLC2R and treated with PMA for 22-24 hrs before CAT activity was determined in cytoplasmic extracts.

5.2. Pairs of augmenting cytokines are not additive in the augmentation of HIV-1 LTR-driven gene expression

5.2.1. The effects of TNF α , IL-1 β and PMA on HIV-1 LTR-driven gene expression in U138MG glioblastoma and U373MG astrocytoma cells

The human glial cell lines, U138MG and U373MG, were examined further for possible synergism between the cytokines found to augment HIV-1 LTR-driven gene expression. The cytokines TNF α and IL-1 β , and phorbol ester were tested individually and in pairs at their optimum concentrations on both cell lines. IL-6 in combination with TNF α was also included with U138MG cells. The augmentation of CAT expression is shown in Figure 5.14.

The results are comparable for both cell lines. Individual agents activate expression from the LTR to similar extent as those reported in Figures 5.1, 5.4 and 5.12, although the absolute level of augmentation is less than previously seen with U373MG. The exposure of cells to TNF α and IL-1 β together produced no increase above that observed for either cytokine alone, in fact the level of augmentation for U138MG was between that of the individual cytokines, and for U373MG it was approximately the same as for TNF α or IL-1 β alone. However, in U138MG cells exposed to TNF α and IL-6 a very slight additive effect of the two cytokines in augmenting CAT expression was observed, IL-6 and TNF α increased expression marginally to from 2.2-fold to 2.8-fold from that seen with TNF α alone. When PMA was added with either TNF α or IL-1 β in U138MG cells it was able to augment further expression from the LTR and appeared to increase the level of augmentation by a similar amount with TNF α or IL-1 β . Although, in U373MG cells the outcome of PMA and IL-1 β was not greater than PMA alone, a similar additive effect to that seen in U138MG was evident with PMA and TNF α , albeit to a lesser extent.

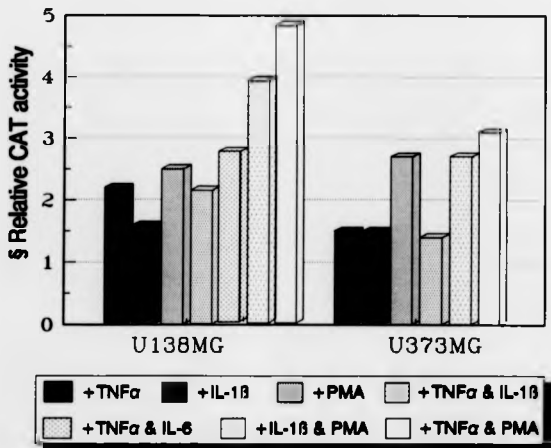


Figure 5.14. CAT activity from the HIV-1 LTR in glial cells after exposure to combinations of cytokines and phorbol ester (PMA) for 22-24 hrs. % Relative CAT activity was calculated from the mean cpm of ^3H -acetyl chloramphenicol in the presence of cytokine/PMA divided by the mean cpm without. Cytokine concentrations (U/ml): TNF α ; U138MG 100, U373MG 2500. IL-1 β ; U138MG 500, U373MG 100; and IL-6 400 U/ml. Phorbol ester, 250 ng/ml.

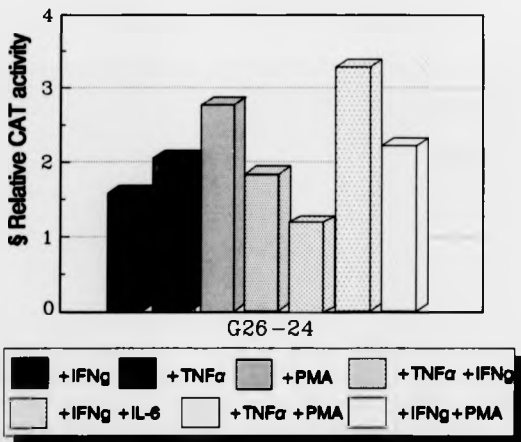


Figure 5.15. CAT activity from the HIV-1 LTR in G26-24 cells after exposure to combinations of cytokines and phorbol ester (PMA) for 22-24 hrs. % Relative CAT activity was calculated from the mean cpm of ^3H -acetyl chloramphenicol in the presence of cytokines/PMA, divided by the mean cpm without. Cytokine concentrations (U/ml): IFNg 1000, TNFa 100, IL-6 400. Phorbol ester, 100 ng/ml.

5.2.2. The effects of TNF α , IFN γ and PMA on HIV-1 LTR-driven gene expression in G26-24 oligodendrogloma cells

The level of augmentation of CAT expression directed by the HIV-1 LTR produced by pairs of activating agents was also examined in G26-24 cells. In this cell line IFN γ , TNF α and PMA had been shown to activate LTR-driven gene expression and these were tested again individually and in pairs. IL-6 plus IFN γ was also included.

The result (Figure 5.15) is similar to that observed for U138MG and U373MG. The combination of TNF α and IFN γ did not have an additive effect on gene expression, the level of augmentation fell between that of each agent alone. PMA was additive to the effect of each cytokine with TNF α and PMA producing a greater activation than either TNF α or PMA alone, although IFN γ plus PMA was only just above that seen for IFN γ alone and below that of PMA alone. Finally, the combination of IFN γ and IL-6 resulted in a significant decrease in CAT expression compared that of IFN γ alone. IL-6 and TNF α together were not tested.

5.3. Augmentation of HIV-1 LTR-driven gene expression by cytokines or PMA in cells co-expressing the transactivator, Tat

5.3.1. Transient co-transfection assays

The effect of individual cytokines on HIV-1 LTR-driven gene expression was investigated in three cell lines, U138MG glioblastoma, U373MG astrocytoma and SK-N-MC neuroblastoma cells transiently expressing Tat by co-transfection with the optimum amount of pSVL α . In U138MG and U373MG cells TNF α , IL-1 β , IL-6 and PMA were tested and in SK-N-MC, IFN α and PMA (Figure 5.16). Comparison of the amount of CAT activity in extracts from Tat transfected cells and those transfected with pLC2R alone, confirmed that Tat was functioning as expected in these experiments.

Exposure of the glioblastoma cell line U138MG transiently co-transfected with Tat and pLC2R to cytokines or PMA produced a slightly increased amount of CAT expression above that observed with Tat alone. This correlated with the ability of each agent, in the absence of Tat, to augment expression from the LTR but neither agent was particularly able to synergize with Tat and enhance expression greatly. IL-6 was also examined in this system to determine if the minimal 1.3-fold augmentation of CAT expression it produced in the absence of Tat would be amplified when Tat was co-expressed. However, this was not evident with U138MG or U373MG cell lines where exposure to IL-6 lead to a slight decrease in the level of CAT expression to below that seen with Tat alone.

The HIV-1 LTR-driven CAT expression in U373MG cells expressing Tat was also enhanced by stimulation with TNF α , IL-1 β and PMA. Here, expression in response to IL-1 β and PMA rose by a small amount to over 1.5-fold and for TNF α this increased further to over 2.5-times the level seen with Tat alone. In this experiment, TNF α was able to synergize with Tat in augmenting LTR-driven gene expression and to a limited extent with IL-1 β and PMA.

In SK-N-MC cells, however, much greater synergy between Tat and agents shown to activate gene expression was found. Both IFN α and PMA enhanced expression greatly, to four-fold and over three-fold, respectively.

5.3.2. The effect of exposure of cell lines permanently expressing Tat to cytokines or PMA

Permanent cell lines expressing Tat were produced by transfection of cells with the retroviral vector, pMoLTR α , which expresses Tat and subsequent selection of cells resistant to the neomycin analogue, G418 (see Chapter 2, Section 2.9.3). Tat expression in bulk cultures of U138MG, U373MG and G26-24 cells was easily confirmed by comparison of the CAT activity directed by the HIV-1 LTR present in 1.0 absorbance unit of extract with that in the same amount of

extract from the parental cells after transfection with pLC2R, although, the level of transactivation of the HIV-1 LTR by Tat in these cells was not formally measured by comparison with parental cells expressing CAT from the LTR in the same transfection. The presence of antigenic Tat protein was detected by indirect immunofluorescence in the nuclei of U373MG cells resistant to G418 several weeks after transfection with pMoLTR α (see Chapter 6).

The effect of individual cytokines known to augment expression driven by the HIV-1 LTR in the parental cells was then determined by transfection of Tat+ cells with pLC2R and treatment with cytokines or PMA in the usual way (see Figure 5.17).

The exposure of U138MG Tat^+ cells to the cytokines TNF α and IL-1 β , and PMA resulted in only a slight increase in CAT expression above that seen in untreated cells with values of 1.3-, 1.4- and 1.2-fold noted, respectively. This was similar to the outcome in U138MG cells transiently transfected with Tat. The combination of TNF α and IL-6 was also included to see if these cytokines were still partially additive to the augmentation of LTR-driven CAT expression in the presence of Tat but this was not the case and TNF α plus IL-6 produced the same 1.4-fold stimulation of CAT activity observed with TNF α alone.

In U373MG Tat^+ cells the cytokines, TNF α and IL-1 β were also only able to increase expression further by a small margin to 1.4-fold. In contrast to the result seen with transiently transfected U373MG cells TNF α did not greatly enhance CAT expression but PMA was able to do so to a limited extent.

Finally, HIV-1 LTR directed CAT activity in G26-24 Tat^+ cells treated with the cytokines TNF α and IFN- γ , and PMA was quite similar to the response of U373MG Tat^+ cells. Cytokines hardly increased CAT expression above the level with Tat alone, only to 1.1-fold each whilst PMA was able to produce a slightly larger augmentation up to 1.5-fold.

Therefore, from these results it would appear that if the Tat protein is present then augmenting cytokines have little effect on LTR-driven gene expression; except notably in SK-N-MC cells.

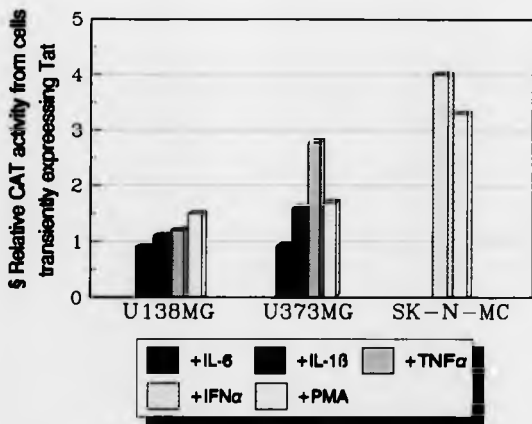


Figure 5.16. CAT activity driven by the HIV-1 LTR in neural cell lines transiently expressing Tat following exposure to cytokines/phorbol ester (PMA) for 22-24 hrs. \$ Relative CAT activity was calculated from the mean cpm of ^3H -acetyl chloramphenicol after treatment with cytokines/PMA divided by the mean cpm without. Cytokine concentrations (U/ml): IL-6, U138MG and U373MG 400; IL-1 β , U138MG 100, U373MG 1000; IFN α , 1000. Phorbol ester (ng/ml), SK-N-MC 50, U373MG and U138MG 100.

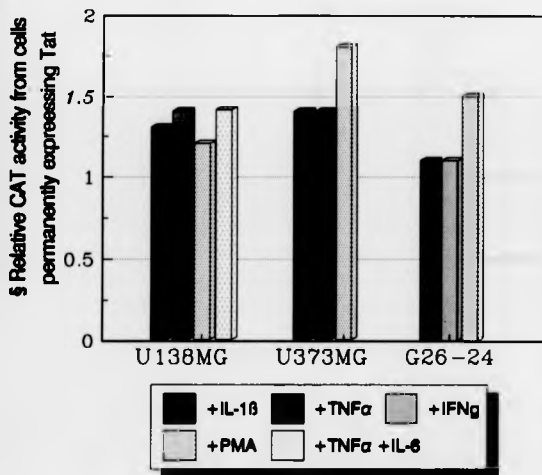


Figure 5.17. CAT activity driven by the HIV-1 LTR in neural cell lines permanently expressing Tat following exposure to cytokines/phorbol ester (PMA) for 22-24 hrs. § Relative CAT activity was calculated from the mean cpm of ^3H -acetyl chloramphenicol after treatment with cytokines/PMA divided by the mean cpm without. Cytokine concentrations (U/ml): IL-1 β , U138MG 100, U373MG 1000; TNF α , U138MG 100, U373MG 1000; IFN γ G26-24 1000, IL-6 U138MG 400. Phorbol ester, all cells 100 ng/ml.

Discussion

Cytokines and expression from the HIV-1 LTR

The exposure of neural cells transiently expressing the CAT reporter gene under the control of the HIV-1 LTR to cytokines clearly identified PMA and one or more cytokines capable of up-regulating expression significantly in each cell type. In summary, TNF α augmented expression in all cells of both neuronal and glial origin, and IL-1 β did so in the human and murine glial cells, except the murine oligodendrogloma. The neuronal cell lines were not tested with IL-1 β . IL-6 enhanced expression in one example only, the primary murine astrocytes. The interferons suppressed expression from the LTR except IFN γ which produced around a two-fold rise in murine primary astrocytes and oligodendrogloma cells and IFN α augmented expression in one neuroblastoma cell line.

Cells were tested with a range of cytokines at the different concentrations detailed in the relevant sections of the text and conclusions were drawn only from the data provided by these assays. Obviously the possibility exists that higher or lower concentrations of cytokine that were not tested could produce effects on HIV-1 LTR-driven gene expression different to those reported.

The experiments were performed as detailed in Chapter 2, by the addition of exogenous cytokine to neural cells transiently transfected with pLC2R, followed by the measurement of reporter gene expression. During the assays neural cells were cultured in medium containing 10% foetal bovine serum to support cell growth. The use of 10% FBS in the cell culture medium meant that the medium was undefined and complete absence of cytokines could not assured. Nevertheless, the levels of specific cytokines in the serum of healthy individuals are undetectable (Gallo *et al.*, 1989) which suggests that the use of serum would not have compromised the results.

Preliminary transfection experiments were performed in a serum-free medium containing insulin, transferrin and bovine serum albumin in DMEM.

Primary astrocytes could be maintained for periods of over one week in this medium but did not appear to proliferate. Unfortunately, cells could not be transfected efficiently in the serum-free medium; it was noted that after the overnight incubation with the calcium phosphate-DNA precipitate, the precipitate adhered strongly to the plastic of the tissue culture dish in the spaces around the cells such that washing with PBS or medium could not remove it. Prolonged exposure to the $\text{Ca}_3(\text{PO}_4)_2$ precipitate was toxic to the cells and inhibited further cell growth. When dishes of these cells were harvested for the measurement of CAT expression there was usually insufficient extract protein to obtain a signal in the assay. This phenomenon would appear to be due to the lack of a serum component which coats the surface of the tissue culture plastic ware and prevents the seemingly irreversible binding of the $\text{Ca}_3(\text{PO}_4)_2$ precipitate. The identity of the serum component was not investigated but bovine serum albumin nor the addition of fibronectin to cells in serum-free medium could prevent it.

Comparisons with lymphocytes and macrophages

These experiments served to demonstrate certain similarities and notable differences in the cytokines involved in the regulation of expression from the HIV-1 LTR in neural cells when compared to T lymphocytes and cells of the monocyte/macrophage lineage. These are discussed below.

TNF α

The activation of HIV-1 gene expression by $\text{TNF}\alpha$ agrees with the central role of this cytokine in activating both HIV-1 gene expression and replication in lymphocytes and macrophages alike (Rosenberg and Fauci, 1990). The effect of $\text{TNF}\alpha$ on HIV-1 was first identified by its ability to increase the cytocidal capacity of HIV-1 grown in both lymphoblastoma cell lines and primary lymphocytes *in vitro* due to the enhanced rate of replication of the virus (Matsuyama *et al.*, 1989b;

Vyakarnam *et al.*, 1990). Subsequently it was demonstrated to exert its effect by increasing expression from the viral LTR (Okamoto *et al.*, 1989).

In similar reporter gene assays performed in the lymphoblastoma cell lines Jurkat and J. Jhan, CAT expression from the HIV-1 LTR in the absence of the transactivator, Tat was augmented by 1.2-fold (Okamoto *et al.*, 1989), seven-fold (Israël *et al.*, 1989) and eight-fold (Osborn *et al.*, 1989), respectively. Therefore, the three-fold enhancement by TNF α found in neural cells is certainly of a comparable magnitude. Further examination of the experimental protocol employed by each group may also offer some explanation as to the differences observed. The length of time transfected cells were exposed to TNF α varied in each study. Okamoto *et al.* treated cells for 12 hrs at 400 U/ml, Israël *et al.* for 16 hrs at 1000 U/ml and Osborn *et al.* for 20 hrs at 100 U/ml which suggests that, in lymphoblastomas, induction of HIV-1 LTR-driven reporter gene expression requires 16-20 hrs before substantial CAT protein is synthesized. Because useful results were obtained in the experiments presented here with cytokine treatment for 22-24 hrs the length of time was not altered in the light of the above published works. However, I would suggest that from previous use of the ^{14}C -chloramphenicol TLC-based CAT assay, which was employed in the above studies from lymphoblastoma cells, this method may lead to an inaccurate level of augmentation by TNF α being reported due to its higher threshold of detection. In lymphoblastoma cells the amount of CAT activity directed by the HIV-1 LTR in transient assays is at the limit of detection of the TLC-based assay, so no acetylated chloramphenicol product is visible in the presented autoradiographs and the claimed percent acetylation (0.4%) is not above background (data from Israël *et al.*, 1989) [I found this to be in the 0.5-1.0% range, from experiments not shown]. Therefore when CAT expression was stimulated by TNF α in these experiments division of a detectable amount of CAT activity by the background level resulted in the reported augmentation being artificially higher. Taking these points into consideration I would suggest that the ability of TNF α to

augment HIV-1 LTR-driven gene expression in neural cells is equivalent to that reported in lymphocytes.

In promonocytic cells transiently transfected with a HIV-1 LTR-CAT plasmid, TNF α was able to augment reporter gene expression considerably (Stanley *et al.*, 1990). A 23-fold increase in CAT expression was observed and, although this may be subject to the same criticism as above due to the use of the TLC-based CAT assay, it suggests that TNF α has a stronger effect on LTR-driven gene expression in monocytic cells compared to lymphocytes or neural cells.

IL-1 β

The ability of IL-1 β to augment HIV-1 LTR-driven gene expression in human glioblastoma and astrocytoma cell lines, and in primary murine astrocytes demonstrates a significant difference in the regulation of HIV-1 gene expression in neural cells compared to lymphocytes and macrophages. IL-1 β has been reported not to augment LTR-driven reporter gene expression in Jurkat lymphoblastoma cells (Osborn *et al.*, 1989), neither will it enhance viral replication in the chronically infected ACH-2 T lymphocyte cell line or its monocyte equivalent U1 (Rosenberg and Fauci, 1990). While in transgenic mice containing integrated but non-expressed HIV-1 LTR-CAT plasmid DNAs there was expression of CAT in differentiated macrophages after exposure to IL-1 (Gendelman *et al.*, 1989), a role for IL-1 and HIV-1 infected macrophages has not been found (Gendelman *et al.*, 1989; Meltzer *et al.*, 1990a; Stanley *et al.*, 1990).

In support of the data presented here, expression from a CAT reporter construct under the control of the HIV-1 LTR in primary rat astrocytes was augmented 4-fold by exposure to IL-1 β at 20 ng/ml (Rodland, 1989). This was equivalent to 20,000 U/ml of IL-1 β used in this study. When assessed by the incorporation of ³H-thymidine the author reports that IL-1 β did not stimulate cellular proliferation of primary astrocytes. This and the data of Lachman *et al.*,

(1987) suggests that the induction of HIV-1 LTR-driven gene expression in astrocytes and astrocytoma cells by IL-1 β and TNF α occurs optimally at concentrations of cytokine that do not promote proliferation of the target cell.

Unfortunately a human oligodendroglioma cell line was not included in this study as examples of this rare tumour were not available from the two main cell culture repositories in Europe or America. Therefore it cannot be determined if the failure of IL-1 β to effect LTR-driven expression in G26-24 cells is a common property of cells derived from oligodendrocytes or simply specific to murine cells.

IL-1 β is also capable of stimulating the synthesis and secretion of another cytokine, IL-6, from a variety of cells including astrocytoma (U373MG; Yasukawa *et al.*, 1987), glioblastoma, astrocytes (Goetzl *et al.*, 1989) and this could modulate expression from the LTR. However, from the data presented in Figure 5.6, IL-6 alone was not capable of augmenting CAT expression in astrocytoma and glioblastoma cell lines and so the effects were assumed to be entirely mediated by direct action of IL-1 β . But in primary murine astrocytes where IL-6 alone augments the possibility exists that IL-6 may have contributed to, or even been responsible for, the stimulation produced by IL-1 β . This was not disproved by the use of neutralizing antibody to IL-6. However, the evidence presented indicates that IL-1 β has an immediate function on HIV-1 gene expression in other neural cells which in turn probably applies to primary murine astrocytes, yet it may operate in conjunction with endogenous IL-6.

IL-6

The strong augmentation seen by IL-6 in primary astrocytes was not evident in the tumour cell line most resembling its human counterpart, the astrocytoma U373MG, or in any other neural cell line tested. Although tumour cells and their primary cell equivalent are not expected to necessarily behave identically, certain similarities were demonstrated in the responses of HIV-1 gene expression to TNF α

and IL-1 β . It is not possible to say if the enhancement of HIV-1 LTR-driven gene expression by IL-6 in primary murine astrocytes would occur in human astrocytes as the question of whether this was a function of primary astrocytes in general or only of those of murine origin could not be addressed.

In general IL-6 did not have much effect on expression from the HIV-1 LTR in neural cells which is similar to the situation in T lymphocytes, but contrary to monocytic cells where IL-6 is a strong activator of HIV expression (Poli *et al.*, 1990a). In chronically infected primary macrophages (derived from blood monocytes) and the promonocytic cell clone U1, IL-6 directly up-regulates production of HIV by, as yet, undefined transcriptional and posttranscriptional mechanisms (Poli *et al.*, 1990a). IL-6 also synergizes with a physiological rise in temperature, GM-CSF, and TNF α , individually in the induction of HIV-1 transcription and replication in these cells (Stanley *et al.*, 1990; Poli *et al.*, 1990a).

IFN

The interferons were quite effective in some neural cell types in reducing HIV-1 LTR-driven gene expression to below the basal level. This suggests that an antiviral state was induced by IFN in these cells which was capable of mediating the effect. Yet, it would be more difficult to relate the data provided from reporter gene assays to a possible function of IFN as an antiviral agent against HIV infected neural cells due to the many points of action of known for IFN. The basic mechanisms are at the post-transcriptional level where mRNAs are subject to degradation by the 2' 5' oligoadenylic acid-induced RNase L, and, via the IFN-regulated protein kinase, the initiation of translation of message is also inhibited in IFN treated cells (Laurence, 1990).

In HIV-1 infected lymphocytic and monocytic cell lines interferon $\alpha\beta$ exhibit a concentration-dependent suppression of viral replication (Hartshorn *et al.*, 1987). IFN α and IFN β will inhibit viral replication in peripheral blood mononuclear cells

(Michaelis and Levy, 1989) and IFN α is particularly effective on monocytic cells where endogenously produced IFN α also restricts HIV-1 replication (Macé *et al.*, 1989). Furthermore the restriction imposed by IFN α on HIV-1 replication in chronically infected monocytic cells was primarily due an inhibition of the release of preformed virions from the plasma membrane (Poli *et al.*, 1989). These findings suggest that the augmentation of HIV-1 LTR-driven gene expression produced by IFN α in SK-N-MC neuroblastoma cells is unique and probably specific to that tumour cell line as it was not evident with the other neuroblastoma. HIV replication or gene expression has not been reported to be enhanced by IFN α in any cell type examined.

IFN γ also restricts HIV-1 replication in lymphocytic and monocytic cell lines but has minimal antiviral activity against HIV-1 infected peripheral blood mononuclear cells (Hartshorn *et al.*, 1987). More recently, the replication of HIV-1 in peripheral blood mononuclear cells and CD4+ T lymphocytes has been shown to induce these cells to secrete TNF α , TNF β and IFN γ . The addition of exogenous IFN γ further enhances HIV-1 replication, albeit weakly, and the addition of neutralizing antiserum to IFN γ decreases viral replication (Vyakarnam *et al.*, 1990). The mechanism of such an effect is unknown but the stimulation of HIV replication by IFN γ may depend upon the endogenous production of TNF in the cultures.

The positive effect of IFN γ on HIV-1 gene expression in the murine G26-24 oligodendroglioma and primary astrocytes is unusual. The augmentation would appear to be a direct consequence of IFN γ stimulation and not mediated by TNF α production, at least in G26-24 cells where neutralizing antiserum to TNF α did not effect the level of augmentation by IFN γ . No information as to the ability of oligodendroglioma cells to secrete TNF α has been reported, which may not be surprising as TNF damages oligodendrocyte cell function (Hofman, 1989). Nevertheless, the combination of lipopolysaccharide and IFN γ or IL-1 β , or IFN γ and IL-1 β together will induce TNF α synthesis in primary astrocyte cultures (Chung

and Benveniste, 1990). Only as little as 10 ng/ml of LPS alone or in conjunction with IL-1 β or IFN γ is necessary (Chung and Benveniste, 1990). For this reason all medium and solutions were made from endotoxin-tested water (Sigma) and there was no significant LPS in the part-purified IFN γ preparations when tested by the Limulus assay (performed by Mr. S. McQuiston in the laboratory of Dr. Alan Morris, Department of Biological Sciences, University of Warwick). The reporter gene experiments performed here were only able to test the function of IFN γ on murine cells as the human equivalents or tissues were not available and whether IFN γ has the same effect on HIV-1 LTR-driven gene expression in normal human astrocytes or oligodendrocytes could not be assessed. However, LTR-driven gene expression was not augmented by IFN γ in the human astrocytoma cell line U373MG.

PMA

The phorbol ester, PMA, was the most effective agent in augmenting CAT expression directed by the LTR in all neural cells. In lymphocytic and monocytic cells PMA will also induce LTR-driven CAT expression and enhance or induce HIV-1 replication in infected cells (Israël *et al.*, 1989; Rosenberg and Fauci, 1990). Specifically, in Jurkat lymphoblastoma cells PMA augments HIV-1 LTR-driven CAT expression by 3.3-fold (Israël *et al.*, 1989) and in U937 promonocytic cells, a 3.9-fold augmentation was reported (Stanley *et al.*, 1990). The data from these two authors indicates that PMA is considerably less efficient in stimulating CAT expression from the LTR in these cell types than TNF α . In neural cells, however, PMA was always able to augment LTR-driven expression more than TNF α .

PMA will also stimulate virus production in U373MG astrocytoma cells infected with HIV-1 (Harouse *et al.*, 1989). PMA at 50 ng/ml augmented virus replication by around 20-fold, measured by the release of p24^{gag} in to the culture supernatant. Although stimulation of U373MG here was only responsible for a

3.3-fold augmentation of LTR-driven gene expression the results of Harouse *et al.*, (1989) suggest that these small rises in the level of expression driven by the HIV-1 LTR will translate into a much greater stimulation of virus production. Surprisingly, PMA had no effect upon primary rat astrocytes transiently transfected with a HIV-1 LTR-CAT reporter gene construct (Rodland, 1989).

Co-stimulation of HIV-1 LTR-driven gene expression with pairs of cytokines

The use of pairs of cytokines found to individually augment HIV-1 LTR-driven gene expression was useful in indicating whether the same or different cellular signalling pathways were invoked to produce the enhanced expression. An additive effect may suggest different pathways whereas non-additive suggests either the same or perhaps mutually exclusive mechanisms operate. For example, in T lymphoblastoma cells HIV-1 gene expression can be augmented by two agents, PHA and PMA, where PHA activates T cell surface antigens and PMA stimulates PKC, which individually produce approximately a 3-fold augmentation of LTR-driven CAT expression but together this rises to 11-fold (Israel *et al.*, 1989); yet some authors report that PHA alone does not augment expression from the HIV-1 LTR and was not additive to the effect of PMA (Tong-Starksen *et al.*, 1989). However, it has been shown that PMA and PHA operate through different intracellular mechanisms in activating T lymphocytes which both regulate HIV-1 gene expression (Siekevitz *et al.*, 1987; Crabtree, 1989). Calcium ionophores are also additive to PMA in augmenting HIV-1 LTR-driven gene expression in lymphocytes (Gruters *et al.*, 1991).

The results obtained in U373MG astrocytoma and U138MG glioblastoma cells treated with TNF α and IL-1 β showed that there was no additive effect on HIV-1 LTR-driven gene expression. TNF α and IFN γ were also not additive with G26-24 oligodendroglioma cells. From the above hypothesis, this might suggest that these cytokines act through a similar mechanism to augment reporter gene

expression directed by the HIV-1 LTR. This expectation was supported by later experiments detailed in Chapters 7 and 9.

The slightly enhanced level of augmentation apparent when U138MG cells were treated with TNF α plus IL-6 compared to TNF α alone cannot really be described as synergistic since IL-6 alone had very little positive effect on LTR-driven expression in these cells, and the small differences in augmentation observed are close to the standard error of the assay (see Introduction). As described previously, in monocytic cells IL-6 alone and in synergy with TNF α was able to augment HIV-1 replication (Poli *et al.*, 1990a). HIV-1 expression was induced to the extent that transcription was stimulated 3- to 4-fold by TNF α and 1.5-fold by IL-6 individually but together produced a 10-fold rise (Poli, *et al.*, 1990a). Obviously the effect of TNF α and IL-6 on LTR-driven gene expression in U138MG cells does not compare with the situation in macrophages and this further disproves any synergistic function for that pair of cytokines and U138MG.

The ability of IL-6 and IFN γ to partially down-regulate CAT expression from the LTR in G26-24 cells compared to IFN γ does not correlate with the slight augmentation evident with IL-6 alone (see Figure 5.6). The result seen was therefore unexpected but probably of little overall significance.

Augmentation of expression from the HIV-1 LTR in the presence of Tat

In lymphoblastoma cells the co-transfection of a Tat expression vector and subsequent treatment with TNF α augments expression from a HIV-1 LTR linked reporter gene more than either agent alone (Irañel *et al.*, 1989; Okamoto *et al.*, 1989). TNF α was reported to enhance expression relative to the amount obtained with Tat in the absence of TNF α treatment by 3-fold (Okamoto *et al.*, 1989) and 19-fold (Irañel *et al.*, 1989) in similar cell lines. As stated earlier, in neural cells HIV-1 LTR-driven gene expression responded only in an analogous fashion in the SK-N-MC neuroblastoma cell line after stimulation with IFN α or PMA. Both these molecules

augmented LTR-driven CAT expression 3- to 4-fold relative to Tat alone when SK-N-MC cells were similarly transiently co-transfected with a Tat expression vector. In U138MG there was only a slight difference when cells transfected with Tat were treated with agents that activate LTR-driven expression. CAT expression from the LTR in these cells, and to a lesser extent in U373MG (see Figure 5.16), appeared to be maximum when Tat was present and there was little or no further stimulation with cytokine or PMA. This appears to be another distinction between lymphoid and neural cells in the response of HIV-1 gene expression to cytokines. This was not due to the loss of a linear relationship between CAT activity and cpm of ^3H -acetylated product in the CAT assay because, although, high levels of CAT are present in extracts from cells co-expressing Tat, only a sufficient amount of extract was used to keep within the limits of the assay. Interpretation of these experiments is further complicated as the promoter driving Tat expression in the transiently transfected cells is also responsive to cytokines (SV40 late promoter, experimental observations) which may effect the level of Tat expression from the vector. However, I do not feel that the extent to which this might have occurred was able to increase Tat expression to such a level that it was responsible for reducing expression from the LTR.

Hoping to clarify the situation further, the response of LTR-driven reporter gene expression in permanent cell lines expressing Tat from an integrated vector (pMoLTR_{cat}) was examined. These cell lines were primarily produced for use in the analysis of RNA transcribed from the LTR, after transfection with pLC2R, and by virtue of the expression of resistance to G418 each cell should also be expressing Tat (confirmed by immunofluorescence data for U373MGAT+ in Chapter 6). An SK-N-MC neuroblastoma cell line permanently expressing Tat was not prepared because with such a low transfection efficiency (experimental observation) detectable amounts of HIV-CAT mRNA would probably not be produced even in the presence of Tat. The response of the LTR to cytokines in the presence of Tat

was assessed in U138MG^{Tat+}, U373MG^{Tat+} and G26-24^{Tat+} cells to also see if there would be a significant difference in the levels of CAT expression to allow the effect of augmenting cytokines to be discerned at the RNA level (see Chapter 6). However, the results for these cell lines (see Figure 5.17) show that stimulation with cytokines in the presence of Tat was marginal. These experiments endorse what the co-transfection experiments had shown and indicate that the results obtained with transient expression of Tat were not on the whole effected by any undue variation in the amount of Tat transfected.

An explanation for the lack of synergy in neural cells between cytokines and Tat may stem from the results described in Chapter 4 on the level of transactivation of HIV-1 LTR-CAT by Tat. The data reported in Table 4.1 demonstrated that Tat does not transactivate with the same efficiency in neural cells as it does in lymphoid cells. Therefore, it is conceivable that Tat, performing less efficiently, may not be able to 'process' all the RNA that is transcribed from the HIV-1 LTR (a review of the proposed mechanisms of Tat functions is given in Chapter 1, Section 1.5.2 iii) and hence becomes saturated to a lesser or greater extent depending upon the cell line. To conform with this suggestion, Tat in SK-N-MC neuroblastoma cells would have to function with the same efficiency as in lymphoid cells which it appeared not to do (see Table 4.1). However, perhaps the highest level of transactivation by Tat was never achieved due the sensitivity of this cell line to the amount of Tat expression vector transfected.

Having determined the functions of certain cytokines on HIV-1 LTR-driven gene expression it was thought important to pursue further some examples at the level of steady-state RNA expression in order to investigate the effect of cytokines on transcription from the LTR.

CHAPTER 6

Chapter 6: Detection of RNA expressed from the HIV-1 LTR in glial cells

Introduction

Experimental technique

Following the analysis of protein expression under the control of the HIV-1 LTR an examination of the steady-state RNA levels in transfected cells was undertaken. This was performed in order to correlate the augmentation of LTR-driven reporter gene expression by cytokines with an increase in steady-state RNA levels. Transient transfection is a fairly inefficient method of introducing foreign DNA into eukaryotic cells with at best 1 to 10% of cells taking up plasmid DNA (Chen and Okayama, 1987), therefore little specific mRNA is likely to be recovered from transfected cells. Only solution hybridization techniques which use partially overlapping complementary probes have the required degree of sensitivity to detect such levels of RNA (Ausubel *et al.*, 1990). Therefore, a ribonuclease protection assay was used with a single-stranded radiolabelled RNA probe corresponding to the 236 bp fragment of the HIV-1 LTR (LTRF, see Chapter 3, Section 3.4) overlapping the start of transcription from the LTR by 77 nucleotides.

Detection of mRNA

The glial cell lines U138MG and U373MG were chosen for the analysis of RNA transcribed from the LTR after transient transfection as they appeared to have the highest efficiencies of transfection judged from the reporter gene experiments, with U138MG being the higher of the two. In order to increase substantially the levels of HIV-CAT mRNA available after co-transfection with pLC2R, cell lines permanently expressing Tat were produced containing pMoLTRTat (a retroviral vector for the expression of Tat) and employed for RNA isolation after transfection

with pLC2R. A clone of G26-24 oligodendroglioma cells, G26(4), expressing CAT under control of the HIV-1 LTR was also created after co-transfection with pLC2R plus a vector encoding resistance to G418 and subsequent selection and screening to find cells expressing CAT. RNA was also prepared from these cells and assayed by the ribonuclease protection method for HIV-specific transcripts.

6.1. HIV-CAT specific RNA levels

6.1.1. Indirect immunofluorescence staining of U373MG Tat+ cells

The expression of Tat was first examined in the bulk culture of U373MG astrocytoma cells created by transfection of pMoLTR_{tat}, to establish what percentage of cells were in fact expressing the protein. The measurement of CAT activity directed by the HIV-1 LTR in these cells already indicated that a functional Tat protein was being expressed (see Chapter 5, Section 5.3.2), nevertheless cell staining by indirect immunofluorescence was performed using a murine monoclonal antibody to Tat and a secondary anti-mouse Ig antibody coupled to biotin. The complex was then detected using the fluorochrome phycoerythrin covalently linked to streptavidin.

Cells were photographed and are shown in Figure 6.1. Bright fluorescence was located to the nuclei of all cells and confirmed that an antigenic protein was produced and localized to the correct cellular compartment. The Tat protein contains a signal for nuclear translocation and retention and is preferentially, and sometimes predominantly, localized to the nucleolus of expressing cells (Hauber *et al.*, 1989). However, Tat in U373MG cells was not entirely restricted to the nucleolus as the whole nuclei of expressing cells was labelled. The fluorescence microscope used did not have sufficient resolution to distinguish the presence of Tat in any sub-nuclear compartment in the cells stained here.



Figure 6.1. Indirect immunofluorescent of U373MG astrocytoma cells permanently expressing HIV-1 Tat. Tat protein was detected using a specific monoclonal antibody, followed by a biotin-conjugated second antibody and phycoerythrin coupled to streptavidin. Magnification $\times 300$.

6.1.2. Analysis of RNA from cells permanently expressing CAT from the HIV-1 LTR and after transient transfection of a Tat expressing cell line

For this assay two sources of cytoplasmic RNA were prepared, one from a bulk culture of U373MG astrocytoma cells permanently expressing Tat 12 hrs after transfection with the HIV-1 LTR-CAT plasmid pLC2R, and the other from the bulk culture of G26-24 oligodendrogloma cells containing and expressing integrated pLC2R DNA, designated G26(4).

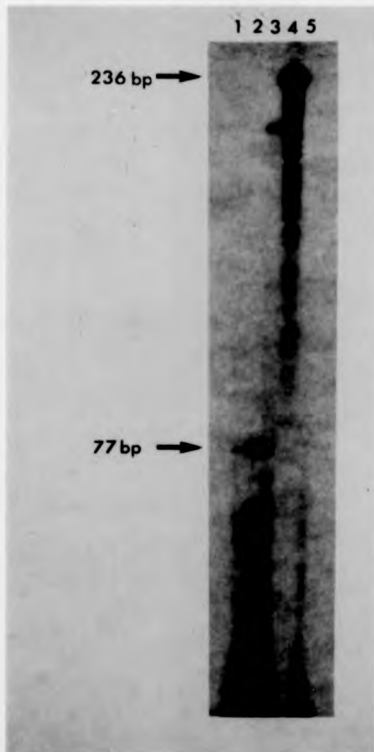
Figure 6.2 shows the autoradiograph from a ribonuclease protection assay after exposure for 2 days. A protected fragment of the correct size (77 bp) was detected with RNA from the CAT+ G26(4) cells (Tracks 1 and 2) but not with RNA from U373MG Tat+ cells transfected with pLC2R (Track 3). The assay was resolved by electrophoresis through a sequencing gel and size markers were provided by Maxim and Gilbert chemical sequencing reactions of the ds DNA footprinting probe, LTRF, from which the riboprobe was derived. Very little difference was observed between the mobility of ss RNA and DNA through the gel and the protected fragment co-migrated with a DNA fragment of approximately 77 nucleotides.

Tracks 4 and 5 were control reactions for the HIV-CAT probe and the absence of the 77 bp fragment in these samples confirms the specificity of the assay for mRNA transcribed from the HIV-1 LTR. There is a major band in track 3, the U373MG RNA, just below the undigested probe present in track 4 (no RNase control), which most likely represents incomplete RNase digestion of the input probe. No contaminating higher molecular weight species were present when RNA from G26(4) was assayed (Tracks 1 and 2, respectively). Autoradiography of the gel for longer periods of time showed minor bands in track 3 (U373MG) below the partially digested probe and extending down the gel which may have masked any specific band of 77 bp. However, a repeat experiment did not demonstrate a

fragment of the correct size in 50 or 100 μ g of the same or different preparations of U373MG RNA.

Figure 6.2.

Ribonuclease protection assay demonstrating specific mRNA from the HIV-1 LTR in cytoplasmic RNA from G26-24 oligodendrogloma cells permanently expressing pLC2R using a 236 bp riboprobe derived from the HIV-1 LTR and overlapping the start of transcription by 77 bp. Track 1: 50 μ g; 2, 100 μ g; 3, 100 μ g of cytoplasmic RNA from U373MG Tat+ cells 12 hrs after transient transfection with pLC2R.



Discussion

Immunofluorescence of Tat expressing cells

Detection of the nuclear antigen Tat in expressing cells was only possible when the high-emission fluorochrome phycoerythrin from the Amersham cytochemical staining kit was used in conjunction with the IgM monoclonal antibody directed against Tat in the immunofluorescence protocol. U373MG^{Tat} cells, in which the function of Tat had recently been indicated by the transactivation of CAT expression from the HIV-1 LTR, were used in cell staining experiments. A murine IgG monoclonal antibody against Tat was unsuccessful as was the anti-Tat IgM antibody when an FITC-conjugated second antibody was used.

The two monoclonal antibodies to Tat react with different portions of the protein. The IgG antibody was raised against the N-terminal 14 aa and the IgM against the C-terminal 14 aa (Dingwall *et al.*, 1989). However, neither epitope is thought to be masked when the protein is expressed in eukaryotic cells, and the difference in the detection is due to the isotype of the antibody (personal communication, Dr. Sheila Green, Laboratory of Molecular Biology, Hills Road, Cambridge). The much larger size of the IgM molecule makes it a better substrate for recognition by the secondary antibody, but successful detection of Tat still required the use of the biotinylated second antibody and the phycoerythrin/streptavidin conjugate which produces a much brighter fluorescence than the usual FITC labels (Amersham cytochemical kit handbook). The difference in detection may suggest that only relatively low levels of Tat are present in the nuclei of expressing cells, nevertheless this was sufficient to transactivate expression significantly from the HIV-1 LTR.

After U373MG^{Tat} cells had been stained satisfactorily the slide was examined under phase contrast microscopy to estimate the proportion of cells

expressing Tat. Unfortunately, there was little cell ultrastructure remaining after the fixing and permeabilization procedures so the same field of view was not photographed under normal light. It could be estimated by eye that the majority, if not all cells in a number of fields examined expressed Tat, as judged from nuclear fluorescence.

Ribonuclease protection assay

The detection of mRNA transcripts from the HIV-1 LTR was possible only using large amounts (100 μ g) of cytoplasmic RNA isolated from a clone of G26-24 oligodendrogloma cells which expressed CAT under the control of the HIV-1 LTR. A sufficient signal was never observed in U373MG^{Tat+} cells which had been transiently transfected with pLC2R. The time of incubation after the transfection of these cells was varied, by harvesting cells at 6, 12 and 24 hrs after the glycerol shock, in order to find the optimum incubation period that allowed the synthesis of largest amount of mRNA. The absence of signal in these transfected cells suggests that the CAT mRNA must be of extremely low abundance or stability. The relative ease with which functional CAT enzyme was assayed in transfected cells perhaps masked the scarcity of the RNA species that encoded it. Enzyme assays are sensitive primarily because an enzymatic function is detected and not individual inert protein. The CAT assays performed here are reported to be extraordinarily so, and capable of detecting less than 200 *femtogrammes* of active enzyme (Eastman, 1987). However, CAT mRNA is notoriously unstable and consequently some laboratories have problems in its detection (personal communication, Prof. Hugh Woodland, Department of Biological Sciences, University of Warwick). Specific transcripts from the HIV-1 LTR were also not be detected in RNA from U138MG and U373MG cells transfected in the absence of Tat (data not shown).

Unfortunately, no further work other than the detection of a correctly initiated RNA species in G26(4) CAT⁺ cells was possible as CAT expression was

not entirely permanent in these cells and all stocks were exhausted in establishing the assay. After the parent G26-24 cells had been transfected with the necessary plasmids and a CAT+ clone obtained by CAT assay, the cells were grown up into one large (175 cm²) flask and aliquots stored in liquid nitrogen. Subsequently it was discovered that cells from clone G26(4) only retained CAT expression for a maximum of approximately two weeks after recovery from liquid nitrogen. Cells kept resistance to G418 but appeared to lose pLC2R sequences as induction of cells with the activating agents PMA and TNF α could not induce CAT expression (data not shown). Interestingly, when these cells expressed CAT from the HIV-1 LTR it was constitutive, unlike the simian Vero cells containing integrated HIV-1 LTR-CAT DNA described by Bednarik *et al.* (1990) where expression was restricted by methylation of the LTR. G26(4) cells that had lost CAT activity were treated with 5' aza-cytidine to suppress methylation of DNA but this did not induce CAT expression.

Experiments therefore could not be performed to demonstrate a correlation between steady-state HIV specific mRNA transcripts and the induction of expression by cytokines. However, a functional system was described for the detection of scarce transcripts from the HIV-1 LTR and with the information provided here it would be possible to embark upon such an investigation after preparation of sufficient stocks of G26-24 or other cells expressing CAT from integrated pLC2R DNA.

CHAPTER 7

Chapter 7: Interaction of cellular proteins with the HIV-1 LTR

Introduction

The analysis of the regulation of gene expression from the HIV-1 LTR was continued at the molecular level by examining the interaction of the LTR with the various cellular proteins involved in the initiation and regulation of transcription. In doing so, it was hoped that information could be obtained about the cellular processes that cytokines activate in neural cells to promote enhanced gene expression from the HIV-1 LTR, and upon the identity of the cellular factors and regions of the LTR important in mediating the effect. Transcription factors regulate gene expression by binding specifically to certain sequence motifs in the LTR and enhancing the activity of RNA polymerase II in transcribing the viral genome. Therefore, experiments to evaluate the binding of nuclear proteins to the LTR were performed using a number of experimental systems. Firstly, in this chapter, the ability of restriction fragments from the HIV-1 LTR to form specific protein-DNA complexes with factors present in nuclear extracts was assessed by gel retardation assays. In Chapter 8, the locations of the sequences involved in the binding of nuclear proteins were determined in the LTR restriction fragment probes using DNase I footprinting, and finally, in Chapter 9, the identification of sequence-specific DNA-binding activities was continued using ds oligonucleotide probes corresponding to specific regions of the LTR in further gel retardation assays.

Preparation of nuclear extracts

Crude nuclear extracts were prepared by the elution of nuclear proteins from isolated nuclei with buffer containing a high concentration of salt as described in Chapter 2, Section 2.12.1. A buffer containing 0.4 M NaCl was used because at this

ionic strength the bulk of non-histone nuclear proteins are extracted from nuclei and the crude protein preparation can be employed directly in experiments without further purification (Goodwin, 1990). Varying the concentration of salt in the buffer allows the extraction of proteins with different binding affinities, such that with increasing ionic strength proteins with higher binding constants are eluted. Although the bulk extraction of nuclear proteins with 0.4 M NaCl was found to be sufficient, the use of a step-wise elution of the nuclei with buffers of increasing ionic strength can be useful in the isolation and fractionation of DNA-binding activities (Plumb *et al.*, 1985). However, as ionic strength gradually increases beyond 0.4 M NaCl the histones are progressively solubilized and introduce a strong non-specific DNA-binding activity into the nuclear extracts which will interfere greatly with the analysis of sequence-specific proteins (Goodwin, 1990).

Gel retardation assay

This method facilitated the characterization of specific nuclear protein-DNA interactions due to the separation of complexes by electrophoresis through a non-denaturing polyacrylamide gel, such that free, unbound DNA migrates quickly through the gel and DNA complexed with protein is retarded to a variable extent depending upon the molecular weight and conformation of the DNA-binding protein(s) involved. The degree with which a fragment of DNA is retarded by the gel matrix can also be markedly dependent on the location of the protein-binding site. Studies on the prokaryotic transcription factor CAP, indicate that the interaction with its recognition site will produce the greatest mobility shift if the site is in the centre of the DNA molecule and a lesser shift if located at either end. This is a consequence of DNA bending by the specific interaction of the transcription factor with its binding site (Garner and Revzin, 1990) and has recently been shown to occur with the eukaryotic transcription factors *fos* and *jun* (Kerppola and Curran, 1991). Complexes were visualized in these assays by the use of a radiolabelled DNA

fragment which was incubated with nuclear protein preparations to recreate the binding of nuclear proteins to LTR sequences *in vivo*. Binding reactions were also performed in the presence of a variable amount of the competitor nucleic acid, poly (dI:dC)-(dI:dC) ds co-polymer, and a fixed amount of pUC13 plasmid DNA, to absorb the non-specific DNA-binding activities present in the nuclear extracts and allow the high affinity, and hence sequence-specific nucleo-protein complexes to be resolved upon electrophoresis. The gel retardation assay is the most sensitive method for the detection of the DNA-binding properties of cellular proteins. This is due primarily to the separation of free and bound DNA during the electrophoresis which allows the use of a relatively high concentration of binding site DNA to facilitate nucleo-protein complex formation even when the concentration of a DNA-binding protein is extremely low. Information is obtained more on the relative rather than the absolute molecular weights of DNA-binding activities and on the relative abundance of each in a given preparation of nuclear proteins.

Cell lines

Experiments on the analysis of DNA-binding proteins in this and following chapters concentrated on three neural cell lines, the astrocytoma U373MG and the two neuroblastoma cell lines, SK-N-MC and SK-N-SH. This was chiefly because more relevant information would be obtained from the use of human cells in the study of HIV. No further work was conducted with the glioblastoma cell line U138MG as preparations of lysed cells were extremely viscous and would most likely make the preparation of nuclear extracts impractical. For each cell line examined nuclear proteins were also prepared from cells stimulated with an appropriate cytokine shown to augment HIV-1 LTR-driven reporter gene expression. Neuroblastoma cells were treated with TNF α at the optimum concentration of cytokine determined from the reporter gene assays (100 U/ml) and for the astrocytoma IL-1 β at 250 U/ml was used. Cells were stimulated for 14-2 hrs

just prior to harvesting for the preparation of nuclear extracts as detailed in Chapter 2, Section 2.12.1.

7.1. Formation of nuclear protein-DNA complexes with restriction fragments from the HIV-1 LTR

7.1.1. Nuclear protein-binding to probe LTRF

Probe LTRF spans the 3' region of the HIV-1 LTR from the nucleotide at position +77 to -158 relative to the start of transcription at position +1 (see Chapter 2, Section 2.12.2) and was used in gel retardation assays with nuclear extracts from unstimulated U373MG, SK-N-MC and SK-N-SH cells. The formation of specific nucleo-protein complexes was determined after a fixed amount of nuclear protein extract was incubated with increasing amounts of the unlabelled competitor nucleic acid, poly (dI:dC)-(dI:dC), and resolved on a 5% polyacrylamide gel. At the higher concentrations of poly (dI:dC)-(dI:dC) shown in Figure 7.1 sufficient competitor was present to absorb out the non-specific DNA-binding activities of the extracts and allow the free probe in each sample to be separated efficiently from the protein-complexed DNA. This required 2 μ g or more of poly (dI:dC)-(dI:dC) for extracts from U373MG and SK-N-SH cells and 6 μ g or more for extracts from SK-N-MC cells. With SK-N-MC extracts even at this concentration of competitor little free probe was separated and the bulk of the DNA remained complexed with protein.

As expected, it was evident that this region of the LTR was capable of specifically binding nuclear proteins present in the extracts from astrocytoma and neuroblastoma cells and a similar pattern of protein-DNA complexes was produced for each cell line. Two major nucleo-protein complexes, marked C1 and C2, are present in tracks 1 and 2 (U373MG), 3 and 4 (SK-N-MC), and 5 and 6 (SK-N-SH) of the autoradiographs of gels photographed for Figure 7.1. The ratio of complex C1 to

C2 appears to vary between the cell lines. In U373MG roughly equivalent amounts of C1 and C2, as judged from the intensity of the bands, were found whereas in the neuroblastoma cell line SK-N-SH complex C1 predominated over C2. In SK-N-MC extracts there appeared to be much more non-specific DNA-binding activity and substantial separation of complexes was not achieved even at the highest concentration of poly (dI:dC)-(dI:dC). The examination of further gel retardation assays between SK-N-MC nuclear extracts and probe LTRF seen in Figure 7.5 did indicate the formation of complexes C1 and C2 similar to those seen with SK-N-SH extracts.

One less intense lower molecular weight complex marked C4 was also visible in gel retardation assays with extracts from U373MG astrocytoma cells present in Figure 7.1 between complex C2 and the free LTRF probe. A fifth complex below C4 may also be present with U373MG cells but due to the proximity of the free probe at the bottom of the gel this was difficult to determine. Complex C4 was also noted in the autoradiographs of gel retardation assays with SK-N-SH cells in Figure 7.1. Complex C4 was not present with SK-N-MC extracts but in another experiment shown later (Figure 7.5) it was demonstrated that C4 could also be formed between LTRF and SK-N-MC extracts.

7.1.2. Nuclear protein-binding to probe 159

Similar gel retardation assays were performed with probe 159 and nuclear extracts from U373MG, SK-N-MC and SK-N-SH cell line in the presence of increasing amounts of poly (dI:dC)-(dI:dC) competitor in order to identify any specific protein-DNA complexes. Probe 159 spans the central part of the HIV-1 LTR from the nucleotide at position -159 to position -305 upstream from the start of transcription (see Chapter 2, Section 2.12.2).

The gel retardation assays in Figure 7.2 demonstrate the formation of one major protein-DNA complex with nuclear extracts from all three cell lines in the

presence of similar amounts of poly (dl:dC)-(dl:dC) competitor as probe LTRF. Comparing the mobility of the complex relative to the free probe with each cell line indicated that a major protein-DNA complex of a similar size was formed with this probe in both neuroblastoma and astrocytoma cells. In extracts from U373MG cells at least one minor complex (C2) was also present below the C1 complex but was not seen in the autoradiographs for SK-N-SH and SK-N-MC.

7.1.3. Nuclear protein-binding to probe 194

The last probe derived from the HIV-1 LTR, 194 corresponds to the 5' region of the LTR from the nucleotide at position -306 to position -488 relative to the start of transcription (see Chapter 2, Section 2.12.2). Gel retardation assays with nuclear extracts from U373MG, SK-N-MC and SK-N-SH cells also demonstrated the formation of specific protein-DNA complexes with this probe (Figure 7.3) when incubated with the same concentrations of poly (dl:dC)-(dl:dC) as required for the other LTR probes.

Nuclear factors binding to probe 194 with extracts from U373MG astrocytoma cells definitely formed three specific complexes of differing intensities, designated C0, C2 and C3 in Figure 7.3. From close examination of the autoradiograph there would appear to be a fainter fourth complex C1, located below C0, which is not clear in the assay shown in Figure 7.3 but more obvious in later oligonucleotide competition experiment (see Figure 7.7, Track 3). With extracts from SK-N-SH neuroblastoma cells three specific complexes were noted with probe 194, the pattern of which was similar to U373MG, as judged by the relative mobilities of the complexes relative to free probe. The complexes were also marked C1, C2 and C3 in Figure 7.3 to indicate this similarity. A protein-DNA complex of similar mobility to C3 was seen with SK-N-MC extracts but the other complexes formed with these extracts were not well resolved because the higher non-specific DNA-binding activity did not appear to be completely competed away. However, in

a later experiment (Figure 7.6) enough competitor was present to allow resolution of all nucleo-protein DNA complexes formed between SK-N-MC extracts and probe 194 and a three complex pattern marked C1, C2 and C3 similar to that described for SK-N-SH was seen.

Figure 7.3 indicated that the nuclear protein(s) interacting with probe 194 DNA to form complex C3 were more abundant compared to those involved in complexes C1 and C2, in U373MG and SK-N-SH extracts, although more so with U373MG. Interpreting the data from Figure 7.3 and Figure 7.6 for the binding of proteins present in SK-N-MC extracts to probe 194 indicates that in this cell line, complexes C1, C2 and C3 were of almost equal intensities and therefore of equal abundance in the extract preparation.

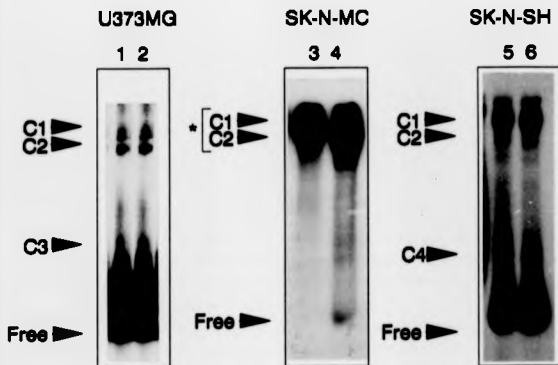


Figure 7.1. Gel retardation assays demonstrating the formation of specific nucleo-protein complexes between a radiolabelled probe, LTRF, spanning the HIV-1 LTR from position +77 to -158 and nuclear extracts from U373MG astrocytoma, SK-N-MC and SK-N-SH neuroblastoma cells in the presence of increasing poly (dl:dC)-(dl:dC) competitor. Tracks: 1 & 2, 3 & 4; 10 μ g of nuclear protein with 2 and 4 μ g of poly (dl:dC)-(dl:dC), respectively; 5 & 6; 20 μ g of nuclear protein with 4 and 6 μ g of poly (dl:dC)-(dl:dC), respectively. Arrows denote the location of probe-specific complexes and free probe at the bottom of each gel. * presumed region of complexes C1 and C2 inferred from Figure 7.5.

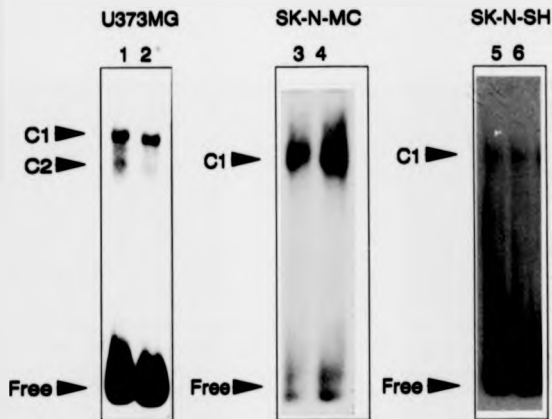


Figure. 7.2. Gel retardation assays demonstrating the formation of specific nucleo-protein complexes between a radiolabelled probe, 159, spanning the HIV-1 LTR from position -159 to -305 and nuclear extracts from U373MG astrocytoma, SK-N-MC and SK-N-SH neuroblastoma cells in the presence of increasing poly (dl:dC)-(dl:dC) competitor. Tracks: 1 & 2, 3 & 4; 10 μ g of nuclear protein with 2 and 4 μ g of poly (dl:dC)-(dl:dC), respectively; 5 & 6; 20 μ g of nuclear protein with 4 and 6 μ g of poly (dl:dC)-(dl:dC), respectively. Arrows denote the location of probe-specific complexes and free probe at the bottom of each gel.

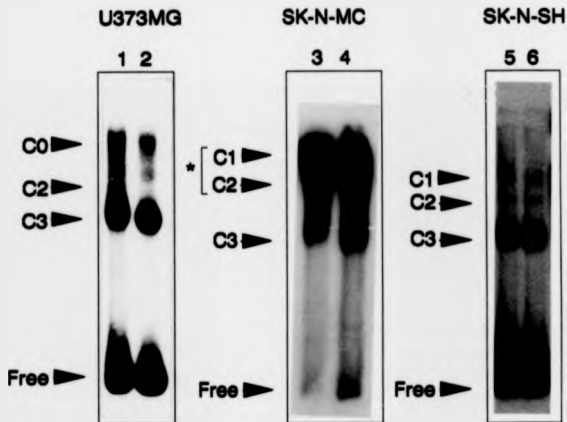


Figure. 7.3. Gel retardation assays demonstrating the formation of specific nucleo-protein complexes between a radiolabelled probe, 194, spanning the HIV-1 LTR from position -306 to -488 and nuclear extracts from U373MG astrocytoma, SK-N-MC and SK-N-SH neuroblastoma cells in the presence of increasing poly (dl:dC)-(dl:dC) competitor. Tracks: 1 & 2, 3 & 4; 10 μ g of nuclear protein with 2 and 4 μ g of poly (dl:dC)-(dl:dC), respectively; 5 & 6; 20 μ g of nuclear protein with 4 and 8 μ g of poly (dl:dC)-(dl:dC), respectively. Arrows denote the location of probe-specific complexes and free probe at the bottom of each gel. * presumed region of complexes C1 and C2 inferred from Figure 7.7.

7.2. Comparison of nuclear protein binding to the HIV-1 LTR with extracts from cells treated with and without cytokines and identification of protein-binding motifs by competition with synthetic oligonucleotides

Further gel retardation assays were performed in order to detect any changes in the factors that bind to the HIV-1 LTR after cells were exposed to a cytokine shown to augment LTR-driven gene expression. Protein extract from U373MG cells treated with IL-1 β and from the neuroblastoma cells treated with TNF α was compared with an equivalent amount from unstimulated cells in the same assay. In some experiments, the protein-binding motifs involved in the formation of the complexes were identified by competition with excess unlabelled synthetic oligonucleotides.

7.2.1. Interaction of nuclear factors with probe LTRF: competition with oligonucleotides to protein-binding motifs located in the HIV-1 LTR and the effects of cytokines

The region of the LTR spanned by probe LTRF is known to contain the binding sites for a number of cellular factors important in the regulation of HIV-1 transcription. These include the enhancer-motif of duplicate NF κ B sites, three binding sites for the transcription factor Sp1 and the TATA box which interacts indirectly with the cellular RNA polymerase II (See Chapter 1, Figure 1.2). In an attempt to identify the participation of any of these factors in the nucleo-protein complexes formed with LTRF, a competition experiment with unlabelled ds oligonucleotides containing the sequences most likely to be involved was performed with extracts from untreated or IL-1 β stimulated U373MG cells. The sequences of the oligonucleotides are given in Chapter 2, Table 2.2. The binding site consensus sequences contained in the NF κ B and TFIID oligonucleotides exactly matches those

present in the HIV-1 LTR. Aliquots of nuclear extracts were incubated with 10 and 25 *picomoles* of unlabelled oligonucleotide for 30 min prior to the addition of the labelled LTRF probe (approximately 5 *femtomoles*), this represented competitor oligonucleotide at a molar excess of approximately 2000- and 5000-fold, respectively. The TFIID oligonucleotide was supplied commercially in a more dilute solution and competition was performed in the presence of 0.44 and 1.75 *picomoles*, equivalent to a 87.5- and 350-fold molar excess.

The results of this gel retardation assay are shown in Figure 7.4. In tracks 1 to 7 nuclear extract from unstimulated U373MG cells and in tracks 8 to 14 extract from U373MG cells exposed to IL-1 β was assayed. This demonstrated the formation of nucleo-protein complexes with LTRF DNA similar to those seen in Figure 7.1, with complexes C1 to C4 visible with extracts from both stimulated and unstimulated cells. However, the ratio of complex C1 to C2 was clearly different between the two sources of nuclear extracts, the intensity of C2 was relatively weak compared to C1 in unstimulated U373MG cells whereas in cells stimulated with IL-1 β C2 was as intense as C1. Moreover, competition with the oligonucleotide containing tandem binding sites for NF κ B, the HIV-1 enhancer, removed most of the protein-binding activity producing C2 and reduced the intensity of the complex down to the level seen with extract from unstimulated cells (Figure 7.4. Tracks 6 and 7 compared to tracks 13 and 14). Competition with the NF κ B oligonucleotide at both concentrations also abolished the formation of complex C3 with extracts from cells treated with IL-1 β , whilst in extracts from unstimulated cells complex C3 was only present very weakly. Competition with the Sp1 oligonucleotide (Figure 7.4. Tracks 2 and 3, and 9 and 10) did not considerably alter the formation of complexes C1 and C2, although, both complexes appeared somewhat fainter with stimulated and unstimulated U373MG extracts when the Sp1 oligonucleotide was used in competition. However, this oligonucleotide was able to prevent the formation of complex C4 in both stimulated and unstimulated U373MG extracts. Incubation of

both sources of extracts with excess oligonucleotide to the TATA box region which binds factor TFIID (Greenblatt, 1991) did not affect the interaction of nuclear factors with LTR fragment. No changes were evident in the protein-DNA complexes seen at either concentration of excess TFIID oligonucleotide (Figure 7.4. Tracks 4 and 5, and 11 and 12).

A similar result was also obtained when this experiment was repeated using excess Sp1 and NF κ B oligonucleotides and different preparations of U373MG nuclear extracts treated with and without IL-1 β . The finding that complex C2 was more abundant than C1 in unstimulated cells was contrary to that described in Figure 7.1 but was confirmed in independent preparations of nuclear extracts from U373MG. The equal amounts of complex C1 and C2 in Figure 7.1 may well be artifactual due to the slower harvesting of U373MG cells from individual flasks rather than roller bottles later used for the preparation of nuclear extracts employed in this and all other experiments. This may have caused the cells undue stress and lead to the activation of NF κ B-like proteins. Subsequently great care was taken to harvest cells quickly from roller bottles and to keep cell suspensions on ice before freezing or nuclear protein preparation.

A limited oligonucleotide competition experiment was also performed with extracts from the neuroblastoma cell lines treated with and without TNF α , to investigate whether additional factor(s) recognized the NF κ B elements in cytokine-stimulated cells. Equal amounts of extract from each source of nuclear protein were compared in the same gel retardation assay and in competition with excess unlabelled oligonucleotide to NF κ B as described above for U373MG. The assay is shown in Figure 7.5. Tracks 1 to 3 and 7 to 9 employed unstimulated extracts from SK-N-SH and SK-N-MC cells, respectively and tracks 4 to 6 and 10 to 12 used extracts from SK-N-SH and SK-N-MC cells treated with TNF α , respectively. Again, a similar pattern of protein-DNA complexes to those seen in Figure 7.1 was formed with probe LTRF with extracts from both cell lines. Complexes C1, C2 and C4 were

clearly visible in SK-N-SH extracts and complex C3 present faintly in TNF α stimulated SK-N-SH extracts. In SK-N-MC only complexes C1, C2 and C4 were seen in extracts with or without TNF α . In both cell lines complex C1 was more intense than C2 in unstimulated extracts yet, with SK-N-SH extracts exposed to TNF α , complex C2 was more intense than C1 (Figure 7.5. Track 4). Extracts from SK-N-MC cells treated with TNF α appeared to indicate a similar result but unfortunately less radioactivity entered the gel in that track (no. 10) thus making interpretation awkward. Competition with excess NF κ B oligonucleotide at either concentration did not alter complex formation between probe LTRF and either source of unstimulated nuclear extract (Figure 7.5. Tracks 2 and 3, and 8 and 9), however, excess NF κ B oligonucleotide considerably reduced the intensity of complex C2 and abolished complex C3 in extracts from SK-N-SH cells treated with TNF α (Figure 7.5. Tracks 5 and 6). In SK-N-MC cells treated with TNF α (Figure 7.5. Tracks 10, 11 and 12) competition with excess NF κ B oligonucleotide (Tracks 11 and 12) may also have reduced the formation of complex C2. In track 10 complex C2 does appear to be more abundant than C1 whereas in tracks 11 and 12, in the presence of excess NF κ B oligonucleotide complex C1 was more intense than C2 and similar to that seen with unstimulated SK-N-MC nuclear extracts.

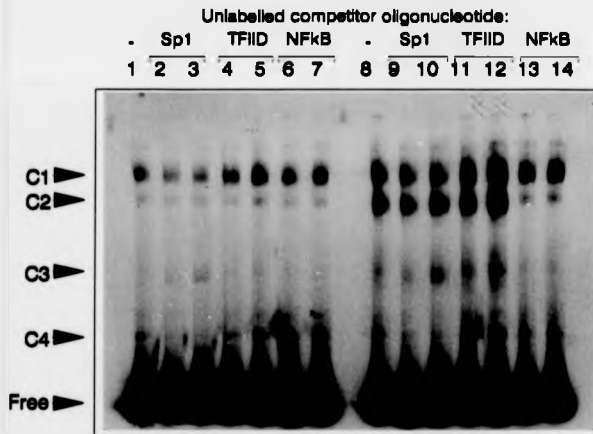


Figure 7.4. Identification of nucleo-protein complexes formed between a radiolabelled probe, LTRF, spanning the HIV-1 LTR from position +77 to -158 and nuclear extracts from untreated and IL-1 β stimulated U373MG astrocytoma cells by competition with oligonucleotides to specific sequence elements located in LTRF. Arrows denote probe-specific protein-DNA complexes and free probe. Tracks: 1-7, untreated; 8-14, stimulated with 250 U/ml IL-1 β for 1½ hrs. Tracks: 1 and 8, no competition; 2 & 9, Sp1 oligonucleotide at 2000-fold molar excess; 3 & 10, Sp1 oligonucleotide at 5000-fold molar excess; 4 & 11, TFIID oligonucleotide at 87.5-fold molar excess; 5 & 12, TFIID oligonucleotide at 350-fold molar excess; 6 & 13, NFkB oligonucleotide at 2000-fold molar excess; 7 & 14, NFkB oligonucleotide at 5000-fold molar excess.

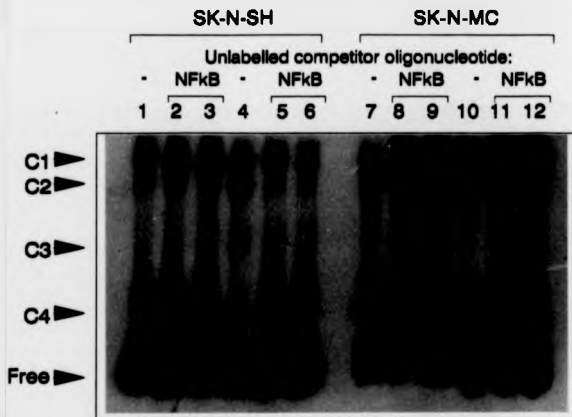


Figure 7.5. Identification of nucleo-protein complexes formed between a radiolabelled probe, LTRF, spanning the HIV-1 LTR from position +77 to -158 and nuclear extracts from untreated and TNF α stimulated SK-N-SH and SK-N-MC neuroblastoma cells by competition with an oligonucleotide to the NFkB elements located in LTRF. Arrows denote probe-specific protein-DNA complexes and free probe. Tracks: 1-3 and 7-9, unstimulated; 4-6 and 10-12, stimulated with 100 U/ml TNF α for 2 hrs. Tracks: 1, 4, 7 & 10 no competition; 2, 5, 8 & 11, NFkB oligonucleotide at 2000-fold molar excess; 3, 6, 9 & 12, NFkB oligonucleotide at 5000-fold molar excess.

7.2.2. Interaction of nuclear factors with probe 194: competition with oligonucleotides to protein-binding motifs located in the HIV-1 LTR and the effects of cytokine

i). The effects of cytokines on the interaction of nuclear factors with probe 194

Specific complex formation with probe 194 and nuclear extracts from all cytokine-treated cells did not reveal any differences compared to those evident with extracts from unstimulated cells (Figure 7.6). Essentially the same number and pattern of protein-DNA complexes were formed in U373MG and SK-N-SH extracts as those seen in Figure 7.3, although in this assay close examination of the autoradiograph did not suggest an additional complex below C0 formed between probe 194 and U373MG extracts. A much clearer result was obtained with SK-N-MC nuclear extracts and probe 194 in this experiment compared to Figure 7.3 and in Figure 7.5 the formation of three nucleo-protein complexes akin to those seen with SK-N-SH extracts were observed and remained unchanged with extracts from cytokine-stimulated cells.

The relative abundance of the protein(s) constituting each complex also remained similar to that described for probe 194 and each source of nuclear extract in Section 7.1.3. Complexes formed with probe 194 and U373MG extracts were more intense with extracts from IL-1 β -stimulated cells but this was not significantly so. And with nuclear extracts from unstimulated and TNF α -treated SK-N-SH cells complex C2 was only faintly visible on the autoradiograph and can just be seen on the photograph in Figure 7.7.

ii). Oligonucleotide competition of factors interacting with probe 194

The 5' region of the HIV-1 LTR has been reported to contain two elements designated Site A and Site B which were important in the interaction with cellular proteins present in lymphoblastoid cells (Orchard *et al.*, 1990). Site A binds as yet unidentified host nuclear protein(s) and Site B consists of two distinct elements

similar to a steroid/thyroid hormone-binding motif (Orchard *et al.*, 1990; Cooney *et al.*, 1991). These are located in the negative regulatory element of the HIV-1 LTR (see Chapter 1 Figure 1.2) and were present at the 5' end of probe 194. Matching ds oligonucleotides were synthesized corresponding to Site A and Site B and one, BM5, in which the 5' element of Site B had been removed by mutation. The sequence composition of these oligonucleotides is given in Chapter 2, Table 2.2 and were identical to those reported by Orchard *et al.* (1990).

Oligonucleotide competition experiments were carried out with nuclear extracts from unstimulated U373MG and SK-N-SH cells by incubation of aliquots of nuclear extracts with excess unlabelled oligonucleotide for 30 min before the addition of labelled probe 194. Similar to Section 7.2.1, oligonucleotides were used at 10 picomoles which represented an approximate molar excess of 2000-fold. In this assay (Figure 7.7), a pattern of complexes was formed with probe 194 and U373MG nuclear extracts similar to those described previously in Figure 7.3, although in this assay a complex just below C0, C1 was just discernible in the photograph (see Track 3). This was slightly clearer on the original autoradiograph. Competition with excess oligonucleotide to Site A did little to effect the formation of complexes C0 and C3 with probe 194, however C2 appeared to be competed away (Figure 7.7, Track 2). More striking was the competition with excess Site B oligonucleotide which almost completely removed complexes C0 and C3 but left C2 and C1 unaltered (Figure 7.7, Track 3). An oligonucleotide containing a consensus protein-binding site for CTF/NF1 which is recognized by a number of cellular factors that bind the nucleotide sequence CCAAT (La Thangue and Rigby, 1988) was also used in competition for factors interacting with probe 194. This was because the Site A binding site located in the LTR of HIV-1 isolate HBX-2 contains a similar CTAAT sequence on the non-coding strand (Orchard *et al.*, 1990) but in the LTR of the LAI isolate of HIV-1 (Wain-Hobson *et al.*, 1985) employed here, there is a point mutation converting this element to the CCAAT motif (La Thangue

and Rigby, 1988). Hence to determine if CCAAT box-binding factors also recognized this sequence an oligonucleotide for the CTF/NF1 family of factors was tested. However, no discernible difference in complexes formed with probe 194 were noted when competition was performed with this oligonucleotide (Figure 7.7. Track 4). Finally, excess unlabelled BMS oligonucleotide which contained only the 3' half-site of Site B (Figure 7.7. Track 5) was able to reduce the relative intensities of complexes C0 and C3 but left complex C2 unchanged.

A similar competition experiment with SK-N-SH nuclear extracts using the same oligonucleotides to sequences involved in complexes with probe 194 is also shown in Figure 7.7. In the absence of oligonucleotide competition three complexes C1, C2 and C3 were resolved (Track 6) and were again similar to those reported in Figure 7.3. When excess Site A oligonucleotide was incubated with the extract before the addition of labelled probe 194, complexes C1 and C2 were not seen in the gel retardation assay (Figure 7.7. Track 7). Excess oligonucleotide to Site B was capable of removing almost all the binding activity which formed complex C3 (Track 8) and the use of Site A and Site B oligonucleotides together, seen in track 9, prevented the formation of almost any protein-DNA complexes with probe 194. The BMS oligonucleotide that contained a mutated Site B 5' half-site also partly reduced the intensity of the complex C3 and did not effect the Site A-specific complexes C1 and C2.

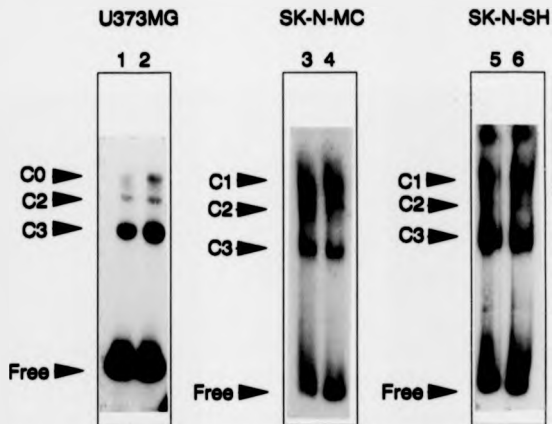


Figure 7.6. Comparison of nucleo-protein complexes formed between a radiolabelled probe, 194, spanning the HIV-1 LTR from position -306 to -486 and nuclear extracts from untreated and IL-1 β stimulated U373MG astrocytoma cells, and SK-N-MC and SK-N-SH neuroblastoma cells, untreated and stimulated with TNF α . Arrows denote probe-specific protein-DNA complexes and free probe. Tracks: 1, 3 & 5, unstimulated; 2, U373MG stimulated with 250 U/ml IL-1 β for 1½ hrs; 4 & 6 SK-N-MC and SK-N-SH stimulated with TNF α for 2 hrs, respectively.

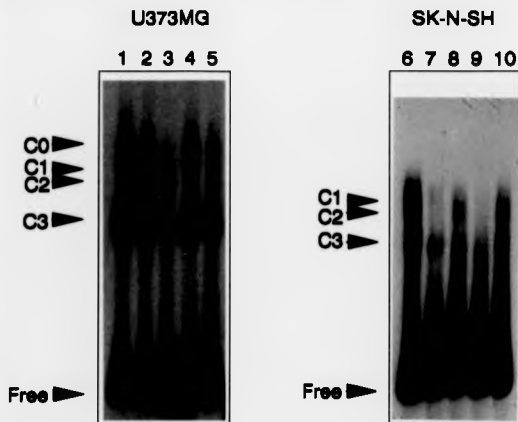


Figure 7.7. Identification of nucleoprotein complexes formed between a radiolabelled probe, 194, spanning the HIV-1 LTR from position -308 to -488 and nuclear extracts from U373MG astrocytoma and SK-N-SH neuroblastoma cells by competition with oligonucleotides to certain sequence elements located in 194. Arrows denote probe-specific protein-DNA complexes and free probe. Tracks: 1 & 6, no competition; 2 & 7, Site A oligonucleotide at 2000-fold molar excess; 3 & 8, Site B oligonucleotide at 2000-fold molar excess; 4, CTF/NF1 oligonucleotide at 700-fold molar excess; 9, Site A and Site B oligonucleotide each at 2000-fold molar excess; 5 & 10, BM5 oligonucleotide at 2000-fold molar excess.

7.2.3. Interaction of nuclear factors with probe 159: competition with oligonucleotides to protein-binding motifs located in the HIV-1 LTR and the effects of cytokines

i). The effects of cytokines on the interaction of nuclear factors with probe 159

Nuclear factor binding to probe 159 in extracts from unstimulated and IL-1 β -treated U373MG astrocytoma cells and unstimulated and TNF α -treated neuroblastoma cells also revealed no change in the number or mobility of the protein-DNA complex formed when cells were exposed to a cytokine shown to augment LTR-driven gene expression. One major complex of the same relative mobility and intensity was apparent with probe 159 in all nuclear extracts (Figure 7.8). The minor complex C2 previously noted in Figure 7.2 formed with U373MG extracts was not seen in either source of U373MG nuclear extract in this assay.

ii). Oligonucleotide competition of factors interacting with probe 159

Further oligonucleotide competition experiments were performed with extracts from unstimulated U373MG and SK-N-MC cells in an attempt to identify the factor(s) that formed the specific complexes previously noted with probe 159 in Figures 7.2. From DNase I footprinting studies, described in Chapter 8, a major protein-binding site occupied by proteins present in neural cell extracts was located in probe 159 (see Chapter 8, Section 8.1.2). The protected sequence of which also contained a CCAAT motif, therefore the CTF/NF1 oligonucleotide was again tested in competition experiments along with the Site A oligonucleotide which possessed a somewhat similar (see Section 7.1.3) but unrelated nuclear protein-binding sequence. The experiments were undertaken in an identical fashion to those competition assays already mentioned, with unlabelled ds oligonucleotide used at 2000-fold molar excess (*ie.* 10 picomoles).

In both U373MG and SK-N-MC extracts one major nucleo-protein complex was formed with probe 159, and neither Site A nor CTF/NF1 oligonucleotides could compete this complex away to any extent (Figure 7.9). The formation of the minor complex just below C1, present with U373MG extracts but not SK-N-MC extracts, was similarly unaffected by competition with either oligonucleotides.

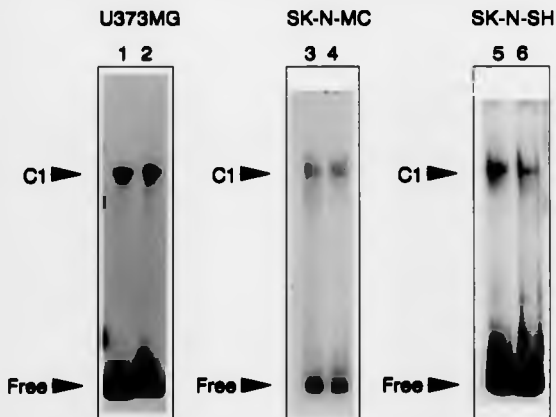


Figure 7.8. Comparison of nucleo-protein complexes formed between a radiolabelled probe, 159, spanning the HIV-1 LTR from position -159 to -305 and nuclear extracts from untreated and IL-1 β stimulated U373MG astrocytoma cells, and SK-N-MC and SK-N-SH neuroblastoma cells, untreated and stimulated with TNF α . Arrows denote probe-specific protein-DNA complexes and free probe. Tracks: 1, 3 & 5, unstimulated; 2, U373MG stimulated with 250 U/ml IL-1 β for 1½ hrs; 4 & 6 SK-N-MC and SK-N-SH stimulated with TNF α for 2 hrs, respectively.

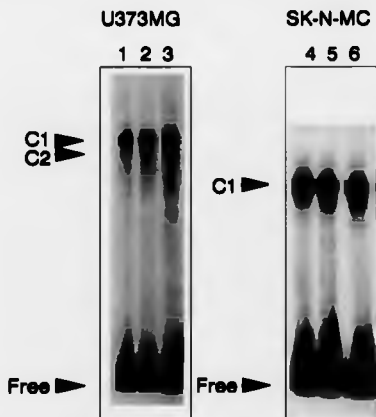


Figure 7.9. Competition of nucleo-protein complexes formed between a radiolabelled probe, 159, spanning the HIV-1 LTR from position -159 to -305 and nuclear extracts from U373MG astrocytoma and SK-N-MC neuroblastoma cells with oligonucleotides to other elements with a degree homology to the nucleotide sequence involved in complex C1. Arrows denote probe specific protein-DNA complexes and free probe. 1 & 4, no competition; 2 & 5, Site A oligonucleotide at 2000-fold molar excess; 3 & 6 CTF/NF1 oligonucleotide at 700-fold molar excess.

Discussion

Experimental analysis of DNA-binding proteins

Gel retardation assays were undertaken using DNA fragments derived from the HIV-1 LTR primarily to provide preliminary information on the interaction of DNA-binding proteins with these sequences and define which probe fragments from the LTR were worthy of further investigation by the more complicated DNase I footprinting technique. The formation of one or more nucleo-protein complexes with each of the LTR probes ensured that it would be worthwhile to footprint the entire long terminal repeat in order to locate the sequences involved.

These gel retardation experiments also served to confirm the ability of the DNA probes, end-labelled on one strand for DNase I footprinting, to bind nuclear proteins. Care was taken during the preparation of the LTR probes to avoid conditions which may cause damage to the DNA and can impair or destroy their interactions with nuclear proteins or create artefactual results. Possible causes include exposure to organic solvents, in particular, the evaporation of ethanol from nucleic acid samples after precipitation (Svaren *et al.*, 1987). Therefore during probe preparation (see Chapter 2, Section 2.12.2) the excess ethanol that remained adhered to the walls of the microcentrifuge tubes after precipitation was always removed by brief re-centrifugation and aspiration with a micropipette and samples resuspended directly in TE buffer without drying by evaporation. The gel purification of the LTR probes also facilitated the separation of denatured DNA from native double stranded probe which can produce aberrant results when used in DNA binding assays (Goodwin, 1990).

Likewise, the gel retardation experiments performed here also confirmed that the nuclear extracts contained functional DNA-binding proteins and that the protein preparations did not suffer proteolytic degradation to any noticeable extent. This was judged from the discrete nature of the majority of the nucleo-protein

complexes visualized in the assays, complexes that smear down the gel often indicate degradation of the extracts. Conversely, the formation of complexes that have an ill-defined upper border suggest a degree of heterogeneity in the factors that recognize the binding site. The identification of a number of distinct DNA-binding activities in the nuclear extracts, by competition with oligonucleotides, compared to the results reported in other studies (Malim *et al.*, 1989; Orchard *et al.*, 1990; Parrott *et al.*, 1991), provided evidence that extraction of nuclear proteins with 0.4 M NaCl performed here produced a nuclear extract that contained most, if not all of the relevant DNA-binding proteins. A slight variation in the recovery of certain DNA-binding activities between extracts from the same cell type was noted. These variations were such that small differences in the relative intensities of nucleo-protein complexes seen in gel retardation assays between extracts from cytokine-treated cells and unstimulated cells were judged not to be significant. Also, different preparations of nuclear extracts often contained variable amounts of non-specific DNA-binding activity so the amount of poly (dI:dC)-(dI:dC) required to absorb this activity had to be determined empirically for each preparation. Although, apart from after prolonged storage of SK-N-MC nuclear extracts (see later in Discussion), the amounts of competitor required remained the same for all preparations.

Nuclear factor binding to the HIV-1 LTR

The HIV-1 LTR was able to form up to eight or nine specific complexes with proteins present in nuclear extracts from astrocytoma and neuroblastoma cell lines. Up to 4 of these complexes were found with probe LTRF which spanned the 3' region of the LTR, one complex with the central domain in probe 159 and three or four with the 5' region present in probe 194. This, in part, reflects the large number of recognition sites for transcription factors that have been reported to be located within the HIV-1 LTR (Greene, 1990) and also confirms that neural cells contain a

significant number of DNA-binding activities that recognize the HIV-1 LTR. The presence of multiple protein-binding sites within each probe fragment may permit the formation of protein-DNA complexes that contain different sequence-specific binding activities and possibly allow cooperation between factors in DNA-binding, such that many different complexes may be resolved in gel retardation assays produced by a lesser number of protein-binding sites. The presence of three binding sites for the transcription factor Sp1 of differing affinities in the HIV-1 LTR (Harrich *et al.*, 1989) could permit the formation of up to three discrete protein-DNA complexes depending upon the concentration of only one transcription factor in the extracts. The situation can be further complicated if any of the nuclear proteins undergo oligomerization on binding their recognition sites. Therefore, some of the individual complexes noted were quite likely due to one or more factors interacting with the LTR at the same recognition sites or a single factor interacting at multiple recognition sites.

Sequence-specific DNA-binding activities

In the light of the oligonucleotide competition experiments carried out, some DNA-binding activities could be assigned as components of one or more of the nucleo-protein complexes seen. In U373MG nuclear extracts two complexes formed with probe 194 could be effectively competed out with excess oligonucleotide specific for Site B. This not only indicated the importance of this region of the LTR in mediating interactions with certain nuclear factor(s) but also that one sequence element formed two distinct complexes. The Site B recognition sequence, as mentioned earlier, was reported to consist of two important regions, closely spaced, which are characteristic of a nuclear site for members of the thyroid/steroid hormone receptor family (Orchard *et al.*, 1990; Cooney *et al.*, 1991). Generally, this class of ligand-bound receptors bind to both half-sites as homodimers and both sites are required for a transcriptional effect to be exerted (Nääär *et al.*, 1991). Competition

with excess BMS oligonucleotide argued against the notion that one complex was specific to each part of Site B as the formation of both complexes was similarly reduced yet neither was removed. The fact that excess BMS did not abolish binding altogether suggests also that the affinity of factor(s) for this mutant oligonucleotide was reduced compared to the wild type sequence and implies the importance for both half-sites in the interaction. Furthermore the finding of two retarded complexes specific for Site B correlates with the data of Cooney *et al.* (1991) who reported two molecular weight forms of a protein, COUP-TF, that interacts with Site B.

With SK-N-SH nuclear extracts, competition with oligonucleotide to Site B was also able to considerably reduce factor binding to one equivalent complex formed with probe 194. The presence of only one complex specific to Site B in these cells demonstrated a difference between neuroblastoma and astrocytoma cell lines. Whilst C3 appeared to be of equivalent mobility in all cell types, the presence of the higher molecular weight complex C0 suggested that there were either multiple activities in astrocytoma cells that recognized this sequence, in agreement with Cooney *et al.* (1991), or some higher structure of Site B-binding protein(s), perhaps due to the observed increased abundance of this DNA-binding activity in astrocytoma extracts. Interestingly, Orchard *et al.* (1990) noted that Site B-binding activity surprisingly decreased in mobility in gel retardation assays after treatment of T cell nuclear extracts with the mild detergents deoxycholate and Brij, indicating that the protein underwent a conformational change or complex formation. However, no detergents were present in the buffers employed in the protein-binding reactions of the gel retardation assays performed here so there were no such factors to promote any similar conformational change or complex formation.

Competition with excess oligonucleotide to the Site A region described by Orchard *et al.* (1990) was also able to prevent formation of complexes with probe 194. In extracts from SK-N-SH neuroblastoma cells both complexes C1 and C2 were competed away, as was complex C2 formed with U373MG nuclear extracts.

Whether excess Site A oligonucleotide also resulted in the loss of C1 in U373MG extracts could not be determined from the assay. This demonstrated again that one region of the LTR was responsible for the formation of up to two distinct protein-DNA complexes in gel retardation assays and that perhaps two (or more) proteins of different sizes recognized the sequence contained within the Site A oligonucleotide. Furthermore, despite the presence of a CCAAT motif in the Site A sequence of HIV LAI, the sequence was recognized by one or more factors that were distinct from the CTF/NF1 family of transcription factors. Another class of DNA-binding proteins that recognize protein-binding sites similar to Site A are the octamer factors (La Thangue and Rigby, 1988). Whilst not examined here, Orchard *et al.* (1990) report that an oligonucleotide containing an octamer consensus sequence did not compete for Site A-binding either.

The protein factor(s) that formed the one major nucleo-protein complex with probe 159 were found to be distinct from those that interact with Site A and the CCAAT box-binding factors CTF/NF1 as neither oligonucleotide was able to compete away the complex. The nature of this protein-DNA interaction is discussed further in Chapter 8 as little information was provided by experiments reported in this chapter; apart from the finding that the nuclear factor or factor(s) involved were relatively abundant in astrocytoma and neuroblastoma cells.

The comparison of complex formation to probes 194 and 159 after cells had been exposed to a cytokine known to augment HIV-1 LTR-driven gene expression did not demonstrate any differences at the level of DNA-binding proteins recognizing either LTR region, and this indicated that these regions were not important in mediating the effects of the cytokines IL-1 β and TNF α . However, extracts were prepared from cells stimulated with cytokines for up to two hours only which would have precluded the discovery of alterations in DNA-binding proteins that occurred after that time. Furthermore, a gel retardation assay can not

distinguish between different protein factors that migrated with the same mobility and interacted with the same regulatory element under different conditions.

Induction of NF κ B-like DNA-binding activity

The interaction of nuclear proteins with sequences located in probe LTRF provided the first indication that elements in the HIV-1 LTR responded to changes in nuclear factors that occurred in cells after exposure to the cytokines IL-1 β or TNF α . The gel retardation assays identified a major nucleo-protein complex, in all cells, and a minor complex, in U373MG and SK-N-SH, (C2 and C3, respectively, Figure 7.4) that were formed strongly with LTRF only in cells exposed to the cytokines and were competed away by excess oligonucleotide containing NF κ B binding sites. Competition with the NF κ B oligonucleotide did not alter the formation of specific complexes between LTRF and extracts from unstimulated cells which indicated that this DNA-binding activity was only present in cytokine-treated cells and that the less intense C2 complex noted with unstimulated cell extracts was not due to the same DNA-binding activity. Therefore, the induction of an NF κ B-like binding activity to the enhancer of HIV-1 by the cytokines TNF α and IL-1 β was evident in neural cells and this is a cellular mechanism that can lead to enhanced gene expression from the LTR (Kawakami *et al.*, 1988; Israel *et al.*, 1989; Osborn *et al.*, 1989). This result was clear with extracts from U373MG astrocytoma cells stimulated with IL-1 β and SK-N-SH neuroblastoma cell extracts after TNF α treatment but was not as definitive in SK-N-MC cells due to a poor experiment. Unfortunately, repeating this for SK-N-MC cells was also unsuccessful due to insufficient competitor nucleic acid in the binding reactions, despite using more than in previous experiments. It was noted that nuclear extracts from SK-N-MC neuroblastoma cells developed increased non-specific DNA-binding activity over the length of time that they were stored, even though this was at -70°C and aliquots

were never re-frozen and used again. This feature was unique to SK-N-MC cells and increased to such an extent that after around two months of storage these extracts could no longer be used.

Competition with an oligonucleotide containing a copy of a high affinity Sp1 binding site was also able to prevent the formation of one complex in gel retardation assays with probe LTRF performed with U373MG nuclear extracts. The corresponding complex was also noted in SK-N-SH extracts but competition with the Sp1 oligonucleotide was not carried out. This also provided evidence for the involvement of the Sp1 transcription factor in HIV-1 gene expression in neural cells which was not unexpected given that Sp1 has been reported to interact with the LTR in a variety of cell types (Harrich *et al.*, 1989; Parrot *et al.*, 1991). The complex was present at approximately equal proportions in extracts from both cytokine-treated and untreated cells and also as expected, Sp1 DNA-binding activity did not appear to be induced by cytokine treatment of U373MG or SK-N-SH cells.

Surprisingly, the oligonucleotide containing the TATA box sequence that interacts with the general transcription factor TFIID did not alter the pattern or intensity of binding to probe LTRF even though the corresponding sequence was present in this part of the HIV-1 LTR. Although a smaller amount of the oligonucleotide was employed in the experiment than the other oligonucleotides, a 350-fold molar excess is still quite considerable. However, the manufacturers (Promega) do report that the oligonucleotide does not perform well when used as a probe for TFIID binding activity with HeLa cell extract in gel retardation assays. Therefore, perhaps insufficient was employed in the competition experiment due to an interaction of lower affinity when TFIID binds its cognate sequence present in an oligonucleotide. A similar phenomenon was noted with Site A and Site B sequences when gel retardation assays were performed using radiolabelled oligonucleotides as probes (see Chapter 9). Binding of these factors was much more easily detected with

less nuclear extract when probe 194 was used rather than the corresponding oligonucleotide. This is discussed further in Chapter 9 but does imply that short stretches of DNA do not bind nuclear factors as efficiently and perhaps flanking DNA sequences are important.

CHAPTER 8

Chapter 8: Localization of nuclear protein-binding sites in the HIV-1 LTR

Introduction

Experimental technique

DNase I footprinting was employed to identify sequences within the HIV-1 LTR which interact with the DNA-binding proteins present in nuclear extracts from the neural cell lines under study. The region of DNA involved in the direct binding of protein to fragments from the LTR was determined by the protection from cleavage afforded to the DNA by the specifically and tightly bound protein in the presence of DNase I. Comparison of the cleavage patterns produced by the limited digestion of protein-bound DNA and protein-free DNA on linear sequencing gels allows the identification of gaps within the ladder of bands which correspond to the 'footprint' from sequence-specific DNA-binding protein. Footprints are often identified by the presence of additional bands, termed DNase stops, caused by an increase in the frequency of enzymatic cleavage of the probe DNA at a particular site due to the influence of bound protein. The identification of protein-induced changes in the pattern of DNase cleavage products can be made more apparent by assaying at least two different concentrations of protein extract so that the effect of increasing protein can be observed. The further inclusion of chemical sequencing reactions of the LTR probe in parallel to the footprinting samples provides the necessary information to read the nucleotide sequence that constitutes the footprint. When conducting this type of experiment the DNA fragments under investigation must be specifically labelled only at one terminus, on one strand, so that the region of protection can be orientated with respect to the position of the labelled nucleotide(s) and complete analysis of a given DNA sequence will require the separate examination of both coding and non-coding strands. Additionally,

DNA-binding proteins may protect either the coding or non-coding strand to the same or different extents or produce a footprint that is more defined on one strand than the other.

DNA probes and nuclear extracts.

The use of high specific-activity radiolabelled probes containing one or more labelled nucleotides allows the detection of extremely small amounts of DNA-binding protein, however, the lack of separation of protein-bound and free DNA molecules in a binding reaction limits the sensitivity of the technique, such that the quality of the footprint depends upon the extent of occupancy of the protein binding site. Thus efficient labelling protocols for the production of high specific-activity probes are required to limit the amount of input DNA and therefore the number of binding sites available for protein-DNA interactions. In order to increase the occupancy of binding sites nuclear protein is included at a large excess to maximize complex formation.

When the DNA sequence under examination contains many potential protein binding sites, as is true for the HIV-1 LTR, the relative abundance of each DNA-binding activity in the nuclear extract will influence which protein-binding sites produce a footprint. The degree of occupancy of any one binding site will be dependent on the concentration of the factors that interact with it and the affinity with which this occurs, such that high concentrations of protein extract will be required if a given activity is scarce or it has low affinity for DNA. The binding constants of sequence-specific DNA-binding proteins vary over a wide range between 10^9 and 10^{14} M^{-1} (Rhodes, 1989) but competitor poly (dI:dC)-(dI:dC) is required to absorb non-specific DNA-binding activity. The use of more than around 150-200 μg of protein (the exact amount depends on the quality of the extract preparation), which may be required to detect rare factors, often introduces substantial non-specific binding activity, inhibiting digestion of the DNA and leading

to the appearance of artefactual protection. Unfortunately, the inclusion of larger amounts of competitor to counter this effect may also compete out the DNA-binding factors of lower affinity. Consideration of the technique to maximize the extent of occupancy of a protein binding site on the target DNA serves to illustrate that footprinting techniques offer only qualitative and not quantitative data on the protein-DNA interactions once a given binding site is fully occupied. Therefore, for this reason and others given above, experiments with both footprinting and gel retardation techniques are usually necessary for a complete analysis of protein-DNA interactions.

Preliminary experiments

Due to the more complex nature of the footprinting procedure the major variables must be established empirically before the assay will produce data from which meaningful results can be drawn. Preliminary experiments were therefore conducted to determine firstly, the time of DNase I digestion of the labelled probe that will produce around the optimum of, on average, one cut per DNA strand, secondly, the concentration of competitor poly (dI:dC)-(dI:dC) required to compete away the non-specific DNA-binding activity and thirdly, the amount of nuclear extract necessary to give protection over the binding sites of interest. Points two and three will be dependent upon the quality of the nuclear extract preparation (see Chapter 7).

Once established, DNase I footprinting was employed to identify regions of the LTR that bound nuclear factors in extracts from unstimulated neural cells and cells stimulated with a cytokine previously shown to augment HIV-1 LTR-driven gene expression. These were U373MG astrocytoma cells stimulated with IL-1 β and SK-N-MC and SK-N-SH neuroblastoma cells stimulated with TNF α . Some experiments were performed with nuclear extracts from unstimulated G26-24 oligodendrogloma cells.

8.1. Identification of sequences within the HIV-1 LTR which bind nuclear proteins

8.1.1. DNase I footprinting of probe LTRF

i). Coding strand

Figure 8.1 illustrates sequences on the coding strand of the HIV-1 LTR (-158 to +78) protected from DNase I digestion by nuclear extracts from unstimulated and cytokine-treated U373MG, SK-N-SH and SK-N-MC cells. Comparison of the pattern of bands produced by DNase I cleavage of the probe in the presence of nuclear protein to that in the absence of nuclear protein (Tracks 1, 6, 11, and 16) demonstrated regions of protection. These were stronger when 150 μ g rather than 75 μ g of extract protein was used. The position of protected sequences was determined from Maxam and Gilbert chemical sequencing reactions (not shown in the figure) and given as the position relative to the start of transcription at +1.

With extracts from U373MG astrocytoma and SK-N-SH neuroblastoma cells there were similar regions of the LTR that exhibited reduced cleavage by DNase I with nuclear extracts from unstimulated (Track 3, U373MG and 8, SK-N-SH) and IL-1 β - or TNF α -treated cells (Track 4, U373MG and 10, SK-N-SH). However, although this appeared to give the LTR partial protection over sequences from position -107 to -80, which correspond to the enhancer of tandem NF κ B sites (Nabel and Baltimore, 1987), it was due to excessive non-specific DNA-binding activity in the extract preparations as no alteration in the DNase I cleavage pattern was observed and neither were any bands in this part of the gel completely absent (see discussion).

The protection seen over two Sp1 sites [designated III and II] (Jones *et al.*, 1986), from -79 to -56, with U373MG and SK-N-SH extracts (Figure 8.1. Tracks 3 and 5, and, 8 and 10, respectively) did partly fulfil the criteria for the interpretation of a genuine region of protection as bands were almost absent from that region.

However, no protein-induced DNase stops were noted in the pattern of DNase cleavage products. This and the partial protection seen over the last Sp1 site (I), which continued to position -36 may also be a consequence of non-specific protein-DNA interactions with U373MG extracts as again no bands were completely absent. Close examination of the autoradiograph over tracks 8 and 10 (150 μ g of extract from SK-N-SH cells) revealed protein-induced DNase stops in the same -55 to -36 region and indicated sequence-specific binding of an Sp1-like factor in SK-N-SH cells. The treatment of U373MG cells with IL-1 β did not increase the artificial protection obtained over the Sp1 sites and the enhancer and therefore the presence of any Sp1-like or inducible enhancer-binding factors could not be determined (Tracks 4 and 5). For similar reasons any TNF α -induced enhancer-binding protein could not be detected in SK-N-SH cells. The degree of protection of the Sp1 sites also did not change when extracts from cytokine-stimulated SK-N-SH cells were used.

The regions of protection seen over sequences in LTRF by extracts from SK-N-MC neuroblastoma cells did, however, completely satisfy the requirements for sequence-specific protein-induced protection (see introduction). With 75 μ g of nuclear extract from unstimulated SK-N-MC neuroblastoma cells there was occupation of Sp1 site III, position -79 to -62, defined by the absence of a strong DNase I hypersensitive site and the presence protein-induced bands at either side of the region that were absent from the no protein control (Figure 8.1. Track 12). Furthermore, what represented partial occupancy of Sp1 site I, position -56 to -36 was also evident and was defined by the greatly reduced intensity of a site of DNase cleavage and the presence of a protein-induced band at the -56 border. With 150 μ g of extract there was complete protection of all three Sp1 sites, from position -79 to -36, (Track 13). At either concentration of extract from unstimulated cells the enhancer was not protected but there was an altered DNase I cleavage pattern slightly dissimilar to the no protein controls at the -80 border due to the presence of

specifically bound Sp1-like proteins. When 75 μ g of extract from TNF α -treated SK-N-MC cells was used there was a similar degree of protection over the Sp1 sites and additional partial protection over the enhancer region which probably represented the weak binding of factors to the proximal NF κ B motif (Track 14). With 150 μ g of nuclear extract from TNF α -treated cells full protection was similarly observed over all three Sp1 sites and protein factor(s) also fully protected the enhancer from position -107 to -80. This demonstrated the presence of TNF α -inducible proteins in SK-N-MC cells which recognize the NF κ B sites of the HIV-1 enhancer.

ii). Non-coding strand

The binding of nuclear proteins to the non-coding strand of probe LTRF was also examined by DNase I footprinting and is shown in Figure 8.2. This probe was radiolabelled at the same 3' end on the non-coding strand (designated NC) to allow the resolution of a similar region of LTR.

In Figure 8.2 the DNase I cleavage pattern of probe LTRF NC in the absence of nuclear protein is present in tracks 1, 6, 11 and 14. There was some fading of the autoradiograph across the samples such that the clearest footprints were obtained in the left hand portion of the gel and with 100 μ g rather than 50 μ g of nuclear extract.

Comparing extracts from U373MG cells unstimulated or treated with IL-1 β (Figure 8.2. Tracks 3, and 5, respectively) there were no discernible differences in the regions of the LTR protected from DNase I cleavage by bound nuclear proteins. When 100 μ g of extract was used protein-induced protection was judged to be present over sequences from position -86 to -63, corresponding to Sp1 site III, by the complete absence of a strong DNase I cleavage point seen with no protein and the increase in intensity of the band at the -63 border. Partial protection also appeared to be evident over the region from -62 to -56 (Sp1 site II) due to the absence of a

weaker DNase I hypersensitive site in the presence of nuclear extract, and protection from -55 to -37 over (Sp1 site I) by similar criteria. Further towards the 3' end of the LTR, sequences from -35 to -21 overlying the TATA box may also be weakly protected, although this could only be determined by the loss of two fainter bands and a reduction in intensity of another within the region. Similarly it was difficult to determine if sequences from -4 to +4 containing the start of transcription and a binding site for the cellular protein LBP-1 (Jones *et al.*, 1988) were also partially protected, a previous assay had shown this to be so (see below and Figure 8.3), but in this example it could not definitely be established. Below this region of the LTR the intensity of the bands on the autoradiograph faded such that no further footprints could be determined.

The footprints obtained with probe LTRF NC and extracts from SK-N-SH and SK-N-MC neuroblastoma cells treated with and without TNF α were regrettably much less clear than those achieved in Figure 8.2 with U373MG extracts, but were also unchanged when extracts from cytokine-treated cells were compared with extracts from unstimulated cells (Figure 8.2). In tracks 8 and 10 the region of the LTR from -86 to -63, overlying Sp1 site III was protected from DNase I digestion and -55 to -37, corresponding to Sp1 site I was partially protected with SK-N-SH nuclear extracts. With SK-N-MC extracts (Tracks 12 and 13) the protected region appeared to cover all three Sp1 sites from -86 to -37 but this was a consequence of fainter exposure as direct examination of the autoradiograph showed a pattern of bands identical to SK-N-SH and indicated protection of Sp1 site III and partial protection of Sp1 site I. Weak protection of sequences surrounding the TATA box (-35 to -21) could not be established with SK-N-SH extracts or SK-N-MC extracts and no further protein-binding sequences of the LTR were determined from this experiment.

In another experiment with nuclear extracts from G26-24 oligodendrogloma cells and U373MG cells (with and without IL-1 β treatment), illustrated in Figure 8.3,

there was extremely good protection from -86 to -37, over all three Sp1 sites, weak protection from -35 to -21 over the TATA region, and also a reasonable degree of protection from -4 to +4 over the cap site and LBP-1 (designated site number I). Note that even when obvious protection of this probe by sequence-specific proteins was evident there were no protein-induced DNase I stops, these, I would suggest, are also influenced by the nucleotide sequences either side of the binding site but nevertheless are often a good marker for genuine protection. There was also reasonable protection over a second LBP-1 site (II) from +6 to +12 and perhaps weak protection of sequences over a third LBP-1 site (III) at +16 to +22.

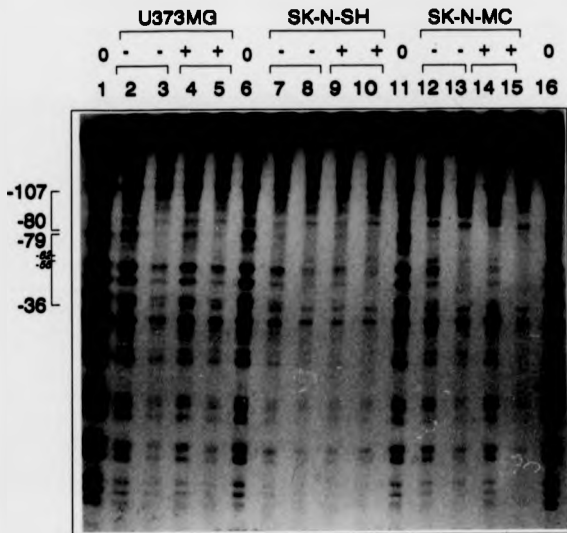


Figure 8.1. DNAase I footprint analysis of the HIV-1 LTR (-158/+78, probe LTRF) labelled on the coding strand, with nuclear extracts from unstimulated or cytokine-treated U373MG astrocytoma and, SK-N-SH and SK-N-MC neuroblastoma cells. Tracks: 1, 6, 11 & 16; no protein. 2 & 3: U373MG; 75 & 150 μ g. 4 & 5: U373MG + IL-18; 75 & 150 μ g. 7 & 8: SK-N-SH; 75 & 150 μ g. 9 & 10: SK-N-SH + TNF α ; 75 & 150 μ g. 12 & 13: SK-N-MC; 75 & 150 μ g. 14 & 15: SK-N-MC + TNF α ; 75 & 150 μ g. Marked sequences indicate the regions of protection.

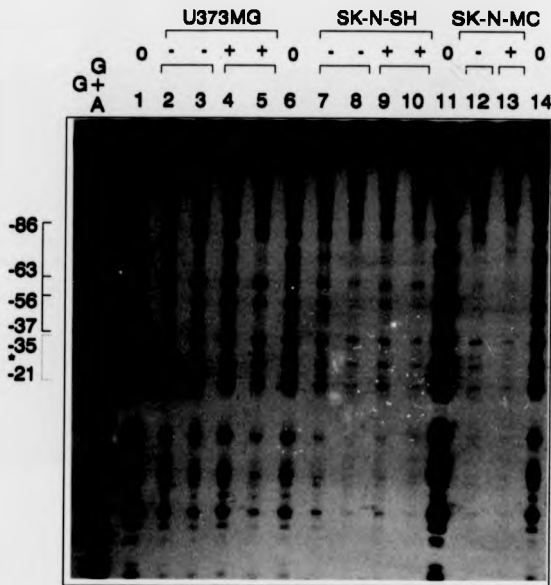


Figure 8.2. DNase I footprint analysis of the HIV-1 LTR (-158/+78, probe LTRF) labelled on the non-coding strand, with nuclear extracts from unstimulated or cytokine-treated U373MG astrocytoma, and SK-N-SH and SK-N-MC neuroblastoma cells. G, G+A: chemical sequencing reactions. Tracks: 1, 6, 11 & 14; no protein. 2 & 3: U373MG; 50 & 100 μ g. 4 & 5: U373MG + IL-1 β ; 50 & 100 μ g. 7 & 8: SK-N-SH; 50 & 100 μ g. 9 & 10: SK-N-SH + TNF α ; 50 & 100 μ g. 12: SK-N-MC; 100 μ g. 13: SK-N-MC + TNF α ; 100 μ g. Marked sequences denote regions of protection. * Indicates region of partial protection.

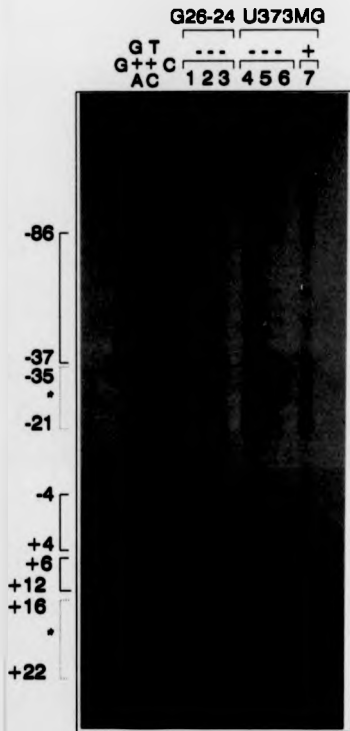


Figure 8.3. DNase I footprint analysis of the HIV-1 LTR (-158/+78, probe LTRP) labelled on the non-coding strand with nuclear extracts from unstimulated G26-24 oligodendrogloma and U373MG astrocytoma cells, and IL-1 β -treated U373MG cells. G, G+A, T+C, C: chemical sequencing reactions. Tracks: 1 & 4; no protein. 2 & 3: G26-24; 100 & 200 μ g 5 & 6; U373MG 100 & 200 μ g. 7: U373MG +IL-1 β ; 200 μ g. Marked sequences indicate the regions of protection. * Indicates region of weak protection.

8.1.2. DNase I footprinting of probe 159

i. Coding strand

Probe 159 (-305 to -159) was labelled on the coding strand (159C) and used initially to examine the DNA-binding activities present in nuclear extracts from unstimulated G26-24 oligodendroglioma, SK-N-MC and SK-N-SH neuroblastoma, and U373MG astrocytoma cells (Figure 8.4).

Examination of the autoradiograph in Figure 8.4 revealed a region of the LTR between positions -285 and -266 relative to the start of transcription that was protected to differing extents by similar amounts of protein extract from all cells. The presence of specific protection was indicated by the reduced intensity or absence of two DNase cleavage points in the centre of the region and the presence of a protein-induced DNase stop at the -285 border, the intensity of which correlated with degree of protection. The definition of the -266 boundary is less well defined due to a region of adenosine-rich sequence that is a poor substrate for DNase I and therefore the exact position of this border may be at some point between -270 and -266. There is also some fading of the DNase I cleavage ladder above the DNase stop at -285, but there is no further evidence to define whether this is a consequence of protein binding to the region below, or genuine protection. A region of weak protection was noted by Shaw *et al.* (1988) over a similar part of the HIV-1 LTR (from -303 to -288) in extracts from activated and resting lymphoblastoma cells. The protected region contains an 8 bp element, AGGCCAAT located at -279 to -272, that differs by only one nucleotide from the consensus octamer-binding motif, ATGCCAAT, first noted in the human histone gene promoters (La Thangue and Rigby, 1988). Figure 8.4 indicates that the factor(s) recognizing the -285 to -266 sequence were much less abundant in the extract from SK-N-MC neuroblastoma cells as the region was extremely weakly protected in this assay, however, the formation of a nucleo-protein complex of similar mobility to the one seen between probe 159 and extracts from U373MG and SK-N-SH cells in gel

retardation assays (Chapter 7, Section 7.2.2), and later footprinting assays supports the evidence for the presence of a factor in these cells capable of recognizing this region.

Comparative analysis of nuclear factor binding to probe 159C between extracts from cytokine-treated and untreated cells is illustrated in Figure 8.5. The footprint over the -285/-266 region of the LTR did not alter when the cells were stimulated with a cytokine known to augment LTR-driven gene expression in either U373MG, SK-N-SH or SK-N-MC cells. The fainter region of the DNase cleavage ladder above -285 was noted and in this assay appeared to be a result of reduced probe digestion in the presence of protein. In agreement with Figure 8.4 no other sequences in probe 159C were observed to be protected by extracts from unstimulated cells, and neither were any additional regions found to be protected with extracts from cytokine-stimulated cells (Figure 8.5).

ii). Non-coding strand

The interaction of nuclear proteins with the LTR was similarly examined on the non-coding strand of probe 159, initially with extracts from unstimulated G26-24, SK-N-MC, U373MG and SK-N-MC cells. Figure 8.6 indicated that a complementary region of the LTR on the non-coding strand of probe 159, from position -285 to -268, also interacted with nuclear factors present in similar amounts of nuclear extract, again from all cells. However, the degree of protection was weaker on the non-coding strand. The protected region was defined by the greatly reduced intensity of several DNase I hypersensitive points with equally strong DNase cleavage of the probe at the 5' and 3' borders. The exact border at the 3' edge was also not well defined due to the sequence composition of the probe and may lie between -271 and -268. Protein(s) protected a region of similar size and the degree to which this occurred was less variable than noted in Figure 8.4, particularly as extract from SK-N-MC cells covered the region more efficiently. Although the gel was over-

exposed to bring up the tracks in the right-hand side which made the footprint over the -285/-268 region in tracks 2, 3 (G26-24) and 4 (SK-N-MC) less clear, this did allow better visualization of 3' sequences and the detection of a smaller region in probe 159NC that was occupied by nuclear protein(s) in extracts from G26-24 and SK-N-MC cells. The region protected by extracts from SK-N-MC from position -249 to -243, was firmly indicated by the absence of several DNase I cleavage points in the probe, and with G26-24 extracts there was a slightly smaller region from -249 to -244 that was similarly defined but also showed a protein-induced DNase stop at the 3' or -244 border. The nucleotide sequences were 'ATGTGGG' and 'ATGTGG' (3' to 5') for SK-N-MC and G26-24, respectively. This sequence of the HIV-1 LTR has not been previously reported to constitute a distinct nuclear protein binding site but the sequence does closely match a region of the SV40 promoter known as the GT1 motif. The sequence protected here is smaller than in SV40 where 'GGTGTGGG' (3' to 5') was defined as the minimal recognition sequence which is known to interact with several ubiquitous cellular proteins (La Thangue and Rigby, 1988). Sequences on the non-coding strand above the -285 border of the octamer-like protected sequence were not observed to interact with nuclear protein.

The equivalent -249 to -243 region of 159NC was not protected by SK-N-SH (Figure 8.6. Tracks 6 and 7) or U373MG extracts (Tracks 8 and 9) but from close examination of the autoradiograph other regions of the LTR, from -242 to -222, did display a weaker pattern of DNase digestion. This was also indicated in Figure 8.6 for G26-24 and SK-N-MC extracts and may be evidence of protein-DNA interactions.

Another region of probe 159NC for which there was some evidence of weak protection by extracts from G26-24 and SK-N-MC cells (Figure 8.6) was located at -212 to -207 and contained the sequence 'CTCTCT' (3' to 5'). The interpretation of protection at this site was difficult as no alteration in the pattern of DNase I digestion was seen at the immediate borders of the site and the region was only

marked by the reduced intensity of a number of bands. Several novel bands were present a little past the 5' end of this region, at position -217/-218, which could indicate that the limited protection seen represented partial protection of a larger site. On the coding strand this region is part of a purine-rich motif from -224 to -198 that is homologous to sequences located -291 to -277 in the IL-2 gene enhancer which bind the inducible factor NFAT-1 (Randak *et al.*, 1990). These LTR sequences are also reported to bind a related constitutive factor, ILF (Li *et al.*, 1991), and by homology of binding sites, other cellular factors may also interact (Klemsz *et al.*, 1990). Further examination with partially-purified nuclear extracts or by gel retardation assay would be required to confirm this region and those from -242 to -222 as a site of nuclear protein interaction.

When footprinting analysis was performed on probe 159NC with nuclear extracts from cytokine-stimulated cells, the results, shown in Figure 8.7, further indicated that the octamer-like motif (-285/-268) was occupied by proteins present in extracts from both unstimulated and IL-1 β -treated U373MG astrocytoma cells. Unfortunately, this latter experiment did not produce such a clear picture due to the probe suffering a certain amount of radiochemical decay, and the data for SK-N-SH and SK-N-MC is poor as sequences around and further downstream of -268/-285 site were not well resolved. But after examination of the autoradiograph no major differences were noted in the sequences of probe 159 between extracts from cytokine-treated and unstimulated neuroblastoma and astrocytoma cells. This can also be said for the putative region from -242 to -222, defined in Figure 8.6, with extracts from unstimulated and IL-1 β -treated U373MG.

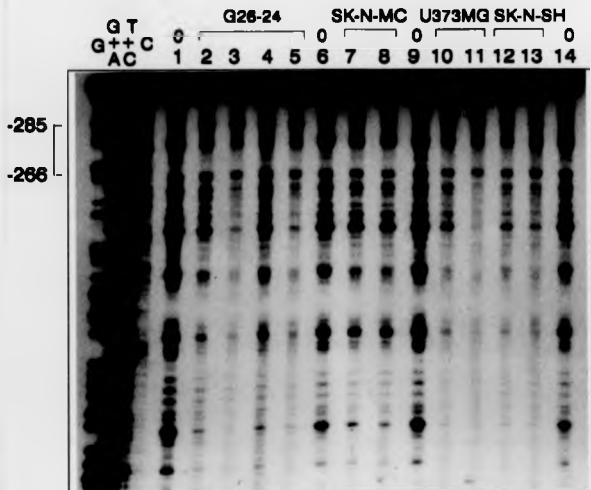


Figure 8.4. DNAase I footprint analysis of the HIV-1 LTR (-306/-159, probe 159C) labelled on the coding strand, with nuclear extracts from unstimulated G26-24 oligodendroglioma, U373MG astrocytoma, and, SK-N-SH and SK-N-MC neuroblastoma cells. G, G+A, T+C, C: chemical sequencing reactions. Tracks: 1, 6, 9 & 14; no protein. 2 & 4: G26-24; 60 μ g. 3 & 5: G26-24; 120 μ g. 7 & 8: SK-N-MC; 50 & 100 μ g. 10 & 11: U373MG; 75 & 150 μ g. 12 & 13: SK-N-SH; 75 & 150 μ g. Sequences marked denote the region of protection.

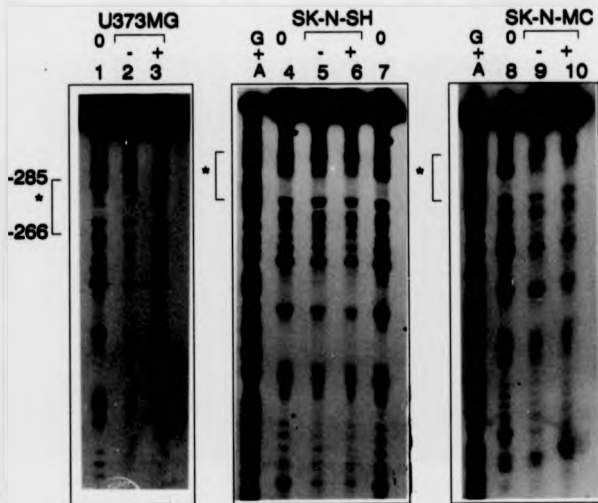


Figure 8.5. DNase I footprint analysis of the HIV-1 LTR (-305/-150, probe 159C) labelled on the coding strand, with nuclear extracts from unstimulated and cytokine-treated U373MG astrocytoma, and SK-N-SH and SK-N-MC neuroblastoma cells. G+A: chemical sequencing reactions. Tracks: 1, 4, 7 & 8; no protein. 2: U373MG; 75 μ g. 3: U373MG + IL-1 β ; 75 μ g. 5: SK-N-SH; 75 μ g. 6: SK-N-SH + TNF α ; 75 μ g. 9: SK-N-MC; 75 μ g. 10: SK-N-MC + TNF α ; 75 μ g. * Sequences marked denote similar region of protection.

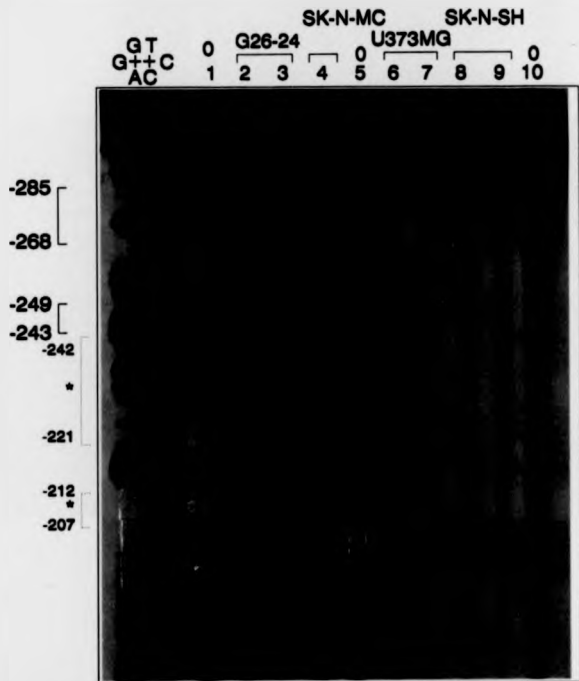


Figure 8.6. DNase I footprint analysis of the HIV-1 LTR (-306/-159, probe 159NC) labelled on the non-coding strand, with nuclear extracts from unstimulated G26-24 oligodendroglioma, U373MG astrocytoma, and SK-N-SH and SK-N-MC neuroblastoma cells. G, G+A, T+C, C: chemical sequencing reactions. Tracks: 1, 5, & 10; no protein. 2 & 3: G26-24; 60 & 120 μ g. 4: SK-N-MC; 100 μ g. 6 & 7: U373MG; 75 & 150 μ g. Sequences marked with a solid line indicate regions of protection. * denotes putative region of protection, see text.

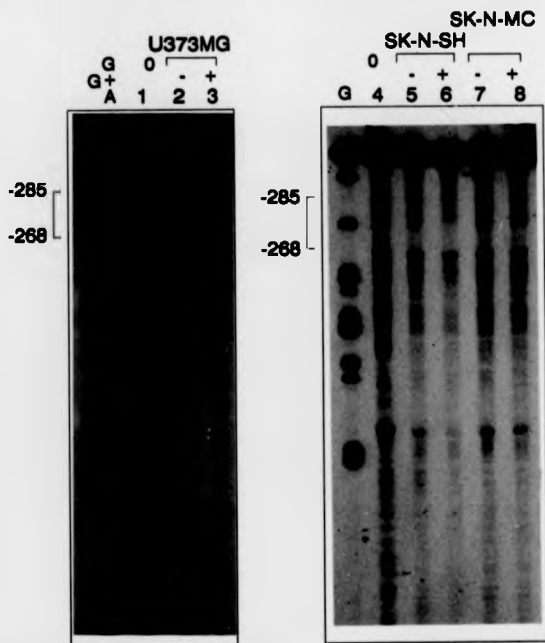


Figure 8.7. DNase I footprint analysis of the HIV-1 LTR (-306/-159, probe 159NC) labelled on the non-coding strand, with nuclear extracts from unstimulated and cytokine-treated U373MG astrocytoma, and SK-N-SH and SK-N-MC neuroblastoma cells. G, G+A: chemical sequencing reactions. Tracks: 1 & 4; no protein. 2: U373MG; 75 μ g. 3: U373MG + IL-18; 75 μ g. 5: SK-N-SH; 75 μ g. 6: SK-N-SH + TNF α ; 75 μ g. 7: SK-N-MC; 75 μ g. 8: SK-N-MC + TNF α ; 75 μ g. Marked sequences indicate the region of protection.

8.1.3. DNase I footprinting of probe 194

ii). Coding strand

Probe 194 spans the farthest 5' sequences of the HIV-1 LTR from position -306 to -488 and was labelled at the 5' end for the analysis of sequence-specific DNA-binding activities present in nuclear extracts from unstimulated and cytokine-treated U373MG astrocytoma and, SK-N-SH and SK-N-MC neuroblastoma cells. Figure 8.8 illustrates the sequences on the coding strand of probe 194C protected from digestion by DNase I. Comparison with the pattern of bands produced in the absence of protein (Figure 8.8. Tracks 1, 6, and 11) revealed a region of the LTR from -382 to -363 that was specifically occupied by nuclear protein(s) present in all extracts from both unstimulated and stimulated cells. The extent of the footprint observed in Figure 8.8 indicated that the proteins which bound to this region were relatively abundant in all cell extracts, although present to a greater excess in neuroblastoma cells as this sequence was fully protected with 75 μ g of extract from either SK-N-SH or SK-N-MC (Tracks 7 and 9, 12 and 13), yet partially protected with an equivalent amount from astrocytoma cells (Tracks 2 and 4). The protected region was well defined by the complete absence of several strong DNase I hypersensitive sites and covered the sequence 'TTCCCTGATTGGCAGAACTA' (5' to 3'), which corresponds to a region of the LTR defined originally by Orchard *et al.* (1990) with nuclear extracts from unstimulated and PMA/PHA-treated Jurkat T lymphoblastoma cells and designated as Site A. In lymphocytes, the factors interacting with this element have not been identified and despite some homology between the sequences involved they are distinct from octamer-binding proteins (Orchard *et al.*, 1990). The results presented here demonstrate that cellular protein(s) with similar sequence-specificities were also present in neural cell lines regardless of activation by the cytokines IL-1 β and TNF α .

Further towards the 3' end of the LTR two regions close to each other were observed to be protected by nuclear proteins, again in extracts from all stimulated and unstimulated neural cells. The regions were from -355 to -346 and -337 to -326 and contain the sequences 'GGGCAGGG' and 'CCACTGACCTTT', respectively. These two regions were also protected to a greater extent by neuroblastoma rather than astrocytoma cell extracts. The -355/-346 region was well defined by protein-induced DNase I stops at both 5' and 3' borders, as well as the partial or full loss of two central bands present in the no protein tracks, and the -337/-326 region was defined by the complete absence or reduced intensity of many strong DNase cleavage points. The protected sequences were similar to those also defined previously by Orchard *et al.* (1990) as one binding site containing two domains designated as Site B. Comparison with the data of Orchard *et al.*, (1990) suggests that only partial protection of Site B was seen here giving the appearance of two separate regions. The sequences reported to be directly involved in the interaction with Site B-binding factor(s) present in T cells were (-347/-343) 'GGTCA' and (-334/-329) 'TGACC' (5' to 3'), spaced 9 nucleotides apart, which make up 5' and 3' half sites of an inverted palindrome with high homology to steroid/thyroid response elements (Beato, 1989; Orchard *et al.*, 1990). The -355/-346 region seen here does not encompass all of the 5' half site as probably only partial protection was evident. This is supported by results reported in Chapter 7 which indicated that factor(s) recognize the entire Site B element of probe 194. Of note is that the two half sites of Site B were recognized as AP-1 binding motifs by sequence homology but the direct binding of AP-1-like factors to the HIV-1 LTR has been referred to only as unpublished results in a paper by Franza *et al.* (1988). The finding that Site B-binding activity was present in unstimulated cells and not only in stimulated cells further suggests Site B proteins are distinct from AP-1. Furthermore, Orchard *et al.*, (1990) could not compete away factor(s) from Jurkat recognizing Site B in gel retardation assays with an oligonucleotide containing an AP-1 binding site.

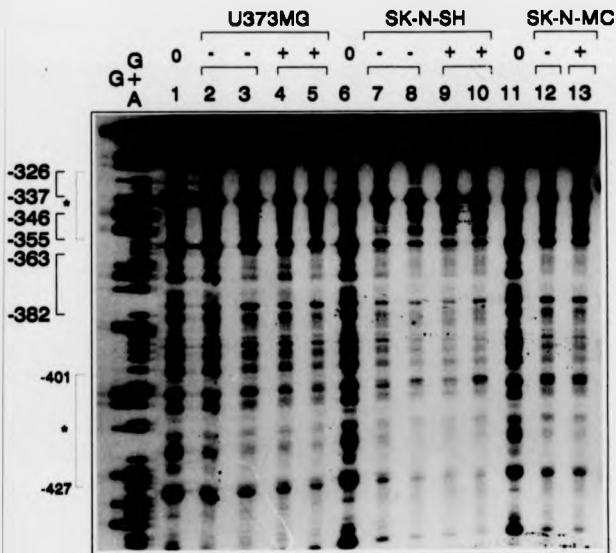


Figure 5.8. DNAase I footprint analysis of the HIV-1 LTR (-488/-306, probe 194C) labelled on the coding strand, with nuclear extracts from unstimulated or cytokine-treated U373MG astrocytoma, and SK-N-SH and SK-N-MC neuroblastoma cells. G, G+A: chemical sequencing reactions. Tracks: 1, 6 & 11; no protein. 2 & 3: U373MG; 75 & 150 μ g. 4 & 5: U373MG + IL-1 β ; 75 & 150 μ g. 7 & 8: SK-N-SH; 75 & 150 μ g. 9 & 10: SK-N-SH + TNF α ; 75 & 150 μ g. 12: SK-N-MC; 75 μ g. 13: SK-N-MC + TNF α ; 75 μ g. Sequences marked with solid lines denote regions of protection. * see text.

ii). *Non-coding strand*

In a similar examination of DNase I footprints on the non-coding strand of probe 194 a region of protection over Site A sequences was noted with extracts from all stimulated and unstimulated cells (Figure 8.9). This covered a similar portion of the non-coding strand from position -381 to -363 over the sequence 'AGGGACTAACCGTC'IIGAT' (3' to 5') and was defined by the absence of several DNase cleavage points, although the exact definition of the 5' -381 border may not have been precise due to the A-rich composition of the probe. This experiment also indicated a greater abundance of Site A-binding factor(s) in extracts from neuroblastoma cells compared to astrocytoma cells as full protection was again seen with the lower concentration of nuclear extracts from SK-N-SH and SK-N-MC, but not from U373MG.

Interaction of Site B-binding factor(s) with probe 194 did not greatly alter the appearance of the DNase I cleavage pattern of a complementary region of the LTR when the probe was labelled on the non-coding strand. A close examination of the autoradiograph was required to reveal changes that suggested any degree of interaction. Small alterations in the pattern of DNase I cleavage products were suggestive of broad region of weak binding between sequences from -352 to -325 over a domain similar to Site B (Orchard *et al.*, 1990). The weak footprint was suggested by the absence of one band at position -325 and the appearance of protein-induced band at position -336, and at -351 with U373MG extracts and -352 with neuroblastoma extracts. These differences are unfortunately difficult to see in a photograph of the autoradiograph. Some disparity between the no protein tracks (number 1 with respect to 6, 11 and 16) made assessment of further protein-induced DNase stops uncertain. The observed changes were similar with extracts from either unstimulated or from cytokine-stimulated cells, except the band at -325 was absent only with extracts from IL-1 β -treated U373MG cells and not unstimulated U373MG cells. This was not considered to be a significant difference. The sequences over the

Site B domain could not be accurately defined as protected, however, the 3' and 5' borders of the proposed region were similar to the footprint described by Orchard *et al.* (1990) with extracts from unstimulated or PHA/PMA-treated lymphoblastoma cells.

iii). Oligonucleotide competition

In order to clarify the interaction of nuclear proteins present in neural cell extracts and the sequences in probe 194 defined as Site B, a DNase I footprint was performed with extracts from SK-N-SH neuroblastoma cells and probe 194 which gave the clearest footprint over this region, after competition with oligonucleotides to specific sequence elements of probe 194 had been performed. Aliquots of nuclear extracts were incubated with an approximate 5,000- or 12,500-fold molar excess of unlabelled oligonucleotide to either Site A, Site B or the Site B mutant BM5 (sequences are given in Chapter 2, Table 2.2) for 30 min to absorb specific DNA-binding activities before the addition of radiolabelled probe 194C. The assay is illustrated in Figure 8.10. Comparison of the no protein track 1 with track 2 shows both regions of protein-induced protection over Site A (-382 to -363) and the two regions corresponding to Site B (-355 to -346 and -337 to -326). The excess of Site A oligonucleotide at either concentration (Tracks 3 and 4) only caused the footprint over Site A to become slightly less protected and allowed the faint appearance of the bands present only in the no protein track 1. In tracks 5 and 6, where Site B oligonucleotide had been pre-incubated with the nuclear extracts, the pattern of bands produced by DNase I digestion was altered over -355/-346 sequences and the protected region was slightly more obscured. Yet there was little difference over the -337/-326 part of Site B where only the borders of this protected region had become more intense and the footprint was marginally less apparent. Competition with the BM5 oligonucleotide that contained mutations to destroy the 5' half site of Site B (located at positions -347 to -343 in the LTR) produced a footprint over the

-355/-346 region that was slightly different to competition with the Site B oligonucleotide (Tracks 7 and 8) in that the pattern of bands defining this domain was more like that observed with no oligonucleotide competition (Track 2). This suggested that cellular proteins were not prevented from binding to the upstream, 5' part of Site B, due to the mutations present in the oligonucleotide, whereas over the -337/-326 region, there was a change in the footprint similar to that noted after competition with Site B oligonucleotide. The use of oligonucleotides to compete away the factors binding to certain regions of the LTR was able to produce only marginal changes in the pattern of protection seen over these domains, and this was probably due to the high degree of non-specific DNA-binding activity found in crude nuclear extract preparations. Therefore, although the changes seen do support the specificity of the protein-DNA interactions further experiments of this nature were not performed.

A summary of the nuclear factor binding sites on the HIV-1 LTR occupied by neural cell proteins indicated by these experiments is shown in Figure 8.11.

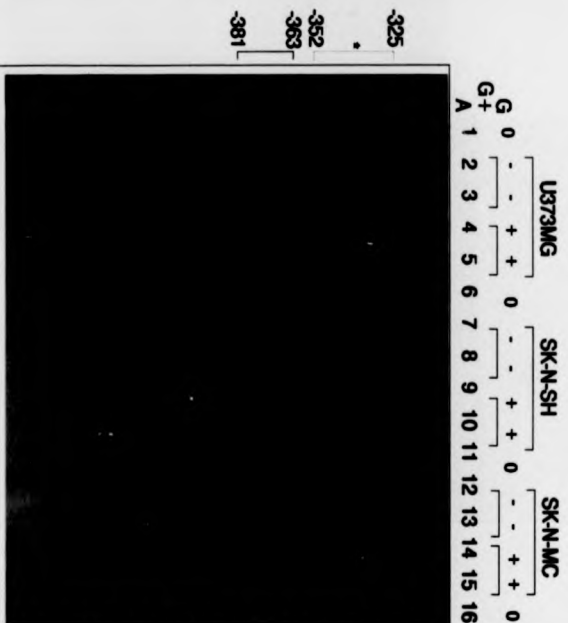
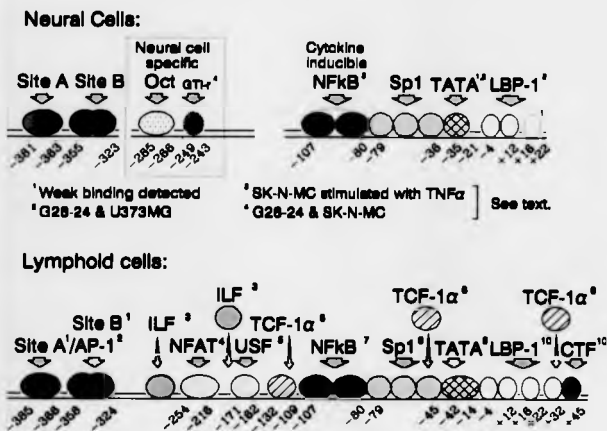


Figure 8.9. DNase I footprint analysis of the HIV-1 LTR

(-489/-306, probe 194NC) labeled on the non-coding strand, with nuclear extracts from unstimulated and cytokine-treated US73MG astrocytoma, and SK-N-SH and SK-N-MC neuroblastoma cells. G, G+A; chemical sequencing reactions. Tracts: 1, 6, 11 & 16; no protein. 2 & 3: US73MG; 75 & 150 μ g. 4 & 5: US73MG + IL-1 β ; 75 & 150 μ g. 7 & 8: SK-N-SH; 75 & 150 μ g. 9 & 10: SK-N-SH + TNF α ; 75 & 150 μ g. 12 & 13: SK-N-MC; 75 & 150 μ g. 14 & 15: SK-N-MC + TNF α ; 75 & 150 μ g. Marked sequences indicate regions of protection.

* see text.

Fig 8.11. Nuclear transcription factor binding sites in the HIV-1 LTR



References:

- ¹ Orchard et al., (1990)
- ² Peterlin, (1991)
- ³ Li et al., (1991)
- ⁴ Shaw et al., (1988)
- ⁵ Smith and Greene, (1989)
- ⁶ Waterman et al., (1991a)
- ⁷ Nabel and Baltimore, (1987)
- ⁸ Jones et al., (1986)
- ⁹ Garcia et al., (1987)
- ¹⁰ Jones et al., (1988)

Discussion

Interpretation of DNase I footprints

The investigation of the sequence-specific protein-DNA interactions occurring with the HIV-1 LTR and extracts from neural cells defined many regions that potentially bind cellular proteins *in vivo*. However, only in a few examples reported was the binding of sequence-specific nuclear factors clearly illustrated, as partial or weak protection of a putative protein binding site was often seen. These were indicated by subtle changes in the pattern of DNase I cleavage products or the reduced intensity of several bands at DNase hypersensitive points. As loss of intensity is a relative property this was judged as a indicative of protein binding only if the bands defining the region were of equal intensity above and below the region and not obviously a consequence of the overall fading of exposure down the gel. Some features were not easily apparent from photographs of the much larger autoradiographs but the majority of conclusions on the interaction of nuclear factors can be determined from data contained in the photographs. Interpretation of marginal data is more convincing by examination of the original autoradiograph as fine detail is lost in reproduction to smaller photographs.

The limitations of footprinting with crude nuclear extracts

The phenomenon of bands fading when nuclear extract was present in the binding reactions was apparent in almost all footprinting assays performed and was almost certainly due to the use of crude nuclear extracts. In order to detect sequence-specific protein-DNA interactions by footprinting techniques it is necessary to use sufficient protein to allow all the probe DNA molecules to become complexed with protein and this requires a low concentration of poly (dI:dC)-(dI:dC) competitor which must still be high enough to compete away the bulk of not all of the non-specific DNA-binding. The probe DNA should be

loosely complexed with the bulk of the extract protein and bound tightly only by sequence-specific factors. The non-specific binding results in reduced DNase I cleavage of the probe, with respect to the no protein controls, due to the relative inaccessibility of the DNA molecule to DNase I in this environment. The intensities of the DNase I cleavage products in a given assay are influenced by the amount of extract employed in the binding reaction and the level of non-specific DNA binding activity in the extract preparation. The latter point I found to be moderately constant when a small amount of extract was assayed with the LTR probe in a gel retardation assay but, as footprinting requires 10 to 30-fold more protein, there was scope for considerable variation and increase in the level of non-specific DNA-binding activity in a footprinting binding reaction. With crude extract preparations these factors profoundly influenced the quality of the footprints produced (experimental observations) and limited the amount of protein extract that could be assayed for binding so that there were often problems in obtaining sufficient occupancy of a given binding site to produce a complete or recognizable footprint. Moreover the high number of binding sites in the HIV-1 LTR, interacting with many cellular proteins (see Figure 8.11) of variable abundance, multiplies these difficulties and the partial or weak protection of one or more of them is inevitable.

To improve greatly the quality of data obtained from footprinting techniques the use of partially or completely purified nuclear proteins is required. Purification techniques for this class of proteins are based on affinity chromatography and preliminary purification uses either DNA or heparin coupled to a solid support. This can then be followed by the other chromatography media related to characteristics of the protein(s) of interest and finally with a DNA affinity resin containing high-affinity binding site DNA. Substantial enrichment of sequence-specific proteins (up to 250-fold) can be achieved after the first step, described above, as 99% of protein in whole cell extracts flows straight through (Sorgor *et al.*, 1989). Partial purification of nuclear extracts from neural cells was attempted by use of a calf thymus

DNA-cellulose affinity column. However, such procedures require inordinately large quantities of starting materials, which when cells have to be grown as adherent monolayers was very time consuming and the purification was also not successful for a number of technical aspects, not least the use of too few cells. Around 7 ml packed cell volume (approximately 2.5×10^9 cells), somewhere in the region of 25 roller bottles of U373MG astrocytoma cells, is a realistic starting point for partial nuclear protein purification. Therefore the use of crude extracts in footprinting was obligatory but this still sufficed to produce a good proportion of meaningful data.

Many of the sites of protein-DNA interaction were positioned over elements that had been defined in other cell systems and were known to be recognized by proteins that regulate transcription (see Figure 8.11). The presence of a footprint over such a region can be interpreted as evidence for the binding of similar factors from neural cells to the LTR as these proteins are, in the most part, defined by the sequences with which they interact. The relationship of these sites to those defined by neural cell extracts is discussed below for each restriction fragment of the HIV-1 LTR.

Nuclear factors binding to sequences located at -158 to +78 in the HIV-1 LTR

NF κ B-like binding activity

DNAse I footprinting studies on the LTR between positions -158 and +78, relative to the start of transcription, demonstrated the interaction of DNA-binding activities from neural cells that interacted with NF κ B, Sp1, TATA and LBP-1 elements (see Figure 8.11). When nuclear extracts were prepared from each cell line after stimulation with a cytokine known to augment HIV-1 LTR-driven gene expression, the only alteration noted in the footprints produced was by extracts from SK-N-MC neuroblastoma cells after stimulation with TNF α , where DNA-binding factors were induced and bound to the pair of NF κ B binding sites constituting the

enhancer of HIV-1. Such nuclear factors were not present in extracts from unstimulated cells and, at least for SK-N-MC, provided further evidence for the involvement of NF κ B-like factors in the induction of HIV-1 gene expression.

A similar change in nuclear factor binding was not detected following the activation of astrocytoma cells with IL-1 β and SK-N-SH neuroblastoma cells with TNF α because of higher levels of non-specific binding in extracts from these cells that resulted in artefactual protection of these sequences (see Section 8.1.1). Previous experiments with an LTR β probe (results not shown), labelled on the coding strand at the 5' rather than 3' termini, indicated that sequences around the enhancer were always partially or fully protected with extracts from unstimulated cells, or cytokine-stimulated cells irrespective of the presence of an NF κ B-like factor, detected by gel retardation assays. This led to the conclusion that this part of the LTR was highly susceptible to non-specific DNA-binding by proteins found in some crude extract preparations. Increasing the amount of competitor in the binding reactions did not alleviate the problem. The incomplete recovery of NF κ B-like activity in the nuclear extract preparations from U373MG and SK-N-SH cells was excluded as aliquots from both were employed in gel retardation assays described in Chapter 7 and demonstrated the induction of NF κ B-like factors by the respective cytokine in these cell lines. In further attempts to maximize the DNA-binding potential of NF κ B, 3 mM rGTP, which is known to stimulate this property of NF κ B (Lenardo *et al.*, 1989), was included in the binding reactions prior to footprinting procedure. The binding buffer already contained sufficient MgCl₂ (5 mM) to stimulate NF κ B binding (Lenardo *et al.*, 1989) yet the addition of GTP did not produce a change in the artefactual footprint observed over the enhancer on the coding strand for either U373MG or SK-N-SH cell lines (results not shown).

The unexpected failure to observe any footprint over the enhancer region when the non-coding strand was examined would appear to be due to the low concentration of the NF κ B-like binding activity in the nuclear extracts as related

factors from HeLa cells will protect the non-coding strand from DNase I digestion after crude extracts have been subject to partial purification (Wu *et al.*, 1988).

Sp1-like DNA-binding activity

Another DNA-binding activity present in extracts from astrocytoma, oligodendroglioma and neuroblastoma cells was analogous to the transcription factor Sp1, for which there are three binding sites in the LTR located in sequences from -77 to -46. Sp1-like activity produced a footprint when both the coding and non-coding strands were examined and was capable of protecting all three Sp1 sites from DNase I digestion. The analysis of DNA-binding with different concentrations of extract demonstrated that the distal Sp1 site (III) was preferentially occupied when DNA-binding activity was limited, indicating that this site possessed a higher affinity for Sp1. When the extract concentrations were increased Sp1-like binding was observed over both sites I and II, although results from footprinting the non-coding strand suggest that Sp1-like factors will bind to site I before site II. The results of Jones *et al.*, (1986) with affinity-purified Sp1 originally indicated that site III has a higher affinity for Sp1 but no preferential occupation of sites I and II by Sp1 was noted. In addition, when the HIV-1 LTR was subject to DNase I footprinting analysis with partially-purified HeLa cell extracts there was no protection of the Sp1 sites even when the binding reactions were supplemented with purified Sp1 (Garcia *et al.*, 1987). This led the authors to conclude that other proteins in HeLa cells may inhibit Sp1 binding, such a finding was not observed in neural cells.

The binding of nuclear factors to the Sp1 sites in the HIV-1 LTR produced a footprint that extended 10 nucleotides past the 3' edge of site I on the coding strand and by 9 nucleotides on the non-coding strand. At the 5' end of Sp1 site III on the non-coding strand the border of the protected sequences was 8 nucleotides past the last residue of the Sp1 site and on the coding strand the footprint merged with that

produced by the enhancer-binding factor. The large molecular size of DNase I (approximately 4 nm; Rhodes, 1989) accounts for a certain amount of this additional protection due to steric hindrance which is advantageous as it amplifies the protection offered by bound protein and results in clearer gaps in the digestion pattern of DNA. However, the 8 to 10 nucleotide space seen here would appear larger than expected as DNase I was capable of detecting bound protein that occupied as few as 6 or 7 nucleotides. The difference may be due to the ability of Sp1 to form dimers (Courey *et al.*, 1989) and perhaps provide a greater degree of steric hindrance. Another characteristic of DNase I as a footprinting reagent is that the rate of cleavage of its substrate is dependent on sequence-determined variations in DNA structure and that some sequences, especially tracts of A or T are not readily digested (Goodwin, 1990). The sequences in the protected region at the 5' end of Sp1 site III are T-rich due to the proximity of the NF κ B site, yet those at the 3' are not, and this along with reports from others of a transcription factor, TCF-1 α identified in T cells by its interaction with the enhancer of the human T cell receptor α subunit, which can also bind to sequences over-lapping the proximal Sp1 site I of HIV-1 (Waterman *et al.*, 1991a) may indicate an additional protein-DNA interaction worthy of further investigation. Especially as the authors report that in mixing experiments purified Sp1 and TCF-1 α can bind concomitantly to the same region of the HIV-1 LTR. Further TCF-1 α sites were also reported by Waterman *et al.* (1991a) to be present overlapping the LBP-1 site in the leader region of the LTR and in sequences located upstream of the distal NF κ B site.

Nuclear factors that interact with TATA and leader sequences

The interaction of nuclear factors with elements of the HIV-1 LTR further downstream of the Sp1 motifs was noted only on the non-coding strand of probe LTRF. Factor(s) may weakly recognize the TATA box whilst other proteins could definitely be demonstrated to bind to the LBP-1 binding sites in the TAR

region of the LTR from nuclear extracts of astrocytoma and oligodendroglioma cells and these sites of interaction were located to similar regions that had been defined previously with nuclear proteins from lymphocytes (Wu *et al.*, 1988) and HeLa cells (Jones *et al.*, 1988). No such binding was observed in extracts from SK-N-MC and SK-N-SH neuroblastoma cells in the assays shown for technical reasons (see Section 8.1.1) but a previous experiment with an LTRF probe radiolabelled on the non-coding strand at the 5' termini had shown partial protection of equivalent sequences over the TATA box with SK-N-MC extracts, but did not permit the visualization of sequences further downstream (results not shown). The preferential protection of sequences on the non-coding strand may also be a consequence of the low abundance of these factors in crude nuclear extracts as in the reports referred to above, where partially-purified extracts were employed, a similar degree of protection was observed on both strands. The LBP-1 protein will also recognize a binding site in the HIV-1 LTR that overlaps the TATA box at an affinity lower than all other LBP-1 sites [see Figure 8.11] and protects a region of the LTR from -38 to -16 (Kato *et al.*, 1991). However the extent of protection observed over the TATA region with neural cell extracts was from -35 to -21. Furthermore the lower affinity interaction is unlikely to have occurred in some experiments performed here due to the low abundance of LBP-1-like DNA-binding activity that did not result in protection of all the high affinity sites.

The protection observed around the TATA box from -35 to -21 with neural cell extracts was marginal and less apparent than that seen with nuclear proteins purified from HeLa or lymphocyte cells where there was a region of protection from -42 to -14 due to the interaction of a TATA box-binding factor (Garcia *et al.*, 1987). The disparity in the size of the protected region between the results from neural cells and other cell types may indicate that different factors recognize these sequences or it may be a consequence of the lower DNA-binding activities present in crude nuclear extracts. DNase I footprinting experiments on deletion and

insertion mutants of the HIV-1 LTR around these sequences suggest that protection of the TATA region is dependent upon the presence of other local protein-DNA interactions, with Sp1 and LBP-1, that stabilize the otherwise weak binding of TATA box factor(s) (Garcia *et al.*, 1987; Jones *et al.*, 1988). No experiments were performed here to address a potentially similar situation in neural cells. However, the failure to observe such an extensive region of protection 3' to the TATA sequences may have been due to an A-rich tract in the non-coding strand that which was not cleaved by DNase I and therefore poorly defined the 3' border.

The LBP-1-like DNA-binding activity found in extracts from oligodendrogloma and astrocytoma cells occupied the LBP-1 site overlying the start of transcription at position -3 to +1 and another at +6 to +12, and also weakly at +16 to +22. The failure to observe LBP-1 binding activity in SK-N-SH and SK-N-MC cells was due to poor DNase I cleavage of the probe in the assay and the absence of an LBP-1-like factor was not established. When sufficient LBP-1 was present in the nuclear extracts from astrocytoma and oligodendrogloma cells the protection of LBP-1 sites I, II and III would suggest that, at least in these cells, an LBP-1-like activity may interact with all sites in the leader region of the LTR. A further two sites for this leader binding protein, which has also been named UBP-1 (Waterman *et al.*, 1991a), exist in sequences further downstream as do single binding sites for the distinct proteins, UBP-2 (Garcia *et al.*, 1989) and CTF/NF1 (Jones *et al.*, 1988) but these were too close to the position of the radiolabel to be resolved in the assays performed. The interaction of the TCF-1 α -like activity could not be distinguished from the LBP-1-like binding activity in the footprinting assays performed here, and whether such an interaction with the LTR would occur in neural cells depends upon the tissue-specificity of the TCF-1 α protein. Waterman *et al.* (1991b) report that TCF-1 α is restricted to T lymphoid cells, although no neural cell types were examined.

Nuclear factors binding to sequences located at -305 to -159 in the HIV-1 LTR

Octamer-binding factors

DNAse I footprinting of the -305 to -159 region of the HIV-1 LTR with extracts from astrocytoma, oligodendroglioma and neuroblastoma cells demonstrated the presence of a constitutive nuclear factor that bound sequences over a region highly homologous to an octamer motif. Octamer factors are ubiquitous transcription factors that can regulate expression in a cell-specific or cell-cycle dependent manner, such as occurs in the B cell specific expression of immunoglobulin genes and histone gene expression (La Thangue and Rigby, 1988). This octamer binding site is located in a region of the HIV-1 LTR that has homology to sequences found in the 5' regulatory region of the IL-2 gene, at positions -63 to -93, that constitute the first antigen response element of the IL-2 gene promoter (Ullman *et al.*, 1991). The sequence in the IL-2 gene promoter protected from DNAse I digestion by extracts from T lymphoblastoma cells has been shown to be analogous to the octamer motif present in histone genes and interacts with the ubiquitous octamer-binding factor, Oct-1, previously known as NF-IL2A (Ullman *et al.*, 1991). Therefore, although a factor apparently capable of recognizing the octamer-like element of HIV-1 is found in T cells it has not been described to interact with these sequences and in this respect the binding of factors to the octamer-like motif may be specific to neural cell lines. This cell-specific difference could be accounted for by preferential occupancy of the additional proximal purine-rich domain located from position -277 to -262 (Li *et al.*, 1991), which overlaps the octamer-like sequence, by nuclear proteins present in T lymphocytes (also see below).

NFAT-1-related and other DNA-binding activities

The region of the HIV-1 LTR from position -254 to -216 is part of a large domain that has been demonstrated to bind the mitogen-inducible transcription factor NFAT-1, or a related factor with similar biological characteristics, present in T lymphocytes but without a recognition sequence highly homologous to the purine-rich NFAT-1 motif of the IL-2 gene promoter (Shaw *et al.*, 1988). In the report of Shaw *et al.* (1988) DNase I footprinting demonstrated the binding of an inducible factor which was related by the sequence it recognized to NFAT-1 to the region described above, and constitutive but unknown protein(s) to sequences of the HIV-1 LTR from -303 to -288 with extracts from T cells. There is also another constitutive nuclear protein that has been demonstrated by others (Li *et al.*, 1991) to bind to either of the conserved distal or proximal purine-rich boxes, located at -276 to -262 and -220 to -206, which lie either side of the NFAT-1 domain of Shaw *et al.* (1988) in the HIV-1 LTR. These purine-rich domains, by homology with the recognition sites, are more closely related to the NFAT-1 motif found in the IL-2 gene promoter (Randak *et al.*, 1989) than the one described by Shaw *et al.* (1988). Such that it would appear quite probable that these purine-rich domains would bind the inducible NFAT-1 factor or another constitutive factor which also recognizes the NFAT-1-binding, purine-rich domain (Klemaz *et al.*, 1990). However, any interaction with the proximal and distal purine-rich domains of the HIV-1 LTR was not found by Shaw *et al.*, (1988) and there seems to be a lack of data on the exact interaction of both inducible and constitutive NFAT-1-related nuclear proteins and the HIV-1 LTR.

No inducible proteins were detected in extracts from either astrocytoma or neuroblastoma cells that bound to sequences in probe 159 and might be analogous to NFAT-1 in lymphocytes. This may be due to there being insufficient DNA-binding activity induced in crude extracts or competition with constitutive

factors such as those described above. The region of the HIV-1 LTR reported by Shaw *et al.* (1988) to interact with NFAT-1 was examined further in Chapter 9 by the use of complementary oligonucleotides in gel retardation assays to confirm if the region of weak protein interaction evident in DNase I footprints between -242 to -222 did indeed bind any constitutive or inducible neural cell proteins. A definite region of protection was demonstrated just upstream (-249 to -243) with extracts from SK-N-MC and G26-24 cells and the footprint produced by nuclear protein(s) was over a sequence of the LTR with some homology to the GTT motif found in SV40 (La Thangue and Rigby, 1988). This is also present in a region of the HIV-1 LTR previously reported to bind NFAT-1. Interestingly, the 'ATGTGGG' sequence of the GTT-related motif is 100% conserved in the LTRs of 23 out of 24 isolates of HIV-1 and, like the interaction with octamer-binding factors, may also be neural-cell specific. Complementary oligonucleotides also containing the protected sequences between nucleotides -249 and -243 were also employed in gel retardation assays to confirm that the footprints were due a specific protein-DNA interaction.

A recent report has shown sequences located within probe 159 to bind recombinant glucocorticoid receptor (GR) at two locations, from -264 to -259 and -255 to -250, which form two half sites of one domain (Ghosh, 1992), characteristic for this class of receptor (Beato, 1989). These sites overlap the distal purine-rich domain (Li *et al.*, 1991) and are located directly adjacent to the octamer- and the GTT-related regions protected by all and two neural cell extracts, respectively. It would appear from the footprinting data presented here that these factors if present in neural cells are of too low abundance to be detected in crude extracts and, in any case, interaction with the HIV-1 LTR would not be expected to occur in the absence of cellular stimulation with a glucocorticoid hormone (Beato, 1989). Nevertheless from the large region of DNA protected by recombinant GR in the report of Ghosh (1992) it would also seem possible that the binding of factors present in neural cells

to either the octamer or GTI-related motif may preclude the interaction of a putative GR-like molecule. The relationship of the GR site to the binding of nuclear factors in lymphoid cells is unclear. However, there is evidence which implicates glucocorticoids and particularly oestrogens in the stimulation of HIV-1 expression (Laurence *et al.*, 1990).

No DNA-binding activity to the reported USF and URS binding sites of the HIV-1 LTR (Lu *et al.*, 1991) was observed in the footprinting experiments with extracts from neural cells. These regions were located too close to the *Ava* I restriction site used to sub-clone and radiolabel the LTR sequences in probe 159 and were not well resolved on the sequencing gels employed. Therefore it was not possible to establish any interaction between similar factors in neural cells and the HIV-1 LTR.

Nuclear factors binding to sequences located at -488 to -306 in the HIV-1 LTR

Factors binding to the Site A element

The footprinting study of sequences within probe 194 demonstrated that neuroblastoma and astrocytoma cells contained a constitutive DNA-binding activity similar to that observed in T lymphoblastoma cells (Orchard *et al.*, 1990) that recognized equivalent sequences known as Site A. The footprint produced with neural cell extracts was slightly larger than that seen with T lymphoblastoma extracts by two residues at the 5' end and three at the 3' end on the coding strand, and one less residue on the 5' end of the non-coding strand and three more at the 3' end. This suggests that slightly different but probably related protein(s) recognize Site A in neural cells compared to lymphocytes. Data from Chapter 7 also indicated that these factors can interact with the Site A element to form two discrete complexes of different molecular weights or conformations, however, there was no evidence from

footprinting experiments to suggest that these did not recognize the same sequences of Site A.

Site B-like binding activity

Constitutive DNA-binding factors were present in astrocytoma and neuroblastoma cells that bound to a region similar to the Site B motif described by Orchard *et al.* (1990). This motif was originally defined by nuclear factors present in T lymphoblastoma cells which, from the palindromic nature of the binding domain and competition experiments with oestrogen and thyroid hormone response elements, were suggested to be members of the steroid/thyroid hormone receptor super-family (Orchard *et al.*, 1990). A subsequent analysis by Cooney *et al.* (1991) demonstrated that the major Site B-binding factor in human T lymphoblastoma cells was analogous to the chicken ovalbumin upstream promoter transcription factor, COUP-TF, which is also member of the steroid/thyroid receptor super-family but distinct from oestrogen or thyroid receptor. Similar COUP-TF-like DNA-binding proteins were also found in HeLa cells where both the low and high molecular weight forms were present. The high molecular weight molecule predominated in lymphoblastoma cells (Cooney *et al.*, 1991).

The partial protection observed over the Site B region with all neural cell extracts (predominantly on the coding strand) was weakest with extracts from U373MG astrocytoma cells. This may have been a consequence of the reduced sensitivity of the footprinting technique as gel retardation assays with probe 194 had indicated that Site B-binding factors were in greater abundance to those recognizing Site A and especially in U373MG (Chapter 7, Section 7.1.3). This prompts the suggestion that the Site B complex was more labile under the conditions used for footprinting even though essentially similar buffers were used for the binding reactions in both gel retardation and footprinting assays. Orchard *et al.* (1990) used a similar buffer for the binding reactions but with a fractionally higher pH (7.9 as

opposed to 7.6) and a greater concentration of glycerol (20% rather than 10% [v/v]). If either of these factors influenced protein stability it may be the increased glycerol concentration which can help stabilize of nuclear proteins (Goodwin, 1990).

The proximity of the protected regions of Site B to the major band representing undigested probe at the top of the autoradiographs made the identification of the protected sequences more difficult as linear sequencing gels do not separate fragments differing by only one or two nucleotides well when larger fragments (200 bp) are being resolved. The distance between the radiolabelled nucleotide(s) at the 5' end of the probe fragment and the Site B sequences was also greater than the optimum 20-150 nucleotide distance for footprinting assays (Rhodes, 1989). Sequences further from, or closer to the radiolabel will not be well resolved on sequencing gels and the ability to discern by eye the differences between the digestion pattern of free DNA and that complexed to specific proteins is reduced. A more accurate footprinting analysis of Site B-binding factors would require the fragment to be re-cloned in order to locate the binding site nearer to the labelled nucleotide(s) and within the central portion of a sequencing gel where the resolving power is greatest. Unfortunately, it was not practical to move the radiolabel to the *Rsa* I restriction site 3' to Site B due to the presence of multiple *Rsa* I sites within the cloning vector.

Oligonucleotide competition

The competition of Site A- and B-binding activity with excess complementary oligonucleotides did not abolish the respective DNA-binding activities as effectively as might be expected, as this was unable to remove completely the respective regions of protection from the DNAse I digestion pattern. The effects seen were specific for the sequence of the oligonucleotide employed but the limited efficiency in competition may have been influenced by the reduced affinity of nuclear factors for the Site A and B sequences observed when these sequences were present in

oligonucleotides rather than in the context of probe 194 (see discussion Chapters 7 and 9). This may not have entirely accounted for the requirement of such a large molar excess of unlabelled oligonucleotide and excessive non-specific DNA-binding activity in crude extracts was almost certainly an additional factor.

The slight effect of competition with the oligonucleotide containing mutations to the 5' half site of Site B, BM5, on the formation of the protected region from -355/-346 provides some evidence for the existence of two protein units that can independently bind to the two motifs of Site B. Comparing the results from gel retardation assays (Chapter 7, Section 7.2.2), where competition with excess BM5 oligonucleotide to factors recognizing probe 194 partially reduced the intensity of proteins producing both Site B-specific complexes without affecting their relative mobilities, with these findings also provides preliminary evidence to suggest that the same DNA-binding protein(s) interact with either half site of Site B. Both of these suggestions are in agreement with the presumption that Site B-binding proteins are related to members of the steroid/thyroid hormone receptor family (Orchard *et al.*, 1990; Cooney *et al.*, 1991).

Summary

From the examination of DNA-binding activities that recognize the HIV-1 LTR in neural cell extracts it is apparent that similar factors to those initially defined in HeLa and lymphocyte cells are present (see Figure 8.11), with the addition of the octamer-related and GTI-related activities that appear neural cell-specific. The ability of cytokines to promote changes in the nuclear factors binding to the HIV-1 LTR was only apparent by the footprinting technique in SK-N-MC neuroblastoma cells exposed to TNF α where factors specifically interacted with the NF κ B sites that were unoccupied with extracts from unstimulated cells. In U373MG astrocytoma cells treated with IL-1 β and SK-N-SH cells treated with TNF α , the presence of inducible factors recognizing these sequences was not apparent as non-specific DNA-binding activity masked any similar interaction with these sequences. However, gel retardation assays performed in Chapter 7 provided evidence for equivalent events in U373MG and SK-N-SH cells.

The gel retardation technique was further exploited in Chapter 9, using oligonucleotides to known or suspected nuclear factor motifs present in the HIV-1 LTR to provide further information on the interaction and induction of DNA-binding proteins.

CHAPTER 9

Chapter 9: The effect of cytokines on the interaction of DNA-binding proteins with the HIV-1 LTR

Introduction

The final investigation of nuclear protein binding sites in the HIV-1 LTR and the DNA-binding activities from neural cells that recognize them was performed using specific radiolabelled, ds oligonucleotides in gel retardation assays with nuclear extracts from astrocytoma and neuroblastoma cells, and each cell type treated with the cytokine IL-1 β or TNF α , respectively. Experiments were performed to further confirm the presence or absence of specific DNA-binding activities and investigate the ability of each cytokine shown to augment expression from the HIV-1 LTR to modulate their interaction with the regulatory regions of the LTR. The data provided by gel retardation assays undertaken with restriction fragments from the HIV-1 LTR (Chapter 7), and from DNase I footprinting (Chapter 8) indicated certain protein-binding motifs and other sequences from in HIV-1 LTR that bound nuclear factors. This information was used to select regions of the LTR for analysis as oligonucleotide probes (a list of oligonucleotides and the nucleotide sequences of each is given in Chapter 2, Table 2.2.).

Competition assays

The primary advantage of the use of synthetic oligonucleotides in gel retardation assays is to allow the interaction of cellular proteins with specific motifs to be assessed in isolation from other sequences as the formation of complexes will be dependent only on the short stretch of DNA that constitutes the protein-binding site. The use of crude nuclear extracts also required the inclusion of the unlabelled competitor nucleic acids, poly (dI:dC)-(dI:dC) and pUC13, in the binding reactions to reduce of non-specific complex formation and allow the specific interactions to be

determined after titration with an increasing amount of competitor. As described previously the end-point is defined as the concentration that allows free probe to be efficiently separated from the bulk of complexed nuclear protein. In practice it was found that competitor did not remove all the non-specific DNA-binding activity present in the nuclear extracts within the range of competitor concentrations that could be practically employed before all DNA-binding activity was competed from the assay (experimental observations). This was apparent much more so when oligonucleotide probes were used rather than restriction fragments from the LTR. Therefore it was important to identify the specific protein-DNA complexes which formed with the oligonucleotide probes by competition analysis. This was facilitated by the higher affinity sequence-specific proteins have for their recognition sites compared to the other proteins in the nuclear extract (Sorger *et al.*, 1989). Inclusion of excess unlabelled oligonucleotide probe (relative to labelled probe) in the binding reaction 20 min prior to the addition of the labelled probe will reduce or abolish the sequence-specific protein-DNA complexes before those arising from the lower affinity binding of abundant proteins. The majority of proteins in crude nuclear extracts bind to DNA in a sequence-independent manner (Goodwin, 1990). Addition of more unlabelled probe oligonucleotide than there is specific ligand for the binding site will begin to compete away complexes in order of the affinity of binding so that sequence-specific complexes will be lost first and non-specific complexes will also be removed at higher concentrations of unlabelled probe. By comparison of the amount of excess unlabelled probe required to compete away a given complex relative to another, the relative affinities of the protein(s) forming each complex with the probe can be ordered. Competition experiments were carried out for each oligonucleotide probe examined with two concentrations of excess unlabelled oligonucleotide to identify sequence-specific interactions.

9.1. Nuclear factor binding to an Sp1 consensus sequence oligonucleotide

Gel retardation assays performed with an oligonucleotide containing a single high affinity binding site for the transcription factor Sp1 (Harrich *et al.*, 1989) and nuclear extracts from U373MG astrocytoma, SK-N-SH and SK-N-MC neuroblastoma cells demonstrated the formation of a number of protein-DNA complexes (Figure 9.1. A). The oligonucleotide competition experiment in Figure 9.1. B indicated that one major sequence-specific complex, marked Sp1, was readily detected in 15 μ g of nuclear extract from each cell line. This complex predominated in intensity over one suggested non-specific complex from U373MG and SK-N-SH extracts (marked ns2) and three such complexes (ns1 to 3) with SK-N-MC extracts (Figure 9.1. A). The non-specific complexes formed with the oligonucleotide probe were of similar mobilities in all cell lines but varied greatly in intensity and may be a consequence of the different cell lines from which the nuclear extracts were prepared.

When compared to the pattern of complexes produced with an equivalent amount of nuclear extract from IL-1 β -stimulated U373MG or TNF α -stimulated SK-N-SH and SK-N-MC cells, no novel protein-DNA interactions were seen and there was generally little change in the relative abundance of Sp1 activity (Figure 9.1. A). The intensity of the Sp1 complex was reduced in extracts from SK-N-SH cells stimulated with TNF α relative to unstimulated cells as was the non-specific complex ns2 and this may have been artefactual. Nevertheless, in agreement with previous results, the activation of neural cells with IL-1 β or TNF α did not appear to significantly alter the DNA-binding properties of the Sp1-like activity.

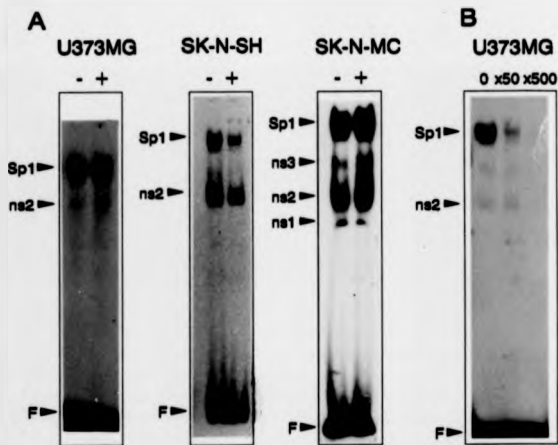


Figure 9.1. A. Nuclear protein binding to an Sp1 oligonucleotide probe in extracts from untreated and IL-1 β -stimulated U373MG astrocytoma, and from untreated and TNF α -stimulated SK-N-SH and SK-N-MC neuroblastoma cells, designated - and +. Sp1 indicates specific complex formation; ns1 to 3, non-specific complexes; F, free probe. B. Competition analysis with excess unlabelled Sp1 oligonucleotide and extracts from U373MG cells. 0, no competition; x50- and x500-fold molar excess of unlabelled Sp1 oligonucleotide.

9.2. Nuclear factor binding to an NF κ B consensus sequence oligonucleotide

The gel retardation assays shown in Figure 9.2 were performed with an oligonucleotide probe corresponding exactly to the sequence of the -104 to -81 region of the HIV-1 LTR which spans the enhancer of two NF κ B motifs and which also represents the consensus NF κ B-binding sequence (Nabel and Baltimore, 1987). As anticipated, a significant difference was seen between the nucleo-protein complexes formed with the NF κ B probe and extracts from unstimulated and cytokine-treated cells. In nuclear extracts from IL-1 β -stimulated astrocytoma and TNF α -stimulated neuroblastoma cells, two slowly migrating complexes of equal intensity, marked kB, were clearly visible and were absent from unstimulated cell extracts (Figure 9.2. A).

When oligonucleotide competition experiments were undertaken with a 50- and 500-fold molar excess of unlabelled NF κ B oligonucleotide (Figure 9.2. B) to determine the specificity of complex formation, the two kB complexes formed with extracts from cytokine-treated astrocytoma or SK-N-SH neuroblastoma cells were eliminated, as was the complex marked ns2 that was also seen with unstimulated cell extracts (Figure 9.2. B). The removal of the ns2 complex was almost certainly due to the use of a molar excess of unlabelled oligonucleotide probe greater than was necessary to compete away only the complexes formed by the proteins with the highest affinity for the NF κ B motifs, as competition with equivalent amounts of the mutant NF κ B oligonucleotide, which will not bind NF κ B or related factors (Schmidt *et al.*, 1990), eliminated ns2 but not the kB complexes (Figure 9.2. B). With extracts from SK-N-SH cells stimulated with TNF α the mutant NF κ B oligonucleotide did cause some reduction of the kB complexes (probably because a greater excess was used than was required). The other non-specific complex ns1, evident in all gel retardation assays in Figure 9.2, was unaffected by either oligonucleotide up to a

500-fold molar excess and was due to more abundant non-specific DNA-binding factors in crude extracts. Therefore DNA-binding activity specific for the enhancer of HIV-1 was induced by the cytokines IL-1 β and TNF α .

To confirm that the absence of an NF κ B-like factor in unstimulated cells was not a consequence of the unequal loss during the nuclear extract preparation of co-factors which are known to stimulate the interaction of these proteins with DNA, the binding reactions were supplemented with a 3 mM final concentration of rGTP and spermidine (Lenardo *et al.*, 1989). Gel retardation assays were performed with extracts from unstimulated U373MG and SK-N-SH cells and compared with an equivalent amount of extract from their cytokine-stimulated counterpart, also after incubation in the presence of rGTP and spermidine (Figure 9.2. C). However, this did not promote the formation of specific complexes with the NF κ B oligonucleotide probe in nuclear extracts from unstimulated cells (Figure 9.2. C) and neither did this appear to enhance greatly the DNA-binding of factor(s) producing the κ B complexes in extracts from cytokine-stimulated cells, although this was not compared in the same assay. Therefore the data indicates that there is no constitutive enhancer binding factor in astrocytoma or neuroblastoma cells and that stimulation by IL-1 β and TNF α , respectively induces an NF κ B-like binding activity.

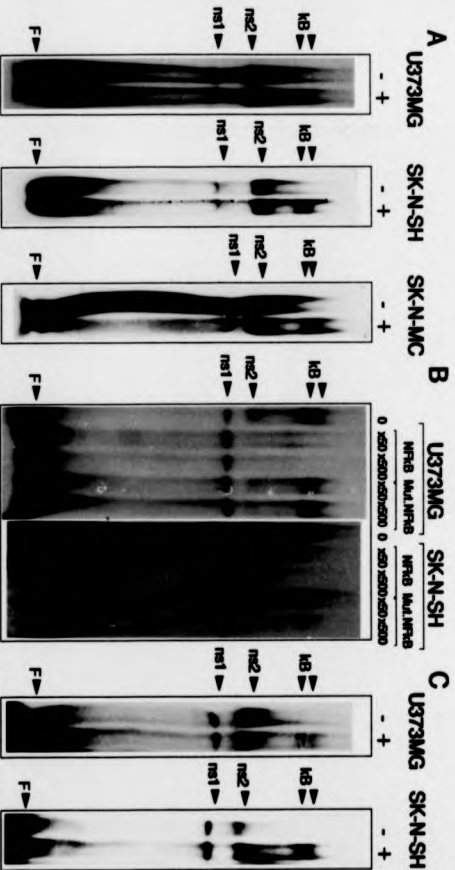


Figure 9.2. A. Nuclear protein binding to an NFkB oligonucleotide probe complementary to the enhancer of HIV-1 in extracts from unstimulated and L-18-stimulated U373MG astrocytoma, and unstimulated and TNF α -stimulated SK-N-SH and SK-N-MC neuroblastoma cells, designated - and +. B. Indicates specific complex formation; ns 1 to 3, non-specific complexes; F, free probe. C. Competition analysis with excess unstimulated NFkB and mutant NFkB (mut.NFkB) oligonucleotides at 0, 50- and 500-fold molar excess with extracts from L-18-stimulated U373MG and TNF α -stimulated SK-N-SH cells. C. Nuclear protein binding to the NFkB oligonucleotide probe in the presence of GTP and Spermidine (2 mM each), see part A for legend.

9.3. Nuclear factor binding to Site A oligonucleotide

The use of a radiolabelled oligonucleotide corresponding to the sequences defined by Orchard *et al.* (1990) as Site A, in gel retardation assays demonstrated extremely variable levels of this DNA-binding activity in the neural cell lines examined and no differences in the pattern of bands observed between extracts from unstimulated and cytokine-treated cells of the same type. With nuclear extracts from U373MG cells the complexes formed were most likely non-specific (see below and Figure 9.3. A). With SK-N-SH nuclear extracts two additional slowly migrating complexes were observed with the Site A probe but were relatively faint, these were marked A1' and A2' in Figure 9.3. These complexes were of a mobility similar to the range observed for the Site A sequence-specific complexes formed with nuclear extracts from G26-24 oligodendroglioma cells (Figure 9.3. B) and were reasonably well separated from each other suggesting they differed appreciably in molecular weight. Also complex A1' was more intense than A2'. However, these complexes were infrequently detected in similar gel retardation assays and could not be confirmed as sequence-specific interactions. In extracts from the SK-N-MC neuroblastoma cells (Figure 9.3. A) complexes were formed similar to U373MG and were similarly suggested to be non-specific by their mobility in comparison to other assays. The oligonucleotide competition with nuclear extracts from unstimulated G26-24 cells performed to identify the specific nucleo-protein complexes formed with the Site A probe demonstrated that in these cells there was readily detectable Site A-binding activity that produced a relatively slowly-migrating, broad complex that smeared partly towards the top of the gel, indicative of a degree of heterogeneity in the protein species recognizing the sequence (Figure 9.3. B). Competition with a 50-fold molar excess of unlabelled Site A oligonucleotide eliminated the broad complex but did not remove the non-specific band marked as nsl.

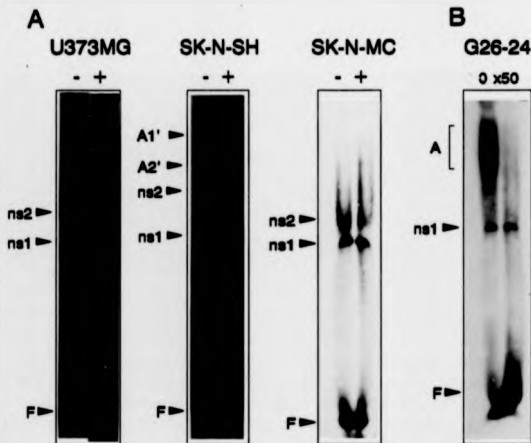


Figure 9.3. A. Nuclear protein binding to a Site A oligonucleotide probe in extracts from untreated and IL-1 β -stimulated U373MG astrocytoma, and from untreated and TNF α -stimulated SK-N-SH and SK-N-MC neuroblastoma cells, designated - and +. A1' and A2' indicate putative specific complexes, see text; ns1 to 3, non-specific complexes; F, free probe. **B.** Competition analysis with excess unlabelled Site A oligonucleotide and extracts from G26-24 oligodendrogloma cells. 0, no competition; x50-fold molar excess of unlabelled Site A oligonucleotide. A indicates specific complex.

9.4. Nuclear factor binding to Site B oligonucleotide

In nuclear extracts from both unstimulated and IL-1 β -treated U373MG astrocytoma cells, similar sequence-specific binding (see below and Figure 9.4. B) to the Site B probe (Orchard *et al.*, 1990) was represented by a broad, slowly migrating complex (Figure 9.4. A). This suggested a heterogeneous mixture of protein factors recognizing the binding motif rather than protein degradation as the complex was quite well defined at its lower but not its upper border. From the oligonucleotide competition with excess unlabelled Site B oligonucleotide and U373MG extracts (Figure 9.4. B), two complexes, marked ns1 and ns2, were indicated to be non-specific, although ns1 was much reduced with U373MG extracts in Figure 9.4. A, perhaps due to the different preparations of nuclear extracts employed. In nuclear extracts from unstimulated or TNF α -treated SK-N-SH neuroblastoma cells there was an extremely faint, ill defined complex above equivalent ns1 and ns2 complexes, that may have indicated specific Site B-binding but which was much less intense and difficult to distinguish from the lower complex below due to the high background in the photograph. A close examination of the autoradiograph did not make the interpretation any more decisive, especially as no binding had been seen in previous assays, but no changes were noted between extracts from unstimulated and TNF α -treated cells. In nuclear extracts from either unstimulated or TNF α -treated SK-N-MC cells specific complexes could also not be determined and there was only a great deal of what was suggested to be non-specific binding activity recognizing the Site B probe, forming complexes analogous to ns1 and ns2 in Figure 9.4 B. Complex ns2 was broad and intense and may have obscured any other complexes.

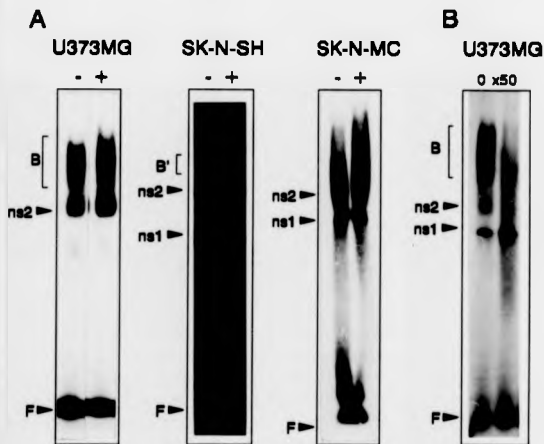


Figure 9.4. A. Nuclear protein binding to a Site B oligonucleotide probe in extracts from untreated and IL-1 β -stimulated U373MG astrocytoma, and from untreated and TNF α -stimulated SK-N-SH and SK-N-MC neuroblastoma cells, designated - and +. B, indicates specific complex formation; B' putative specific complex, see text; ns1 to 2, non-specific complexes; F, free probe. B. Competition analysis with excess unlabelled Site B oligonucleotide and extracts from U373MG cells. 0, no competition; x50-fold molar excess of unlabelled Site B oligonucleotide.

9.5. Gel retardation assays with oligonucleotides containing other sequences from the HIV-1 LTR

9.5.1. Nuclear protein interactions with 5C159 and 159 oligonucleotides

Oligonucleotide 5C159 contained complementary sequence to the HIV-1 LTR from position -261 to -240, that was shown by DNase I footprinting analysis (Chapter 8, Section 8.1.2) to contain a small region, from -249 to -243, protected by nuclear protein(s) in extracts from G26-24 oligodendroglioma and SK-N-MC neuroblastoma cells.

Oligonucleotide 5C159 was used as a probe to detect any similar binding activity in U373MG astrocytoma and SK-N-SH neuroblastoma cells, as well as SK-N-MC, in the gel retardation assays shown in Figure 9.5. In an initial experiment titrating the amount of poly (dI:dC)-(dI:dC) competitor required using extracts from both unstimulated neuroblastoma cells, two complexes were noted in assays performed in the presence of 0.1 to 0.5 μg of poly (dI:dC)-(dI:dC) competitor which were of similar mobilities in either cell line. Figure 9.5. A, illustrates binding to the 5C159 probe with 0.3 μg and 0.4 μg of poly (dI:dC)-(dI:dC). From this assay the slower migrating and relatively faint complex, marked 5C in the figure, was suggested to be specific because as the concentration of poly (dI:dC)-(dI:dC) was increased the faster migrating complex (ns2) began to decrease in intensity, an effect noted across the range of poly (dI:dC)-(dI:dC) concentrations from 0.1 to 0.5 μg (not shown). The higher concentration of competitor was able to bind more non-specific activity but did not alter the intensity of the 5C complex suggesting it to be specific. Oligonucleotide competition shown in Figure 9.5. B, with nuclear extracts from unstimulated SK-N-MC cells and a 50- or 500-fold excess of unlabelled 5C159 was not successful in further identifying complex 1 as sequence-specific because at the lower concentration no complexes were competed away and at the higher concentration both complexes 1 and 2 were removed. Complex 2 appeared

analogous to ns2 in Figure 9.5. A, which was suggested to be non-specific. A third quickly migrating complex not found in Figure 9.5. A, could, however, be identified as non-specific.

Comparison of protein-DNA complex formation to probe 5C159 with extracts from TNF α -stimulated SK-N-MC cells did not reveal any changes compared to those described above for unstimulated cells, SK-N-SH cells were not examined (results not shown).

A second oligonucleotide to a region of the HIV-1 LTR suggested to perhaps weakly interact with nuclear factors by DNase I footprinting (Chapter 8, Section 8.1.2) was also examined in gel retardation assays. Oligonucleotide 159 contains sequences from -249 to -219 in a region that has been described previously by Shaw *et al.* (1988) to bind the inducible transcription factor NFAT-1 found in mitogen-stimulated T lymphocytes.

In gel retardation assays performed with extracts from unstimulated SK-N-MC neuroblastoma cells, two faint complexes of different mobilities were formed with radiolabelled 159 oligonucleotide probe (Figure 9.5. B). Unfortunately the correct level of oligonucleotide competition that would abolish either complex individually was not found (Figure 9.5. B. Tracks 4, 5 and 6), and neither complex could be identified as sequence-specific. However, when nuclear factor binding to oligonucleotide 159 was compared in equal amounts of extract from unstimulated or TNF α -treated SK-N-MC cells, a similar pattern of complexes to that illustrated in Figure 9.5. B was observed and there was no differences with extracts from cytokine-stimulated cells (results not shown).

Oligonucleotides 5C159 and 159 were also used to assay for any similar DNA-binding activity in extracts from unstimulated and IL-1 β -treated U373MG astrocytoma cells. In gel retardation assays with oligonucleotide probe 159 three complexes, marked 1, 2 and ns1, were observed with extracts from both unstimulated and IL-1 β -treated U373MG cells (Figure 9.5. C. Tracks 1 and 7). Competition with

excess unlabelled probe indicated that complex ns1 was non-specific but complexes 1 and 2 could not be identified as sequence-specific as oligonucleotide competition with excess unlabelled 159 abolished both of the slower migrating complexes (Tracks 2 and 8). An indication of the lack of sequence specificity in factors forming complex 2 in Figure 9.5. C, was given by the ability of competition with a similar unlabelled excess of oligonucleotide 5C159 to reduce the intensity of complex 2 and dissociate factors forming this complex, such that an additional complex of higher mobility was also observed below complex 2 and the non-specific complex ns1 became larger and more intense. Excess unlabelled 5C159 did not affect complex 1 which was therefore suggested to be specific (Tracks 6 and 12). Complex 1 formed with oligonucleotide 159 was quite broad and not sharply defined and this also implied that a number of proteins may recognize these sequences in extracts from U373MG cells. No cytokine-inducible factors were identified.

Gel retardation assays with oligonucleotide probe 5C159 and extracts from unstimulated and IL-1 β -treated U373MG cells also formed a pattern of three complexes (Figure 9.5. C, Tracks 3 and 9). The complex with the highest mobility was judged to arise from non-specific interactions from the competition with excess unlabelled oligonucleotide (Figure 9.5. C, Tracks 4 and 10). Competition also abolished the two upper complexes and an identification of any sequence-specific binding could not be made. Although in keeping with the result from SK-N-MC cells the treatment of U373MG cells with IL-1 β did not alter the formation of nucleoprotein complexes with probe 5C159.

The sequences contained in oligonucleotides 5C159 and 159 overlap by 9 nucleotides such that both probes contain the sequence from -249 to -243, the GTI-related motif described in Chapter 8 to be protected from DNase I digestion with SK-N-MC and G26-24 nuclear proteins in footprinting analysis. Therefore reciprocal competition experiments with these oligonucleotides were performed with extracts from unstimulated or IL-1 β -treated U373MG cells to evaluate if the

same protein factors were interacting with each probe via the sequences common to them. As already mentioned, competition of complex formation to oligonucleotide 159 by excess unlabelled 5C159 oligonucleotide did not affect the intensity of the slowest migrating complex 1 which itself was suggestive of a specific interaction (Figure 9.5. C. Tracks 6 and 12) but the reverse; radiolabelled 5C159 competed with excess unlabelled 159, prevented proteins forming complexes with 5C159 (Tracks 5 and 11) as effectively as the equivalent amount of unlabelled 5C159 probe. This indicated that the factors interacting with oligonucleotide probe 159 may be distinct from 5C159, at least in U373MG cells, and that those forming complexes with 5C159 were either bound via the sequences common between the two oligonucleotides, the GTT-related motif or weak, probably non-specific, interactions.

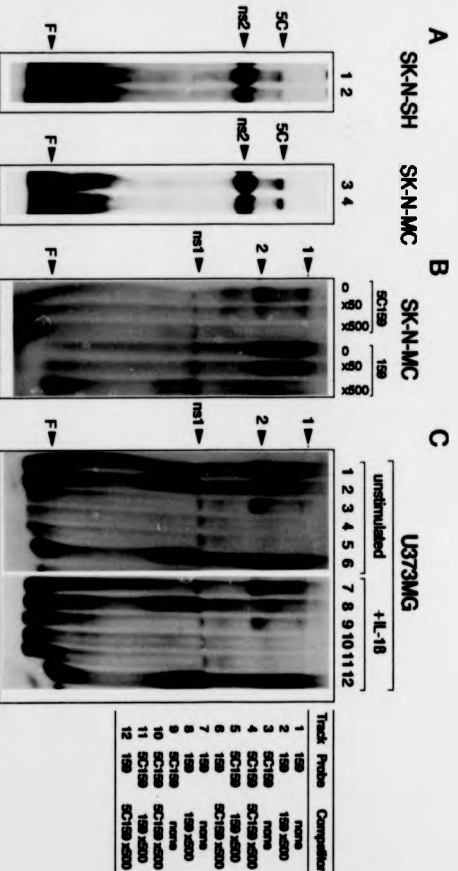


Figure 8.5. A: Nuclear protein binding to oligonucleotide probe SC159 in extracts from unstimulated SK-N-SH and SK-N-MC neuroblastoma cells. SC indicates specific complex (see text); ns2, non-specific complex; F, free probe. Tracks: 1 & 3, 0.3 μ g and 2 & 4, 0.4 μ g poly (dIdC)- (dIdC) competitor (also see text). **B:** Competition analysis with excess unlabeled SC159 and 159 oligonucleotides at 0, 50 and 500-fold molar excesses with extracts from unstimulated SK-N-MC cells. Arrows indicate complexes formed; ns1, non-specific complex; F, free probe. **C:** Nuclear factor binding to SC159 and 159 oligonucleotide probes in extracts from unstimulated and IL-18-treated U373MG astrocytoma cells and competition analysis with either unlabeled oligonucleotide probe at the fold molar excesses given in the table. Legend as part B.

Discussion

Sequence-specific DNA-binding activities and the HIV-1 LTR

The analysis of the DNA-binding factors present in nuclear extracts reliably demonstrated that in astrocytoma and neuroblastoma cell lines there was a constitutive Sp1-like DNA-binding activity, and a cytokine-inducible NF κ B-like activity after stimulation of astrocytoma cells with IL-1 β and neuroblastoma cells with TNF α . Other DNA-binding factors reported in Chapters 7 and 8 to interact with the Site A and Site B elements of the HIV-1 LTR were not always detected by the use of complementary radiolabelled oligonucleotide probes in gel retardation assays, and Site B activity was confirmed only in U373MG astrocytoma cells and Site A in G26-24 oligodendroglioma cells. Nuclear protein binding to an oligonucleotide complementary to HIV-1 LTR sequences containing the GTT-related motif was detected in the other neural cell lines U373MG and SK-N-SH, as well as the neuroblastoma SK-N-MC. The specificity of this interaction was suggested by competition with increasing poly (dI:dC)-(dI:dC) competitor in neuroblastoma but not astrocytoma cells. The interaction of constitutive sequence-specific factors with another oligonucleotide probe containing sequences complementary to GTT-related motif and sequences reported to bind the T cell inducible transcription factor, NFAT-1 (Shaw *et al.*, 1988) was also suggested by the gel retardation assays with U373MG extracts but could not be conclusively demonstrated. However, no cytokine-inducible DNA-binding activities other those recognizing the enhancer of NF κ B motifs were found to operate in neural cells.

NF κ B DNA-binding activity

An oligonucleotide probe containing the two NF κ B sites of the HIV-1 enhancer interacted with sequence-specific nuclear proteins to form two retarded complexes of slightly different mobilities. Comparison with similar gel retardation

assays performed with nuclear extracts from T lymphoblastoma cells after stimulation with mitogens or TNF α , and using an identical enhancer probe, also demonstrated the formation of two closely migrating specific complexes essentially the same as those observed here with neural cell extracts (Franza *et al.*, 1987; Bohnlein *et al.*, 1988; Osborn *et al.*, 1989; Schmidt *et al.*, 1990). Such complexes appear characteristic of the inducible DNA-binding activity that recognizes the HIV-1 enhancer and whether each complex represents single and double occupancy of the NF κ B sites by exactly the same protein factors is unclear but studies by UV-crosslinking (Molitor *et al.*, 1990; Hansen *et al.*, 1992), microscale DNA-affinity precipitation technique (Franza *et al.*, 1987) and direct cloning (Bours *et al.*, 1992) have demonstrated up to four related polypeptides which form these inducible complexes in T lymphoblastoma cells. Identification of the NF κ B-like factors induced in neural cells could most easily be achieved by the use of specific antiserum to members of the NF κ B and *c-rel* family of proteins to produce a 'super-shift' or inhibit binding in gel retardation assays.

The detection of an enhancer-binding activity was always in the form of two complexes and these were never observed in nuclear extracts from unstimulated cells. A minimum of two independent preparations of nuclear extracts from unstimulated and cells stimulated with the respective cytokine were examined by this technique and despite the precaution of adding additional GTP or spermidine, NF κ B-like proteins capable of binding DNA were not detected in nuclear extracts from unstimulated cells. The fact that these two molecules also did not appear to stimulate greatly the binding of NF κ B-like factors to the probe DNA either suggests that close to maximum DNA-binding was seen in the assays performed or that GTP and spermidine cannot stimulate DNA-binding of these factors from neural cells. No distinction can be made on the basis of the experiments carried out here. In experiments reported by Lenardo *et al.* (1989) GTP or spermidine considerably enhanced binding of NF κ B to its recognition site.

Sp1-like DNA-binding activity

The gel retardation assays performed the Sp1 oligonucleotide probe further supported results obtained from gel retardation assays with LTR restriction fragments as probes and DNase I footprinting experiments on the constitutive nature of this DNA-binding activity. A single high-affinity Sp1 binding site was used as the probe to maximize the detection of Sp1-like factors and to simplify the interpretation of the complexes formed in gel retardation assays over the use of an oligonucleotide containing all three Sp1 sites present in the HIV-1 LTR (Jones *et al.*, 1986). Mutational analysis carried out by Harrich *et al.* (1989) showed that the high affinity consensus sequence probe will bind transcriptionally active Sp1 in the context of the HIV-1 LTR.

Site A- and Site B-binding activity

The ability of the complementary oligonucleotides to Site A and Site B to bind related factors found in neural cells was demonstrated earlier in Chapter 7 by the use of excess unlabelled oligonucleotide to compete away the corresponding protein-DNA complexes formed with the HIV-1 LTR probe 194. However, except for Site A-like activity in G26-24 cells and Site B-like activity in U373MG cells these factors were undetectable when radiolabelled oligonucleotides were used as probes. These DNA-binding factors appear to interact with oligonucleotide probes at lower affinity than when their recognition site is present in a DNA fragment and the difference emphasizes the importance of examining both the individual protein-binding motifs in the form of oligonucleotides and restriction fragments. A number of factors could influence this interaction but it is not likely to be due to a requirement for cooperative binding of Site A and Site B to their respective sites in the HIV-1 LTR because a complex that corresponded to both activities bound to the LTR was never observed in gel retardation assays (Chapter 7). Indeed the data of Orchard *et al.* (1990) similarly implies that binding of either Site A or Site B factors

is enhanced in the absence of the other. One explanation for the differential binding of these factors to oligonucleotide and restriction fragment probes may lie in the recently confirmed ability of transcriptional factors to bend their cognate DNA on binding, such that the target DNA bends by about 90° around the protein factors (Lilley, 1991). Were the additional sequences to further stabilize the protein-DNA interaction this would provide one mechanism to account for the observed difference in binding independent of the sequence of the binding site.

The interaction of Site B-binding activity from U373MG astrocytoma cells demonstrated that at least when factors were more abundant (estimated from the gel retardation assays with LTR probe 194 in Chapter 7) the corresponding radiolabelled oligonucleotide probe could detect substantial binding. In the neuroblastoma cells less Site B-specific activity was present when assayed with LTR probe 194 and subsequently none was detected with the oligonucleotide probe. The heterogeneity in the protein factors present in U373MG extracts for Site B is supported by the existence of multiple COUP-TF proteins of at least three different molecular weights, which were shown by Cooney *et al.* (1991) to bind to Site B. The authors describe low M_r COUP-TFs of 46,000 and 47,000 daltons and high M_r forms of 68,000 daltons, although the high M_r form predominates in T cells and was shown to interact strongly with Site B. Unpublished data from Orchard *et al.* presented at the 1991 MRC AIDS directed programme meeting indicated that the Site B-binding factors purified from T cells have molecular weights of 97,000 to 105,000 daltons by UV-crosslinking and gel renaturation experiments. This suggest that there are a number of factors present in the same cell line (both groups examined the T lymphoblastoma Jurkat) that will bind to this recognition site.

Site A-binding activity has not yet received similar attention from other research groups but data presented in Chapter 7, demonstrating two Site A specific complexes, suggests proteins of two molecular weights or one or more factors that have different conformations. Unfortunately these were not sufficiently abundant to

be reliably detected with a Site A oligonucleotide probe, although if the two complexes observed between the Site A probe and SK-N-SH nuclear extracts were sequence-specific then this would be in agreement with earlier results. The finding of a very broad complex between G26-24 oligodendroglioma extracts and the Site A probe indicates that in this cell line there are multiple factors capable of recognizing the binding motif.

DNA-binding activities that interact with the neural cell specific GTI-related motif and the putative NFAT-1 binding site

Gel retardation assays with oligonucleotide 5C159 further indicated that the GTI-related motif bound a sequence-specific nuclear factor or factors found in neuroblastoma cells and, by comparison of the gel retardation assays performed, a similar factor may also be present in astrocytoma cells. The gel retardation assays reported in Chapter 7 with LTR probe 159 demonstrated the formation of one major protein-DNA complex that was certainly due to factors recognizing the octamer-like motif, although assays with U373MG cells did infrequently detect another faint complex of higher mobility (see Chapter 7, Figure 7.9). However, experiments to investigate if this additional complex with LTR probe 159 was due to protein factors interacting with GTI-related motif were not performed.

The binding of nuclear factors to oligonucleotide 159 was inconclusive except for experiments with U373MG cells where the data indicated that some factors may specifically interact and were distinct from factors recognizing the GTI-related motif. Moreover, the use of oligonucleotide probe 159 to detect inducible protein factors that potentially interact with the NFAT-1 domain (Shaw *et al.*, 1988) indicated that none were present in extracts from cytokine-stimulated U373MG and SK-N-MC cells. A result consistent with there being no additional protein-DNA complexes formed between LTR probe 159 and extracts from all three cytokine-

stimulated neural cell lines when compared with nuclear extracts from untreated cells (Chapter 7, Figure 7.8).

CHAPTER 10

Chapter 10: Concluding remarks

Cytokine augmentation of HIV-1 gene expression

The regulation of HIV-1 LTR-driven reporter gene expression by cytokines in neural cells described in this thesis provides evidence to indicate that expression of HIV-1 in these cells may also respond to the activation signals delivered by similar cytokines. This assumption parallels the situation reported previously in T lymphocytes (Israel *et al.*, 1989; Okamoto *et al.*, 1989; Osborn *et al.*, 1989) and monocytic cells (Stanley *et al.*, 1990) where similar augmentation of LTR-driven expression by cytokines corresponded to a like augmentation of HIV-1 replication (Matsuyama *et al.*, 1989a; Poli *et al.*, 1990a; Rosenberg and Fauci, 1990). In human neural cells, from ~~where the data~~ is most relevant to the AIDS dementia complex (ADC), TNF α was capable of augmenting significantly expression from the LTR in neuronal and glial cell lines. Similarly IL-1 β augmented expression in glial cell lines.

This is supported by the results of Tornatore *et al.* (1991) who demonstrated that TNF α and IL-1 β also induced productive HIV-1 replication in latently infected primary human foetal astroglial cells, particularly in those cells positive for glial fibrillary acidic protein and morphologically resembling astrocytes. These data provided by Tornatore *et al.* (1991) complement the use of molecular techniques *in vitro* described here and suggests that results obtained from LTR-driven reporter gene assays may parallel the expected response of a quiescent HIV-1 provirus in neural cells. The response of the human astrocytoma and glioblastoma cells most closely modelled that of primary glial cells infected with HIV-1, whereas primary murine astrocyte cultures, also investigated here, did not. The additional response of LTR-directed gene expression to IFN γ and IL-6 in these cells implied a role for these cytokines in the induction of HIV-1 replication that was not supported by the equivalent primary human cell culture (Tornatore *et al.*, 1991) and indicates that

transfected or infected murine neural cells will not provide a suitable model for the study of the control of HIV-1 gene expression.

In contrast there have been no previous studies with primary neuronal cells parallel to those of Tornatore *et al.* (1991) to define the signals that lead to the induction or augmentation of HIV-1 replication. However, from the function of TNF α on HIV-1 replication in infected lymphocytes (Matsuyama *et al.*, 1989a), monocytes (Poli *et al.*, 1990a) and foetal glial cells (Tornatore *et al.*, 1991) it is likely that this cytokine similarly regulates virus expression in infected neuroblastoma cells and by analogy, neurons. And this is implied by my results. There is evidence to indicate that neurons respond to IL-1 β , and perhaps IL-1 β may stimulate the HIV-1 LTR with a comparable effect to TNF α . Studies show that normal brain demonstrates immunocytochemical staining for IL-1 associated with neurons and a subpopulation of neurons have been noted to stain for IL-1 receptors (Hofman, 1989). Furthermore, experiments indicate that IL-1 is a trophic factor for certain neurons in which somatostatin acts as a neurotransmitter (Scarborough *et al.*, 1989).

The augmentation of HIV-1 LTR-driven gene expression in astrocytoma and glioblastoma cells by TNF or IL-1 β was not additive to the stimulation achieved by co-expression of the HIV-1 transactivator protein Tat. Similar results were obtained from transfection of a permanent murine oligodendrogloma cell line expressing *tax* and the subsequent stimulation with TNF α and IFN γ . This suggests that activating cytokines would not enhance the rate of HIV-1 replication in an infected cell once active virus production had begun and rather that they function primarily as a signal to induce latent HIV-1. In the neuroblastoma cell line SK-N-MC, cytokines may operate as the initial cellular stimuli for the activation of HIV but results suggest that they would also perpetuate viral replication.

Activation of HIV-1 gene expression in neural cells was investigated from the aspect of cytokine control in relation to the strong association between the state of virus expression and immunological response mechanisms. It would be interesting to

investigate whether other signals involved in the neurological functions of astrocytes and neuronal cells, such as the many neuro-active chemicals and peptides that astrocytes and neurons synthesize and are responsive to, also regulate either the latent or activated state of HIV-1. Some of these compounds, such as signals from β -adrenergic receptors (Melner *et al.*, 1990) and VIP (Tseng and O'Dorisio, 1989) to astrocytes, induce a cAMP-dependent PKA pathway of signal transduction and would therefore not be expected to modulate expression from the HIV-1 LTR (Paya *et al.*, 1991). Additional signalling pathways could operate in an analogous way to the stimulation of HIV-1 expression through cell surface antigens expressed on lymphocytes (Tong-Starksen *et al.*, 1989; Gruters *et al.*, 1991). One of these molecules capable of weakly activating the HIV-1 LTR, CD44, is found also on the surface of astrocytes in human brain and expression is increased during reactive gliosis (Girgah *et al.*, 1991).

Modulation of viral transcription by specific DNA-binding protein interactions with the HIV-1 LTR

The augmentation of HIV-1 LTR-driven gene expression by TNF α and IL-1 β in neuroblastoma and astrocytoma cells was found to correlate with the induction of transcription factors specific for the HIV-1 enhancer. These protein factors were rapidly inducible, within 1 to 2 hrs following cytokine stimulation, and recognized the consensus but not mutant NF κ B sequences. Although no experiments were performed to determine the direct effect of such proteins on transcription driven by the HIV-1 LTR there is substantial evidence (see Chapter 1, Section 1.5.3. iv) to suggest that such proteins enable TNF α and IL-1 β to augment HIV-1 LTR-driven gene expression in astrocytoma and neuroblastoma cells.

Confirmation that the induction of proteins specific for the NF κ B motifs will also augment viral replication through enhanced transcription from the LTR would require further experiments involving the manipulation of whole virus. This has been

examined previously in a lymphocytic cell line (Lu *et al.*, 1991). Deletion of the NF κ B sites from a HIV-1 proviral clone and transfection into unstimulated or PMA-treated lymphoblastoma cells indicate that in unstimulated cells the NF κ B sites are not obligatory for viral growth but are important for augmented replication in activated lymphocytes (Lu *et al.*, 1991). Both the NFAT-1 and NF κ B motifs were found to mediate this effect, yet deletion of either site resulted in higher levels of virus production in activated cells than wild type virus and to a much greater extent with an NFAT-1-deleted provirus (Lu *et al.*, 1991). Only deletion of both sites resulted in reduced viral replication (Lu *et al.*, 1991). Moreover the authors presented data to suggest that the NFAT-1 motif interacts with the same PMA-inducible proteins that recognize the NF κ B sites (Lu *et al.*, 1991). The use of two distinct motifs in the LTR by enhancer-binding factors was not observed in astrocytoma and neuroblastoma cells.

The investigation of the DNA-binding proteins in neural cells was restricted to one time point following cytokine stimulation (for reasons of available time) even though the effect of TNF α and IL-1 β on protein expression was determined after 22-24 hrs stimulation when reporter gene activity was maximal (data for this is not shown). Therefore it is possible that other protein factor(s) specific for sequences within the HIV-1 LTR were induced later during the course of reporter gene expression and these may modulate the effects of cytokines. It may be anticipated that transcription factors which require protein synthesis, unlike the NF κ B/*rel* family, would not be detected a short time after cytokine stimulation. The inducible transcription factor NFAT-1, associated with enhanced HIV-1 expression in lymphocytes (Shaw *et al.*, 1988; Siekevitz *et al.*, 1987), is detected in these cells within 30 min of stimulation and levels peak after 1 to 2 hrs (Hivroz-Burgaud *et al.*, 1991). Therefore if a similar factor requiring protein synthesis had been present in neural cells following cytokine stimulation it would have been detected in the assays performed, assuming it followed similar kinetics of induction.

A further inducible factor suggested to interact with the HIV-1 LTR is AP-1, which is similar to the NF κ B/*rel* proteins in that it does not require protein synthesis and is rapidly activated by agents such as phorbol ester (Franza *et al.*, 1988). There is evidence to suggest that this factor is induced in lymphocytes following physiological activation (Crabtree, 1989) but no indication that it plays an important role in the induction of HIV-1 gene expression (see Chapter 1, Section 1.5.3. iv). In nuclear extracts from neural cells no changes were noted in the mobility or number of protein-DNA complexes formed between Site B/AP-1 sequences present in the HIV-1 LTR (see Figure 1.2) after cytokine stimulation. It is interesting to speculate whether, following recent reports, a competitive interaction also exists between AP-1 and the steroid hormone-like receptor factors that interact with Site B, such that Site B-factors repress activation mediated by AP-1 proteins and prevent the formation of an active AP-1 complex. This would be similar to a novel mechanism described for the regulation of collagenase and AP-1-dependent promoters where ligand-bound retinoic acid receptors inactivate AP-1 complexes and suppress expression (Desbois *et al.*, 1991). This process requires the hormone receptor to be competent in binding its cognate DNA target, a property which Site B or COUP-TF molecules are thought to possess in the absence of a known ligand (Wang *et al.*, 1989). In the chicken ovalbumin promoter COUP-TF activates transcription in conjunction with a second protein, S300-II, and it is possible that either no ligand is necessary or additional protein(s) replace the usual ligand-receptor interaction in this class of steroid hormone receptor-like molecules (Wang *et al.*, 1989).

There are now known to be many proteins that recognize the NF κ B sequence motif, forming a growing family of transcriptional activators with the majority of these protein related to the proto-oncogene product c-Rel which bind DNA as heterodimers or pairs of heterodimers (Baeuerle, 1991; Bours *et al.*, 1992; Ryseck *et al.*, 1992; Hansen *et al.*, 1992; Muchardt *et al.*, 1992). Data relating to the identification of nuclear factors specific for the NF κ B binding site induced in

lymphocytes following PMA stimulation indicates that the polypeptides contributing to these protein-DNA complexes also change with time following stimulation (Molitor *et al.*, 1990). UV-crosslinking experiments demonstrated that proteins of different molecular weights exhibit bi-phasic kinetics of induction; a complex analogous to NF κ B (containing p65 and p50 or p50B) is translocated to the nucleus within 20 min, whereas a second, additional complex consisting of proteins similar to p50 or p50B and *c-rel* [p85] (Schmid *et al.*, 1991) is induced after stimulation for 16 hrs (Molitor *et al.*, 1990). Therefore the NF κ B and *c-rel* containing transcription complexes appear to be subject to differential regulation in human lymphocytes and a similar phenomenon may occur on NF κ B motifs located in other promoters in different human cell lines (Hansen *et al.*, 1992). Therefore the nuclear protein assays performed on neural cells may not have detected all the inducible complexes specific for NF κ B sequences. In addition, a brain-specific transcriptional activator recognizing the NF κ B motif has been identified in neurons and astrocytes of rat grey matter. The relationship of this factor to NF κ B and *rel* has not been precisely determined but in preliminary experiments it appeared distinct from NF κ B (Korner *et al.*, 1989) and certain similarities with the data of Molitor *et al.*, (1990) suggest it may represent a protein equivalent to *c-rel*.

The identity of the nuclear factors recognizing the HIV-1 enhancer may also hold the explanation for the lack of synergism between activating cytokines and Tat in the augmentation of LTR-driven gene expression in most neural cell lines. The idea that Tat-transactivation may become saturated in neural cells by the additional mRNA initiated due to the (presumed) actions of NF κ B-like factors on the HIV-1 LTR may be possible (see Chapter 5, discussion). Recent experiments point to a more complex interaction between Tat and the transcriptional machinery and show that although *c-rel* transactivates HIV-1 LTR-driven reporter gene expression up to 15-fold in hepatoma cells, it does not add to the transactivation effect of Tat (Muchardt *et al.*, 1992).

In addition to inducible enhancer-binding factors, neural cell lines also expressed several constitutive DNA-binding activities which bound to other elements located in the negative regulatory element of the LTR. Potentially interactions from Site B, the octamer and GTT¹-binding factors may contribute to any negative influence this region of the LTR is likely to have on HIV-1 LTR-driven gene expression and viral replication in neural cells. No DNA-binding activity corresponding to the USF factor, responsible for a considerable proportion of the effect of the NRE on viral replication (Lu *et al.*, 1991) and gene expression (Giacca *et al.*, 1992) could be identified for technical reasons. However, constitutive sequence-specific factors may interact with the NFAT-1 region in astrocytoma and SK-N-MC neuroblastoma cells and this region is known to confer an additional significant negative influence on HIV-1 replication in unstimulated lymphoblastoma cells (Lu *et al.*, 1991).

Octamer-binding factors are present in many cell types including those of lymphoid origin (Sive and Roeder, 1986) but it has not previously been reported that the octamer site in the HIV-1 LTR can be protected by nuclear factors, as my data demonstrate. The identity of the factor(s) responsible was not pursued but several octamer-binding proteins, including one identical to the lymphoid specific factor Oct-2, have been identified in brain extracts and a glioma cell line (Korner *et al.*, 1989). No additional information was found on the other neural specific DNA-binding activity, GTT¹, although the related motif in SV40 is known to bind a number of constitutive or cell-specific factors including an Sp1-like molecule (La Thangue and Rigby, 1988).

The functions of the other sequence-specific DNA-binding activities, Sp1 and LBP-1, observed in neural cells, by analogy with other systems, could contribute to the basal level of expression from the HIV-1 LTR (Waterman *et al.*, 1991a). The interaction of these factors, predominantly Sp1, with the LTR is required for

In addition to inducible enhancer-binding factors, neural cell lines also expressed several constitutive DNA-binding activities which bound to other elements located in the negative regulatory element of the LTR. Potentially interactions from Site B, the octamer and GTT^F-binding factors may contribute to any negative influence this region of the LTR is likely to have on HIV-1 LTR-driven gene expression and viral replication in neural cells. No DNA-binding activity corresponding to the USF factor, responsible for a considerable proportion of the effect of the NRE on viral replication (Lu *et al.*, 1991) and gene expression (Giacca *et al.*, 1992) could be identified for technical reasons. However, constitutive sequence-specific factors may interact with the NFAT-1 region in astrocytoma and SK-N-MC neuroblastoma cells and this region is known to confer an additional significant negative influence on HIV-1 replication in unstimulated lymphoblastoma cells (Lu *et al.*, 1991).

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efficient viral replication in both lymphocytic and monocytic cell lines (Gaynor, 1991; Parrot *et al.*, 1991).

Cytokines and the AIDS dementia complex

Fragmentary evidence is beginning to accumulate consistent with the pathological findings observed in the CNS tissue of those suffering symptoms of the AIDS dementia complex supporting the proposal that neural cell infection by HIV may have a direct role in the pathogenesis of ADC. The low frequency of HIV detection in neural cells *in vivo* is not explained by an intrinsic resistance of neural cell lines and primary neural cells to HIV-1 infection *in vitro* (Cheng-Mayer and Levy, 1990). Moreover, the infection of primary brain cell cultures results in a persistent, latent infection in which viral replication and HIV-1 antigen expression is absent (Tornatore *et al.*, 1991) or restricted (Christofinis *et al.*, 1987; Rytik *et al.*, 1991). Along with the discovery of mechanisms that lead to the induction of HIV-1 gene expression and replication (Tornatore *et al.*, 1991) in neural cells, all of these findings suggest that HIV-1 may not be detected by immunocytochemistry or *in situ* hybridization for mRNA in resident neural cells unless activated by interactions with immune cells or through cytokines. The examination of brain explants from AIDS and ADC patients by dual labelling immunocytochemistry after culturing in the presence of TNF α or IL-1 β may provide confirmation of the true extent of neural cell involvement in HIV-1 infection.

Furthermore, the importance of cytokines in ADC is indicated by the finding that the levels of IL-1 β , TNF α and IL-6 are elevated in the CSF of patients suffering symptoms of ADC: in four out of five patients, IL-1 β and IL-6 were detected in CSF samples, although in this study TNF α was not found (Gallo *et al.*, 1989), and Grimaldi *et al.* (1991) reported TNF α to be elevated to more than twice the level in plasma in the CSF of 15 out of 19 patients in the later stages of HIV-1 disease and suffering neurological symptoms of ADC. The levels of TNF α in CSF were

comparable with that necessary to augment HIV-1 replication in lymphocytes (Ito *et al.*, 1989; Matsuyama *et al.*, 1989a) and at ≥ 1 U/ml would transactivate expression significantly from the LTR in several neural cell lines. The measurement of cytokine concentrations in the CSF may not accurately reflect the levels in the CNS parenchyma, as demonstrated by the high levels of TNF α on the surface of astrocytes and macrophages in patients with multiple sclerosis, without significant levels in the CSF (Franciotta *et al.*, 1989; Hofman, 1989). Cell-associated TNF α will also augment HIV-1 LTR-driven gene expression (Tadmori *et al.*, 1991).

The production of cytokines within the CNS may arise from infiltrating immunologically competent lymphoid and myeloid cells (Hamblin, 1988) and endogenous cells of the CNS (see Chapter 1, Section 1.6.2). In addition, HIV-1 gene products, envelope gp120 and the precursor gp160 will induce IL-6 production from CD4+ T lymphocyte (Oyaizu *et al.*, 1991) and IL-1 β , IL-6 and prostaglandin secretion from mononuclear cells (Meltzer *et al.*, 1990b). Whether envelope products will have a similar effect on cytokine production by microglial or astrocyte cells is at present under investigation in the laboratory of Dr. Jean Merrill (Department of Neurology, University of California, Los Angeles). In addition, macrophages have receptors for a number of neuropeptides which can induce the secretion of oxygen metabolites (Goetzl *et al.*, 1989) and the cytokines IL-1 β , TNF α and IL-6 (Lotz *et al.*, 1988), several or all of which can augment HIV-1 replication in macrophages, lymphocytes and glial cells (Ito *et al.*, 1989; Matsuyama *et al.*, 1989a; Matsuyama *et al.*, 1989b; Poli *et al.*, 1990a; Tornatore *et al.*, 1991).

In conclusion, the evidence I have presented here implies that (as with lymphoid and myeloid cells) cytokines, particularly TNF α and IL-1 β , may positively influence the levels of expression from the HIV-1 LTR in infected neural cells. Furthermore these cytokines may function as a signal to induce expression from latent proviral genomes and initiate productive replication within the central nervous system.

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Appendix A: List of Suppliers

Advanced Protein Products, Unit 18H, Premier Partnership
Estate, Lews Road, Briery Hill, West Midlands DY5 3UP.
Foetal calf serum.

American Type Culture Collection (ATCC), Rockville, Maryland, USA.
Cell lines SK-N-SH and SK-N-MC.

Amersham International plc., Aylesbury, Buckinghamshire, UK.
Goat anti-mouse Ig biotin conjugated antibody, human placental RNase
inhibitor, phycoerythrin/streptavidin immunocytochemical reagent, ³²P
Radioisotopes, restriction enzymes.

BDH Laboratory Supplies Limited, Fourways, Carlyn Industrial Estate,
Atherstone, Warwickshire, CV9 1JG.
General chemicals.

Bio-Rad Laboratories limited, Caxton Way, Watford Business Park, Watford,
Hertfordshire WD1 8RP.
Protein assay reagent.

Boehringer Mannheim (Diagnostics & Biochemicals) Limited, Bell Lane, Lewes,
East Sussex, BN7 1LG.
Calf intestinal alkaline phosphatase, *E.coli* tRNA, restriction enzymes.

British Biotechnology Limited, Watlington Road, Cowley, Oxford, OX4 5LY.
Recombinant human IL-1 β .

Camlab limited, Nuffield Road, Cambridge, CB4 1TH.
Phenol and phenol/chloroform.

Chiron Corporation, The Biocine Company, 4560, Horton Street, Emeryville,
California 94608, USA.
HIV-1 molecular clone pARV2.7A.

Costar UK Ltd., Victoria House, 28-38, Desborough Street, High Wycombe, Bucks.
Cell-freezing vials and tissue culture flasks.

Dako Ltd., 16, Manor Courtyard, Hughenden Avenue, High Wycombe, Bucks,
HP13 5RE.
Rabbit anti-cow glial fibrillary acidic protein antibody.

Difco Laboratories,
Bacto-agar and Bacto-tryptone.

Du Pont (UK) limited, Biotechnology Systems Division, NEN Research Products,
Wedgewood Way, Stevenage, Hertfordshire, SG1 4QN.
³H-acetyl Coenzyme A and Econofluor scintillation fluid.

Eastman Kodak limited, Acornfield Road, Knowsley Industrial Park North,
Liverpool L33 7UF.
135 mm transparency and print film, X-ray developer and fixative,
photographic paper Kodabrome F2RC.

Fisons Limited, Bishop Meadow Road, Loughborough, Leicestershire LE11 0RG.
Acrylamide, Butan-1-ol, chloroform, diethyl ether, dimethyl sulphoxide,
iso-amyl alcohol, N,N-methylene bis-acrylamide.

Fluka AG, CH-9470, Buchs.
Hydrazine.

Fuji Photo Film Company (UK) Ltd, 125, Finchley Road, London.
X-ray film.

Genzyme, 75, Kneeland Street, Boston, Massachusetts, MA 02111 USA.
Polyclonal anti-serum to murine TNF α , recombinant human TNF α and
recombinant human IL-6.

Gibco-BRL, PO Box 35, Trident House, Renfrew Road, Paisley, PA3 4EF Scotland.
DNAse I, 1 kb DNA ladder, trypsin-EDTA, glutamine,
penicillin/streptomycin, modification and restriction enzymes.

HT Biotechnology Ltd., Unit 4, 61, Ditton Walk, Cambridge.
Ribonuclease protection assay kit (Ambion Inc.).

Life Sciences Inc., 2900, 72nd Street North, St. Petersburg, Florida, USA.
AMV reverse transcriptase.

May and Baker Ltd, Eccles, Manchester.
Acetic and formic acid.

Medical Research Council AIDS Reagent Project, National Institute for Biological
Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts, EN6 3QG.
ATCC Cell lines glioblastoma U138MG and astrocytoma U373MG and
HIV-1 molecular clone PBH10aR3.

Pharmacia Biosystems limited, Biotechnology Division, Davy Avenue, Knowhill,
Central Milton Keynes, MK5 8PH.
Plasmids pSVL and pCH110, poly (dl:dC)-(dl:dC), restriction enzymes.

Polaroid (UK) Ltd., Ashley Road, St. Albans, Herts.
Type 55 land film.

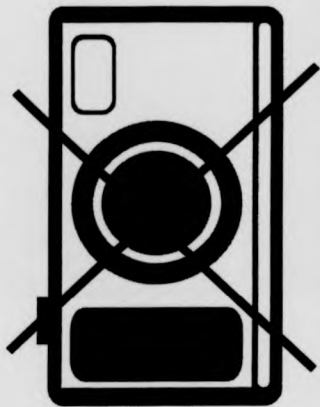
Prolabo, 12, rue Pelée, F70511, Paris, France.
Glycerol.

Promega (UK) limited, Epsilon House, Enterprise Road, Chilworth Research
Centre, Southampton SO1 7NS.
Modification enzymes, RNase-free DNase I, TFIID and CTF/NF1
consensus sequence oligonucleotides.

Sigma Chemical Company Ltd, Fancy Road, Poole, Dorset BH17 7TG.
Agarose, ampicillin, 5'-azacytidine, CAT enzyme, chloramphenicol,
chloroquine, DEAE-dextran, DMEM, ethidium bromide, Ficoll, goat anti-
rabbit Ig FITC antibody, Geneticin, Hepes buffer, (ag)- natural murine IFN α ,
PMA, protease inhibitors: pepstatin A, leupeptin, antipain and PMSF.

Siratagene limited, Cambridge Innovation Centre, Cambridge Science Park, Milton
Road, Cambridge, CB4 4GF.
Plasmids pBSII-KS+, pSV2CAT and pWLneo.

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**Cytokine regulation of human immunodeficiency virus type 1 gene
expression in cells of neural origin**

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AUTHOR **Simon Swingler,**

DEGREE

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