Post transcriptional regulation of HSV gene expression and studies of the mutagenic properties of HSV-1

by

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Summary

During herpes simplex virus (HSV) infection expression of the different classes of viral genes is co-ordinately regulated and sequentially ordered. The three major classes of genes - immediate-early (IE), early and late can be separated on the basis of the kinetics of their expression and requirements for ongoing DNA synthesis. At the core of this regulation are three of the IE proteins IE110, IE175 and IE63. Both IE175 and IE63 are essential for viral growth and IE110 while non-essential does confer growth advantage in cell culture. While much is known about transcriptional control of gene expression and the interplay of these three IE proteins, little is known of any post-transcriptional regulatory mechanisms that may be employed by the virus. Recent interest has focused on this area and the aim of this study has been the investigation of a virus induced factor, termed LPF (late processing factor), which was previously shown to selectively increase the 3' processing efficiency of the herpes simplex virus type-2 (HSV-2) UL38 late poly(A) site. In this study an *in vitro* 3' processing assay was used to examine the 3' processing efficiencies of a selection of herpes simplex virus type-1 (HSV-1) poly(A) sites from the three temporal classes of genes. It was demonstrated that LPF selectively increased processing at the poly(A) sites of two late HSV-1 genes while having no effect on the processing efficiencies of another four poly(A) sites from the IE and early classes of genes. In addition both LPF responsive poly(A) sites were shown to be inherently less efficient 3' processing sites that the non-responsive sites. No common factor, which could be responsible for the reduced

efficiency of these two sites, was identified by sequence or two dimensional structural analysis. Examination of the protein binding properties of each of the test poly(A) site RNAs revealed that three protein bands A, B and C were consistently bound to the poly(A) site RNAs. These proteins were of similar size to components of the 3' processing complex required for efficient cleavage and polyadenylation of precursor mRNAs. In addition the level of protein binding was shown to be increased by HSV infection.

As stated previously the HSV-1 IE63 gene is essential for viral growth and it has been shown by a number of studies to be required for late gene expression. There is evidence that it can exert this influence at both the transcriptional and post-transcriptional levels and also that it may or may not influence late gene expression via an effect on DNA synthesis. In this study IE63 has been shown to be required for LPF activity and the expression of selected true-late genes (UL38, UL44 and US11). IE63 also appears to be required for the efficient expression of three additional genes, two early genes UL29 and UL42 components of the viral DNA synthesis machinery and the IE gene IE110.

The evidence presented here supports a central role for IE63 in the regulation of gene expession. At least part of the mechanism of this regulation is at the post-transcriptional level mediated by its ability to increase the processing efficiency of selected poly(A) sites, with specific deficiencies in individual poly(A) sites making them targets for regulation. It is clear this is not the whole story and that IE63 may exert an additional influence via regulation of viral DNA synthesis.

HSV-1 and HSV-2 can transform mammalian cells to a tumourigenic phenotype and it has been proposed that transformation occurs via a hit-and-run mechanism since the continued presence of viral DNA or proteins is not required for the maintenance of the transformed state. One way in which such a hit-and-run mechanism might operate is by increasing the frequency of mutations and there is evidence that HSV-1 can act as a mutagen. The aim of the second part of this study was to identify the properties of HSV-1 which could induce such mutations using a mutagenesis assay based on the shuttle vector pZ189. This study was the continuation of work initiated in the Institute by P. Clarke and the first step was to increase the efficiency of transformant recovery in the assay. To this end each aspect - plasmid preparation, transfection, infection and tranformation was optimized in turn and transformant recovery increased 3 to 5-fold. Using this assay HSV-1 infection was shown to increase the mutation frequency by 2.5-fold, however this was not considered to be significantly different from the spontaneous mutation frequency and further use of the assay to determine the mutagenic properties of HSV-1 was not pursued.

Abbreviations

A	- adenine
ATP	- adenosine-5'-triphosphate
BHK	- baby hamster kidney
bp	- base pair
BSA	- bovine serum albumin
C	- cytosine
CAV	- cell associated virus
CAT	- chloramphenicol acetyl transferase
cDNA	- complementary DNA
Ci	- curie
CIP	- calf intestinal phosphatase
cm	- centimetre
cpm	- counts per minute
cps	- counts per second
CPSF	- cleavage and polyadenylation specificity factor
CRV	- cell released virus
CstF	- cleavage stimulation factor
C-terminal	- carboxy terminal
CTP	- cytidine-5'-triphosphate
dATP	- 2'-deoxyadenosine-5'-triphosphate
dCTP	- 2'-deoxycytosine-5'-triphosphate
dl	- deletion
DMSO	- dimethylsulphoxide
dNTP	- 2'-deoxyribonucleoside-5'-triphosphate
dNDP	- 2'-deoxyribonucleoside-5'-diphosphate
dGTP	- 2'-deoxyguanosine-5'-triphosphate
DSE	- downstream element
DTT	- dithiothreitol
dTTP	- 2'-deoxythymidine-5'-triphosphate
dUTP	- 2'-deoxyuridine-5'-triphosphate
dUMP	- 2'-deoxyuridine-5'-monophosphate
E. coli	- Escherichia coli
EDTA	- sodium ethylenediamine tetra-acetic acid
G	- guanine
gm	- gram
GM	- growth medium
GSHV	- ground squirrel hepatitis virus
GTP	- guanosine-5'-triphosphate
h	- hour(s)
HBS	- hepes buffered saline
Hepes	- 4-(-2-hydroxyethyl)-1-piperazine ethane
HIV	- human immunodeficiency virus

hnRNP - heterogeneous nuclear ribonucleoprotein particle
HRP - horse radish peroxidase
HSV - herpes simplex virus
HSV-1 - herpes simplex virus type-1
HSV-2 - herpes simplex virus type-2
HTLV-1 - human T-cell lymphotropic virus
ICP - infected cell protein
IE - immediate-early
INF - HSV infected
in - insertion
kb, kbp - kilobase pair(s)
kD - kilodalton
KV - kilovolts
L - litre
LacZ - β-galactosidase gene
LAT - latency associated transcript
LB - loading buffer
LMT - low melting temperature
LPF - late processing factor
M - molar
mg - milligram(s)
MI - mock infected
min - minute(s)
mJ - millijoule
ml - millilitre(s)
MLTU - major late transcription unit
mm - millimetre(s)
mM - millimolar
moi - multiplicity of infection
mRNA - messenger RNA
msec - millisecond(s)
mtr - morphological transforming region
NBCS - new born calf serum
ng - nanogram
nm - nanometre
NPT - non-permissive temperature
NP40 - nonidet P40
nt - nucleotide
N-terminal - amino terminal
NTP - nucleoside-5'-triphosphate
OD - optical density
ORF - open reading frame
ori - origin of replication
PABP - poly(A) binding protein
PABII - poly(A) binding protein II
PAP - poly(A) polymerase

PBS	- phosphate buffered saline
PCR	- polymerase chain reaction
PCV	- pack cell volume
PEG	- polyethylene glycol
pfu	- plaque forming unit(s)
PK	- proteinase K
pi	- post infection
pmol	- picomole(s)
PMSF	- phenylmethyl sulphonyl fluoride
Poly(A)	- polyadenylation
R	- purine
RNase	- ribonulcease
rNDP	- ribonucleoside diphosphate(s)
RNP	- ribonucleoprotein particle
rpm	- revolutions per minute
RT	- room temperature
SDS	- sodium dodecyl sulphate
sec	- second(s)
snRNP	- small nuclear ribonucleoprotein particle
SV40	- simian virus 40
T	- thymidine
TEMED	- N,N,N,N,- tetramethylethylene diamine
TK	- thymidine kinase
αTIF	- alpha transinducing factor
TMPD	- 2,6,10,14-tetramethylpentadecane
tRNA	- transfer RNA
ts	- temperature sensitive
U	- uridine
USE	- upstream element
UTP	- uridine-5'-triphosphate
UTR	- untranslated region
UV	- ultraviolet
VHS	- virion host shut off
Vmw	- virion-specific polypeptide of apparent molecular weight (X)
VP	- virion protein
VZV	- varicella-zoster virus
v:v	- volume : volume
wt	- wild type
w:v	- weight : volume
Y	- pyrimidine
µg	- microgram
µl	- microlitre
µM	- micromolar
µCi	- microcurie

Contents

Chapter 1

Introduction

1.1 Herpes simplex virus type-1 and type-2

The large family of Herpesviridae consists of more than 80 distinct herpesviruses isolated from a wide variety of animal species. Herpes simplex virus type-1 (HSV-1) and type-2 (HSV-2) are part of a small group of human herpesviruses of which seven have been isolated to date. These include the well characterised Epstein-Barr virus (EBV), human cytomegalovirus (HCMV) and varicella-zoster virus (VZV) plus the more recently identified and less well characterised human herpes virus type-6 and type-7 (HHV-6 and HHV-7). The main focus of this study are the herpes simplex viruses and the aim of this Introduction is therefore to give a general understanding of the properties of HSV-1 and HSV-2.

Herpes simplex virus (HSV) infections constitute an extremely prevalent communicable disease in humans. There are a range of clinical manifestations of infection from localised ulcerative vesicular lesions of the lips, nasal membranes or genital skin, to more severe but thankfully rarer infections such as keratoconjunctivitis, encephalitis, eczema, respiratory tract and gastro-intestinal tract involvement. Transmission of HSV occurs via close contact with virus at the mucosal membranes or skin break, in either the oral or genital area. Primary infection is normally localised and self-limiting in the immunocompetent individual, however severe infections of longer duration, which

can also be disseminated, occur in the immunocompromised or immunologically immature individual (Whitely, 1985). In general HSV-1 is the predominant cause of oral infections and HSV-2 is responsible for genital infection, however a variable proportion of genital infections are due to HSV-1.

Following initial infection with HSV, the virus rapidly enters the peripheral nerve endings and is transported to the neuronal cell bodies of the sensory ganglia where the virus assumes a life-long latent state (reviewed by Rock, 1993). The latent virus can be reactivated at widely varying intervals and is transported ante-retrograde along the sensory axon from the ganglion back to the epithelial cells. In general recurrent disease is of shorter duration and milder than the primary infection.

1.1.1 Morphology

All members of the Herpesviridae, including HSV-1 and HSV-2, show the same morphology, which is the primary basis of identification. Each virion is between 120-200nm in diameter and is made up of the following four components (reviewed by Rixon, 1993) (Figure 1.1).

The core was originally thought to be composed of a fibrillar spool around which the viral DNA was wrapped. However recent studies suggest that there is little or no protein present in the core and that the volume is taken up entirely by DNA, the liquid state of which gives the featureless appearance seen by electron microscopy (Booy *et al.*, 1991).

The capsid is an icosahedral structure (100-110nm in diameter) which surrounds the core and is composed of 150 hexameric and 12 pentameric capsomeres.

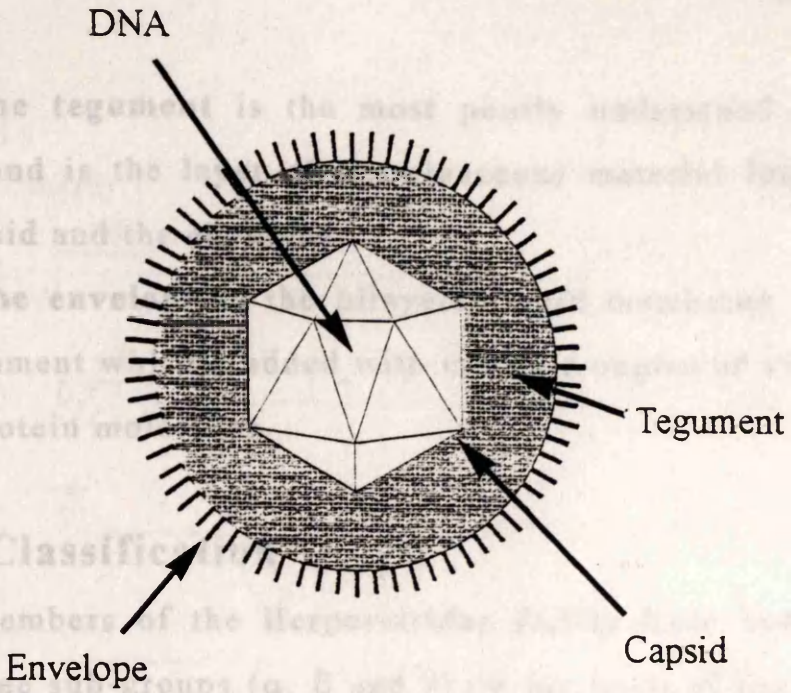


FIGURE 1.1:

Representation of the virion morphology of the Herpesviridae, showing the relative positioning of the outer envelope layer containing glycoprotein molecules, the tegument and the capsid surrounding the central core of double stranded DNA.

The tegument is the most poorly understood part of the virion and is the layer of proteinaceous material found between the capsid and the envelope.

The envelope is the bilayered lipid membrane surrounding the tegument which is studded with multiple copies of virus encoded glycoprotein molecules.

1.1.2 Classification

Members of the Herpesviridae family have been classified into three sub-groups (α, β and γ) on the basis of their biological properties. The other main system of classification is based on the arrangement of reiterated DNA sequences within the virus genome, with the Herpesviridae being divided in to six sub-groups on this basis (A to F) (Figure 1.2). The biological properties of each newly isolated herpesvirus are easy to establish and this method has therefore been adopted as the dominant criterion for classification (reviewed by Roizman, 1993).

(a) Alpha herpesviruses

Alpha herpesviruses can have either a narrow or wide host range both *in vivo* and *in vitro*. The reproductive cycle is characteristically short (<24h) with rapid spread of infection in cell culture resulting in the mass destruction of susceptible cells. Latent infection can be established and is frequently but not exclusively in sensory ganglia. Included in this group are HSV-1, HSV-2, bovine herpes virus-2 and VZV.

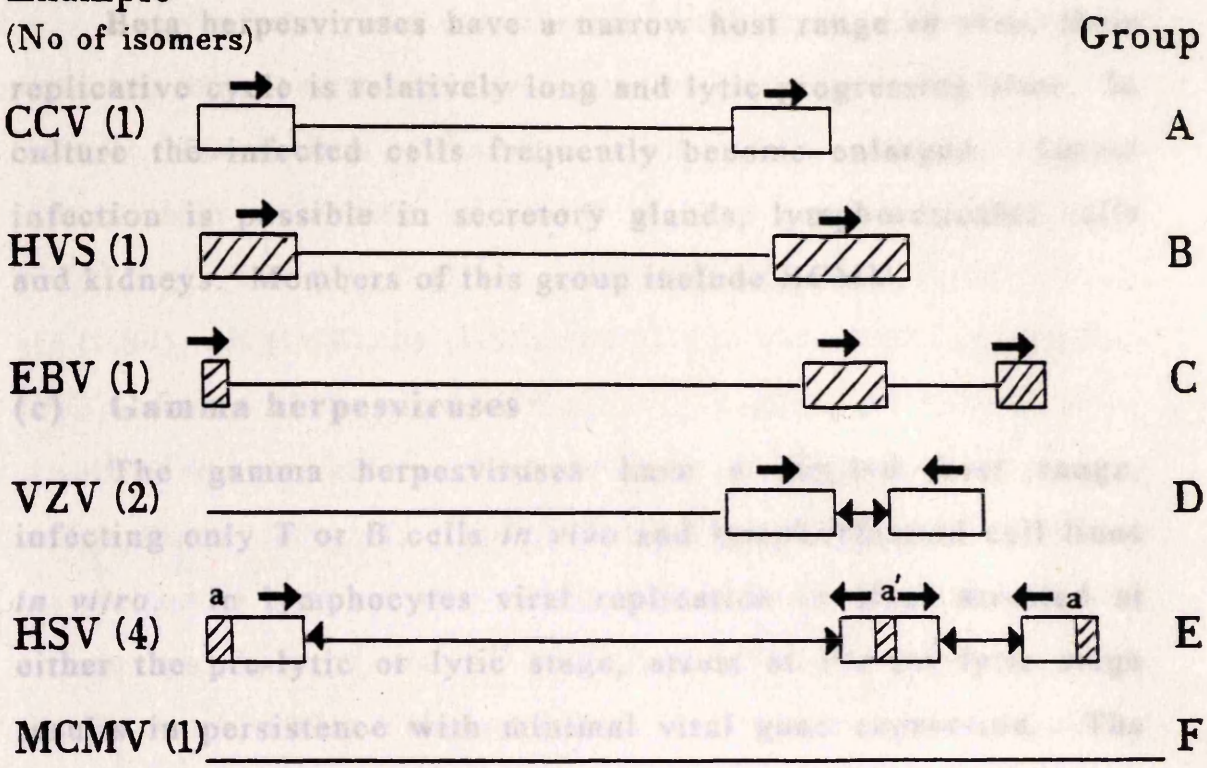


FIGURE 1.2:

A schematic diagram of the DNA sequence arrangements of the six classes (A-F) of genomes of the Herpesviridae. Lines represent unique sequences and arrowed lines show possible inversions. Repeats of >1kb are represented by open boxes and small reiterated sequences are represented by lined boxes. Arrows above the boxes denote whether the repeats are direct or inverted. The relative positioning of the *a* sequences are indicated. The number of possible isomers are given in parenthesis. CCV : channel catfish virus, HVS : herpesvirus saimiri, EBV : Epstein-Barr virus, VZV : varicella-zoster virus, HSV : herpes simplex virus, MCMV : murine cytomegalovirus. (adapted from Roizman, 1993).

(b) Beta herpesviruses

Beta herpesviruses have a narrow host range *in vivo*, their replicative cycle is relatively long and lytic progression slow. In culture the infected cells frequently become enlarged. Latent infection is possible in secretory glands, lymphoreticular cells and kidneys. Members of this group include HCMV.

(c) Gamma herpesviruses

The gamma herpesviruses have a limited host range, infecting only T or B cells *in vivo* and lymphoblastoid cell lines *in vitro*. In lymphocytes viral replication is often arrested at either the pre-lytic or lytic stage, arrest at the pre-lytic stage results in persistence with minimal viral gene expression. The length of the reproductive cycle is variable and latent infection is frequently demonstrated in lymphoid tissue. A classic example of this grouping is EBV.

1.1.3 The virus life cycle

HSV infection of mammalian cells, the subsequent replication of the virus with production of numerous progeny and the ultimate lysis of the host cell takes a relatively short time (18-20h in fully permissive tissue culture). This time span does not reflect the complex and incompletely understood nature of the series of steps required for this occurrence. The following section gives a brief outline of the viral replicative cycle as we understand it to date (reviewed by Roizman and Sears, 1993).

(a) Entry

Entry of the virus into the host cell occurs in two phases, viral attachment with rapid transition to penetration of virus into the cell. The surface of the virus is covered by multiple copies of glycoprotein molecules, of which 10 have been identified : gB (UL27), gC (UL44), gD (US6), gH (UL22), gK (UL53), gL (UL1), gG (US4), gE (US8), gJ (US5) and gI (US7) (reviewed by Spear, 1993). Entry of the virus is thought to require the sequential or simultaneous interaction of the virion envelope glycoproteins with cell surface receptors (Fuller and Lee, 1992). The initial interaction is between the virion glycoprotein C molecules and heparan sulphate on the cell surface (McClain and Fuller, 1994; Herold *et al.*, 1991). The subsequent interaction of glycoproteins B, H and D with the cell surface results in stable attachment of the virion and fusion with the plasma membrane and finally release of the nucleocapsid into the cell cytoplasm (Cai *et al.*, 1988; Fuller and Spear, 1987; Desai *et al.*, 1988). Both glycoproteins B and C are capable of binding to the cell surface heparan sulphate molecules (Fuller and Lee, 1992; Herold *et al.*, 1991; Kuhn *et al.*, 1990), however other cell surface receptors involved in this process remain to be identified. The precise roles of these glycoproteins are not known and it is probable that other surface glycoproteins are also involved in the process. Two likely candidates are glycoprotein K, which is thought to play a role in controlling virus-induced cell fusion and glycoprotein L which forms a heterodimer with glycoprotein H an association which appears to be required for the processing and intracellular transport of both the glycoprotein H and glycoprotein L proteins (Hutchinson *et al.*, 1992). Although virus entry of the cell can

occur by endocytosis this route is thought to be non-productive and results in the destruction of the virus in the intracellular lysosomes (Spear, 1993).

(b) Release of viral DNA

The now de-enveloped virus capsid is transported to the nuclear pores via the cellular cytoskeleton where its DNA is released into the nucleus of the cell. Release of the viral DNA requires a viral protein, thought to be the product of the UL36 gene (Batterson *et al.*, 1983). In addition, there is a requirement for certain virion components to make their way into the nucleus. Host macromolecular synthesis is subsequently shut off by the virion host shut off factor (VHS), a component of the virion tegument, whilst transcription of viral IE genes, by the cellular RNA polymerase II, is induced by the virion tegument protein Vmw65 (VP16, αTIF, UL48).

(c) Viral gene expression

Transcription of viral DNA by host cell RNA polymerase II takes place in the nucleus of the cell, the mature transcripts are transported to the cytoplasm where protein synthesis is carried out. A detailed description of the co-ordinate regulation of HSV gene expression (Honess and Roizman, 1974; Clements *et al.*, 1977; Watson *et al.*, 1979) is given in Section 1.1.6.

(d) Viral DNA synthesis

Viral DNA synthesis occurs in the host cell nucleus, can be detected from 3h post infection (pi) and continues for at least another 9-12h pi. DNA is replicated by a rolling circle

mechanism producing head-to-tail concatamers of unit-length viral DNA, which are cleaved and packaged into pre-formed capsids. The viral proteins involved in the process of DNA synthesis and their various functions are discussed in Section 1.1.8.

(e) Capsid assembly

Three basic capsid forms are evident from electron microscope examination of HSV infected cells (reviewed by Rixon, 1993). 'A' capsids which have no internal structure (Gibson and Roizman, 1972; Cohen *et al.*, 1980), 'B' intermediate capsids which lack viral DNA but possess a core consisting of a second proteinaceous layer inside the outer capsid shell (Sherman and Bachenhiemer, 1988; Rixon *et al.*, 1988), and 'C' full capsids containing the viral genome. 'B' capsids are thought to be the progenitors of the fully mature 'C' capsids, with the core proteins of the B capsids being lost prior to DNA packaging. The additional proteins found in 'B' capsids are thought to have protease and scaffolding functions (Rixon, 1993), and 'A' capsids are generally thought to be the end products of defective packaging (Schrag *et al.*, 1989) The proteins encoded by the HSV-1 genes that have been identified as components of the virus capsid are as follows : UL19, UL38, UL26.5, UL26, UL18 and UL35, with the UL26 and UL26.5 proteins only present in the 'B' capsids. Recently Tatman *et al.*, (1994) demonstrated, using a baculovirus expression system, that the polypeptide products of UL19, UL38 and UL18 genes (VP5, VP19C and VP23) are required for formation of the major structural components of the outer capsid shell, with UL26 and UL26.5 encoding proteins involved in scaffold formation. However the exact relationship of

the three capsid forms and their roles in assembly and DNA packaging remain to be clarified.

(f) Envelopment and viral egress

Different theories exist regarding the mechanisms by which the virus capsid becomes enveloped and leaves the cell (reviewed by Rixon, 1993). The first, and undisputed stage, is budding of the capsid through the inner nuclear membrane, the virus then follows one of two possible routes. (a) The newly acquired envelope fuses with the outer nuclear membrane, or with the contiguous endoplasmic reticular membrane and the capsid is released into the cytoplasm. Envelopment of the capsid ocurring by budding through a cytoplasmic membrane (Cheung *et al.*, 1991; Whealy *et al.*, 1991). (b) The envelope acquired in the first step is retained and the virion enters the cytoplasm in a vacuole formed by the outer nuclear membrane. Both theories suggest that final maturation of the virion is achieved in the golgi with egress of the virion by exocytosis from golgi-derived vesicles. Much of the information available on capsid envelopment is based on electron microscopic examination of virus infected cells, which have been treated with Brefeldin A, which results in dissassembly of the golgi complex. The results of these experiments show that Brefeldin A inhibits the formation of viral particles without affecting nucleocapsid formation, with aberrant accumulation of enveloped virions within the perinuclear cisternae and rough endoplasmic reticulum, lending support to theory (a) (Eggers *et al.*, 1992; Cheung *et al.*, 1991; Whealy *et al.*, 1991). There is as yet no clear evidence to indicate whether the final stage of

tegumentation takes place in the nucleus or the cytoplasm of the cell.

(g) Light-particles

Cells infected with HSV-1 and a number of other α herpesviruses release not only virions but in addition non-infectious virion related particles, termed light or L-particles (Szilagyi and Cunningham, 1991; McLauchlan and Rixon, 1992). In cell culture infected cells release mature virions and L-particles in approximately equal amounts. L-particles are composed of tegument surrounded by envelope components but lack capsid and viral DNA. The pathway of L-particle formation is unclear, however the ability of L-particles to attach to and fuse with cells and release tegument proteins has been shown to be similar to wild type (wt) virus (McLauchlan *et al.*, 1992a; Rixon *et al.*, 1992), this would suggest that virions and L-particles are produced by related pathways. Although L-particles have not been shown to occur *in vivo*, equivalent structures have been demonstrated in infections with other α herpesviruses (McLauchlan and Rixon, 1992), the role of L-particles could therefore constitute a general feature of herpesvirus infections. L-particles have been demonstrated to deliver two tegument proteins, Vmw65 and VHS, to the cell cytoplasm with an efficiency equal to the wt virion (McLauchlan *et al.*, 1992a). A possible advantage of L-particles *in vivo* would be to increase the supply of tegument-derived helper functions thereby enhancing the infectious process.

(h) Virion host shut off (VHS)

During HSV-1 or HSV-2 infection of permissive cells, viral gene expression and polypeptide synthesis occurs against a background of declining host macromolecular synthesis and inhibition of host DNA synthesis (reviewed by Fenwick, 1984). The efficiency with which host shut off occurs varies between the two virus strains, HSV-2 in general is more efficient than HSV-1, with effects being seen from 2-4h pi. There are two distinct phases of host protein synthesis suppression (Kwong *et al.*, 1988), the initial phase, dissociation of mRNA from the polysomes, is mediated by a virion tegument component, the product of the UL41 gene (Kwong *et al.*, 1988; McGeoch *et al.*, 1988). This first phase does not require viral gene expression, having been shown to occur in cells infected in the presence of actinomycin D (preventing viral gene expression), and in cells infected with UV-irradiated virus (Kwong and Frenkel, 1987). The second mechanism, reduction of the remaining levels of protein synthesis, similarly does not require viral gene expression and results in the degradation of mRNA to non-hybridizable fragments and a decline in DNA synthesis (Fenwick and Clark, 1982; Fenwick, 1984). Kwong *et al.*, (1988), have mapped virus mutations which affect both phases of shutoff of host protein synthesis to a single 265bp region of the virus genome and propose therefore that both these activities are mediated by a single viral gene (UL41). This function has been shown to shorten both cellular and viral mRNA half lives (Strom and Frenkel, 1987) and while VHS is not essential for virus growth in cell culture it appears to confer growth advantage to the wt virus.

(i) Latency

A significant and as yet incompletely understood property of herpesviruses is their ability to establish latent infections in their natural hosts (reviewed by Rock, 1993, Fraser *et al.*, 1992). The α herpesviruses are neurotropic and can be maintained in the non-dividing cells of the neurons and therefore are not required to replicate to maintain their latent state. The normal infective cycle consists of viral replication at the site of primary infection followed by axonal transport of the virus to the neuronal cell bodies. After acute ganglionic infection subsides virus persists in the neuron, during this time infectious virus is not recoverable from ganglionic homogenate. Reactivation of the virus can be induced by a variety of stimuli, stress, UV irradiation, nerve root section, trauma at the site of primary infection, hyperthermia or explant culture of ganglia. During reactivation the virus is transported centrifugally via the nerve cell axon to the original site of infection.

In latency the virus genome is present in a non-integrated episomal state (Mellerick and Fraser, 1987). Restricted viral transcription has been demonstrated in the latently infected neurons of rabbits, mice and humans. Three collinear latency associated transcripts (LATs) of 2.0 kb, 1.5 kb, and 1.45kb have been detected by Northern analysis of RNA isolated from latently infected ganglia (Spivack and Fraser, 1987, Stevens *et al.*, 1987, Wagner *et al.*, 1988), the 2.0 kb transcript can also be detected in productively infected non-neuronal cells (Spivack and Fraser, 1987, Stevens *et al.*, 1987), and recently an approximately 8kb RNA species homologous to the LAT region has been observed in productively infected cells (Zwaagstra *et al.*, 1991; Devi-Rao *et*

al., 1991). The LATs map to the long repeat region of the viral genome, are anti-parallel to and overlap the 3' terminus of the immediate early IE110 gene (Figure 1.3). The small predominantly nuclear poly(A)$^-$ RNAs are related by splicing (Spivack *et al.*, 1991), and are thought by some to represent stable introns of the larger 8kb transcript (Farrell *et al.*, 1991, Rader *et al.*, 1993). To date expression of LAT related HSV-1 proteins has not been convincingly demonstrated and it is possible that the LATs are functional RNAs which do not encode proteins. A number of LAT deletion mutant viruses have been used to study the function of these transcripts however the exact role of the LATs in latency has not been established. The general consensus of opinion is that the LATs are not required for the establishment or maintenance of latent infection but may play a role in reactivation from latency (Block *et al.*, 1990; Dobson *et al.*, 1989; Lieb, 1989a; Javier, *et al.*, 1988; Deshmane *et al.*, 1993; Hill *et al.*, 1990). There is some conflicting *in vitro* and *in vivo* evidence emerging to suggest that the LATs may be involved in both of these functions (Sawtell and Thompson, 1992). Differences in the reactivation phenotypes of the LAT mutant viruses appears to be related to the extent and positioning of the deletions involved. Deletion mutants missing a 168bp region upstream of the putative promoter (Batchelor and O'Hare, 1990) and the TATA element (Nicosia *et al.*, 1993), in which no LAT transcripts were detectable, showed normal explant reactivation (Block *et al.*, 1990). Whereas deletion of the promoter and portions of the 5' region of the major 2kb LAT transcript, produced viruses which were unable to reactivate (Block *et al.*, 1993) or reactivate poorly (Hill *et al.*, 1990) from the latent state. It was initially proposed

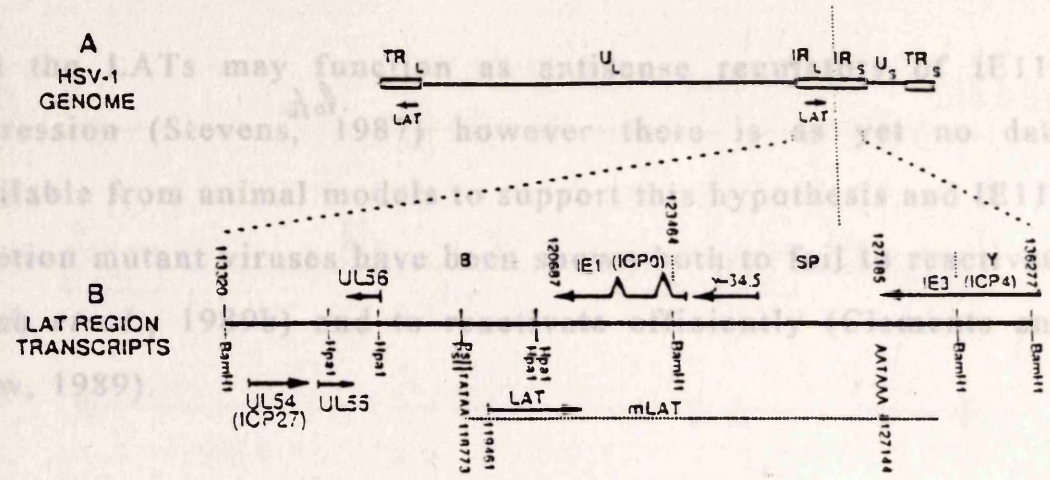


FIGURE 1.3:

The LAT region of HSV-1, (taken from Fraser *et al.*, 1992).

A : conventional prototype map of the HSV-1 genome, showing the position and orientation of the latency associated transcripts (LATs) within the genome.

B : The LAT region, showing the positions (in bp) of the smaller LAT transcripts (LAT), the larger transcript (mLAT), the adjacent genes UL54, UL55 and UL56, the overlapping IE110, the IE175 gene and neurovirulence factor γ 34.5 (RL1), relative to the 152kb genome. The TATA box of the promoter is located at position 118773.

that the LATs may function as antisense regulators of IE110 expression (Stevens, *et al.*, 1987) however there is as yet no data available from animal models to support this hypothesis and IE110 deletion mutant viruses have been shown both to fail to reactivate (Lieb *et al.*, 1989b) and to reactivate efficiently (Clements and Stow, 1989).

(j) Transformation

The effect of HSV infection on cellular transformation and the association of HSV with cervical cancer will be discussed in Chapter 5.

1.1.4 Genome structure

The HSV genome is approximately 150kb in length and has a G+C content of 68% (HSV-1) or 69% (HSV-2) (Roizman and Sears, 1993). It consists of two covalently linked components designated L (long) and S (short). Each segment contains unique sequences (U_L and U_S) flanked by a pair of repeat sequences, one of which is terminal (TR_L and TR_S) and the other internal (IR_L and IR_S). The molecule contains regions of terminal redundancy, *a* sequences, which vary in size from strain to strain (400bp in HSV-1), one or more of which are located internally at the L/S joint *a'* and lie in the opposite orientation to the terminal *a* sequences (Sheldrick and Berthelot, 1975) (Figure 1.4). In addition to the *a* sequences and inverted repeats HSV-1 DNA contains a number of small tandemly reiterated sequence elements varying in copy number (McGeoch *et al.*, 1988). Preparations of HSV virion DNA contain equivalent amounts of four isomers (Figure 1.4) which differ in the orientation of the L and S

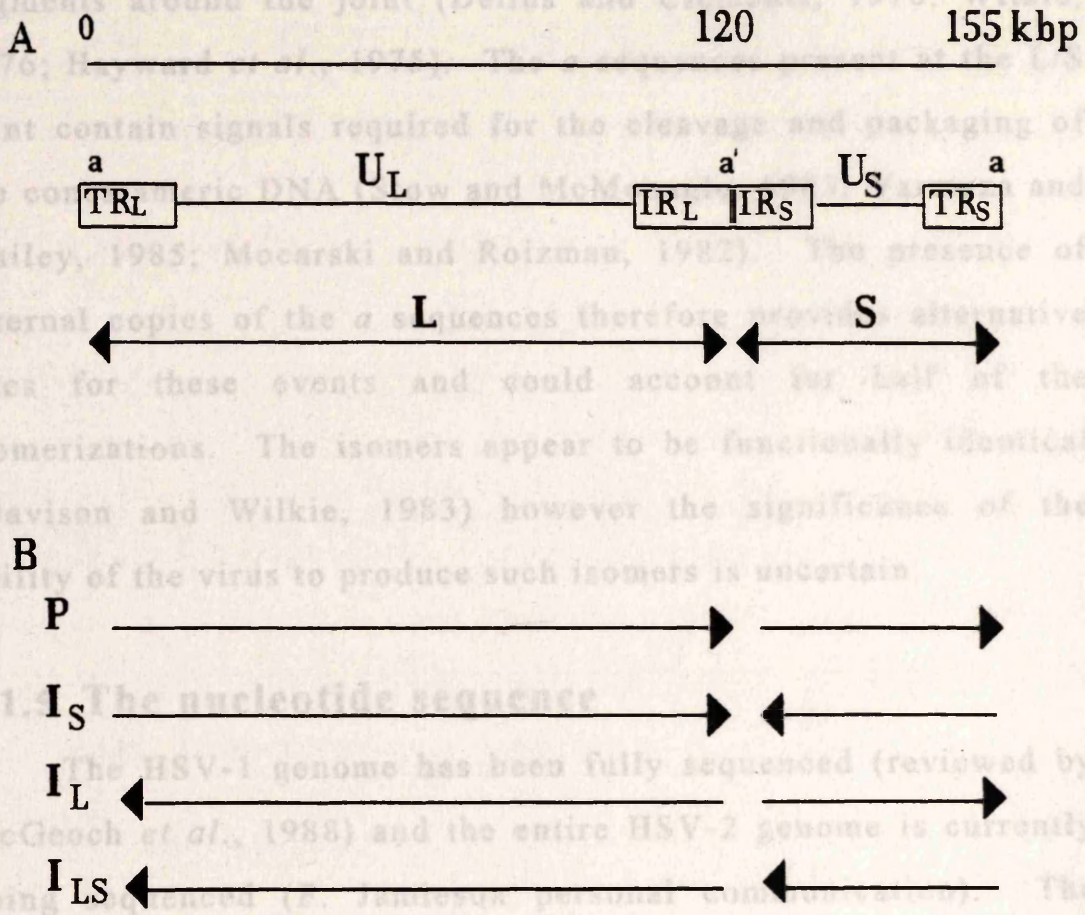


FIGURE 1.4:

Shows the structure of the HSV-1 genome.

A : the unique sequences are illustrated as solid lines (U_L and U_S), the repeats TR_L, IR_L, IR_S and TR_S are illustrated as boxes, showing the relative positions of the *a* sequences.

B : the isomers of HSV-1. P (prototype), I_S (S inverted with respect to P), I_L (L inverted with respect to P), I_{LS} (L and S inverted with respect to P).

segments around the joint (Delius and Clements, 1976; Wilkie, 1976; Hayward *et al.*, 1975). The *a* sequences present at the L/S joint contain signals required for the cleavage and packaging of the concatameric DNA (Stow and McMonagle, 1983; Varmuza and Smiley, 1985; Mocarski and Roizman, 1982). The presence of internal copies of the *a* sequences therefore provides alternative sites for these events and could account for half of the isomerizations. The isomers appear to be functionally identical (Davison and Wilkie, 1983) however the significance of the ability of the virus to produce such isomers is uncertain.

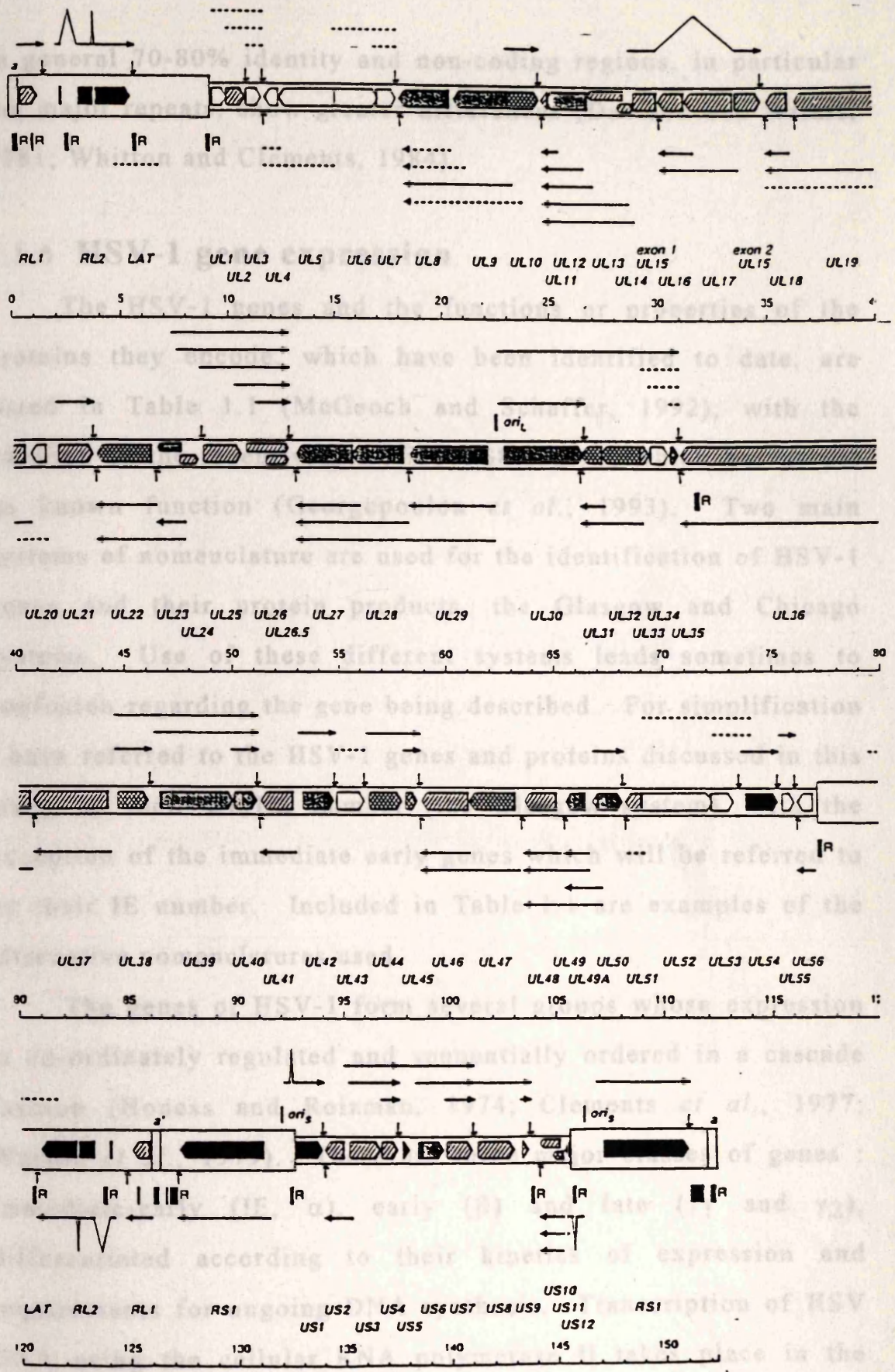
1.1.5 The nucleotide sequence

The HSV-1 genome has been fully sequenced (reviewed by McGeoch *et al.*, 1988) and the entire HSV-2 genome is currently being sequenced (F. Jamieson personal communication). The HSV-1 genome is estimated to encode at least 74 unique genes, three of which are present in the inverted repeat regions and are therefore duplicated. The U_L region contains 58 genes and the U_S region 12 genes with an additional 2 genes in the U_L inverted repeats and one gene in the U_S inverted repeats (McGeoch *et al.*, 1985; 1986; 1988). A diagram of the HSV-1 genome map taken from the study of McGeoch and Schaffer (1992), is shown in Figure 1.5. In general transcripts start close upstream of the ORFs and terminate adjacent to poly(A) sequences located either immediately downstream of the ORF to be expressed or further downstream after another similarly orientated ORF or ORFs.

The HSV-1 and HSV-2 genomes are generally similar (Kieff *et al.*, 1971; Kudler *et al.*, 1983), 50% of their sequences exhibit homology and the coding sequence of corresponding genes show

FIGURE 1.5:

Diagrammatic representation of the genetic content of HSV, (adapted from McGeoch and Schaffer, 1992), showing the scale of the DNA sequence in kbp. The location and direction of proposed protein coding reading frames are indicated by broad arrows : Immediate-early (IE, α) genes ; early (β) genes ; early-late (γ_1) ; and true late (γ_2) genes ; genes of unknown class are white. The gene designations are given below the relevant open reading frames (ORFs) and polyadenylation sites are indicated by small vertical arrows. RNA transcripts are shown as thin arrows, reasonably well characterised transcripts are shown as solid lines and poorly characterised transcripts or hypothetical transcripts are shown as dashed lines. Positions of major families of short tandem reiterations are shown as filled boxes beneath the gene layouts and are marked "R". *ori* ; origin of replication.


RL1 RL2 LAT UL1 UL3 UL2 UL4 UL5 UL6 UL7 UL8 UL9 UL10 UL12 UL13 UL11 exon 1 UL15 UL14 UL16 UL17 exon 2 UL15 UL18 UL19
0 5 10 15 20 25 30 35
ori_L
UL20 UL21 UL22 UL23 UL24 UL25 UL26 UL26.5 UL27 UL28 UL29 UL30 UL31 UL32 UL34 UL33 UL35 UL36
40 45 50 55 60 65 70 75 80
UL37 UL38 UL39 UL40 UL41 UL42 UL43 UL44 UL45 UL46 UL47 UL48 UL49 UL49A UL50 UL51 UL52 UL53 UL54 UL55 UL56
80 85 90 95 100 105 110 115
ori_S
ori_S
a'
a
LAT RL2 RL1 RS1 US1 US2 US3 US4 US5 US6 US7 US8 US9 US10 US11 US12 RS1
120 125 130 135 140 145 150

in general 70-80% identity and non-coding regions, in particular the major repeats, show greater differences (Davison and Wilkie, 1981; Whitton and Clements, 1984).

1.1.6 HSV-1 gene expression

The HSV-1 genes and the functions or properties of the proteins they encode, which have been identified to date, are listed in Table 1.1 (McGeoch and Schaffer, 1992), with the addition of the recently identified US8.5 gene, which has as yet no known function (Georgopoulou *et al.*, 1993). Two main systems of nomenclature are used for the identification of HSV-1 genes and their protein products, the Glasgow and Chicago systems. Use of these different systems leads sometimes to confusion regarding the gene being described. For simplification I have referred to the HSV-1 genes and proteins discussed in this study by their UL/US number (the Glasgow system), with the exception of the immediate early genes which will be referred to by their IE number. Included in Table 1.1 are examples of the alternative nomenclatures used.

The genes of HSV-1 form several groups whose expression is co-ordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman, 1974; Clements *et al.*, 1977; Watson *et al.*, 1979). There are three major classes of genes : immediate-early (IE, α), early (β) and late (γ_1 and γ_2), differentiated according to their kinetics of expression and requirements for ongoing DNA synthesis. Transcription of HSV DNA using the cellular RNA polymerase II takes place in the nucleus of the cell, HSV mRNAs are similar to host cell mRNAs in capping, methylation and polyadenylation, however very few

TABLE 1.1 :

A list of HSV-1 genes, indicating the function of the proteins they encode (where known) and whether the gene is essential (e) or non-essential (ne) for viral growth in tissue culture, (taken from McGeoch and Schaffer, 1993).

TABLE 1.1 :

HSV-1 gene	Protein/Function (alternative nomenclature)	Status
a	400bp terminal redundancy : sequence contains signals for processing/packaging of nascent DNA	e
RL1	Neurovirulence factor (ICP34.5 γ 34.5)	ne
RL2	IE 110 (ICP0; Vmw110; α0, IE1)	ne
LAT	Latency associated transcripts	ne
UL1	Glycoprotein L	e?
UL2	Uracil-DNA glycosylase	ne
UL3	Unknown	ne
UL4	Unknown	ne
UL5	Component of the DNA helicase- primase complex	e
UL6	Role in virion morphogensis (VP11-12?)	e
UL7	Unknown	-
UL8	Component of the DNA helicase- primase complex	e
UL9	Ori-binding protein	e
UL10	Probable integral membrane protein	ne
UL11	Myristylated tegument protein ; role in envelopment and transport of nascent virions	ne
UL12	Deoxyribonuclease ; role in maturation and packaging of nascent DNA virions	e
UL13	Tegument protein (VP18.8)	ne
UL14	Unknown	-
UL15	Unknown; possible NTP-binding motifs	e?
UL16	Unknown	ne
UL17	Unknown	ne
UL18	Capsid protein (VP23)	-
UL19	Major capsid protein (VP5)	e
UL20	Integral membrane protein; role in egress of nascent virions	e/ne

TABLE 1.1 continued

UL21	Unknown	-
UL22	Glycoprotein H	e
UL23	Thymidine kinase	ne
UL24	Unknown	ne
UL25	Virion protein	e
UL26	Capsid protein (VP24) ; proteinase	e
UL26.5	Internal protein of immature capsids (VP22a)	e?
UL27	Glycoprotein B	e
UL28	Role in capsid maturation/DNA packaging	e
UL29	ssDNA binding protein (ICP8)	e
OriL	Origin of replication	ne
UL30	Catalytic subunit of DNA polymerase	e
UL31	Unknown	-
UL32	Unknown	-
UL33	Role in capsid maturation/DNA packaging	e
UL34	Membrane associated phosphoprotein ; substrate for US3 protein kinase	-
UL35	Capsid protein (VP26)	-
UL36	Very large tegument protein (VP1-2, Vmw273)	e
UL37	Unknown : forms a complex with UL29 protein ; may have a DNA binding role	-
UL38	Capsid protein (VP19c)	e
UL39	Ribonucleotide reductase large subunit (ICP6, Vmw136, R1)	e/ne
UL40	Ribonucleotide reductase small subunit (Vmw38, R2)	e/ne
UL41	Virion host shut off factor	ne
UL42	Subunit of DNA polymerase; complexes with UL30 protein	e
UL43	Unknown ; probable integral membrane protein	ne
UL44	Glycoprotein C	ne
UL45	Virion protein	ne
UL46	Modulates IE gene transactivation by the UL48 protein (Vmw65)	ne

TABLE 1.1 continued

UL47	Tegument protein (VP13-14?) ; modulates IE gene transactivation by the UL48 protein	ne
UL48	Major tegument protein ; transactivates IE genes (Vmw65, VP16, α TIF)	e
UL49	Tegument protein (VP22)	-
UL49A	Possible membrane glycoprotein	e?
UL50	Deoxyuridine triphosphatase	ne
UL51	Unknown	ne
UL52	Component of the DNA helicase-primase complex	e
UL53	Glycoprotein K	e?
UL54	IE63; post-transcriptional regulator of gene expression (ICP27, Vmw63, IE2)	e
UL55	Unknown	ne
UL56	Unknown	ne
RS1	IE175; transcriptional regulator (ICP4, Vmw175, IE3)	e
OriS	Origin of replication	e?
US1	IE68; regulatory protein (ICP22, Vmw68, IE4)	e/ne
US2	Unknown	ne
US3	Protein kinase; phosphorylates UL34	ne
US4	Glycoprotein G	ne
US5	Glycoprotein J?	ne
US6	Glycoprotein D	e
US7	Glycoprotein I	ne
US8	Glycoprotein E	ne
US9	Virion protein	ne
US10	Virion protein	ne
US11	Virion protein; ribosome-associated in the infected cell	ne
US12	IE12; function unknown (ICP47, Vmw12, IE5)	ne

viral mRNAs are derived by splicing compared to host mRNAs, where the majority are spliced (Wagner, 1985). Only five HSV ORFs IE110, US12 (IE12), US1 (IE68) and UL15, contain introns (Watson, *et al.*, 1981, Rixon and Clements, 1982; Perry *et al.*, 1986).

A number of 5' and in particular 3' co-terminal "families" of mRNA transcripts have been described (McLauchlan and Clements, 1982; Wagner, 1985; Wagner and Roizman, 1969; Murchie and McGeoch, 1982; Frink *et al.*, 1981; Watson and Vande Woude, 1982) (Figure 1.5) and a few RNAs are known to extend beyond the usual polyadenylation site (Holland *et al.*, 1984; Chou and Roizman, 1986; Anderson *et al.*, 1981). In addition to these observations selective and possibly regulated use of polyadenylation signals has been described (McLauchlan *et al.*, 1989; 1992b).

(a) Immediate-early (α) gene expression

The five IE genes IE110 (IE1), IE63 (IE2), IE175 (IE3), IE68 (IE4) and IE12 (IE5), are the first viral genes to be transcribed and do not require de-novo viral protein synthesis for their expression (Honess and Roizman, 1974; Clements *et al.*, 1977; Wagner, 1985). IE110 and IE175 are encoded within the terminal and internal repeat sequences of the genome and are therefore diploid (Watson *et al.*, 1979; Anderson *et al.*, 1980; Mackem and Roizman, 1980; McGeoch *et al.*, 1988). IE68 and IE12, which contain identical promoter and 5' leader sequences are located in the short repeat region with their coding sequences in the US region (Watson *et al.*, 1981). IE63, the only IE gene not associated with the repeat sequences, is encoded entirely

within the UL region of the genome (Figure 1.5). The regulatory regions of the IE gene promoters contain elements found in most eukaryote promoters, such as the TATA box and SP1 site (GC rich elements) (Figure 1.6). In addition all IE genes contain variable copy numbers of a unique DNA consensus motif, TAATGARAT, required for response of the promoter to the virus encoded transactivator Vmw65 (α TIF, VP16, UL48) (Roizman and Sears, 1993, Gaffney *et al.*, 1985, reviewed by O'Hare, 1993). Vmw65, a protein component of the virus tegument, by itself binds DNA poorly, it has been shown however to interact with two cellular factors, Oct-1 and an additional factor variously termed HCF (host cell factor ; Greaves and O'Hare, 1990), C1 factor (Kristie *et al.*, 1989), VCAF (Vmw65 complex assembly factor ; Xiao and Capone, 1990) or CFF (complex forming factor ; Katan *et al.*, 1990), which direct the binding of Vmw65 to the TAATGARAT consensus. Oct-1, which stimulates the transcription of a number of cellular genes, binds to a sequence known as the octamer motif, ATGCAAAT, which overlaps with the 5' (TAAT) portion of the TAATGARAT element. Oct-1 and Vmw65 form a low affinity complex with TAATGARAT (Kristie *et al.*, 1989; La Marco *et al.*, 1991), and the additional host factor has been shown to stimulate complex formation by several orders of magnitude (Kristie *et al.*, 1989). The transcriptional activities of Vmw65 appear to be separate from its ability to complex with Oct-1 (Ace *et al.*, 1988; Greaves and O'Hare, 1989; Triezenberg *et al.*, 1988), with evidence that the acidic C-terminal domain of Vmw65 is essential for the *in vivo* transactivation of the IE genes (Greaves and O'Hare, 1989; Triezenberg *et al.*, 1988; Werstuck and Capone, 1989). It has been suggested that the C-terminal domain of

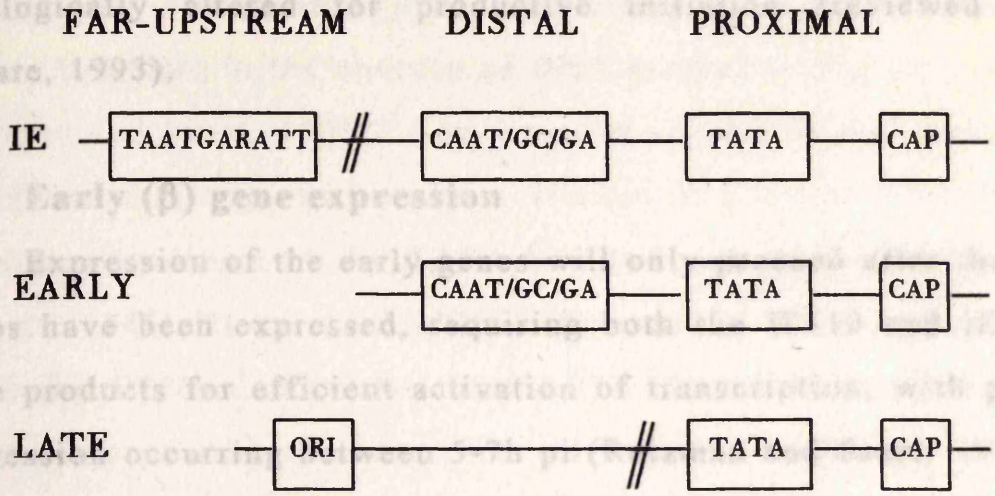


FIGURE 1.6 :

Diagrammatic representation of the promoters of the three classes of HSV-1 genes (adapted from Johnson and Everett, 1986). All classes of promoter contain a capsite and TATA box. IE promoters contain additional upstream promoter sequences and the TAATGARAT regulatory region required for activation of IE gene expression by the virion protein Vmw65. Early promoters require only the upstream sequences and late promoters consist of the TATA box and capsite linked to a functional origin of replication.

Vmw65 activates IE gene expression by promoting assembly of an initiation complex containing the TATA box binding protein topologically altered for productive initiation (reviewed by O'Hare, 1993).

(b) Early (β) gene expression

Expression of the early genes will only proceed after the IE genes have been expressed, requiring both the IE110 and IE175 gene products for efficient activation of transcription, with peak expression occurring between 5-7h pi (Roizman and Sears, 1993). Information regarding the DNA sequence elements required for early promoter activation has been gained mainly from studies of the thymidine kinase promoter (UL23). Mutational analysis has shown that the promoter consists of four regions located immediately upstream of the cap site : the TATA box, an inverted CAAT motif, and two GC rich elements which the bind the cellular transcription factor SP1 (McKnight *et al.*, 1981; Jones *et al.*, 1985) (Figure 1.6). These sequences are also important for activation of transcription in the absence of viral proteins, which suggests they interact with cellular factors (Everett, 1987).

(c) Late (γ) gene expression

DNA replication is required for the maximal expression of this group of genes (Honess and Roizman, 1974; Swanstrom and Wagner, 1974; Powell and Courtney, 1975; Marsden *et al.*, 1976), with peak expression occurring from 10-16h pi, approximately 2h after the peak of viral DNA synthesis (Roizman, 1969; Wilkie, 1973). The late genes can be divided into two groups depending on the stringency of their requirement for DNA replication. The

γ_1 genes - sometimes described as leaky late or early late, have reduced but detectable levels of expression when DNA synthesis is inhibited. The γ_2 genes - the 'true' late genes, their expression is hard to detect in the absence of DNA synthesis (Wagner, 1985; Roizman and Sears, 1993). The promoter regions of this group of genes are minimal, with only a limited requirement for DNA sequences upstream of the TATA box and cap site (Johnson and Everett, 1986; Homa *et al.*, 1986) (Figure 1.6). Recent studies, involving deletion and mutation of the UL38 (γ_2) promoter region, have demonstrated that sequence elements downstream of the TATA box and spanning the transcriptional start site of this promoter are functionally different from the early and γ_1 promoters. They show that this γ_2 promoter consists of a core of sequences from the TATA box to sequences up to and including the transcription start, linked to a downstream activating element within the nontranslated leader region (Guzowski and Wagner, 1993). Transfection studies have shown that, like early gene promoters, late promoters are activated by IE proteins, with evidence for the involvement of IE63, IE68 and IE175 in late gene expression (Sears *et al.*, 1985; Sacks *et al.*, 1985; Dennis and Smiley, 1984; De Luca and Schaffer, 1985; Everett, 1986). The role of IE63 in this process will be discussed in the following Section. There are two main theories explaining the dependence of late gene expression on DNA synthesis. The first suggests that late genes are self regulated during the early phase of viral infection, either by the binding of trans-acting negative factors or by physical constraints placed by the DNA secondary structure in the vicinity of the late genes. In this case DNA replication would relieve the block allowing full expression of the late genes

(Mavromara-Nazos and Roizman, 1987). The second theory suggests that a trans-activator is produced or modified during viral DNA replication thus allowing activation of late gene expression (reviewed by Roizman and Sears, 1993). To further complicate our understanding of late gene expression it has been shown that HSV-1 temperature sensitive (ts) mutants deficient in the major binding protein (UL29), which are blocked at the level of viral DNA synthesis, express low levels of a γ_2 protein glycoprotein C (UL44) (Godowski and Knipe, 1983), which would suggest that, in this case at least, late gene expression is not entirely dependent on the activation of DNA synthesis.

1.1.7 Protein expression

The majority of HSV proteins examined to date appear to be extensively post-translationally modified. Such modifications include cleavage (eg. UL26), phosphorylation (a number of HSV-1 proteins are phosphorylated including the IE proteins IE175, IE110, IE68 and IE63) (Purves *et al.*, 1991; 1992; Purves and Roizman, 1992), sulphation (glycoproteins) (Erickson and Kaplan, 1973; Hope and Marsden, 1983), glycosylation (glycoproteins) (Campadelli-Fiume and Serafini-Cessi, 1985), myristylation (UL11) (MacLean *et al.*, 1992) and poly(ADP-ribosyl)ation (IE175, UL18) (Preston and Notarianni, 1983; Blaho *et al.*, 1992). However the relationship between these modifications and the functions of the various proteins is unclear, although the glycoproteins must at least be partially glycosylated to function, and virions produced in the presence of tunicamycin (a drug which inhibits N-linked glyscoylation) are noninfectious (Kousoulas *et al.*, 1983). Phosphorylation of proteins has been

shown in other systems to modulate a variety of macromolecular events, such as transcription, translation and viral transformation (Hunter and Karin, 1992). Phosphorylation can also modulate protein function by inducing allosteric conformational changes (Hurley *et al.*, 1990; Sprang *et al.*, 1988). Eleven HSV induced phosphoproteins have been detected, which include four of the IE proteins, the UL41 (VHS) protein, the UL39 protein (the large sub-unit of ribonucleotide reductase), and the UL42 protein (part of the viral DNA polymerase complex) (Marsden *et al.*, 1987). Pulse chase studies have shown that the phosphates of several of these proteins (IE175, IE68 and IE63) cycle on and off during viral replication (Wilcox *et al.*, 1980). However, the functional role of phosphorylation for most of these viral proteins remains unknown.

(a) The immediate-early proteins

Synthesis of the IE proteins reaches peak rates at 2-4 h pi, with mRNA and protein continuing to accumulate until late in infection. To date all IE proteins with the exception of IE12, which has no known role as yet, have been shown to have regulatory functions (Roizman and Sears, 1993). All the IE polypeptides are phosphorylated and located predominantly in the nucleus of infected cells (Hay and Hay, 1980; Periera *et al.*, 1977), with the exception of IE12 which is not phosphorylated and is found in the cytoplasm of infected cells (Preston, 1979; Marsden *et al.*, 1982).

IE110 (IE1) plays an important although non-essential role during productive infection in cell culture. Mutant viruses

containing deletions of IE110 do not grow as efficiently as wt in cell culture and the replication efficiency of these mutants appears to be dependant on host range and multiplicity of infection (moi). Infection of Vero and HeLa cells at a low moi produced a lower virus yield from Vero cells than from HeLa cells, while at high moi virus yield was the same in both cell types (Chen and Silverstein, 1992). Transfection studies have demonstrated that IE110 is able to activate IE, early and late gene expression (Gelman and Silverstein, 1985; Cai and Schaffer, 1992; O'Hare and Hayward, 1985), with co-ordinate expression of IE175 and IE110 being required for the efficient activation of early genes (Gelman and Silverstein, 1986; Everett, 1984). In addition co-transfection studies have shown that IE110 is capable of enhancing IE175 expression from transfected viral DNA, and can thus activate IE gene expression in the absence of other viral proteins. It has been proposed therefore that IE110 could, by this ability to induce IE175 expression, be involved in the reactivation of the virus from the latent state (Cai and Schaffer, 1992; Russell *et al.*, 1987; Lieb *et al.*, 1989a).

IE63 (IE2) is one of only two IE genes whose expression is essential for virus replication (Sacks *et al.*, 1985), the other is IE175. A number of studies have indicated that the IE63 protein is involved in the regulation of viral gene expression (Sacks *et al.*, 1985; Smith *et al.*, 1992; Sekulovich *et al.*, 1988; Hardwicke *et al.*, 1989, McMahon and Schaffer, 1990) and is specifically required for late gene expression. The plethora of conflicting information available and the apparently multifunctional nature of the IE63 protein makes identification of the specific mechanism of

this regulation difficult. Initial studies of a range of virus mutants deficient or lacking in IE63 expression, showed generally similar phenotypes, overexpression of IE proteins, significant levels of early proteins and reduced levels of late proteins. Thus demonstrating a role for IE63 in the regulation of HSV gene expression (Sacks *et al.*, 1985). Transient transfection assays have demonstrated that IE63 acts synergistically with IE175 and/or IE110 to trans-repress or trans-activate transcription from the promoters of selected HSV-1 genes, while having little or no effect alone (Sekulovich *et al.*, 1988, McMahon and Schaffer, 1990, Hardwicke *et al.*, 1989). However Rice and Knipe (1988), have shown that IE63 alone can stimulate at least one HSV promoter, that of glycoprotein B (UL27). In general IE and early promoters were repressed and late promoters stimulated by IE63.

There is also evidence for IE63 mediated post-transcriptional regulation of viral gene expression (Sandri-Goldin and Mendoza, 1992; Smith *et al.*, 1992; McLauchlan *et al.*, 1992b). Sandri-Goldin and Mendoza demonstrated that (a) poly(A)$^+$ RNA levels were increased by IE63 where the target gene had only the minimal poly(A) signal and no further downstream regulatory sequences and that (b) spliced mRNAs containing 5' or 3' introns were reduced 5- to 10-fold in the presence of IE63. A comparsion of the levels of transcription of wt HSV-1 with an IE63 ts mutant (Smith *et al.*, 1992) showed that while synthesis of IE transcripts was reduced and synthesis of late transcripts continued in the mutant at NPT, this did not correlate with levels of mRNA and protein present. IE mRNA and protein accumulated and late mRNAs and proteins were reduced. McLauchlan *et al.*, (1992b) demonstrated that IE63 expression

was required for the activity of a virus induced factor, termed LPF, which increased the 3' processing efficiency of the HSV-2 UL38 late poly(A) site mRNA. Taken together these results provide strong evidence for IE63 mediated post-transcriptional regulation of these mRNAs.

It has been suggested (Sandri-Goldin and Mendoza, 1992) that IE63 down regulates host cell gene expression post-transcriptionally via an effect on splicing, this would be a convenient regulatory mechanism since very few virus transcripts are spliced in comparison to the host cell. During virus infection distribution of components of the splicing machinery, small nuclear ribonulceoproteins (snRNPs), are found to condense into distinct foci in the nucleus of the infected cell (Martin *et al.*, 1987), and recent studies have demonstrated that IE63 is required for this localisation and in fact co-localises with the redistributed snRNPs (Phelan *et al.*, 1993). In addition during HSV infection it has been shown that there is a decrease in splicing of the cellular genes GAPDH, actin and β-tubulin which requires the expression of IE63 (Sandri-Goldin and Mendoza, 1992). In addition, IE63 deficient virus mutants show greatly impaired host shut off, although this is most likely due to the absence of a late gene product required for this function (Sacks *et al.*, 1985).

The expression of late viral genes requires ongoing viral DNA synthesis and it has been proposed that IE63 affects late gene expression via an effect on DNA synthesis. Direct involvement of IE63 in DNA synthesis has been difficult to demonstrate conclusively, the series of ts and deletion virus mutants studied, show a range of DNA synthesis abilities, from little or no DNA synthesis in the deletion mutants (18% of wt

HSV) (McCarthy *et al.*, 1989), to moderate (46% of wt HSV), or unaltered synthesis (100% of wt HSV) in the ts mutants (Sacks *et al.*, 1985). These differences are likely to be dependent on the nature of the mutations, the deletion mutants producing little or no IE63 protein and the ts mutants producing aberrant forms of the IE63 protein. Curtin and Knipe (1993) have noted that in IE63 mutant virus infected cells, assembly of pre-replicative complexes in the nucleus is defective, and may be due to an observed conformational change in the UL29 protein, which is required for formation of these structures. It has also been reported that the electrophoretic mobility of IE175 is altered in cells infected with IE63 mutant viruses (Su and Knipe, 1989). It is possible that IE63 could therefore be influencing gene expression indirectly by affecting the activities of components required for gene trans-activation and DNA synthesis.

The range of defects in protein expression seen in cells infected with IE63 mutant viruses demonstrate the multifunctional nature of this protein. It would seem likely therefore that it contains several domains which affect gene expression both positively and negatively. Deletion analysis has identified several functional domains within the 512 amino acid polypeptide product of the IE63 gene (Figure 1.7). The trans-activating property of the protein has been mapped to its C-terminal half, and the trans-repressing function localised to the C-terminal third of the protein (Hardwicke *et al.*, 1989, Rice and Lam, 1994). In addition sequences which resemble zinc-finger metal binding domains, which are capable of binding zinc and single-stranded DNA *in vitro*, have been identified in the C-terminal 105 amino acids (Vaughan *et al.*, 1992). Analysis of the acidic N-terminus has

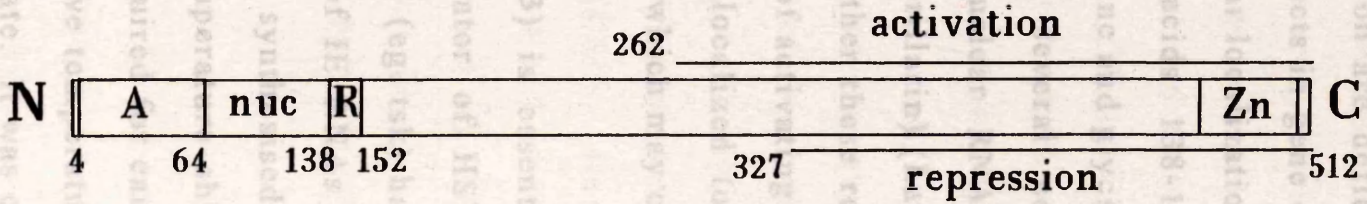


FIGURE 1.7 :

Representation of the functional domains of the IE63 protein (512 amino acids). N : N-terminal domain, C : C-terminal domain, A : acidic region, nuc : putative nuclear localization signal, R : RGG box, ZN : zinc finger region.

shown that whilst not required for the trans-activating and trans-repressing functions, amino acids 12-63 are required for an essential lytic function of the virus (Rice *et al.*, 1993). Mutants lacking this region are deficient in DNA replication but do not show severe defects in gene expression. The N-terminal half also contains a nuclear localization signal within amino acids 109-138. Finally amino acids 138-152 encompass a region composed entirely of arginine and glycine residues, this RGG-box region has been found in several cellular proteins which have been implicated in nuclear RNA processing (core snRNP proteins, nucleolin and fibrillarin) (Lam *et al*, 1992).

Taken together these results provide a picture of a complex protein capable of activating and repressing gene expression, with these activities localized to the C-terminus and quite separate from the signals which may confer its nuclear localization.

IE175 (IE3) is essential for HSV replication and is the major transactivator of HSV genes. Studies using HSV-1 ts mutants in IE175 (eg. tsk) have demonstrated that at NPT there is overexpression of IE RNAs and polypeptides and early and late proteins are not synthesised (Courtney *et al.*, 1976; Marsden *et al.*, 1976). Temperature shift experiments showed the IE175 is continuously required for early gene transcription, since a shift to the non-permissive temperature ends early gene expression and IE mRNAs accumulate. It was concluded from this data that IE175 is required for transcription of later classes of genes (Watson and Clements, 1980) and is involved in autoregulation of IE gene transcription (Preston, 1979; Dixon and Schaffer, 1980). Alone IE175 is capable of activating transcription, however the effect is

increased substantially when IE110 is present (Everett, 1984). Additional evidence suggests that IE63, IE110 and IE175 act co-operatively to regulate viral gene expression (Everett, 1986; Gelman and Silverstein, 1985, McMahon and Schaffer, 1990; O'Hare and Hayward, 1985). IE175 has been implicated in the regulation of IE gene expression, plasmids expressing IE175 can repress both basal and activated levels of transcription from the IE175 promoter (Michael and Roizman, 1993; O'Hare and Hayward, 1985) In addition increased levels of IE175 have been shown to reduce transcription from IE175, IE68 and IE12 promoters (DeLuca and Schaffer, 1985). Although IE175 is clearly required for the expression of early and late genes the exact mechanism of this action has not been determined. Tedder *et al.*, (1989) have shown that *in vitro* the presence of IE175 binding sites enhance the transcription of the early gene, glycoprotein D (US6). However another study has shown that mutation of the IE175 binding sites within the glycoprotein D promoter region had no effect on transcription (Smiley *et al.*, 1992). Current theories suggest that IE175 has a role in the stabilisation of TATAA box dependent factors (Roizman and Sears, 1993).

IE68 (IE4) As yet no clear function has been assigned to IE68. There is however some evidence to suggest it may play a role in late gene expression of the virus. A deletion mutant missing the carboxy terminal of the protein, has varied growth characteristics depending on the cell type infected, appearing to grow normally in some and poorly in others, with the activity of

at least one true late promoter reduced (Post and Roizman, 1981; Sears *et al.*, 1985).

IE12 (IE5) There appears to be no obvious or essential role for this protein during tissue culture infections, mutants lacking the entire gene remaining viable (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

(b) The early proteins

The majority of early genes encode proteins which are involved in DNA metabolism and replication, their expression signaling the onset of viral DNA synthesis. DNA replication and the involvement of the following β gene products UL30 (DNA polymerase), UL29 (the major DNA binding protein), UL9 (ori binding protein), UL8 (component of the helicase primase complex), and UL42 (subunit of the DNA polymerase) are described in detail in the following Section. In this Section therefore only a brief description is given of those β gene products involved in the optimal utilization of the deoxyribonucleoside pools required for efficient DNA synthesis. The majority of these enzymes, with the exception of ribonucleotide reductase, are non-essential for virus growth in cell culture, however many of the enzymes involved in nucleotide metabolism appear to confer an advantage to the virus during infection of the natural host (Morrison, 1991).

Alkaline DNase, encoded by the UL12 gene, is thought to supply preformed nucleotides to the replicative machinery of the virus by hydrolysis of cellular DNA (Weller *et al.*, 1990). It has been proposed that this enzyme has an additional role in the

cleavage of viral DNA concatamers prior to packaging of the viral DNA (Morrison, 1991).

Thymidine kinase, the enzyme encoded by the UL23 gene phosphorylates nucleoside analogues. The substrate specificity of the viral enzyme is much lower than its cellular counterpart and as such many of the anti-viral drugs used in the treatment of herpes infection are nucleoside analogues.

Ribonucleotide reductase is formed as a tetramer of two subunits encoded by the UL39 gene (large subunit) and the UL40 gene (small subunit) (Ingemarson and Lanikien, 1987). The enzyme catalyses the reduction of rNDPs to dNDPs a crucial step in the synthesis of deoxyribonucleotides.

dUTPase encoded by the UL50 gene hydrolyses dUTP to dUMP, the role of this enzyme appears to be suppression of the size of the dUTP pool relative to that of dTTP ensuring that the ratio of U:T incorporated into newly synthesised DNA is kept low (Williams and Parris, 1987).

Three of the β proteins which have known functions encode virus structural proteins, UL49 is a constituent of the tegument, US6 encodes glycoprotein D and UL27 encodes glycoprotein B, the US6 and UL27 proteins have also been assigned to the γ_1 class of proteins (Roizman and Sears, 1993).

(C) The late proteins

The majority of the structural components of the virus tegument, capsid and envelope are late proteins. For example : the tegument proteins encoded by UL48 (Vmw65) and UL41 (VHS) are classified as γ1 proteins : the capsid proteins encoded by UL19 (the major capsid protein, VP5) and UL38 are members

of the γ_1 and γ_2 groups respectively. The surface glycoprotein components are divided between the γ_1 and γ_2 groups, US8, US4, US5 and US7, are γ_1 genes which encode glycoproteins E, G, J and I respectively. The UL22 and UL44 γ_2 genes encode glycoproteins H and C respectively, and finally the UL45 γ_2 gene encodes a structural protein which is non-essential for growth and most likely an envelope protein (Visalli and Brandt, 1991; 1993).

1.1.8 DNA replication

The HSV genome contains three origins of replication (*ori*), two *ori* elements map to the repeat sequences flanking the US region and one maps to the UL region, situated between the transcription start sites of UL29 and UL30 (McGeoch *et al.*, 1988). The three *ori* elements are functionally equivalent, with early studies showing that none of the *ori* elements are uniquely required for viral replication (reviewed by Roizman and Sears, 1993). Transient complementation assays, in which cloned segments of HSV DNA were tested for their ability to support replication of co-transfected plasmids containing the *ori*S and *ori*L, have identified seven proteins which are required for *ori* dependent DNA synthesis (Wu *et al.*, 1988; Challberg, 1986). These studies have recently been substantiated by Stow (1992), using a recombinant baculovirus system expressing the seven proteins. These proteins which are encoded by the HSV genes UL5, UL8, UL9, UL29, UL30, UL42 and UL52 are thought to form multiprotein complexes, held together by protein-protein and/or DNA-protein interactions such as those involved in other eukaryotic DNA replication systems (Bramhill and Kornberg, 1988). Figure 1.8 shows a schematic summary of the biochemical

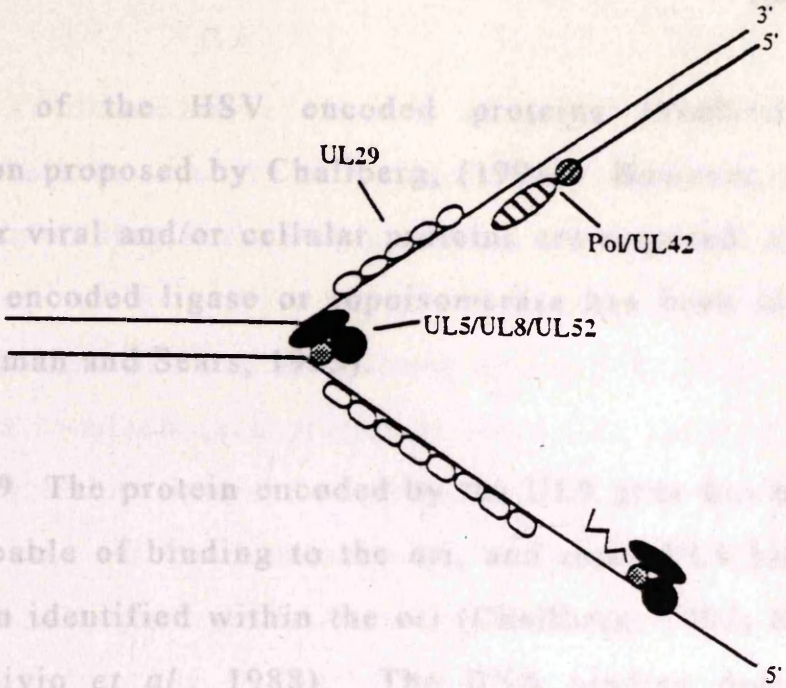


FIGURE 1.8:

Schematic summary of the activites of the HSV-1 encoded proteins required for viral DNA replication and their functions in formation of the replication fork, proposed by Challberg (1991).

activities of the HSV encoded proteins involved in DNA replication proposed by Challberg, (1991). However, it is likely that other viral and/or cellular proteins are required, for example no virus encoded ligase or topoisomerase has been identified as yet (Roizman and Sears, 1993).

UL9 The protein encoded by the UL9 gene has been shown to be capable of binding to the ori, and three UL9 binding sites have been identified within the ori (Challberg, 1991; Elias *et al.*, 1990; Olivio *et al.*, 1988). The DNA binding domain of the protein has been localised to the C-terminal third of the polypeptide chain (Weir *et al.*, 1989), however the exact interaction of UL9 with the ori has not been fully determined. The events following binding of UL9 to the ori, that lead to initiation of DNA synthesis, are even less well understood : before intiation of synthesis of the DNA daughter strand can occur the two parental strands must be unwound and it has been proposed that the binding of UL9 triggers this in some way. Consistent with this idea is the finding that UL9 has an intrinsic helicase activity (Bruckner *et al.*, 1991). Boehmer *et al.*, (1993) have demonstrated that UL9 can catalyse the ATP-dependent unwinding of a DNA duplex, alone UL9 has only limited DNA unwinding ability, their studies showed that the addition of the UL29 protein (the major DNA binding protein) had a stimulatory effect. Furthermore the UL9 protein has been shown, by the use of protein affinity-chromatography, to form a tight complex with UL29 (Boehmer and Lehman, 1993a). It has been proposed therefore that the UL29/UL9 protein complex plays an important role in the recognition of the *ori* and unwinding of the DNA

duplex. To date however there is no experimental evidence that the UL9 helicase activity plays an essential role in the initiation of DNA unwinding at the origin of replication.

UL5/UL8/UL52 The proteins encoded by these genes form a complex in which each protein is present in equimolar amounts. This complex has been shown to function as both a primase and a helicase, and its biochemical properties suggest that it primes lagging strand synthesis as it unwinds DNA at the replication fork (Crute *et al.*, 1988; 1989). Both the primase and helicase activities of this complex require the presence of the UL29 protein, which may serve to stabilize the ssDNA or localize the complex to the replication fork (Crute and Lehman, 1991). Studies using recombinant baculovirus expressed proteins have demonstrated that a subcomplex of the UL5/UL52 proteins contains both helicase and primase activity with no apparent requirement for UL8 (Calder and Stow, 1990; Dodson and Lehman, 1991). It has been proposed that UL8 may therefore function by stabilizing the association of the RNA primers with template DNA (reviewed by Challberg, 1991).

UL30/UL42 The DNA polymerase exists as a heterodimer of the UL30 and UL42 polypeptides (Hernandez and Lehman, 1990; Gottlieb *et al.*, 1990; Crute and Lehman, 1989; Monahan *et al.*, 1993). UL30 contains the catalytic activity and in addition to its polymerase activity has an intrinsic 3' to 5' exonuclease activity, which may serve as a proof-reading function (O'Donnell *et al.*, 1987) and a 5' to 3' exonuclease/RNase H activity (Crute and Lehman, 1989). During the semi-discontinuous synthesis of

the lagging strand, this activity may serve to remove the RNA primers from the 5' end of Okazaki fragments. UL42 has been shown to increase the processivity of the polymerase enzyme (Gallo *et al.*, 1989; Hernandez and Lehman, 1990). For this increase to occur it has been demonstrated that the DNA binding protein of UL29 must be complexed to the single stranded DNA (Hernandez and Lehman, 1990).

UL29 The DNA binding protein (ICP8) encoded by the UL29 gene is a central component of the replication machinery. Three of the HSV-1 replication enzymes are stimulated by the UL29 protein, the DNA polymerase (Hernandez and Lehman, 1990; Ruychan and Weir, 1984), the helicase-primase (Crute and Lehman, 1991), and the origin-binding protein (Boehmer *et al.*, 1993). In addition UL29 appears to be involved in the correct localisation of the replication proteins within the infected cell (Quinlan *et al.*, 1984; De Bruyn Kops and Knipe, 1988; Curtin and Knipe, 1993). The UL29 protein has also been shown to lower the melting temperature of synthetic polynucleotides (Wang and Hall, 1990; Powell *et al.*, 1981), de-stabilise partial DNA duplexes (Boehmer and Lehman, 1993b). It has also been shown to bind more tightly to single stranded DNA than double stranded DNA (Ruyechan, 1983; Ruyechan and Weir, 1984: Lee and Knipe, 1985) where binding is co-operative and independent of sequence. It has been proposed therefore that UL29 functions by unwinding the parental duplex DNA thus facilitating the use of these strands as templates for the DNA polymerase (reviewed by Challberg, 1991), as yet however no direct evidence for this exists. Ellis-Dutch and Lehman (1993), have demonstrated that

the UL29 protein can promote renaturation of complementary single stranded DNA and suggest a role for this protein in the high level of recombination that occurs during HSV-1 infection. Trans-dominant UL29 HSV mutants have been identified which can inhibit viral replication and significantly inhibit the production of wt HSV-1. Expression of the mutant UL29 protein inhibits wt UL29 function and results in a reduction of viral DNA replication and late gene expression (Gao and Knipe, 1991). In addition, the UL29 protein appears to have a negative regulatory effect on IE175 expression (Godowski and Knipe, 1986). UL29 therefore appears to play an important role in maintenance of the cascade of viral gene expression.

1.2 mRNA 3' end formation

Eukaryotic gene expression requires the activities of complex biochemical machinery to transcribe, process and transport mature mRNA from the nucleus to the cytoplasm of the cell for translation into a functional protein product. The areas within the nucleus to which the transcriptional and post-transcriptional events are localised and the mechanism by which mRNA is transported into the cytoplasm for translation have not yet been fully identified. There is evidence however, which suggests that splicing and polyadenylation of nascent RNA transcripts occurs close to the site of transcription and fully processed transcripts, in particular spliced transcripts, are transported along tracks to the nuclear pores, where they exit into the cytoplasm. (reviewed by Rosbash and Singer, 1993). Regulation of gene expression could occur at a number of levels, with the majority of past investigations focusing on transcriptional control. Over the past decade an increasing understanding of post-transcriptional events has stimulated interest in these processes as a means of regulating gene expression.

During synthesis of the primary transcripts transcription continues across the polyadenylation site and termination of transcription can occur at a considerable distance downstream of the DNA sequences coding for the 3' mRNA termini (Citron *et al.*, 1984; Le Meur *et al.*, 1984; Birnstiel *et al.*, 1985).

Formation of the mRNA 3' end (Manley, 1988; Wahle and Keller, 1992; Leff and Rosenfeld, 1986) is carried out by an RNA processing reaction which involves the endonucleolytic cleavage of the RNA followed by addition of a poly(A) tract by the poly(A)

polymerase. These poly(A) 'tails' play an important role in the life of the mRNAs, with evidence emerging that they are involved in the control of mRNA stability, export of the RNA from the nucleus and mRNA translation (Jackson and Standart, 1990; Atwater *et al.*, 1990; Wickens, 1990b; Bacharova, 1992).

Regulation of these events could therefore occur at any or all of the following stages; transcription termination, 3' end formation, splicing, stability and translation. In this study we have examined the effect of 3' processing efficiency on the regulation of gene expression. In this introduction the mechanisms of cleavage and polyadenylation of mRNA are considered in detail and a brief outline is given of the other post-transcriptional events which influence gene expression.

1.2.1 Cleavage and polyadenylation

Nearly all mammalian mRNAs, with the exception of the major histone mRNAs, are polyadenylated at their 3' ends. Polyadenylation of precursor mRNA occurs in a two-step reaction, endonucleolytic cleavage followed by the addition of a 200-300nt stretch of A residues. The development of *in vitro* systems, that faithfully reproduce these events (Manley, 1983; Moore and Sharpe, 1984; 1985), has been an important step forward in their investigation. The ability to study the cleavage and polyadenylation steps separately has in addition allowed the individual requirements of these two events to be investigated.

1.2.2 Sequence requirements

(a) Poly(A) signal

The hexanucleotide sequence AAUAAA, a pre-requisite for cleavage and polyadenylation, is found in virtually every eukaryotic mRNA that has been isolated or sequenced. Its distance from the mRNA 3' end, while not precise, is found in the vast majority of cases to be 10 to 30nt upstream of the actual cleavage site, which is usually at an A or CA residue (reviewed by Manley, 1988; Leff and Rosenfeld, 1986; Wahle and Keller, 1992). Deletion of this sequence has been shown to prevent stable 3' end formation of mRNA (Fitzgerald and Shenk, 1981), with mutational analysis of the sequences demonstrating an absolute requirement for the U at position 3. Mutation of the AAUAAA to AAUACA results in an 8-fold reduction in cleavage efficiency, the AAUUAA mutation a 25-fold reduction, and the AAUGAA and AACAAA mutations a 50-fold reduction in cleavage efficiency (Wickens and Stephenson, 1984). Computer assisted analysis, of 269 vertebrate cDNA sequences, compiled the following consensus sequence for the poly(A) signal :-

$$A_{98}\ A_{86}\ U_{100}\ A_{99}\ A_{99}\ A_{98}$$
$$U_{12}$$

The numbers indicating the percentage frequency of occurrence of the nucleotide shown at that position (Wickens, 1990a).

The only natural variant, AUUAAA which is present in 12% of the mRNAs compared, functions nearly as efficiently as the canonical sequence (Wilusz *et al.*, 1989; Hagenbuchle *et al.*,

1980; Jung *et al.*, 1980). While the cleavage reaction requires additional sequences, the hexanucleotide appears to be sufficient for the polyadenylation reaction (Ryner *et al.*, 1989; Conway and Wickens, 1987), with efficient polyadenylation occurring on pre-cleaved mRNA substrates containing the AAUAAA hexanucleotide (Zarkower *et al.*, 1986; Manley *et al.*, 1985; Moore and Sharpe, 1985). The studies of Wigley *et al.*, (1990) demonstrated that there must be a minimum distance of 11nt between the hexanucleotide and the cleaved 3' end to allow polyadenylation to occur, and that the 2' hydroxyl of the hexanucleotide U is required for the binding of specific processing factors. They propose that on shorter substrates the poly(A) polymerase cannot simultaneously contact the specificity factor and the mRNA 3' end.

(b) Downstream elements

The realisation that sequences in addition to the hexanucleotide were required for efficient 3' end formation arose from the observation that the AAUAAA sequence was found within protein coding regions where it was apparently never utilized. Sequences downstream of the AAUAAA, which enhance the efficiency of 3' end formation, have been identified in a number of systems, with the SV40 late poly(A) site receiving considerable attention. Deletion analysis has shown that sequences between 3 and 60nt downstream of the AAUAAA, of the SV40 late poly(A) signal, are required for the efficient utilization of the normal polyadenylation site, and a functional element has been identified that is contained within the sequence AGGUUUUUU (Sadofsky and Alwine, 1984; Sadofsky *et al.*,

1985). While Conway and Wickens (1987), agree that sequences downstream of the AAUAAA are necessary for 3' end formation, their chemical modification analysis, suggests that the critical features of the element are either diffuse or redundant. Further to this it has been demonstrated that these downstream elements are required not only for cleavage and polyadenlyation but also for the accuracy of cleavage (Ryner *et al.*, 1989). It has been suggested that the differences observed in these studies result from the use of different cell lines. However it has been shown that deletion of sequences from 6nt downstream of the AAUAAA, dramatically reduced 3' end formation in both hamster and monkey cell lines and in addition deletion of the consensus proposed by Sadofsky had no effect on processing efficiency (Gimmi *et al.*, 1988). Similarly, examination of the rabbit (Gil and Proudfoot, 1987) and mouse (Chen and Nordstrom., 1992) β globin poly(A) sequences has identified elements downstream of the AAUAAA which are critical for efficient 3' end formation. In HSV, deletion of the region 2 to 20nt 3' of the thymidine kinase gene poly(A) signal resulted in a 4-fold decrease in its processing efficiency (Cole and Stacy, 1985; Zhang *et al.*, 1986). The transfection studies of McLauchlan *et al.* (1985), showed that deletion of sequences located around 30nt downstream of the AAUAAA of the HSV-2 IE12 poly(A) signal, containing the consensus motif YGTGTTYY (Y=pyrimidine), reduced expression from a CAT reporter gene, fused upstream of this poly(A) site, to negligible levels. Computer analysis showed that this sequence was present in 67% of the mammalian 3' mRNA termini which were examined.

In addition a number of these studies demonstrated that the activity of the downstream element was dependent on its

proximity to the polyadenylation site. Increasing the natural distance of the element from the AAUAAA by 10 to 40 nt abrogated any effect the element had on 3' end formation (Cole and Stacy, 1985; Zhang *et al.*, 1986, Gil and Proudfoot, 1987; McDevitt *et al.*, 1986).

It is apparent from the evidence presented in these studies that two distinct types of downstream element exist, either GU- or U-rich in composition, and studies which examined both the SV40 early and adenovirus E2A poly(A) sequences, which contain GU- and U-rich elements respectively, found them to be functionally interchangeable. Sequence compilation analysis has identified U-rich TG/ANNNTTTTT (Renan, 1987), AGUUUUUU (Sadofsky *et al.*, 1985), or GU rich YGTGTTYY (McLauchlan *et al.*, 1985; Taya *et al.*, 1982) elements to be present in the 3' termini of the majority of mammalian mRNA on which data is available.

(c) Upstream elements

In a number of studies, sequence elements located upstream of the AAUAAA hexanucleotide, have also been shown to affect the efficiency of 3' end formation. Mutational analysis of the SV40 late mRNA poly(A) sequences has identified a region, 13-51nt 5' of the AAUAAA, which influences the efficiency of the 3' RNA processing. This region consists of three core elements of the sequence AUUUGURA (R=purine) which function in a distance dependent and co-operative manner. Increasing the distance between the elements and the AAUAAA decreases the efficiency of processing and the effect of multiple copies of the sequence is additive (Schek *et al.*, 1992). Deletional analysis has shown that sequences 5' of the L_1 poly(A) site of the adenovirus

major late transcription unit (MLTU) are part of a selector element which promotes predominantly L_1 site usage (DeZazzo and Imperiale, 1989). The ground squirrel hepatitis virus (GHSV) poly(A) sequences contain elements, located within 107nt upstream of the less efficient hexanucleotide variant UAUAAA, that increase the efficiency of 3' processing of this site from <10% to 50-60% (Cherrington *et al.*, 1992). A number of studies of HIV 3' processing have identified a sequence element, (CUUUUUG) 56-93nt 5' of the AAUAAA, which enhances the processing efficiency at the 3' LTR poly(A) sequences (Valsamakis *et al.*, 1992; Cherrington and Ganem, 1992; Gilmartin *et al.*, 1992; Weichs an der Glon *et al.*, 1993). In yeast two upstream sequence elements, UAGUAGUA and UAGUCUGUA, have been identified which enhance the efficiency of 3' end formation (Hou *et al.*, 1994). In the retroviral systems the situation is further complicated by the fact that the genomic RNA contains the canonical poly(A) signal at both the 5' and 3' ends, production of this RNA requires that the 5' copy must be ignored. Processing at the correct 3' end may therefore result from occlusion of the 5' AAUAAA by the proximity of the capsite (Weichs and der Glon *et al.*, 1991), or by an increase in processing efficiency of the 3' site, mediated by the upstream elements (Valsamakis *et al.*, 1992; Gilmartin *et al.*, 1992). In addition it has been suggested that both mechanisms operate to specify usage of the 3' AAUAAA (Cherrington and Ganem, 1992; Weichs and der Glon *et al.*, 1993).

These upstream sequence elements appear to be functionally similar and show little or no sequence homology just a general U-richness. This has led to the suggestion, unconfirmed as yet, that

they may function in a manner similar to that of the downstream elements, possibly interacting with the same factors (Valsamakis *et al.*, 1991).

(d) Secondary structure

With computer assistance it is now possible to look at the predicted RNA secondary structures of poly(A) sites. As yet, only limited information is available on a very few sites. In particular the poly(A) sequences of HTLV-1 and HIV-1 have been predicted to form stable stem-loop structures, which bring essential downstream (HTLV-1) and upstream (HIV-1) elements into close proximity to the AAUAAA hexanucleotide (Ahmed *et al.*, 1990; Toyoshima *et al.*, 1990; Gilmartin *et al.*, 1992; Seiki *et al.*, 1983). This is especially striking in the case of HTLV-1, where the polyadenylation site is more that 250nt downstream of the only evident AAUAAA. Examination of synthetic poly(A) sequence constructs, has demonstrated that insertion of sequences which increase the distance between the AAUAAA and a downstream GU-rich element, but form a stem-loop structure, results in normal utilisation of the poly(A) signal, insertion of sequences which simply increase the distance with no stable stem-loop being formed result in decreased usage (Brown *et al.*, 1991). These results are in agreement with evidence which, as previously discussed, demonstrates that the distance from the AAUAAA to additional upstream or downstream elements is critical for efficient usage of the poly(A) site. Examination of the RNA secondary structure of the bovine growth hormone poly(A) sequences (Woychik *et al.*, 1984) has shown that they are capable of forming a stem-loop structure. While no evidence is presented

that indicates these structures exist *in vivo*, there is good correlation between the position of the stem-loop and the observed poly(A) site, with the AAUAAA occupying a single-stranded hairpin loop just upstream of a single-stranded region containing the site of cleavage and polyadenylation. In histone mRNAs, which are not polyadenylated, the presence of a highly conserved stem-loop structure, with a six base stem and 4 base loop, has been observed, which is thought to provide some of the functions of the poly(A) tail found in other mRNAs (Pandey *et al.*, 1994; Pandey and Marzluff, 1987; Sun *et al.*, 1992). In contrast however, Cherrington and Ganem (1992) have shown that a predicted stem-loop structure in the GSHV poly(A) sequences, which is within the upstream element, is not necessary for efficient utilisation of the poly(A) site.

The central theme of these preliminary studies seems to be the ability of the RNAs of the poly(A) site to form stem-loop structures. However, these predictions do not take into account the effect that protein bound to the RNA would have on the overall conformation of the structures. Extensive mutational analysis will be necessary to evaluate what physiological role if any, these structures play in 3' processing.

1.2.3 The biochemistry of mRNA 3' processing

The establishment of *in vitro* systems (Moore and Sharpe, 1984; 1985) which faithfully reproduce the events of cleavage and polyadenylation in crude cell extracts using RNAs that resemble authentic *in vivo* pre-mRNAs, was a huge step forward on the road to deciphering the mechanisms and components required for pre mRNA cleavage and polyadenlyation.

By the addition of pre-cleaved mRNAs to *in vitro* reactions, the process of polyadenylation can be examined uncoupled from the cleavage reaction. The inclusion of ATP analogues, such as cordycepin (3' dATP), which prevent poly(A) tail elongation allow examination of the cleavage reaction in the absence of polyadenylation. More recently biochemical fractionation of the nuclear extracts, used in these *in vitro* reactions, has begun to provide insight into the nature of the processing factors involved.

(a) Cleavage

Efficient cleavage and subsequent polyadenylation of pre-mRNAs requires the assembly of a complex consisting of at least nine proteins (Zarkower and Wickens, 1987; 1988; Takagaki *et al.*, 1988; 1990; Christofori and Keller, 1988; Gilmartin and Nevins, 1989). At least three separable activities have been identified, the poly(A) polymerase (PAP), an activity which confers AAUAAA specificity (CPSF) and another which stabilises complex formation (CstF).

CPSF : The cleavage and polyadenylation specificity factor, variously termed CSF, PF2 and SPF, is required for cleavage as well as conferring AAUAAA specificity to the subsequent polyadenylation reaction (Bienroth *et al.*, 1991; Keller *et al.*, 1991; Weiss *et al.*, 1991). The complex consists of four proteins of approximately 160kD, 100kD, 74kD and 30kD (Bienroth *et al.*, 1991; Wahle and Keller, 1992; Keller *et al.*, 1991). There is however some disagreement between studies regarding the existence of the smallest protein, the studies of Gilmartin and

Nevins (1989), found no such protein and Keller *et al.* (1991) assigned a molecular weight of 35kD.

CstF : Cleavage stimulation factor, or CF1, is a complex of three proteins of 77kD, 64kD and 50kD (Takagaki *et al.*, 1990). This factor is required for cleavage but is dispensable for polyadenylation (Takagaki *et al.*, 1989; Gilmartin and Nevins, 1989). While CPSF and PAP are sufficient to reconstitute accurate cleavage and polyadenylation, addition of CstF to a mixture of the factors significantly enhances the efficiency of these events (Takagaki *et al.*, 1989). Binding of CstF to pre-mRNA requires the presence of the AAUAAA hexanucleotide, but no direct interaction occurs, binding being dependent on sequences downstream of the cleavage site (Weiss *et al.*, 1991; Takagaki *et al.*, 1990).

(b) Polyadenylation

The *in vitro* addition of the poly(A) tail to the cleaved 3' end of mRNA successfully mimics the *in vivo* reaction and as such occurs in two phases : the initial phase the addition of 10 adenylate (A) residues is slow, the second phase the further elongation of the poly(A) tail, with the addition of up to 200 A residues, is rapid. The initial phase is AAUAAA dependent (Wahle and Keller, 1992) and requires two factors, the poly(A) polymerase (PAP) and a factor which confers AAUAAA specificity, the elongation phase requiring an additional protein, the poly(A) binding protein II (PAB II) (Beinroth *et al.*, 1993; Wahle *et al.*, 1993).

PAP : which has been purified to near homogeneity, exists in several chromatographically and electrophoretically distinct forms, of 57-60kD, and behaves as a monomer in solution (Wahle, 1991; Wahle and Keller, 1992). The enzyme can non-specifically polyadenylate any RNA in the absence of other factors, with no primer specificity except a slight preference for poly(A) or a 3' terminal A residue, which is in agreement with the presence of an A at the cleavage site of most transcripts (McLauchlan *et al.*, 1985; Wahle and Keller, 1992). The N-terminal of the enzyme contains a ribonucleoprotein particle (RNP) consensus, similar to that of RNA-binding proteins. In addition to being essential for polyadenylation PAP is believed to play a role in cleavage of some pre-mRNAs (Takagaki *et al.*, 1988; Christofori and Keller, 1988; 1989).

PAB II : This 49kD nuclear protein, which behaves as a monomer in solution, has a specificity for single-stranded purine polyribonucleotides and like PAP, contains an RNP-consensus. PAB II is responsible for oligo(A) dependent, PAP catalyzed, polyadenylation. Although this can occur in the absence of the AAUAAA specificty factor, only a complex formed from PAP, PAB II and the specificity factor functions with maximum efficiency (Bienroth *et al.*, 1993; Wahle, 1993; Wahle and Keller, 1992). The properties of the specificity factor will be discussed in the following Section.

(c) Complex formation

Formation of the processing complex on pre-mRNA is rapid, ATP dependent, and occurs only on RNAs containing the

AAUAAA hexanucleotide and the appropriate downstream elements. It has been proposed that CPSF and PAP form an initial complex on the pre-mRNA, via direct interaction with the AAUAAA (Gilmartin and Nevins, 1989; Wahle and Keller, 1992). This association has been shown to be unstable and transient, the subsequent addition of CstF stabilises the complex and thereby commits it to processing of the poly(A) site (Weiss *et al.*, 1991). Since the binding of CstF is dependent on downstream elements which are variable in composition, it has been proposed that these elements determine the poly(A) site efficiency by variability in their capacity to bind CstF and therefore stablise the complex (Wahle and Keller, 1992). Two additional factors have been described, CFI and CFII, which are required for catalysis of the cleavage reaction. These additional factors are as yet only poorly characterised (Takagaki *et al.*, 1989; Manley *et al.*, 1993).

The mechanisms by which the proteins present in these complexes interact with the RNA and each other are not as yet understood. However, UV cross-linking analysis of crude nuclear extracts and the previously identified CPSF and CstF fractions has shown that several proteins bind directly to the RNA. The studies of Moore *et al.*, (1988), identified three proteins of 155kd, 68kD and 38kD, present in nuclear extracts which were UV cross-linked to the adenovrius L_3 poly(A) sequences. Cross-linking of the 160kD and 30kD components of CPSF (Gilmartin and Nevins, 1991; Keller *et al.*, 1991) and the 64kD component of CstF (Takagaki *et al.*, 1990; Wilusz and Shenk, 1988) to pre-mRNA substrates has been demonstrated. Isolation and characterisation of cDNAs encoding the 64kD and 50kD components of the CstF, has further elucidated their roles in complex formation. The N-

terminus of the 64kD protein, which contains an RNP-consensus, has been found to bind RNAs in an AAUAAA dependent manner (Takagaki *et al.*, 1992). The 50kD component contains a number of transducin repeats, similar to those of the β subunit of G-proteins and the yeast pre-mRNA splicing protein, which may be responsible for interactions of the CstF complex with the processing factors (Frankel *et al.*, 1991; Takagaki and Manley, 1992).

Taken together these results suggest that complex formation requires the direct interaction of specific complex components with the RNA substrate and co-operative interaction of the components themselves. Such a model of complex formation is shown in Figure 1.9 (taken from Takagaki *et al,*. 1990; Manley *et al.*, 1993).

1.2.4 The role of snRNPs and hnRNPs in 3' end formation

An important and unresolved question is whether or not snRNP particles are involved in 3' end formation. In agreement with snRNP involvement, anti Sm antibodies directed against a common epitope of all U-type snRNPs have been shown to inhibit the 3' processing reaction *in vitro* (Moore and Sharpe, 1984; 1985; Sperry and Berget, 1986). However, Berget and Robberson (1986), demonstrated that site-directed cleavage of the U1, U2, U4 and U6 snRNPs did not affect 3' processing *in vitro*. In addition an RNA fragment containing the AAUAAA sequence has been immunoprecipitated by snRNP specific antibodies (Hashimoto and Steitz, 1986; Stefano and Adams, 1988). The U11 snRNP has also been shown to co-purify with CPSF activity in

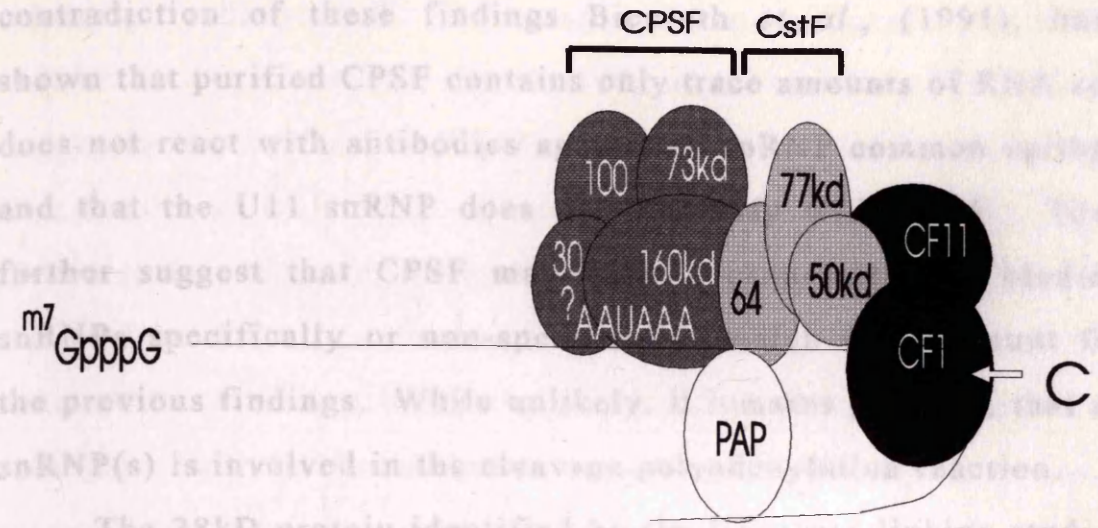


FIGURE 1.9 :

A model representing assembly on pre-mRNA of the 3' processing complex, showing the factors required for cleavage and polyadenylation. CPSF : cleavage and polyadenylation specificity factor, CstF : cleavage stimulation factor, PAP : poly(A) polymerase, with two additional poorly characterised cleavage factors CFI and CFII. The cleavage site C is indicated by an open arrow, ^{m7}GpppG : trimethyl cap. (taken from Manley *et al.*, 1993).

nuclear extracts (Christofori and Keller, 1988). In direct contradiction of these findings Bienroth *et al.*, (1991), have shown that purified CPSF contains only trace amounts of RNA and does not react with antibodies against an snRNP common epitope and that the U11 snRNP does not co-purify with CPSF. They further suggest that CPSF may have a propensity for binding snRNPs specifically or non-specifically, which may account for the previous findings. While unlikely, it remains possible, that an snRNP(s) is involved in the cleavage polyadenylation reaction.

The 38kD protein identified by the UV cross-linking studies of Moore *et al.*, (1988), has been identified as a heterogeneous nuclear ribonucleoprotein particle C (hnRNP) by immunoprecipitation. This protein, one of a set which are thought to be involved in the packaging and post-transcriptional processing of all pre mRNAs (reviewed by Spector, 1993), has a strong affinity for RNA and possibly has an important role in splicing. However the significance of this finding has still to be established.

The exact relationship of the splicing reaction to the cleavage/polyadenylation reactions has still to be determined, with no clear evidence to suggest whether splicing occurs pre or post polyadenylation. There is evidence that splicing can enhance polyadenylation, it has been demonstrated that *in vitro* the presence of an intron enhanced the rate of polyadenylation at a donwnstream poly(A) site (Niwa *et al.*, 1990) and in addition, mutation of the poly(A) site inhibited splicing of the upstream intron (Niwa and Berget, 1991). They propose therefore that splicing and polyadenylation factors interact to recognise the terminal exons containing the poly(A) sequences. However there

is contrasting evidence which shows that splicing components can inhibit polyadenylation. The binding of two molecules of the U1 snRNP U1A protein, to the 3' UTR of U1 mRNA, reduced its expression by inhibition of polyadenylation (Boelens *et al.*, 1993; Van Gelder *et al.*, 1993). This inhibition has subsequently been shown to be mediated by the interaction of the U1A protein with mammalian PAP (Gunderson *et al.*, 1994). It remains to be determined whether these findings are a general feature of splicing and polyadenylation reactions and whether such interactions form part of a mechanism by which the two processes could influence each other.

1.2.5 mRNA transcription termination

Until recently there was relatively little known about the sequence requirements for transcriptional termination, only that termination occurred on a stretch of DNA that may extend hundreds or thousands of nt downstream of the cleavage site (Citron *et al.*, 1984; Le Meur *et al.*, 1984; reviewed by Birnstiel *et al.*, 1985). However, there is now evidence to suggest that transcription termination at the 3' end of genes encoding poly(A)+ mRNAs, requires two distinct cis-acting sequence elements, the poly(A) signal and a downstream transcription pause site (Whitelaw and Proudfoot, 1986; Logan *et al.*, 1987).

There are two proposed models for poly(A) signal mediated termination of transcription - (a) 3' end cleavage generates an unprotected downstream product which is degraded 5' to 3' by exonucleases and/or DNA/RNA helicases, thereby destablising the transcription complex : (b) the poly(A) signal causes factors present in the transcription complex (which prevent termination)

to be released rendering it prone to termination (reviewed by Eggermont and Proudfoot, 1993). Using an *in vitro* coupled polyadenylation-transcription system it was shown that transcription termination at the SV40 early polyadenylation region was dependent on the presence of a functional poly(A) signal and downstream GT-rich element (Miralles, 1991). In addition they demonstrated that there was no degradation of the uncapped 5' transcripts generated by cleavage, lending support to model (b). In the same study transcription termination did not occur at the adenovirus L_3 poly(A) region, suggesting that in this case at least, other factors must be involved. It was recently shown (Edwalds-Gilbert *et al.*, 1993) that the efficiency of termination is directly correlated to the efficiency of 3' end formation. Constructs of a β major globin gene, linked to either the adenovirus L_1 or L_3 poly(A) sequences, were tested for the efficiency of transcription termination. Transcription termination was more efficient at the L_3 site which is also the most efficient 3' processing site. There is growing evidence therefore to suggest that cleavage/ polyadenylation and transcription termination are linked, and that the efficiency of both these processes may be related to the presence of sequence elements downstream of the poly(A) signal.

While regulation at the level of transcription termination is relatively unknown, it has been shown that transcription of one gene may down regulate the transcription of another downstream gene, which is in the same 5' to 3' orientation (Cullen *et al.*, 1984; Proudfoot, 1986). It is possible therefore that the efficiency of transcription termination, directed by 3' processing efficiency, could control the expression of some genes.

1.2.6 mRNA stability

For cells to respond rapidly to changes in transcription rates, mRNAs must be capable of rapid turnover, in addition it is important that the mRNAs of constitutive structural genes are long lived. Therefore not only must turnover of mRNAs from different genes be regulated but mRNAs from the same gene must also be regulated, with the mRNAs from eukaryote cells exhibiting a range of stabilities which vary over several orders of magnitude (Sachs, 1993). The search for sequences which confer stability have so far been fruitless, however recent studies have noted that the presence of AU-rich sequences, within the 3' UTRs of some short lived mRNAs, play a role in their rapid degradation (Shaw and Kamen, 1986; Bohjanen *et al.*, 1991; Vakalopolou *et al.*, 1991). The effect of these sequences appears to be mediated by deadenylation of the poly(A) tails, which is a prerequisite for mRNA degradation, with final degradation being carried out by exo- or endo-nucleases. However, the exact mechanism of this deadenylation has not been determined (reviewed by Sachs, 1993; Atwater *et al.*, 1990). The stabilising effect of the poly(A) tail has been shown to require the poly(A) binding protein PABP (Bernstein *et al.*, 1989), but again the exact relationship of PABP binding and stability has not been determined.

1.2.7 Translation of mRNA

The majority of available information on mRNA translation has been gained from studies of the translational changes which occur during oocyte maturation (Bacharova, 1992; Fox *et al.*, 1989; Vassalli *et al.*, 1989; Jackson and Standart, 1990; Wickens, 1990b). A large fraction of mRNAs synthesised in growing

oocytes are not translated immediately, but remain dormant or masked, and stored for future translation (reviewed by Bacharova, 1992; Wickens, 1990b). In general, stored messages contain short poly(A) tails (15-90 adenylate residues), activation is accompanied by cytoplasmic polyadenylation and an increase in tail length (>150 adenylate residues). Poly(A) tail length appears to have a quantitative effect on mRNA translation with short A tails dictating low levels of translation (Wickens, 1990b; Bacharova, 1992; Vassalli *et al.*, 1989). Cytoplasmic polyadenylation requires the separable activities of the PAP, and an RNA binding activity that recognises the AAUAAA poly (A) signal and a U-rich element, which is located in the 3' UTR of the mRNA. This element sometimes referred to as the cytoplasmic polyadenylation element has the proposed consensus sequence UUUUUNU (Fox *et al.*, 1992; Vassalli *et al.*, 1989; Paris and Richter, 1990). These elements therefore resemble the U-rich downstream elements (Sadofsky and Alwine, 1984; Sadofsky *et al.*, 1985; Manley, 1988) and upstream elements (Valsamakis *et al.*, 1992) identified in a number of eukaryotic mRNAs which are required for efficient 3' end formation. It is possible though unproven as yet that these elements may be identical in function (Fox *et al.*, 1992).

1.2.8 Regulation of 3' processing

Alternative RNA processing is known to be employed in a number of instances to generate diversity in gene expression. For genes in which alternative protein products are predicted to have an important consequence to the cell phenotype, a means of selectively regulating mRNA processing is therefore required.

This regulation may in turn be subject to developmental or tissue-specific control. There are a number of well documented situations where production of more than one mRNA from a single transcriptional unit involves the usage of alternative 3' processing signals, three of which are described here.

(a) Adenovirus 2 major late transcription unit (MLTU)

The major late promoter of adenovirus 2 initiates synthesis of pre-mRNAs that are processed into mRNAs which fall into five 3' co-terminal families, L1 to L5 (Figure 1.10). These mRNAs all share a common tripartite 5' leader with a capped terminus encoded at the RNA initiation site. Interestingly the promoter functions at early times of infection producing L1 transcripts as early as 1h pi, prior to DNA replication and the early-late switch of viral gene expression at 6-8h. In contrast neither the L2 or L3 mRNAs are detectable until 5-6h pi (Shaw and Ziff, 1980). A number of conflicting theories have been proposed to explain this regulation. The first which suggested that transcription termination or pausing downstream of the L1 site, prevents the use of the L2-L5 sites early in infection, has been ruled out (Wilson-Gunn *et al.*, 1992; Falck-Pedersen and Logan, 1989). The second, proposed that at early times the L1 site, which is the most 5' site, is used preferentially and that the switch at late times requires transcription from a replicating viral template. Evidence in support of this has been provided by Falck-Pedersen and Logan (1989), who demonstrated that cis-acting and not trans-acting factors are involved. This implies that direct interaction of the processing machinery with the DNA template occurs and that differences in transcription rates could therefore be involved.

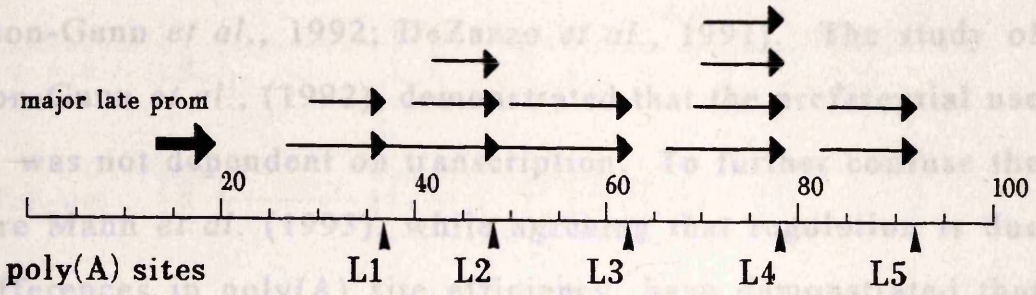


FIGURE 1.10 :

Adenovirus-2 major late transcription unit. L1-L5 are 3' co-terminal families of mRNAs which have a common tripartite 5' leader and promoter. Poly(A) sites are indicated by small arrowheads (adapted from Shaw and Ziff, 1980). The genome position of these transcripts is given in map units.

The third, proposed that regulatory elements located upstream of the L1 site promote its use at early times, and this preferential use is mediated by trans-acting factors present in the nuclear extracts (Wilson-Gunn *et al.*, 1992; DeZazzo *et al.*, 1991). The study of Wilson-Gunn *et al.*, (1992), demonstrated that the preferential use of L1 was not dependent on transcription. To further confuse the picture Mann *et al.* (1993), while agreeing that regulation is due to differences in poly(A) site efficiency, have demonstrated that CstF interaction with the L1 site is less stable than the L3 interaction, in addition they found that there was a substantial decrease in the level of CstF activity as adenovirus infection proceeded to the late phase. They imply that the L3 site is used in preference to the L1 site at late times of infection in direct contradiction of the findings of Wilson-Gunn *et al.*, (1992), who propose that the L1 site is the most efficient.

It is not possible at this stage to make a definitive statement regarding the mechanism of early to late switch of this transcription unit, although there is a strong case for regulation being mediated by poly(A) site strength and the differential interaction of the five poly(A) sites with the available processing factors.

(b) The immunoglobulin heavy chain locus

Primary transcripts from the IgM heavy chain (μ) transcription unit are differentially processed to produce two distinct mRNAs in a developmentally-regulated and cell specific manner. The membrane (μm) and secreted (μs) forms are produced from a single primary transcript which is alternatively processed at its 3' end (Figure 1.11). Cleavage at the proximal

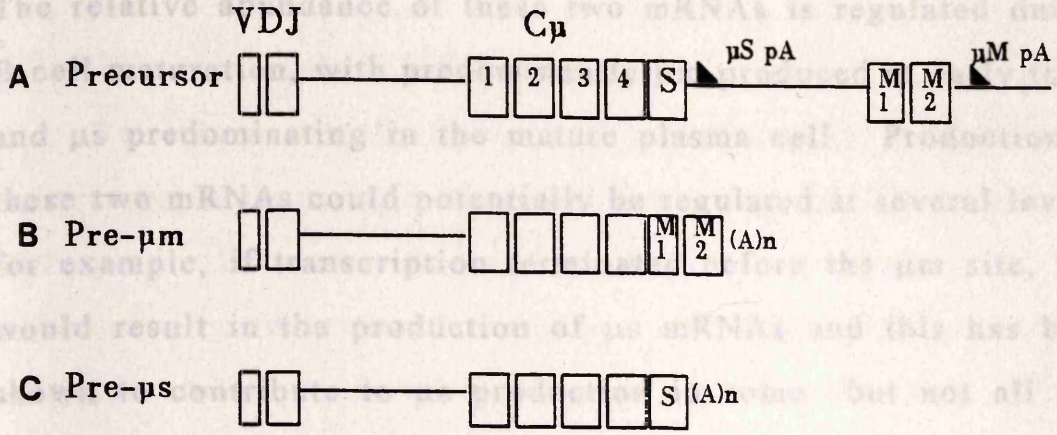


FIGURE 1.11 :

Representation of the proposed 3' processing scheme for the production of the membrane bound and secreted forms of the immunoglobulin heavy chain μ RNA (adapted from Peterson and Perry, 1989). VDJ : productively rearranged immunoglobulin variable, diversity and joining segments. Cμ 1-4 : μ Constant region exons, S : secretory exons, M : membrane exons, (A)n : poly(A) tail. Polyadenylation sites are indicated by large arrowheads.

A : IgM pre mRNA

B : membrane bound form of μ pre-mRNA, produced by splicing of exon M to exon Cμ4, with removal of the secretory exon and poly(A) site, and cleavage and polyadenylation at the membrane poly(A) site (μM pA).

C : secretory form of μ pre-mRNA, produced by cleavage and polyadenylation at the secretory poly(A) site (μS pA).

poly(A) site producing μs mRNA, and splicing of the Cμ4 exon to the M exons, which removes the μs site, producing μm mRNA. The relative abundance of these two mRNAs is regulated during B-cell maturation, with predominantly μm produced at early times and μs predominating in the mature plasma cell. Production of these two mRNAs could potentially be regulated at several levels: for example, if transcription terminated before the μm site, this would result in the production of μs mRNAs and this has been shown to contribute to μs production in some but not all cell lines representative of the mature plasma cell (Guise *et al.*, 1988; Galli *et al.*, 1987). Competition for cleavage and polyadenylation between the less efficient μs and the efficient μm poly(A) site would result in preferential production of μm RNAs under limiting conditions and production of μs mRNAs under non-limiting conditions (Galli *et al.*, 1988). This example pre-supposes that splicing can occur only after cleavage and polyadenylation at the μm site and that the splicing reaction is therefore not involved in regulation. However the relative kinetics of splicing vs cleavage/polyadenylation are not known at this time. Similarly, competition between cleavage/polyadenylation at the μs poly(A) site and splicing at the suboptimal Cμ4 to M exon splice site would result in μs mRNA production depending on the relative strengths of these two reactions. This assumes that splicing can occur either before or after cleavage/polyadenylation at the μm site (Lassman and Milcarek, 1992; Petersen and Perry, 1989). Both models however, predict that regulation depends on increasing usage of the less efficient μs site and that factors involved in the

cleavage/polyadenylation reaction are therefore limiting in B-cell and not limiting in the plasma cell.

(c) The calcitonin/CGRP gene

Calcitonin or calcitonin gene related peptide (CGRP) arise from a single genomic locus in which the primary RNA transcript undergoes tissue-specific processing, which results in the differential production of calcitonin or CGRP mRNAs. CGRP mRNA, which is produced in neuronal cells, results from splicing of the first three exons to the 5th and 6th exons, with use of the distal poly(A) site 3' to the 6th exon. Calcitonin mRNA, produced in thyroid cells, contains the first four exons and utilises the poly(A) site immediately 5' to exon 4 (Amara *et al.*, 1984) (Figure 1.12). Expression experiments, using the wild type gene and a series of mutated calcitonin/CRGP genes, have shown that mRNA regulation is not determined by poly(A) site selection but by choice of either the exon 4 or exon 5 splice site (Leff *et al.*, 1987). It has been demonstrated that CGRP mRNA production is dependent upon the efficient utilisation of the intrinsically weak calcitonin specific 3' site of exon 4 (Yeakley *et al.*, 1993). This tissue specific splicing must reflect differences in the splicing machinery of different cells.

(d) The HSV-1 late processing factor (LPF)

mRNA transcripts which are 5' co-terminal and utilise different poly(A) sites are not an uncommon finding in HSV (Wagner, 1985), it has been suggested therefore, that HSV gene expression may be influenced by the efficiency of poly(A) site usage. In support of this theory McLauchlan *et al*, (1989; 1992b),

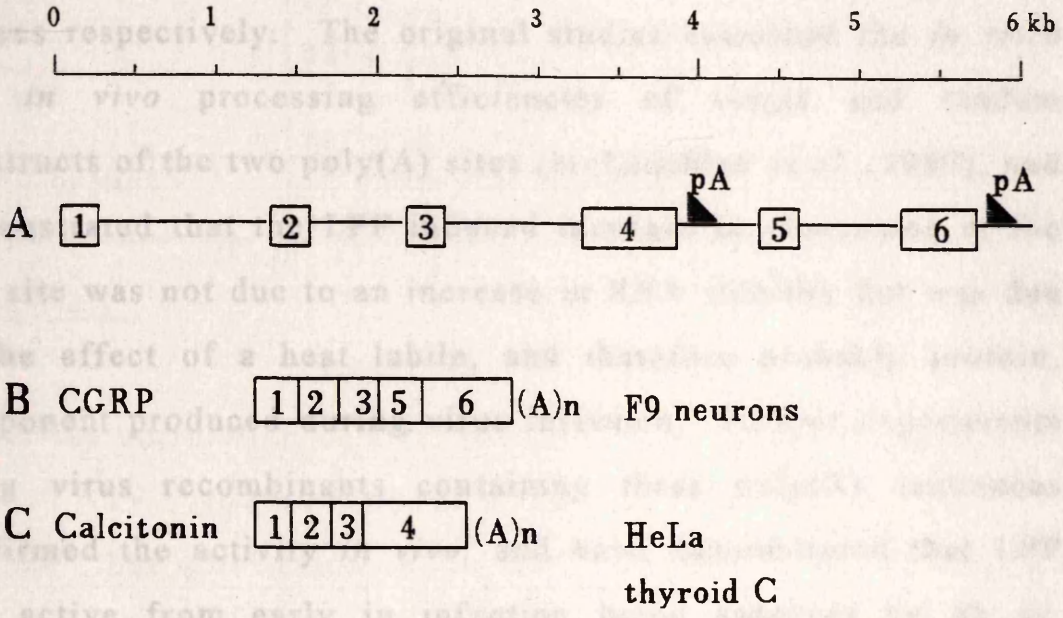


FIGURE 1.12 :

A : organization of the calcitonin/CGRP gene, exons are represented by open boxes and introns by solid lines.The size of the gene (kb) is shown at the top of the figure and the poly(A) sites (pA) are indicated by large arrowheads, (A)n poly(A) tail (adapted from Yeakley *et al.*, 1993).

B : CGRP is produced in neuronal cells by the splicing of exon 4 to exon 6 and usage of the poly(A) site immediately 3' of exon 6, with removal of exon 5 and the poly(A) site immediately 3' of exon 4.

C : calcitonin is produced in HeLa and thyroid C cells by cleavage and polyadenylation at the poly(A) site immediately 3' of exon 4.

identified an activity, termed LPF, induced in HSV infected cells which selectively increased 3' processing efficiency at an HSV-2 late poly(A) site (UL38) and did not increase 3' processing efficiency at the HSV-2 IE12 poly(A) site, common to the US10 and US11 genes which are from the IE, early and late temporal classes respectively. The original studies examined the *in vitro* and *in vivo* processing efficiencies of single and tandem constructs of the two poly(A) sites (McLauchlan *et al.*, 1989), and demonstrated that the LPF induced increase in processing at the late site was not due to an increase in RNA stability but was due to the effect of a heat labile, and therefore probably protein, component produced during virus infection. Further experiments using virus recombinants containing these poly(A) sequences confirmed the activity *in vivo*, and have demonstrated that LPF was active from early in infection being apparent by 4h pi. Infection in which protein synthesis was prevented by treatment with cycloheximide, indicated that gene expression was required for LPF activity. Using a range of virus mutants deficient in IE gene expression the IE63 gene product was identified as essential for LPF activity (Table 1.2). Finally transient transfection experiments, where constructs containing either the IE or late poly(A) site were co-transfected with an IE63 expression plasmid, confirmed that IE63 was required for induction of a five fold increase in expression of the construct containing the L poly(A) site. Thorough though these studies were it remains to be determined whether this virus activity has a more widespread effect on HSV poly(A) site processing efficiency other than the two examples studied. In addition the fragment sizes of the two poly(A) sites examined were quite different, the late poly(A) site

Virus	Virus gene(s) expressed	LPF activity
wt HSV-1 (17^+)	IE, early and late	+
*ts*1204	None	-
*ts*K	IE (non-functional IE175)	+
*ts*1207	IE, early	+
*dl*1403	IE (except IE110), early and late	+
27-LacZ	IE (except IE63), early and late	-
HSV-1 (F)Δ325	IE (truncated IE68), early and late	+

TABLE 1.2 :

Shows the correlation of LPF activity with virus gene expression from wt HSV-1 strain (17+) and a range of virus mutants.

***ts* 1204** : has a *ts* lesion in the UL25 gene, virions can bind to cells but fail to penetrate and release the capsid into the cytoplasm, no viral proteins are produced.

***ts*K** : has a *ts* lesion in IE175 and produces only four functional IE polypeptides IE110, IE63, IE68, IE12 and a non-functional IE175.

***ts*1207** : has a *ts* mutation is the UL39 gene and produces IE and early proteins and no late proteins..

***dl*1403** : has a deletion within both copies of IE110 and produces all IE proteins (except IE110) and early and late proteins..

27-LacZ : contains a LacZ gene inserted into the 5' untranslated leader region of the IE63 gene and produces all IE proteins (except IE63), early proteins and some late proteins.

HSV-1 (F) Δ325 : contains a deletion in the IE68 gene and produces all IE proteins, (with a truncated version of IE68), early and late proteins. (Taken from McLauchlan *et al.*, 1992b).

construct containing sequences 118nt upstream and 141nt downstream of the UL38 poly(A) signal and the IE12 poly(A) site construct containing sequences 26nt upstream and 62nt downstream of the poly(A) signal, it is possible therefore that some elements required for LPF regulation of processing were missing from the IE site.

There are a number of questions raised by these initial studies regarding the range and specificity of LPF activity, the involvement of IE63 and the overall role of this activity in the regulation of viral gene expression. It is the purpose of this project therefore to address these questions.

Aims of the project

1. To determine the range and specificity of LPF activity.

2. To study the role of IE63 in:-
   a) the LPF mediated increase in 3' processing efficiency of selected HSV-1 poly(A) sites
   b) the relationship of this activity to the expression of proteins from the three temporal classes of HSV-1 genes.

3. To investigate the properties which determine whether a particular HSV-1 poly(A) site responds or does not respond to LPF by :-
   (a) examination of the protein binding properties of selected HSV-1 poly(A) sites using mock infected and HSV-1 infected nuclear extracts
   (b) by analysis of HSV-1 poly(A) site mRNAs for sequence and structural elements which may influence 3' processing efficiency.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Cells

BHK C13 Baby hamster kidney cells were used for the propagation and titration of both wild type (wt) viruses and recombinant viruses.

HeLa cells, derived from a human cervical carcinoma cell line, were used for the production of nuclear extracts.

Vero cells, derived from African green monkey kidney, were used as a negative control for 27-LacZ infections (Smith *et al.*, 1992) and for the growth of HSV-1 strain KOS.

Vero 2.2 cells are derived from Vero cells by co-transfection of a plasmid containing the neomycin resistance gene (G418), under the control of the SV40 promoter, and the plasmid pSG130B/S containing the wild type ICP27 (IE63) gene (Smith *et al.*, 1992). These cells were used for the assay and growth of 27-LacZ.

2.1.2 Bacterial cells

E.Coli K12 strain DH5α (hsd R17, lac U169, gyr A96, thi-1, rel A1, rec A1, end A1, supE44, ϕ80, lacZ M15) were used for the propagation of plasmids.

2.1.3 Viruses

HSV-1 (wt) strain 17^+ and HSV-1 (wt) strain KOS were obtained from stocks maintained in the Institute by Mary Murphy. HSV-1 1802 which contains a unique Xba-1 site (Rixon and McLauchlan, 1990) a gift from Dr. V. Preston, was used for the production of recombinant virus. 27-LacZ the HSV-1 ICP27 (IE63) insertion mutant was a gift from R.M. Sandri-Goldin. This virus was made from HSV-1 strain KOS by insertion of a cassette, containing the lacZ gene under the control of the HSV UL39 promoter, into the 5' untranslated leader region of the ICP27 (IE63) coding sequences, resulting in inactivation of the IE63 gene, therefore no IE63 protein is produced (Smith *et al.*, 1992).

2.1.4 Tissue culture media

BHK-C13 cells were grown in Glasgow's modified eagle's medium (GibcoBRL) supplemented with 0.25% sodium bicarbonate, 4mM L-glutamine, 100units/ml penicillin, 100units/ml streptomycin, 10% tryptose phosphate broth and 10% new born calf serum (NBCS).

Vero cells were grown as for BHK-C13 cells, 10% foetal calf serum replacing the 10% NBCS.

Vero 2.2 cells were grown as for Vero cells with a supplement of G418 (neomycin) as a selection agent for stable transfectants. G418 was omitted during experimental work.

2.1.5 Bacterial culture media

Bacteria were propagated in 2YT broth (88.5mM $NaCl_2$, 1.6% (w:v) Bacto tryptone, 1% (w:v) yeast extract). Solid media

for the isolation of bacterial colonies consisted of 1.5% (w:v) agarose in L-broth (1% (w:v) Bacto tryptone, 1% yeast extract, 177mM $NaCl_2$). Media was supplemented where appropriate with ampicillin.

2.1.6 Enzymes

Restriction enzymes and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim. DNase and RNaisin (ribonuclease inhibitor) from Promega. SP6 RNA polymerase and DNA T4 ligase from Gibco BRL. Proteinase K from Sigma and Taq DNA polymerase from Perkin Elmer.

2.1.7 Radiochemicals

The following radiochemicals were obtained from Amersham:- (α-^{32}P) UTP, specific activity 800Ci/mmol. ^{14}C chloramphenicol (CAT assay grade), specific activity 50-62 mCi/mmol. ^{35}S dATP, specific activity >1000Ci/mmol. Methyl ^{3}H thymidine, specific activity 25Ci/mmol.

2.1.8 Chemicals and miscellaneous reagents

General chemicals were obtained from Sigma and BDH. TEMED from Bio-Rad. The ECL detection system and Rainbow coloured protein molecular weight markers from Amersham.

2.1.9 Antibodies

The following mouse monoclonal antibodies were produced in the Institute and were a gift from Dr. A. Cross. Mouse monoclonal antibodies were raised against the protein products of the following HSV genes : IE110, IE175, UL29, US6, US8 and

UL42. Rabbit polyclonal antipeptide antibodies to HSV UL45 and IE63 were produced in the Institute and were a gift of G. McVey. Rabbit polyclonal antibody to purified HSV UL44 was a gift from Dr. G. H. Cohen and Dr. R. J. Eisenberg. The rabbit polyclonal antipeptide antibody to HSV-2 UL38 was a gift of Dr. W.S.M. Wold (Yei *et al.*, 1990). Peroxidase conjugated goat anti-mouse antibody (IgG) was obtained from Sigma and peroxidase conjugated protein-A was obtained from Bio-Rad.

2.1.10 Plasmids

pLW2 (Gaffney *et al.*, 1985), was used as a marker of transfection efficiency in transient assays. This plasmid carries the CAT reporter gene under the control of the HSV-2 IE12/68 promoter with the polyadenylation sequences from IE12 and was a gift from Dr. J. McLauchlan.

The constructs generated during this study were based on the plasmids pGEM1 and pCAT (Promega), and pFJ10 (Rixon and McLauchlan, 1990).

2.1.11 Commonly used buffers and solutions

LB: 50% (w:v) glycerol, 445mM Tris.,
10mM EDTA, 445mM Boric acid,
0.1% bromophenol blue,0.1% xylene cyanol.

50x DHS: 1% Ficoll, 1% polyvinylpyrrolidone,
1% bovine serum albumin (BSA).

PBS: 170mM NaCl, 3.4mM KCl, 10mM $NaHPO_4$,
2mM KH_2PO_4 (pH7.2).

2X PKB: 100mM Tris.Cl (pH8.0),
20mM EDTA (pH8.0), 20m MNaCl, 0.4% SDS.

STET: 1% Triton-X100, 10mM Tris.Cl (pH8.0), 234mM Sucrose, 50mM EDTA.

20xSSC: 3M NaCl, 300mM NaCit (pH7.4).

20xSSPE: 3M NaCl, 200mM NaH_2PO_4, 20mM EDTA.

TAE: 40mM Tris., 0.1% acetic acid, 50mM EDTA.

TBE: 89mM Tris., 2mM EDTA, 89mM Boric Acid.

TBS (A): 4mM Tris., 100mM NaCl (pH7.5).

TTBS: 0.05% Tween-20 in TBS(A).

TE: 10mM Tris.Cl, 1mM EDTA (pH7.1).

TBS(B): 140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1mg/ml dextrose, 25mM Tris.Cl (pH7.4), 0.005% (w:v) phenol red, 100units/ml penicillin, 100units/ml streptomycin.

Trypsin: 25% (w:v) trypsin in TBS(B).

2.2 Methods

The methods used in this work were essentially as described by Maniatis *et al.*, (1989).

2.2.1 Cell culture

All cell lines were maintained as monolayers in the appropriate growth medium (GM), at 37°C in 5% CO_2. Harvesting of the cells was performed by washing the cell monolayer once in versene, then once with a (1:3) trypsin:versene mix. Cell stocks were stored in 1ml aliquots of 10^8 cells/ml at -70°C in a (2:1:2) mixture of serum: glycerol: growth medium.

2.2.2 Plasmid preparation

(a) Small scale method

1ml of a 10ml overnight culture of bacteria, inoculated from a single transformed colony, was centrifuged at 13,000rpm for 1min. The pellet was resuspended in 100µl of STET and lysozyme (1mg/ml) and boiled for 1min. The lysate was cleared by centrifugation (13,000rpm, 10min). An equal volume of isopropanol was added to the lysate and the precipitated nucleic acids pelleted by centrifugation (13,000rpm, 10min). The pellet was washed in 70% ethanol, lyophilised, resuspended in H_2O and stored at -20°C. Restriction endonuclease digests of DNA prepared by this method included 0.2µl of RNase A (1mg/ml) to remove any contaminating RNA, facilitating band visualisation following gel electrophoresis.

(b) Large scale method

Bacterial cells were harvested from a 300ml saturated overnight culture by centrifugation (5000rpm, 10min). The cell pellet was resuspended in 3ml of pre-lysing solution (25% sucrose, 50mM Tris.Cl pH 8.0), 1ml Lysozyme (5mg/ml), 1ml 250mM EDTA (pH8.0) and left on ice for 5min. After the addition of 5ml lysis solution (1% (w:v) Brij, 0.4% (w:v) deoxycholate, 62.5mM EDTA, 500mM Tris.Cl) the mixture was left to lyse on ice and was finally cleared by ultracentrifugation (48,000rpm, 30min). Caesium chloride (0.9gm/ml) and ethidium bromide (0.5ml of 5mg/ml) were added to the clear lysate and plasmid DNA was banded by ultracentrifugation (40,000rpm, 64h). Covalently closed circular plasmid DNA was removed from the caesium chloride gradient using a hypodermic needle and syringe. Ethidium bromide was removed by repeated extractions with iso-butanol. Caesium chloride was removed by ethanol precipitation, 1/10th volume of 6M sodium acetate and 2.5 volumes of ethanol were added and the mixture precipitated overnight at -20°C. Plasmid DNA was pelleted by centrifugation (13,000rpm, 20min) washed once in 70% ethanol, lyophilised and resuspended in H_2O.

2.2.3 Quantification of DNA

Determination of DNA concentration was performed by spectrophotometry as described by Maniatis *et al.*, (1989).

2.2.4 Storage of recombinant bacteria

Transformed bacteria were stored as a frozen glycerol stock. 0.9ml of overnight culture was mixed with 0.1ml sterile glycerol and stored at -70°C.

2.2.5 Sequencing of plasmid DNA

Sequencing of plasmid DNA was performed using Sequenase version 2.0 DNA sequencing kit (United States Biochemical). This method is based on the dideoxy-mediated chain termination method. Denaturation and sequencing of plasmid DNA was carried out in accordance with the manufacturers recommended protocol. Electrophoresis of the sequencing products was performed on an 8% polyacrylamide gel prepared using Sequagel sequencing system reagents (National Diagnostics).

2.2.6 Transfection of BHK-C13 cells

(a) Calcium phosphate precipation (DMSO)

This method is as described by Stow and Wilkie (1976). 10μg of plasmid DNA was mixed with 500μl of HBS (0.137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 5.5mM D-glucose, 21mM hepes pH7.05) containing 10μg calf thymus carrier DNA. 35μl of 2M $CaCl_2$ was added and the solution mixed thoroughly. Growth medium was removed from a 50mm culture dish containing a 70% confluent monolayer of BHK cells and the calcium DNA precipitate used to overlay the cells. The culture plates were incubated at 37°C in 5% CO_2 for 40min after which 4ml of growth medium was added and the cells incubated for a further 4h at 37°C. The medium was removed and the cell monolayer washed once in serum free medium. 1 ml of 25% (v:v) DMSO in HBS was

then added to the cells for exactly 4min after which the cell layer was washed carefully three times in serum free medium, overlayed with normal growth medium and incubated for a further 48h.

(b) Lipofection

30µl of Lipofection reagent (Rose *et al.*, 1991), which contains 1ml Ptd Etn (L-a-phosphatidylethanolamine dioleoyl) and 4mg DDAB (dimethyldioctadecylammonium bromide), was mixed with 70µl of HBS (20mM hepes, 150mM NaCl pH7.4). In a separate tube 5µg of plasmid DNA was suspended in 100µl of HBS, both solutions were mixed together and left at room temperature (RT) for at least 10min. The transfection mixture was then added to 2ml of Optimem (low serum medium, Gibco BRL) and was used to overlay a 50mm culture dish containing a 70% confluent monolayer of BHK cells. After incubating the cells at 37°C for 4h, 2ml of GM containing 20% NBCS was added to the monolayer and the cells incubated for a further 48h. The Lipofection reagent was a gift from C. Addison.

2.2.7 Standard cloning techniques

(a) Restriction endonuclease digestion

Restriction endonuclease digests were performed using the buffers and conditions recommended by the suppliers.

(b) Purification of DNA fragments

Purification and isolation of DNA fragments was carried out on low melting temperature (LMT) agar gels using the method described by Garabedian *et al.*, (1993). Samples suspended in loading buffer (LB) were electrophoresed on LMT agarose gels at

6V/cm for 1 h. The gels consisted of TAE and the appropriate percentage (0.7-1%) LMT agarose and 0.5µg/ml ethidium bromide. The DNA fragments were visualised by long wavelength UV light and the band containing the DNA fragment excised. Three volumes of 200mM NaCl and 20mM EDTA were added to the gel slice and heated to 68°C to melt the agar. DNA was removed from the mixture by phenol extraction (x2) followed by phenol/chlorofrom extraction (x1). The DNA was finally recovered by ethanol precipitation.

2.2.8 Polymerase chain reaction - PCR

(a) Oligonucleotide primers

For each of the regions of HSV-1 DNA to be amplified by PCR, two 30mer primers (one 5' and one 3' to the sequence required), were designed according to the recommendations of Innis and Gelfand, (1990). Each primer contained a 10 nucleotide 5' degenerate end encoding an appropriate restriction endonuclease site which would allow simple cloning of the fragment into the required vector, pGEM1. The oligonucleotides were produced in the Institute on a Cruachem PS250 DNA synthesiser.

(b) Deprotection of oligonucleotides from the synthesis column

A 2ml disposable syringe containing 1.5ml concentrated ammonium hydroxide was attached to one end of the column and a second empty 2ml syringe was attached to the other end of the column. One column volume of ammonium hydroxide was gently pushed into the column which was then left at RT for 20min.

Another column volume of ammonium hydroxide was then pushed into the column and left a further 20min. This procedure was repeated until all the ammonium hydroxide had been pushed through the column. The ammonia solution was then pushed back and forth through the column several times, mixing the solution thoroughly. Deprotection of the oligonucleotide was carried out by incubation of the mixture in a securely capped vial at 55°C for 5h, followed by overnight lyophilisation.

(c) Oligonucleotide purification

Half of the lyophilised aliquot of oligonucleotide was resuspended in sample loading buffer (90%(w:v) deionized formamide, 89mM Tris., 2mM EDTA, 89mM boric acid) and was heat denatured at 90°C for 10min, then cooled on ice. The sample was loaded onto a polyacrylamide gel (15% acrylamide, 0.6% bis.acrylamide, 42% urea, in TBE) and electrophoresed at 30w for 1h. Tracking dye (0.1% bromophenol blue, 0.1% xylene cyanol in formamide) was loaded in parallel with the sample to allow the progress of the oligonucleotide down the gel to be followed. The oligonucleotides were visualised by shadowing with long wavelength UV light then cut from the gel and shredded finely. The DNA was eluted from the gel into 200µl H_2O by incubation in a shaking incubator at 37°C for 2h. The mixture was filtered through a Spin-X column, phenol/chloroform extracted and the oligonucleotides recovered by ethanol precipitation. The concentration of the oligonucleotides was determined as described in Maniatis *et al.*, (1989) using the following formula.

$$\text{Concentration } (\mu M) = \frac{OD_{260}}{10x \text{ length of oligonucleotides}}$$

All oligonucleotides were resuspended in sterile H_2O at a concentration of 50pmol/µl.

(d) Polymerase chain reaction - PCR

PCR was carried out using Taq DNA polymerase, under the conditions recommended by Innis and Gelfand (1990). The reaction mixture consisted of the following, PCR buffer (no $MgCl_2$), 100µM of each dNTP (dATP, dCTP, dGTP, dTTP), 100ng DNA template, 50pmol of each oligonucleotide primer, 1mM $MgCl_2$, 2.5 units Taq DNA polymerase, in a total volume of 100µl of sterile H_2O. The optimal concentration of $MgCl_2$ was determined by examination of a range of concentrations between 1-4mM. The reaction mixture was covered with a layer of mineral oil and amplification carried out on an automated PCR machine (Hybaid, Omnigene). The following conditions were used in all cases, further optimisation of the system was not required.

The PCR program used was as follows:-

Step 1	95°C	5min	
2	50°C	1min 30sec	denaturation 1 cycle
3	72°C	2min	
4	95°C	1min 10sec	
5	55°C	1min 30sec	amplification 30 cycles
6	72°C	2min	

7	95°C	1min 10sec	
8	55°C	1min 30sec	extension 1 cycle
9	72°C	5min	

To check that the correct size of fragment had been produced a 10µl aliquot of the PCR product was run on a 1% agarose gel (TBE), containing 0.5µg/ml ethidium bromide and the DNA visualised using short wave UV light. DNA was retrieved from the remainder of the PCR product by phenol/chlorofrom extraction followed by ethanol precipitation.

2.2.9 Ligation of DNA fragments

DNA fragments with cohesive ends were produced in two ways. (a). PCR products were cleaved with the appropriate restriction endonucleases according to the enzyme site encoded in the primers, ethanol precipitated and reconstituted in 10µl H_2O. (b). 10µg plasmid DNA was cleaved with restriction endonucleases and the appropriate fragment purified from a LMT gel. Vector plasmid DNA was cleaved with restriction endonucleases to generate compatible cohesive ends. To prevent reannealing of the vector plasmid ends, after cleavage was complete, the digest was treated with 25units CIP (calf intestinal phosphatase) at 37°C for 30min. The appropriate fragment of vector DNA was then purified as before. DNA fragment and vector plasmid were mixed in ligation buffer (50mM Tris.Cl pH7.6, 10mM $MgCl_2$, 10mm DTT, 5mM ATP, 50µg/ml BSA, 5 units T4 DNA ligase) at a ratio of 10:1 and incubated overnight at 16°C.

2.2.10 Transformation of E.coli

(a) Preparation of competent bacteria for electroporation

This method follows the Bio-Rad manual recommendations. 10ml of a saturated overnight culture of bacteria was added to 1L of 2YT broth and grown at 37°C in a shaking incubator until the OD_{600} reached 0.6. The cells were transferred to pre-cooled tubes and centrifuged at 4,000rpm for 15min. The supernatant was discarded and the pellet resuspended in 1L ice cold sterile H_2O. This step was repeated once more, the pellet resuspended in 20ml ice cold 10% glycerol, centrifuged once again and the pellet finally resuspended in ice cold 10% glycerol to give a total volume of 0.5ml. This suspension was divided into 40µl aliquots, frozen on dry ice and stored at -70°C.

(b) Transformation - Electroporation

Transformation of ligated plasmid DNA was carried out after heat inactivation of the ligase enzyme at 65°C for 10min (Ymer, 1991). 1-3µl of the ligation mixture was added to 40µl of competent cells, transferred to a cold 0.2mm electroporation cuvette and one pulse of 12.5KV/cm (time constant 4-5msec) applied to the cuvette. The cells were immediately resuspended in 1ml 2YT broth and the suspension incubated at 37°C for 1h. Solid medium plates containing ampicillin, were inoculated with 100µl aliquots and incubated overnight to allow the formation of colonies.

2.2.11 Preparation and assay of virus

(a) Preparation of virus stocks

Monolayers of BHK cells in 2L roller bottles were infected with HSV at a moi of 0.003 pfu/cell in 40ml GM. The virus infected cells were incubated at 31°C for 3-4 days until complete CPE (cytopathic effect) was observed. The cells were shaken into the GM and pelleted by centrifugation (1500rpm, 10min). To concentrate the cell released virus (CRV) the supernatant was further centrifuged (12,000rpm, 2h) the pellet resuspended in 5ml GM and sonicated for approximately 5min. The pellet of cell associated virus (CAV) from the initial low speed centrifugation step was resuspended in a small volume of GM, sonicated briefly, centrifuged at low speed, and sonicated twice more until the cell clumps were dispersed. Both the CRV and CAV viral suspensions were checked for sterility, titrated on BHK cells and stored at -70°C.

(b) Titration of virus

Serial ten fold dilutions of virus samples were made in PBS with 10% NBCS. Duplicate tissue culture plates (50mm) of BHK monolayers were infected with 400µl aliquots of the 10^{-4} to 10^{-9} dilutions. GM was removed from the plates and the virus left to adsorb to the cells for 1h at 37°C. The inoculum was then removed and the cells overlayed with 4ml of Eagle's A+B containing 0.5% agar and 10% NBCS. The cells were incubated at 37°C for 3-4 days to allow plaques to develop. Before staining with Geimsa the cells were fixed and the virus killed with 2ml glutaraldehyde solution (Cidex). The plaques were then counted and the virus titre established.

(c) Preparation and purification of viral DNA

10 2L roller bottles of BHK monolayers were infected as before (0.003pfu/cell). Once CPE was complete the cells and virus were shaken into the GM and centrifuged (2,500rpm, 30min, 4°C) to remove cell debris. The supernatant was further centrifuged (12,000rpm, 2h, 4°C) and the pellet resuspended in 1ml Eagle's A+B and placed overnight on a rotary mixer at 4°C. The virus suspension was then separated on a 5-15% ficoll gradient (12,000rpm, 2h, 4°C). The tight lower band of virion DNA was removed by dripping through a disposable syringe needle. Virion DNA was mixed with Eagle's A+B and this suspension pelleted by centrifugation (15,000rpm, 2h, 4°C) (Rixon *et al.*, 1988). The pellet was resuspended in 100µl TE, treated with Proteinase K (50µg/ml) and 0.5% SDS for 1h at 37°C (Stow and Wilkie, 1976), phenol/chloroform extracted three times, ethanol precipitated overnight and resuspended in 50µl H_2O. The DNA concentration of the sample was estimated by running an aliquot alongside known DNA standards on a 0.7% agarose TBE gel.

2.2.12 Production and assay of recombinant virus

(a) Construction and screening of recombinant virus

Plasmid DNA cleaved with Xba-1 was electrophoresed on a 0.7% LMT agarose TAE gel and the appropriate fragment isolated and purified. The plasmid DNA fragment (approximately 0.5µg) was ligated to Xba-1 digested HSV-1 1802 DNA (approximately 0.5µg). Half of the ligation mix was used to transfect a monolayer of BHK cells (50mm culture plate), using either the calcium phosphate precipitation or Lipofection method. The cells

were overlayed with 4ml GM and left at 37°C for 2-4 days until plaques were seen. A mini preparation of virus stock was made from the transfection plate by scraping the cells into the GM. Titrations were performed on this stock and single plaques picked and resuspended in 200µl PBS containing 10% NBCS. Further mini stocks of virus were made by infecting BHK monolayers (1 well of a 24 well Lindbro cell culture plate containing $5x10^5$ cells). These mini virus stocks were assayed for CAT activity and positive plaques went through further rounds (2-3) of purification, to produce a preparation of homogeneous virus. Stocks of virus were made as described previously using aliquots of the mini virus stock (approximately 10^7pfu/ml) to infect five 2L roller bottles of BHK cells. The final stock of virus was titrated as described previously. One set of duplicate titration plates was stained for the presence of β-galactosidase (the β-galactosidase gene is carried on the plasmid DNA insert). Comparison of the virus titres from both sets of plates confirmed the purity of the stock.

(b) Chloramphenicol acetyl transferase (CAT) assay

BHK cells were infected at a moi of 10 pfu/cell with the appropriate virus. The cells were harvested at 2, 4, 8, 12, 18 and 24h post infection, washed once in PBS, scraped into 1ml of PBS, pelleted (13,000rpm, 2min) and resuspended in 50µl of 250mM Tris.Cl pH7.8. The cells were lysed by freeze thawing three times on dry ice and cell debris removed by centrifugation (13,000rpm, 5min). Extracts were assayed for CAT activity using the solvent extraction method of Seed and Sheen, (1988). 10µl of extract was added to 40µl of reaction mix (0.05µCi ^{14}C chloramphenicol,

0.5mM butyryrl co-enzyme A and 200mM Tris.Cl pH7.8) and incubated at 37°C for 1h. 200µl of a solution of TMPD and mixed xylenes (2:1) was added to each reaction tube, mixed thoroughly and centrifuged (13,000rpm, 2min) 180µl of the top layer containing the butyrylated form of the chloramphenicol was added to 5ml scintillation fluid and the counts per minute (cpm) measured. CAT activity was expressed as the percentage ^{14}C chloramphenicol converted to the butyrylated form per µg of protein per hour.

(c) Thymidine kinase (TK) assay

Cell extracts were prepared as described in the previous method by lysis of the cells in either CAT buffer (250mM Tris.Cl pH7.8) or thymidine kinase assay (TK) buffer (20mM Tris.Cl pH7.5, 2mM $MgCl_2$, 10mM NaCl, 6.5mM β- mercaptoethanol, 0.5% (v:v) NP40). 10µl of extract was added to 40µl of reaction mix to give final concentrations of 100mM sodium pyrophosphate pH6.0, 10mM $MgCl_2$, 100µM dTTP, 5mM ATP, 100µCi/ml methyl ^3H thymidine. The mixture was incubated at 30°C for 2h. The reaction was terminated by the addition of 100mM EDTA and 33µM thymidine and heating to 90°C for 4min. After cooling on ice for 5 min the mixture was centrifuged (13,000rpm, 2min) and the supernatant spotted onto filter paper discs (Whatman's DE81). The discs were washed three times in 4mM ammonium formate pH4.0 with 10mM thymidine at 37°C, then twice in 100% ethanol. The discs were dried, placed in 5ml scintillation fluid and the cpm of ^3H thymidine measured. TK activity was expressed as cpm/µg protein.

2.2.13 *In vitro* mRNA processing

(a) Preparation of nuclear extracts

HSV infected or mock infected nuclear extracts were made in parallel using the small scale method of Lee and Green, (1990). HeLa cells were grown in 140mm culture plates to 70% confluency and either infected for 8h with 10pfu/cell of the appropriate virus in 3ml GM or mock infected with 3ml of GM. Cells were harvested in PBS and pelleted by centrifugation (2,000rpm, 5min, 4°C). The pellet was washed once in 30 volumes of PBS, resuspended in 1 pack cell volume (PCV) of buffer A (10mM hepes pH8.0, 1.5mM $MgCl_2$, 10mM KCl, 1mM DTT) and left to swell on ice for 15min. The cells were lysed by passing the suspension through a narrow-gauge needle 5-8 times (produces approximately 80% lysis). A crude nuclear pellet was produced by centrifugation of the lysate (13,000rpm, 20sec). Nuclei were resuspended in two thirds of the original PCV in buffer C (20mM hepes pH8.0, 1.5mM $MgCl_2$, 25% (v:v) glycerol, 420mM NaCl, 0.2mm EDTA, 1mM DTT, 0.5mM PMSF (phenyl methyl sulphonyl fluoride)) and incubated at 4°C for 30 min on a rotary mixer. The suspension was centrifuged (13,000rpm, 30min, 4°C) and the supernatant aliquoted and stored at -70°C.

(b) *In vitro* transcription/labelling of plasmid DNA

Precursor RNAs for *in vitro* polyadenylation reactions were synthesised using SP6 RNA polymerase and labelled with ^{32}P UTP. Plasmid DNA was linearised by cleavage with EcoR1 and approx 1µg of DNA was incubated in a reaction mix of SP6 buffer, 2.5mM DTT, 2µg BSA, 0.1mM each NTP (ATP, CTP, GTP, UTP), 12.5µM CAP ($^{m7}G[5']ppp[5']G$), 3 units RNaisin,

20μCi ^{32}P UTP and 15 units SP6 RNA polymerase for, 1h at 37°C. The labelled precursor mRNA was purified by phenol/chloroform extraction followed by chloroform extraction and concentrated, with tRNA as a carrier, by ethanol precipitation overnight at -20°C. The precipitated precursor mRNA was resuspended in H_2O to give an activity of 200 counts per second (cps) per μl.

(c) Gel purification of precursor mRNA

When required, precursor mRNAs were further purified by electrophoresis of the radio-labelled mRNA on a 6% acrylamide gel. The band of radioactive mRNA was localised by short exposure of the gel to X-ray film. The band was excised and the mRNA eluted from the gel by incubation overnight at RT in elution buffer (400mM NaCl, 50mM Tris.Cl pH7.5, 0.1% SDS, 0.1μg/ml Proteinase K). Gel was removed from the sample by centrifugation through a Spin-X column. Precursor mRNA was then purified and concentrated as above.

(d) *In vitro* processing of precursor mRNA

In vitro processing reactions were performed using 11μl of nuclear extract incubated with 1mM cordycepin (3' dATP), 5mM creatine phosphate, 2.5mM Tris.Cl pH7.6, 1-2x10^4 cpm of RNA and 2.5% PEG, at 30°C for 2h (McLauchlan *et al.*, 1988). RNA was isolated for analysis as follows: 100μl of 2x PK buffer, 50μg of Proteinase K and 40μg yeast tRNA were added to the reaction mix and the volume adjusted to 200μl with H_2O. Proteinase K digestion was performed at 37°C for 15min. The RNA was

purified by phenol/chloroform extraction and ethanol precipitation.

(e) Analysis of the *in vitro* processing products

The RNA precipitate obtained as described above was resuspended in 6µl of loading buffer (98% formamide. 0.02% (w:v) xylene cyanol, 0.025% (w:v) bromophenol blue, 10mM EDTA pH8.0) and the products of the *in vitro* processing reaction separated on a 6% acrylamide gel. The bands of processed and unprocessed mRNA were visualised by auto-radiography or by the use of a Molecular Dynamics Phosphorimager. The relative quantities of processed and unprocessed mRNA were determined by quantification of the radioactivity present in each band using phosphorimage technology.

2.2.14 Southern blotting

(a) Random prime labelling of probe

Labelling of the DNA probe was carried out using the Mega-prime DNA labelling system of Amersham in accordance with manufacturers recommendations. 25ng of DNA was used for each probe which was labelled with ^{32}P ATP (50µCi/reaction). The probe was purified from unincorporated ^{32}P ATP by passage through a Sephadex G50 column.

(b) Transfer of DNA

Southern blotting was performed essentially as described by Southern, (1975). 2-3µg of viral DNA, either HSV-1 1802 or recombinant virus DNA, was digested with BamH1. The digest was electrophoresed on a 0.7% agarose TBE gel, at 100mV for

5-6h. Pre-heating of the digest to 56°C for 2-3min disrupted any pairing of cohesive ends. The DNA was then denatured by soaking the gel in buffer (1.5M NaCl, 0.5M NaOH) for 45min. The gel was neutralised by soaking in buffer (1M Tris.Cl, pH7.5, 1.5M NaCl) for 30min. Transfer of the DNA to a nylon membrane (Hybond-N, Amersham) took place by capillary action, overnight in 20xSSC. The DNA was cross-linked to the membrane by UV light using a UV Stratalinker 1800 (120mJ/30sec).

(c) DNA:DNA hybridization

The membrane was incubated in pre-hybridization buffer (5xSSPE, 4xDenhardt's (DHS), 0.5% SDS) for 1h at 65°C in a rotary oven (Hybaid maxi-oven). The radiolabelled probe was added in fresh hybridization buffer and the membrane incubated overnight at 65°C. After hybridization was complete the membrane was washed at 65°C in successive changes of decreasing concentrations of SSPE buffer with 0.1% SDS (2xSSPE to 0.1xSSPE). The membrane was then wrapped in cling film to prevent drying and autoradiographed.

2.2.15 Protein extraction and Western blotting

Mock infected or HSV (10pfu/cell) infected cells were harvested by washing and pelleting in PBS. Protein was extracted by boiling the cell pellet for 5 min in boiling mix (4% SDS, 125mM Tris.Cl pH6.7, 39% (v:v) glycerol, 0.002% (w:v) bromophenol blue, 2% (v:v) β–mercaptoethanol). Proteins were separated by electrophoresis on 8-12% SDS-polyacrylamide gels. Transfer of the proteins to a nitrocellulose membrane was carried out overnight at 100mA in blotting buffer (25mM Tris., 192mM

glycine, 20% (v:v) methanol), using Bio-Rad blotting apparatus. Free protein binding sites on the membrane were blocked by incubation in TBS containing 5% dried milk, for 4-5h. This and all subsequent incubations and washing were carried out with gentle agitation. The membrane was then washed thoroughly in TBS with 5% tween-20, before being incubated overnight with the appropriate dilutions of specific primary antibodies in TBS-tween. Three 10min TBS-tween washes removed the excess primary antibody, the membrane was then incubated for 1h with a 1:1000 dilution of the appropriate secondary antibody in TBS-tween, either protein-A HRP or goat anti-mouse HRP conjugate. Detection of antibody labelled proteins was performed using the ECL detection kit of Amersham in accordance with the recommended protocol. Membranes were stripped of antibody and reprobed as follows. Incubation at 50°C in stripping buffer (100mM β–mercaptoethanol, 2% SDS, 62.5mM Tris.Cl). The membrane was then washed at least three times (10 mins) in TBS-tween, blocked in 5% dried milk and TBS and reprobed as described previously.

2.2.16 RNA-protein UV cross-linking

Nuclear extracts were prepared as for *in vitro* polyadenylation, and were finally dialysed for 3h against binding buffer (60mM KCl, 20mM hepes, 1mM $MgCl_2$, 10% glycerol), with 3 changes of buffer during that time (Vakalopolou *et al.*, 1991). The protein concentrations of the HSV infected and mock infected nuclear extracts were estimated by Bradfords (1976) method and equilibrated to 10-15 μg/μl. Radiolabelled precursor mRNA was prepared as before and reconstituted in binding buffer

to give an activity of 300cps/µl. The following reaction mixture was incubated for 30min at RT (8µl binding buffer, 1µl radiolabelled precursor mRNA and 1µl nuclear extract). The mRNA and proteins were irreversibly cross-linked using UV irradiation, 250mJ/cm^2 (Bohjanen *et al.*, 1991). Unprotected RNA was removed by digestion with RNase A (1mg/ml, for 15min at 37°C). An equal volume of protein boiling mix was then added and the mixture boiled for 5min. The proteins, radiolabelled with mRNA, were separated on a denaturing SDS-acrylamide gel and visualised using a Phosphorimager. Rainbow coloured, protein molecular weight markers were run alongside the samples on each gel to give an estimation of the protein sizes.

Chapter 3

Results

3.1. The effect of an HSV-1 induced factor (LPF) on the *in vitro* processing efficiencies of selected HSV-1 poly(A) sites

3.1.1 Introduction

In the last few years considerable attention has been paid to the mechanisms of mRNA 3' end formation (Manley, 1988; Gilmartin and Nevins, 1989; Wahle and Keller, 1992). The complex picture emerging from these studies indicates great scope for regulation of these events. One example of a regulatory role for 3' mRNA processing is the production of the immunoglobulin μ mRNAs during B-cell maturation. The switch from production of the membrane bound form of IgM to production of the secretory form of IgM appears to result, at least in part, from alternative poly(A) site selection (Galli *et al.*, 1988; Petersen and Perry, 1989; Lassman and Milcarek, 1992; for review see Guise, 1989). Another well documented example of post-transcriptional regulation of gene expression is found in adenovirus where the differential use of the L1 and L3 poly(A) sites of the major late transcription complex, at early and late times respectively in the virus growth cycle, has been described (Falck-Pedersen and Logan, 1989; Wilson-Gunn *et al.*, 1992; Mann *et al.*, 1993).

Studies of HSV have identified a virus induced factor, LPF, which selectively increases the efficiency of 3' processing at the poly(A) site of a late gene of HSV-2 (UL38), but does not

increase 3' processing efficiency at another poly(A) site common to the US10, US11 and US12 genes which are members of the early, late and immediate-early, gene classes of HSV-2 respectively (McLauchlan *et al.*, 1989). The aim of this study is the determination of the specificity of LPF activity by examination of a range of HSV poly(A) sites.

In vitro processing systems, which faithfully cleave and polyadenylate exogenous pre-mRNA at the authentic poly(A) site, have allowed analysis of the mechanisms and components required for 3' end formation of RNA (Moore and Sharp, 1984; 1985). In this way, the regulation of gene expression can be examined at a post-transcriptional level without the additional influence of transcriptional regulation. In the following experiments the *in vitro* processing efficiencies of a range of poly(A) sites have been examined using an *in vitro* processing system. The poly(A) sites selected, were from representative members of the different temporal classes of HSV-1 genes, the IE, early and late gene classes. Individual plasmids containing these poly(A) sites were constructed to produce runoff precursor mRNA transcripts from a phage SP6 promoter. Analysis of the effect of virus infection on the processing efficiencies of these poly(A) sites was studied by incubation of precursor mRNAs with mock-infected or HSV infected nuclear extracts, which contain all the soluble components required for efficient cleavage and polyadenylation.

3.1.2 HSV-1 poly(A) sites

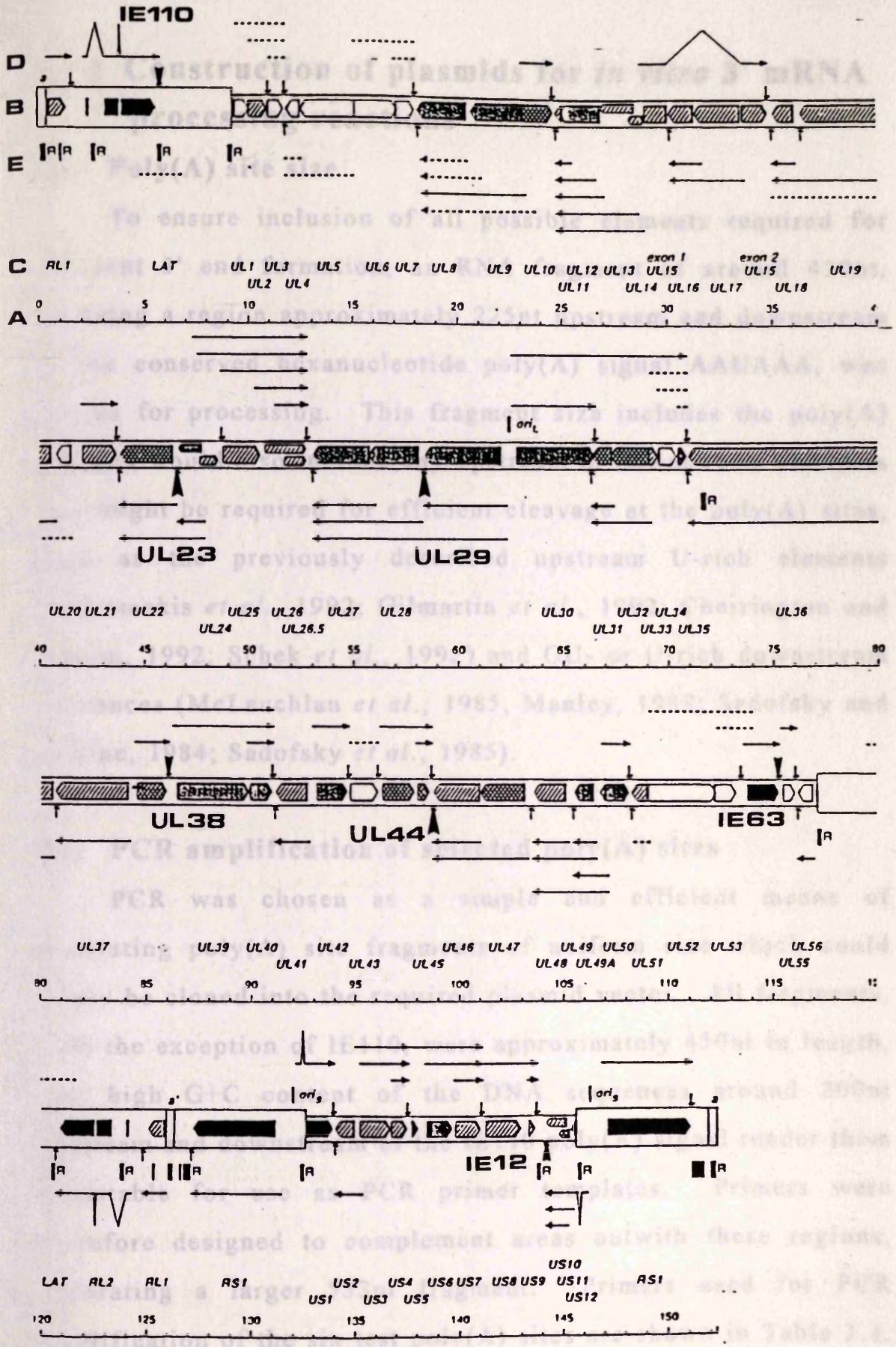
Poly(A) sites from the IE110 and IE63 HSV-1 genes were chosen to represent the IE class of HSV-1 genes. IE110 is a trans-inducer of IE, early and late genes and IE63 (UL54) is a

multifunctional protein involved in the regulation of late gene expression. The early class of HSV-1 genes was represented by the poly(A) sites of the UL23 and UL29 genes. UL23 encodes the enzyme thymidine kinase and UL29 encodes the major DNA binding protein. The late class of HSV-1 genes was represented by the poly(A) sites of the UL38 and UL44 genes. UL38 encodes a protein component of the virion, and is the HSV-1 equivalent of the HSV-2 late poly(A) site used in the studies of McLauchlan *et al.*, (1989; 1992b), while UL44 encodes glycoprotein C a virion surface glycoprotein (McGeoch *et al.*, 1985). The poly(A) sites chosen therefore represent genes of each of the temporal classes of HSV-1 (Roizman and Sears, 1993).

The HSV-1 genome map (McGeoch and Schaffer, 1992) of Figure 3.1 shows the map locations, in kbp, of the six selected poly(A) sites . The duplicated tandem poly(A) signals of IE110 are situated at positions 5638, 5678, 120693 and 120734 (Perry *et al.*, 1986). The locations of the other poly(A) signals are as follows : IE63 at position 115277 (Whitton *et al.*, 1983), UL23 at positions 46613 and 46626 (McKnight, 1980), UL29 at position 58414 (Quinn and McGeoch, 1985), UL38 at position 86016 (Anderson *et al.*, 1981) and UL44 at position 98663 (Frink *et al.*, 1983). The IE110, IE63, UL23, UL29 and UL38 poly(A) sites are all utilised by single RNA transcripts. The UL44 poly(A) site is also utilised by the 3' co-terminal UL45 transcript, UL45 has recently been classed as a late gene (Visalli and Brandt, 1991; 1993). Transcripts, corresponding to read through of the poly(A) sites of UL23, UL29 and UL38 have been isolated but no protein or function has as yet been assigned to them (Figure 3.1).

FIGURE 3.1:

A diagram of the genetic content of HSV, adapted from McGeoch and Schaffer (1992). (A) Scale of the 152kbp DNA sequence. (B) Representation of the DNA sequence features:- Locations of the polyadenylation sites are indicated by small vertical arrows, locations of the HSV-1 poly(A) sites selected for examination in this study are indicated by large arrowheads and the gene designations are in bold type : locations and directions of proposed protein coding reading frames are indicated by broad arrows : immediate-early (IE, α) genes ; early (β) genes ; early-late (γ1) ; and true late (γ2) genes ; genes of unknown temporal class are white. (C) Gene designations. (D) Rightward and (E) Leftward transcribed RNA species : reasonably well characterised transcripts are shown as solid lines and poorly characterised transcripts or hypothetical transcripts are shown as dashed lines. Positions of major families of short tandem reiterations are shown as filled boxes beneath the gene layouts and are marked "R". *ori*; origin of replication.


IE110
D
B
E
C
A
RL1
LAT
UL1 UL3
UL2 UL4
UL5
UL6 UL7
UL8
UL9
UL10
UL12 UL13
UL11
exon 1
UL15
UL14 UL16 UL17
exon 2
UL15
UL18
UL19
0
5
10
15
20
25
30
35
ori_L
UL23
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UL20 UL21
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ori_S
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RS1
US1
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RS1
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3.1.3 Construction of plasmids for *in vitro* 3' mRNA processing reactions

(a) Poly(A) site size

To ensure inclusion of all possible elements required for efficient 3' end formation, an RNA fragment of around 450nt, spanning a region approximately 225nt upstream and downstream of the conserved hexanucleotide poly(A) signal AAUAAA, was chosen for processing. This fragment size includes the poly(A) site and would also include any upstream or downstream elements that might be required for efficient cleavage at the poly(A) sites, such as the previously described upstream U-rich elements (Valsamakis *et al.*, 1992; Gilmartin *et al.*, 1992; Cherrington and Ganem, 1992; Schek *et al.*, 1992) and GU- or U-rich downstream sequences (McLauchlan *et al.*, 1985, Manley, 1988; Sadofsky and Alwine, 1984; Sadofsky *et al.*, 1985).

(b) PCR amplification of selected poly(A) sites

PCR was chosen as a simple and efficient means of generating poly(A) site fragments of uniform size which could easily be cloned into the required plasmid vector. All fragments, with the exception of IE110, were approximately 450nt in length. The high G+C content of the DNA sequences around 200nt upstream and downstream of the IE110 poly(A) signal render them unsuitable for use as PCR primer templates. Primers were therefore designed to complement areas outwith these regions, generating a larger 553nt fragment. Primers used for PCR amplification of the six test poly(A) sites are shown in Table 3.1. The 5' degenerate ends of the primers contain restriction endonuclease sites which allowed easy cloning of the fragments

TABLE 3.1:

Oligonucleotide primers used in the PCR amplification of the HSV-1 poly(A) sites IE110, IE63, UL23, UL29 UL38 and UL44. P1 upstream primer, P2 downstream primer. Sizes of the amplified fragments are shown in parenthesis and map positions of the primers are given; UL23 and UL29 are encoded on the opposite strand of the DNA. The restriction sites of HindIII (AAGCTT), BamHI (GGATCC), PstI (CTGCAG) and EcoRI (GAATTC) are underlined.

OLIGONUCLEOTIDE PRIMERS FOR PCR AMPLIFICATION OF HSV-1 DNA

Poly (A) Site (fragment size nt)	HSV-1 Map Position	Primer	Nucleotide Sequence
IE110	5417-5437	P1	TTTGAAGCTTTTCCGCAGCCTGCGGTCTCG
(553)	5570-5550	P2	AATTGGATCCTCCATGCCAGTGGCAGGATG
IE63	115074-115094	P1	GGGTTAAGCTTCGACTACGCGACCCCTTGGTG
(415)	115469-115449	P2	TGTGTGGATCCGGGAAGCTCCTAAGGGCAC
UL23	46849-46830	P1	TGGTAAGCTTCCTCCGTCCCATGCATGTC
(458)	46413-46434	P2	TTTGGGGATCCGTGGCTATGGCAGGGCTTGC
UL29	58613-58593	P1	GGGGAAGCTTGGCTGCGAGGACGACAAC
(431)	58200-58221	P2	TGTGGGATCCACCGAGGCGGTCCGTACGCTAC
UL38	84521-84540	P1	TGGTGAAGCTTGATGCCCGAGGATAGTCCC
(427)	86236-86217	P2	GTGTGTGGATCCAAGCACCCACCTGTGGTAC
UL44	98430-98450	P1	TTGGGCTGCAGCGTCGGCGGTATTGACCAG
(470)	98890-98870	P2	GTTGGAATTCAGCCAACGCCCTGACCAACG

into the vector pGEM1, used for the production of run-off transcripts. Primers used for amplification of the poly(A) site fragments of IE110, IE63, UL23, UL29 and UL38, encode a HindIII site in the upstream primer P1 and a BamHI restriction site in the downstream primer P2 (Figure 3.2). As discussed later choice of these sites facilitated construction of the plasmid vectors used in the production of the recombinant viruses (Results 3.2.2 b). As the UL44 poly(A) site DNA fragment contains an internal HindIII site, primer P1 contains a PstI site and primer P2 an EcoRI site, (Figure 3.2). The six poly(A) site DNA fragments were produced from a template of HSV-1 strain 17^+ DNA by 30 cycles of PCR.

(c) Cloning of PCR amplified poly(A) site DNA

The PCR amplified poly(A) sites of IE110, IE63, UL23, UL29 and UL38, were digested with HindIII and BamHI and ligated to the vector pGEM1 which had been pre-cleaved with HindIII and BamHI. Similarly the UL44 poly(A) site fragment was digested with PstI and EcoRI and ligated to pGEM1 pre-cleaved with PstI and EcoRI. The PCR primers were designed to produce DNA fragments which could be cloned into the pGEM1 vector such that the SP6 promoter could be used to produce poly(A) RNA transcripts in the correct 5' to 3' rightward orientation (Figure 3.2). The plasmids constructed in this way were termed pG110, pG63, pG23, pG29, pG38 and pG44 respectively. The DNA inserts in all six plasmids were sequenced using primers to both the SP6 and T7 promoters which flank the insert. Only one mismatched nucleotide was found in the poly(A) sequence of UL23, this G for T mismatch occurred 98nt 3' to the

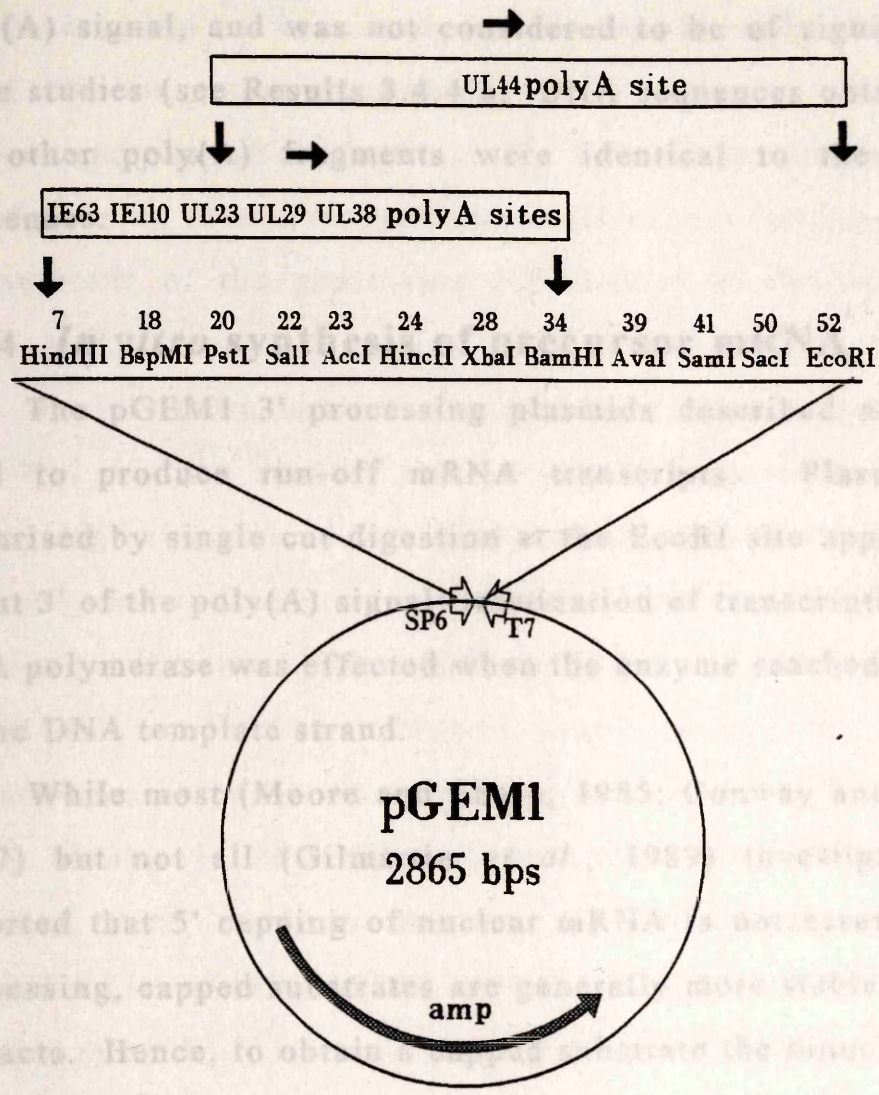


FIGURE 3.2:

Construction of plasmids pG110, pG63, pG23, pG29, pG38 and pG44. Poly(A) site fragments from the IE110, IE63, UL23, UL29 and UL38 HSV-1 genes were inserted at the HindIII and BamHI sites of the poly cloning region of pGEM1. The UL44 poly(A) site fragment was inserted at the PstI and EcoRI sites of the poly cloning region of pGEM1.

poly(A) signal, and was not considered to be of significance to these studies (see Results 3.4.4 a) DNA sequences obtained from the other poly(A) fragments were identical to the predicted sequences.

3.1.4 *In vitro* synthesis of precursor mRNA

The pGEM1 3' processing plasmids described above were used to produce run-off mRNA transcripts. Plasmids were linearised by single cut digestion at the EcoRI site approximately 220nt 3' of the poly(A) signal: termination of transcription by SP6 RNA polymerase was effected when the enzyme reached the 5' end of the DNA template strand.

While most (Moore and Sharp, 1985; Conway and Wickens, 1987) but not all (Gilmartin *et al.*, 1989) investigators have reported that 5' capping of nuclear mRNA is not essential for 3' processing, capped substrates are generally more stable in nuclear extracts. Hence, to obtain a capped substrate the dinucleotide cap analogue ^{m7}GpppG was included in these *in vitro* transcription reactions.

Transcription of the linearised poly(A) site plasmids with SP6 RNA polymerase in the presence of ^{32}P labelled UTP resulted in production of a single species of radiolabelled precursor mRNA around 450nt in length.

3.1.5 Standardisation of the 3' processing reaction

Mock infected (MI) and HSV-1 infected (INF) nuclear extracts were prepared by the method of Lee and Green (1990). The basal levels of 3' processing activity of extracts used varied from one paired set of MI and INF nuclear extracts to another,

this observation has been made by other workers (R. McWilliams ; Dr. A. Phelan, unpublished data), and is likely due to differences in the condition of the cells at the time of extract preparation. The following measures were therefore taken to ensure that valid comparisons of the processing efficiencies of the test poly(A) sites could be made.

Nuclear extracts were prepared from monolayers of MI and INF HeLa cells, seeded and harvested in parallel. *In vitro* processing was carried out with paired sets of these extracts. The reconstituted volumes and therefore the concentration of the nuclear extracts were determined by the packed cell volume of the initial cell suspension. Protein concentrations of the nuclear extracts, measured by the method of Bradford (1976), were found to be similar in both MI and INF extracts (Table 3.2). All extracts were used to process a control plasmid pSAU5, (McLauchlan *et al.*, 1989), which contains the HSV-2 IE12 poly(A) site (IE) 5' in tandem to the HSV-2 UL38 late (L) poly(A) site, inserted into the multicloning site of pGEM1, under the control of the SP6 promoter (Figure 3.3). Initial studies, which identified the HSV induced processing factor LPF, demonstrated an alteration in the processing efficiencies of the SAU5 poly(A) sites. The processing efficiency of the L site increased markedly and that of the IE site decreased slightly when processing was carried out with INF nuclear extracts : all extracts used in this study showed this change in 3' processing (Figure 3.4 and Table 3.3). Each pair of extracts was used to process as many of the test poly(A) sites as possible, each extract being used to process at least four of the six test poly(A) sites examined.

PROTEIN CONCENTRATION OF HeLa CELL NUCLEAR EXTRACTS

EXTRACT	No. Extracts	Mean Conc. (µg/µl)	Range of Conc.
MOCK INFECTED	6	7.45	5.25-9.4
HSV INFECTED	6	7	4.75-8.2

TABLE 3.2:

Mean protein concentrations of a sample of six mock infected and HSV infected HeLa cell nuclear extracts used in the *in vitro* processing reactions. Protein concentration measured by the method of Bradford is expressed as µg protein/µl of extract.

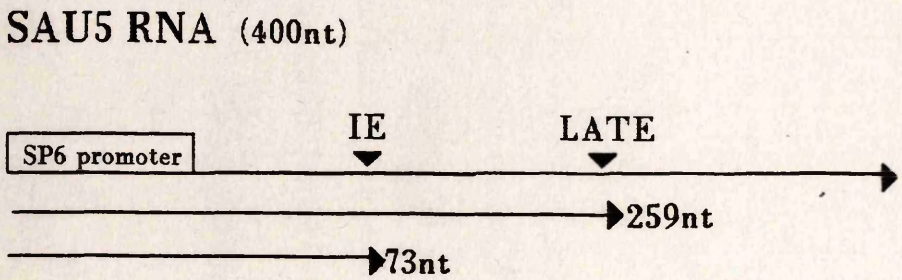


FIGURE 3.3:

Diagram of the tandem arrangement of poly(A) sites in the control plasmid pSAU5. The HSV-2 poly(A) site common to US10, US11 and US12 (IE) is 5' to the HSV-2 UL38 poly(A) site (L). Production of run off mRNA transcripts is under the control of the SP6 promoter. Cleavage at the IE site produces mRNA species of 73nt, cleavage at the L site mRNA species of 259nt.

FIGURE 3.4:

An Autoradiograph of typical processing reactions, using mock infected (MI) and HSV infected (IN) nuclear extracts. Pr-unprocessed precursor mRNA, P- uncleaved precursor mRNA (approx. 400nt), L- mRNA product (259nt) obtained by cleavage at the L poly(A) site of the control plasmid SAU5. IE- mRNA product (73nt) obtained by cleavage at the IE poly(A) site of the control plasmid SAU5. C- mRNA species (200-250nt) obtained by cleavage of the test poly(A) sites from HSV-1 genes of IE110, IE63, UL23, UL29, UL38 and UL44.

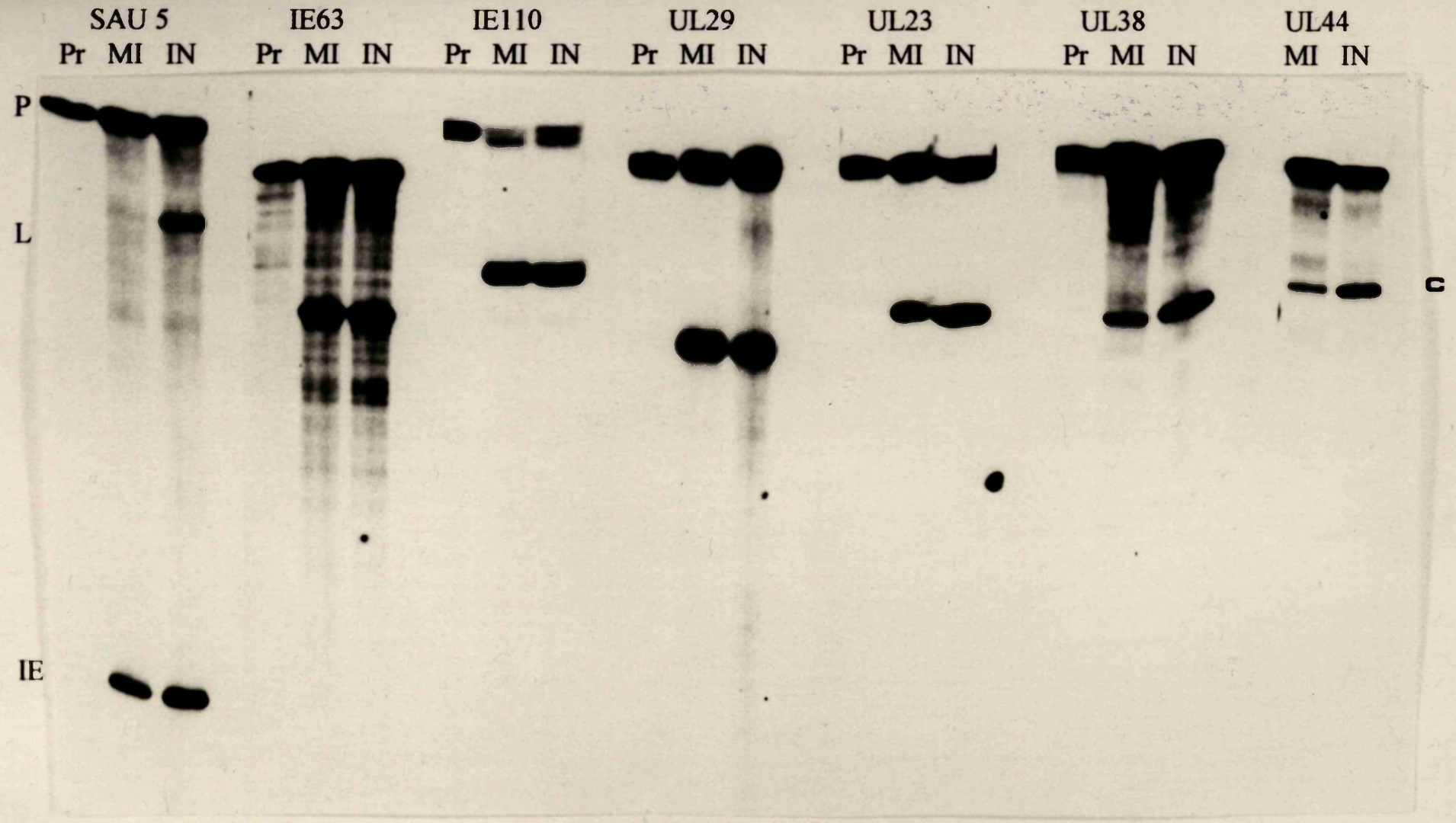

SAU 5
Pr MI IN
IE63
Pr MI IN
IE110
Pr MI IN
UL29
Pr MI IN
UL23
Pr MI IN
UL38
Pr MI IN
UL44
MI IN
P
L
IE
C

TABLE 3.3:

Shows the % 3' RNA processing efficiencies at the IE110, IE63, UL23, UL29, UL38, UL44 and SAU5 poly(A) sites. *In vitro* processing of each precursor was performed a minimum of six times using sets of paired MI and INF nuclear extracts prepared on different days. The mean and standard deviation (S.D.) and the mean of the % difference of the MI and INF results of each set of results is displayed at the bottom of each column.

Mean - mean of each column of results, SD - standard deviation of each column of results, Mean diff - mean of the % difference between the MI and INF processing efficiencies, p - two sided p value for the hypothesis that % processing in the MI extract equals the % processing in the INF extract based on the binomial distribution, NS - not significantly different, SIG - significantly different.

				% 3' PROCESSING EFFICIENCIES OF HSV POLY (A) SITES												
POLY(A) SITES	IE110		IE63		UL23		UL29		UL38		UL44		SAU5 L		SAU5 IE	
EXTRACT DATE	MI	INF	MI	INF	MI	INF	MI	INF	MI	INF	MI	INF	MI	INF	MI	INF
08/09/92			65	68			75	63	8	13	22	30	5	27	72	29
			25	18												
21/09/92	95	92			50	44			6	9	26	37	9	16	60	34
04/12/92			40	41	12	21	54	50			12	18	9	12	53	50
											14	29				
01/02/93									9	13	9	21	4	8	44	29
02/02/93	75	74							12	19	7	13				
16/02/93					9	15	32	23					8	18	56	45
02/03/93	81	78	35	44					13	22	14	24	5	12	28	25
									10	18	12	19				
									6	13	7	15				
01/04/93	70	78	37	34					12	20						
22/06/93	80	81					25	36	4	11	13	16				
01/04/93			45	19					8	15	26	22	6	12	38	55
14/09/93					38	36	46	45	7	30						
22/11/93	82	87			30	44							12	16	45	52
27/11/93			40	28	30	20	48	24			13	13	8	20	42	56
MEAN	80.50	81.67	41.00	36.00	28.08	29.92	46.58	40.17	8.65	16.55	14.54	21.40	7.33	15.61	48.67	41.67
S.D.	8.41	6.65	12.26	17.29	15.41	12.81	17.69	15.59	2.82	5.97	6.67	7.37	2.55	5.70	13.07	12.39
MEAN DIFF		2		-13		23		-9		81		62		133		-9
S.D.		5		26		42		29		37		42		115		33
p value		NS		0.46		NS		0.22		SIG		SIG		SIG		0.51

3.1.6 *In vitro* 3'processing

Radiolabelled precursor RNAs, corresponding to RNA transcripts from the test poly(A) sites, were cleaved *in vitro* by incubation with either MI or INF nuclear extracts, cordycepin and creatine phosphate, for 2h at 30°C. The reaction mixture was then treated with proteinase K to remove any proteins bound to the RNA. Under normal conditions, the products of 3' end formation of a single precursor are of varying sizes, depending on the length of poly(A) tail added. These products, when separated on a polyacrylamide gel, appear as a smear and determination of processing efficiencies of the poly(A) sites under these conditions is difficult. Accurate cleavage was therefore assayed in these studies by substitution of ATP with cordycepin (3' dATP) an analogue of ATP - addition of the cordycepin residue to the cleaved 3' terminus of RNA prevents further elongation of the poly(A) tail. While inclusion of cordyecepin is known to reduce the overall efficiency of precursor cleavage this effect is universal and does not depend on the individual properties of the precursor and as such will have no influence on the outcome of these studies (McWilliams, unpublished data; Moore and Sharpe, 1985). To prevent addition of ATP present in the nuclear extracts to the cleaved RNA, ATP was depleted by pre-incubation of the extracts at 30°C for 20min. A source of energy other than ATP was therefore required to allow the cleavage reactions to proceed and creatine phosphate, which has a higher phosphate group transfer potential than ATP, was used. Transfer of the phosphoryl group being catalysed by the endogenous creatine phosphokinase of the nuclear extracts (Moore and Sharp, 1985). The efficiency of substrate RNA cleavage was facilitated by the inclusion of PEG

in the *in vitro* cleavage reactions (J. McLauchlan, personal communication), this effect appeared to be due to concentration of trans-activating factors by reducing the apparent reaction volume.

Typical 3' RNA processing of the test poly(A) sites resulted in a mixture of uncleaved precursor RNA (approximately 400nt) and cleaved precursor product (200-250nt), appearing as discrete bands when separated on polyacrylamide gels. *In vitro* 3' RNA processing of the control plasmid pSAU5 produced a mixture of uncleaved precursor RNA (400nt), RNA cleaved at the IE poly(A) site (73nt) and RNA cleaved at the L poly(A) site (259nt) (Figure 3.4).

3.1.7 The cleavage efficiencies of selected HSV-1 poly(A) sites are increased by LPF

(a) Autoradiography

Products of the *in vitro* 3' processing reactions of the six test RNAs were separated by electrophoresis on 6% polyacrylamide gels and the radiolabelled bands of uncleaved and cleaved precursor mRNA were visualised by autoradiography. Figure 3.4 shows a typical autoradiograph obtained from these processing reactions. The processing efficiencies of the HSV-1 UL38 and UL44 late poly(A) sites were consistently increased with INF nuclear extracts. By contrast, the HSV-1 poly(A) sites of IE110, IE63, UL23 and UL29 showed no discernable change in processing efficiency when processed with either MI or INF nuclear extracts. The controls of pSAU5 showed the predicted increase in processing efficiency at the L poly(A) site and little or no change in processing efficiency at the IE poly(A) site with INF nuclear extracts.

(b) Quantification of 3' processing data

Quantification of the 3' processing efficiencies of the six poly(A) sites was performed using a Molecular Dynamics Phosphorimager. The total level of radioactivity present in the uncleaved precursor and cleaved product bands was measured by volume integration. This measures the total radioactive counts within a delineated area of the gel minus the background radioactivity which was determined for individual bands by measurement of an area of the gel nearest to the band being counted. Processing efficiency was expressed as the % of precursor RNA cleaved during the *in vitro* processing reaction, using the following formula.

$$\text{Processing efficiency} = \frac{\text{*Cleaved Product counts} \times 100}{\text{Total counts}}$$

Total counts = counts in uncleaved precursor band plus counts in *cleaved product band

* These values were adjusted to account for U content of the cleaved band relative to each uncleaved precursor band, assuming that efficiency of ^{32}P UTP incorporation was proportional to the U content. A sample calculation is given below:-

Uncleaved precursor RNA (UL38) * * 100Us * *

cleaved product * 51Us *

RNA tail degraded and lost * * *

The number of Us present in the cleaved product can therefore be expessed as a % of the total number of Us present in the uncleaved precursor, in this case 51%.

uncleaved precursor RNA = 267470 (radioactive counts)

cleaved product (actual) = 13223 (radioactive counts)

(adjusted) = $\frac{13223 \times 100}{51}$

Us in cleaved product

=25927.5

PROCESSING EFFICIENCY= $\frac{25927.5}{25927.5+267470}$

= 8.8%

Quantification of the RNA processing efficiencies eliminates apparent effects caused by differences in sample loading of the polyacrylamide gels. The processing efficiency of each poly(A) site was determined a minimum of six times using six different paired MI and INF nuclear extracts. Results are shown in Table 3.3 and Figure 3.5 A,B,C,D. The 3' processing efficiencies of the UL38 and UL44 precursors were consistently increased when processing was carried out with INF nuclear extracts. The mean processing efficiency of UL38 showed an 81% increase and that of UL44 showed a 61.5% increase above that obtained with the MI nuclear extracts. The IE63, UL29 and UL23 precursors showed some slight differences in processing efficiencies when processed with INF nuclear extracts - the mean processing efficiency of

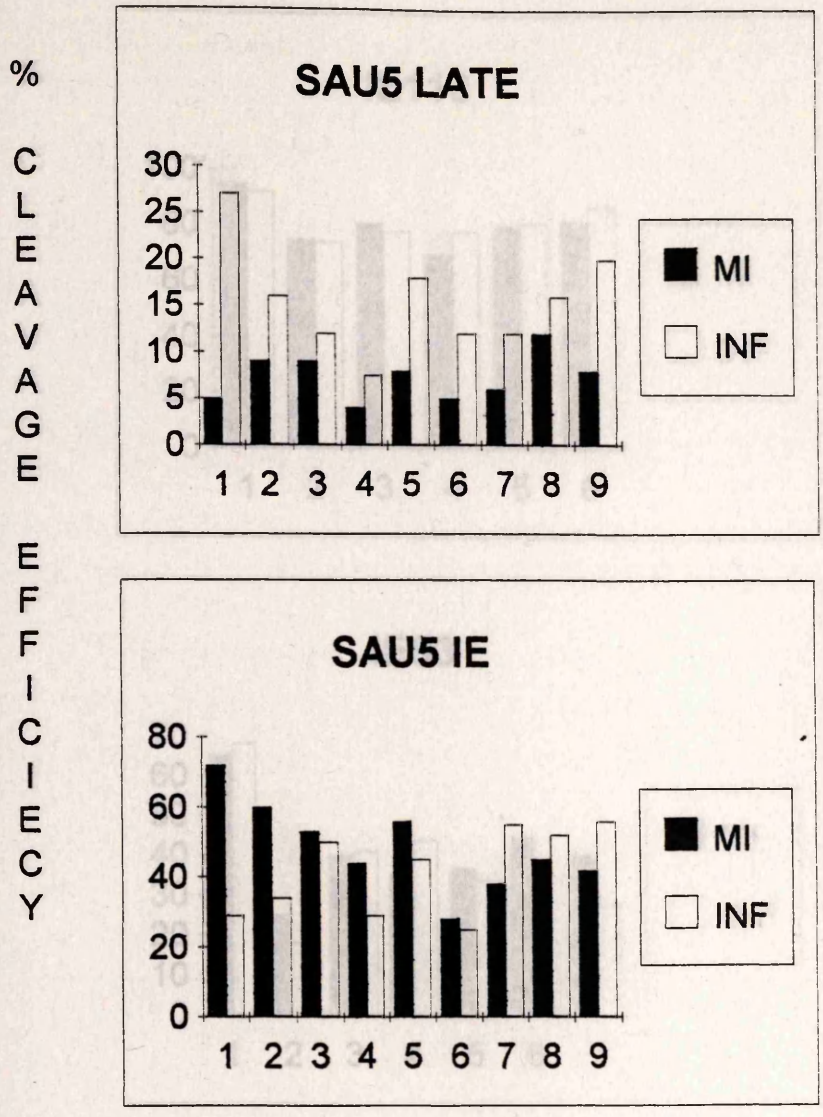


FIGURE 3.5 (A):

The % 3' RNA processing efficiencies of the control plasmid SAU5 poly(A) sites. IE: HSV-2 IE12 poly(A) site, L: HSV-2 UL38 poly(A) site. Graphical representation of the results presented in Table 3.3. Y-axis : % processing efficiency. X-axis : the number of individual processing reactions carried out. MI - mock infected nuclear extract, INF - HSV-1 infected nuclear extract.

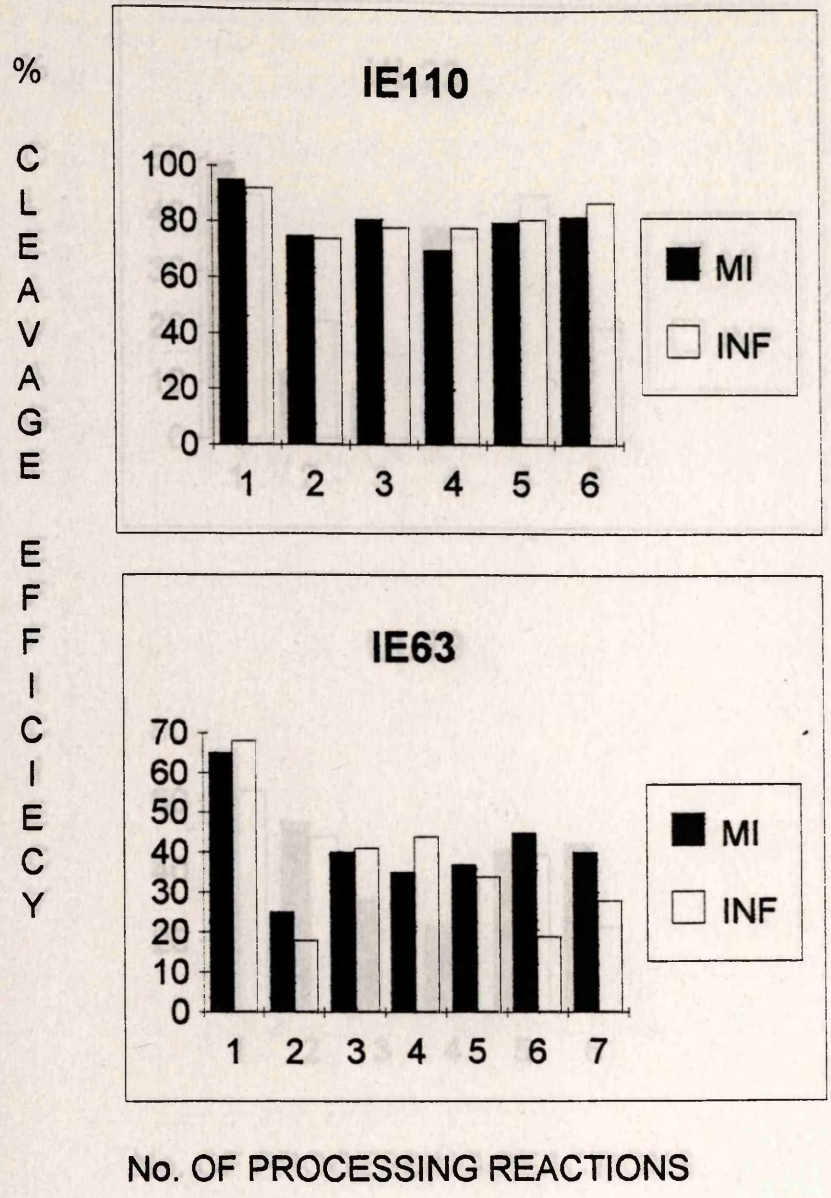


FIGURE 3.5 (B):

The % 3' RNA processing efficiencies of the poly(A) sites of the HSV-1 IE genes IE110 and IE63. Graphical representation of the results presented in Table 3.3. Y-axis : % processing efficiency. X-axis : the number of individual processing reactions carried out. MI - mock infected nuclear extract, INF - HSV-1 infected nuclear extract.

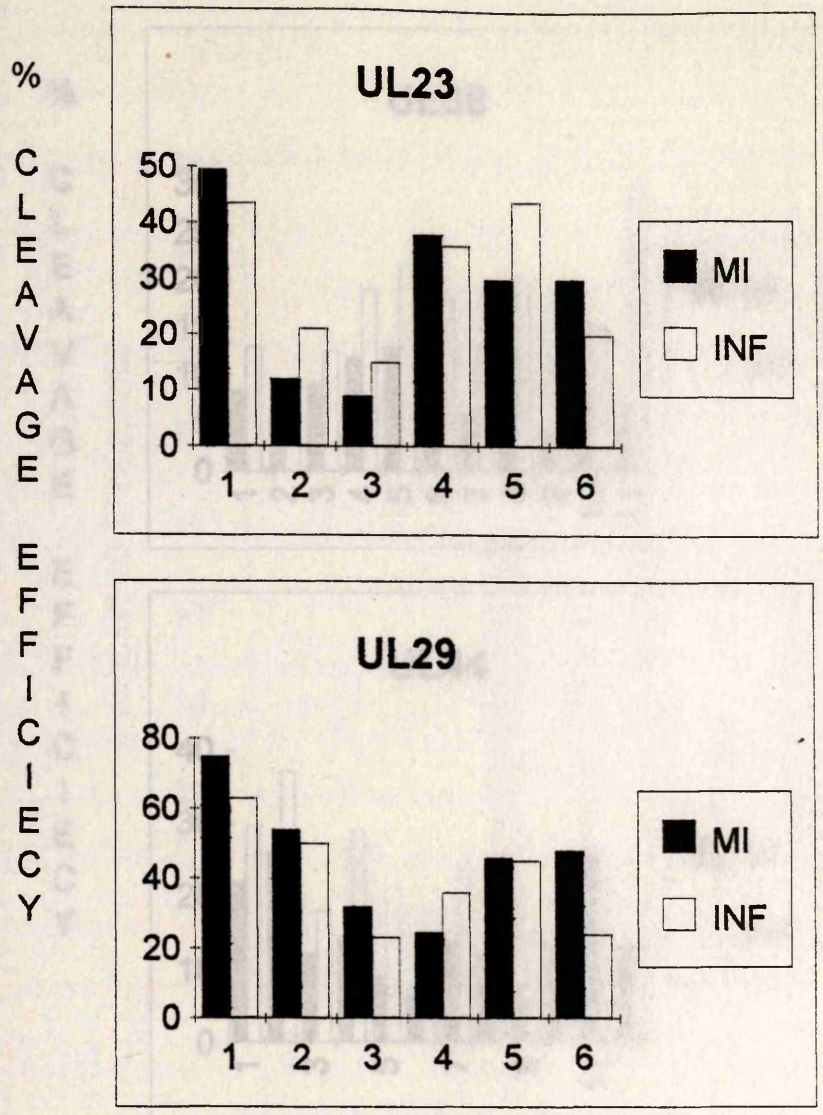


FIGURE 3.5 (C):

The % 3' RNA processing efficiencies of the poly(A) sites of the HSV-1 early genes UL23 and UL29. Graphical representation of the results presented in Table 3.3. Y-axis : % processing efficiency. X-axis : the number of individual processing reactions carried out. MI - mock infected nuclear extract, INF - HSV-1 infected nuclear extract.

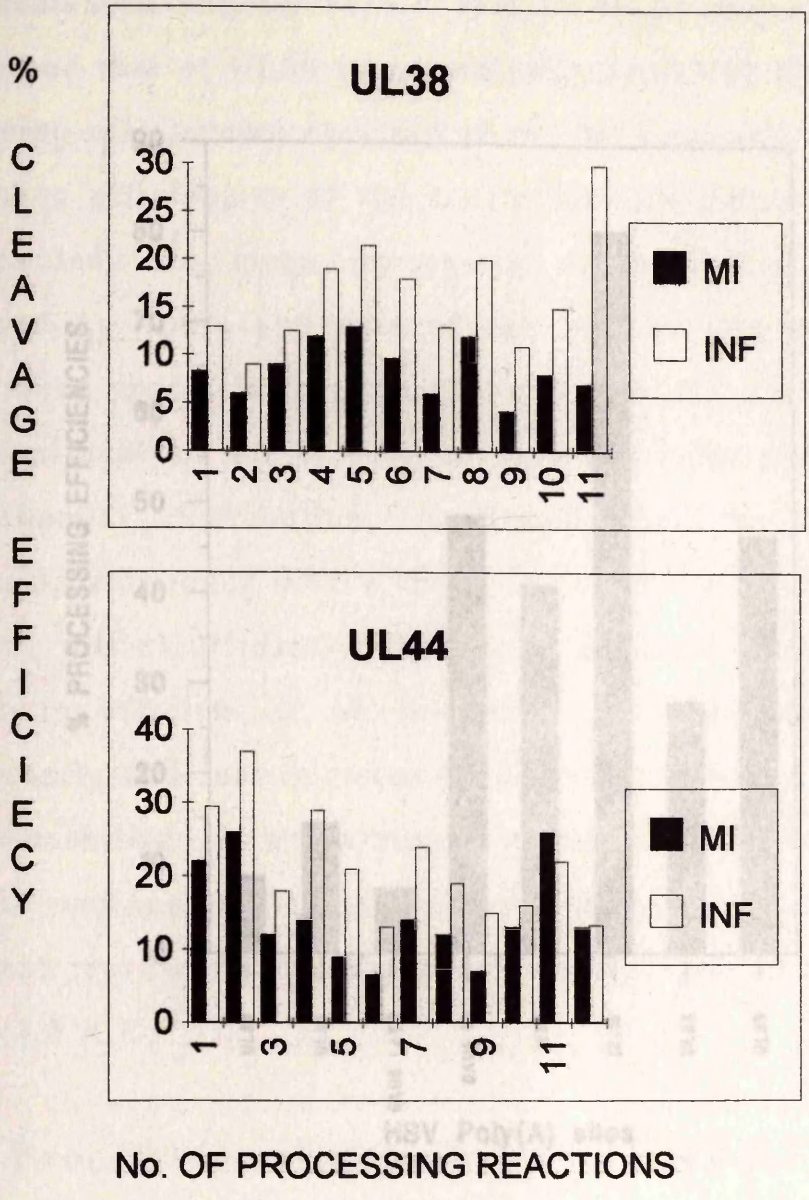


FIGURE 3.5 (D):

The % 3' RNA processing efficiencies of the poly(A) sites of the HSV-1 late genes UL38 and UL44. Graphical representation of the results presented in Table 3.3. Y-axis : % processing efficiency. X-axis : the number of individual processing reactions carried out. MI - mock infected nuclear extract, INF - HSV-1 infected nuclear extract.

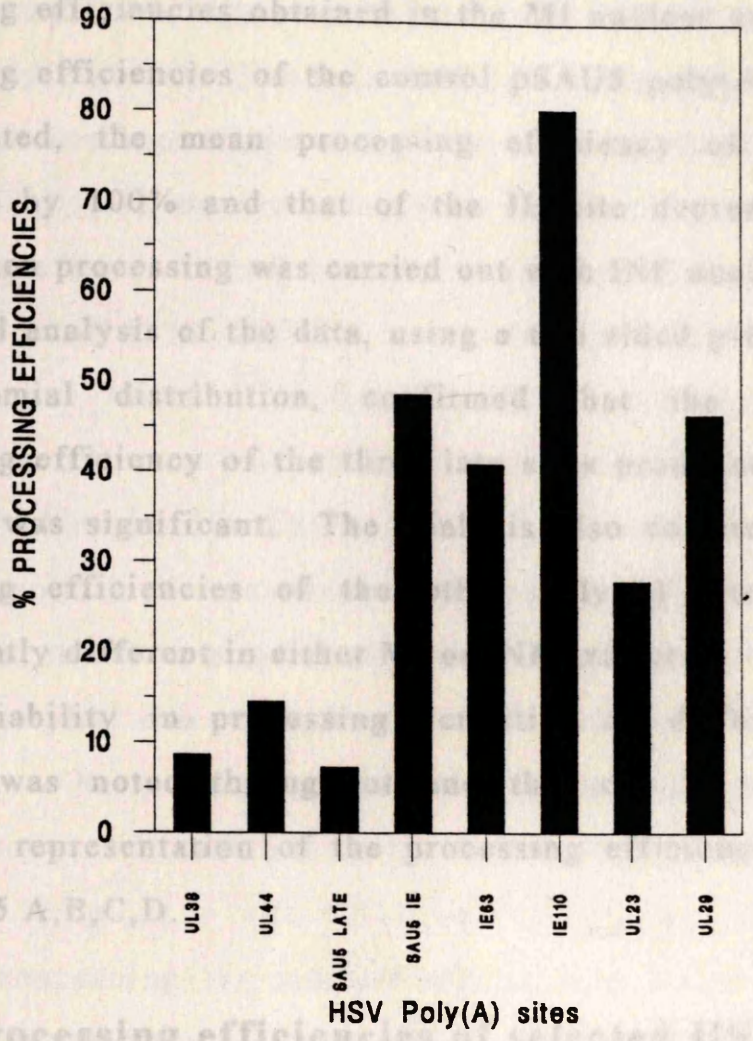


FIGURE 3.6:

3' processing efficiencies of the test poly(A) sites using mock infected nuclear extracts. The percentage processing efficiency is expressed as the mean of the set of results presented in Table 3.3.

IE63 decreased slightly (13%), that of UL29 decreased slightly (9.2%) and that of UL23 increased slightly (23%) from the mean processing efficiencies obtained in the MI nuclear extracts. The processing efficiencies of the control pSAU5 poly(A) sites were as expected, the mean processing efficiency of the L site increased by 100% and that of the IE site decreased slightly (17%) when processing was carried out with INF nuclear extracts. Statistical analysis of the data, using a two sided p-test based on the binomial distribution, confirmed that the increase in processing efficiency of the three late sites produced in the INF extracts, was significant. The analysis also confirmed that the processing efficiencies of the other poly(A) sites were not significantly different in either MI or INF extracts.

Variability in processing activities of different nuclear extracts was noted throughout and this can be seen in the graphical representation of the processing efficiencies given in Figure 3.5 A,B,C,D.

3.1.8 Processing efficiencies of selected HSV-1 poly(A) sites with MI nuclear extracts

Examination of the processing efficiencies of the six test HSV-1 poly(A) sites with MI nuclear extracts revealed striking differences in the basal levels of processing efficiency of these sites. The mean processing efficiencies, using MI extracts, of the UL38, UL44 and pSAU5 L poly(A) sites were lower than the mean processing efficiencies of the IE110, IE63, UL23, UL29 and SAU5 IE poly(A) sites (Figure 3.6). The poly(A) sites which respond to LPF therefore appear to be inherently less efficient than those which do not respond.

3.1.9 Discussion

These *in vitro* studies have confirmed that an HSV-1 induced factor, termed LPF, alters the 3' processing efficiencies of certain HSV-1 poly(A) sites. The action of LPF appears to be selective, only the processing efficiencies of the late poly(A) sites of HSV-1 (UL38 and UL44) are increased with INF nuclear extracts. The processing efficiencies of the poly(A) sites from the IE110, IE63, UL23 and UL29 genes of HSV-1 are unaffected by LPF. We have shown by *in vitro* studies that the HSV-1 poly(A) sites which respond to LPF are less efficient processing sites than those which do not respond to LPF.

Examples of post-transcriptional regulation of gene expression, in which poly(A) site choice is influenced both by the inherent poly(A) site strength and the concentration of processing factors, have been described in other systems. In adenovirus, the switch from early to late gene expression is thought to be regulated by poly(A) site selection. There are currently two theories concerning the mechanisms of this selection; the first proposes that poly(A) site choice is determined by its spatial arrangement. At early times of infection the L1 poly(A) site, of the major late transcriptional unit, being the most 5' site, is used preferentially. Later in infection, by an as yet unidentified mechanism, RNA cis-acting sequences override this preference and all poly(A) sites are used equally efficiently (Falck-Pedersen and Logan, 1989). While Wilson-Gunn *et al.*, (1992), suggest that regulatory elements in the L1 site are required for its selective use at early times. The second theory proposes that poly(A) site selection is determined by the differing strengths of the poly(A) sites. Mann *et al.*, (1993), propose that at late times, when one of

the RNA processing factors is present at a limiting concentration, the late L3 poly(A) site being the strongest site is used in preference to the weaker L1 site. A second example is found during B-cell differentiation, which requires a switch from the production of membrane bound (μm) to the secretory (μs) form of IgM, this switch is thought to be regulated by poly(A) site choice. In the mature B-cell the μm site is used preferentially whereas in the terminally differentiated plasma cell the μs site is used preferentially. Two main models exist to explain this change in poly(A) site usage. In the first, poly(A) site choice is dependent on the relative strengths of the two poly(A) sites (Galli *et al.*, 1987), use of the weaker of the two sites, μs, is increased in plasma cells due to an increase in concentration of the processing factors present. In the second model, cleavage and polyadenylation at the μs site competes with splicing at a suboptimal 5' splice site (Lassman and Milcarek, 1992; Petersen and Perry, 1989). Like the first model, an increase in cleavage efficiency at the μs poly(A) site results in its preferential use. Transcription termination is thought to play an additional role in IgM secretion, termination occurring 3' to the μs site more frequently in plasma cells than B-cells, thereby increasing the number of mRNA transcripts containing only the μs poly(A) site (Guise *et al.*, 1989; Peterson and Perry, 1989).

I present two explanations of the LPF associated increase in 3' processing efficiencies at the UL38 and UL44 poly(A) sites. Firstly, there could be structural or sequence elements present in the responding poly(A) sites and absent from the non-responding poly(A) sites which interact directly with LPF or LPF induced factor(s). These factors could be the cell derived processing

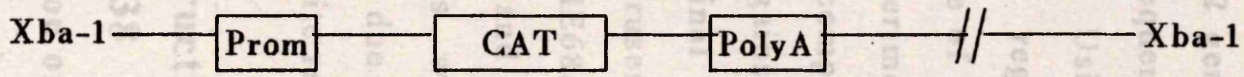
components or factors which can either substitute for them or stabilise their interactions. Secondly, LPF could increase the processing efficiencies of all virus poly(A) sites, and the poly(A) sites at which this would be apparent would be those which function sub-optimally in the absence of LPF activity. In this study we have been able to show that the three poly(A) sites which resond to LPF are inherently less efficient. It is perhaps not coincidental that the sites which respond to LPF are from genes which are expressed only at late times of infection, this could be a means of regulating their expression. Any generalised increase in processing efficiency again could be brought about by the direct involvement of LPF with the processing machinery of the cell either by increasing the level of processing components, substituting for them or stabilising their activity. LPF could thus give a boost to the processing efficiencies of all poly(A) sites at late times of infection when levels of cell derived processing factors are declining.

3.2 The *in vivo* effect of LPF

3.2.1 Introduction

The *in vitro* experiments described in the previous Section were designed to examine effects of LPF on the processing efficiencies at selected HSV-1 poly(A) sites. Processing efficiency was determined by the measurement of cleavage efficiency without the additional influence of posttranscriptional events such as polyadenylation, splicing and translation of mRNA : events which could have a dramatic effect on the regulation of gene expression *in vivo*. It was important therefore to confirm that the LPF effect was not an artefact of the *in vitro* assay, but a genuine phenomenon ongoing during virus infection *in vivo*. Previous *in vivo* studies, carried out by McLauchlan *et al.*, (1989; 1992b), used virus recombinants to confirm their *in vitro* findings, these recombinant viruses contained a CAT reporter gene fused upstream of the IE and late poly(A) sites used in their *in vitro* studies, which were present either singly or in tandem. Examination of the mRNA species produced from these recombinant viruses (McLauchlan *et al.*, 1989) and comparison of the levels of CAT activity obtained during infection with the virus recombinants (McLauchlan *et al.*, 1992b) demonstrated that LPF functioned *in vivo* to increase 3' mRNA processing at the HSV-2 UL38 late poly(A) site above that obtained at the IE poly(A) site.

In the following experiments a number of virus recombinants were used to confirm these *in vivo* studies utilising the previously constructed vFJ7, vFJ10 (Rixon and McLauchlan, 1990) and vSAU3 (McLauchlan *et al.*, 1992b) virus recombinants (Figure 3.7) and a newly constructed virus recombinant vFJM38. Viruses vFJ7 and vSAU3 both contain promoter sequences from the HSV-2



vFJ7	HSV-2 early	HSV-2 IE12
vSAU3	HSV-2 early	HSV-2 UL38 late
vFJ10	HSV-2 IE68/12	HSV-2 IE12
vFJM38	HSV-2 IE68/12	HSV-1 UL38 late

FIGURE 3.7:

Features of the CAT reporter cassettes used in the construction of the virus recombinants vFJ7, vSAU3, vFJ10 and vFJM38. Prom - promoter sequences, Poly(A) - poly(A) sequences.

early UL40 (the small subunit of the ribonuclease reductase) gene, linked to the CAT reporter gene, with poly(A) site sequences from HSV-2 IE12 and HSV-2 UL38 genes respectively. Viruses vFJM38 and vFJ10 both contain promoter sequences from the HSV-2 IE68/12 gene, linked to the CAT reporter gene, with the poly(A) site sequences from HSV-1 UL38 and HSV-2 IE12 genes respectively. Using vFJ7 and vSAU3, differences in post-transcriptional regulation of viral gene expression due to differential use of the inserted poly(A) sites was studied. In the following experiments, CAT activity of the four recombinant viruses was compared over a time course of infection and standardised against viral thymidine kinase levels.

The original aim of this study was to produce a series of recombinant viruses with the CAT reporter gene under the control of the HSV-1 IE68/12 promoter, with the HSV-1 poly(A) sites of UL38, UL44 and IE63. These HSV-1 poly(A) sites would represent sites which differed in their response to LPF. Unfortunately, due to lack of time, construction of the UL44 and IE63 poly(A) site recombinant viruses was not completed.

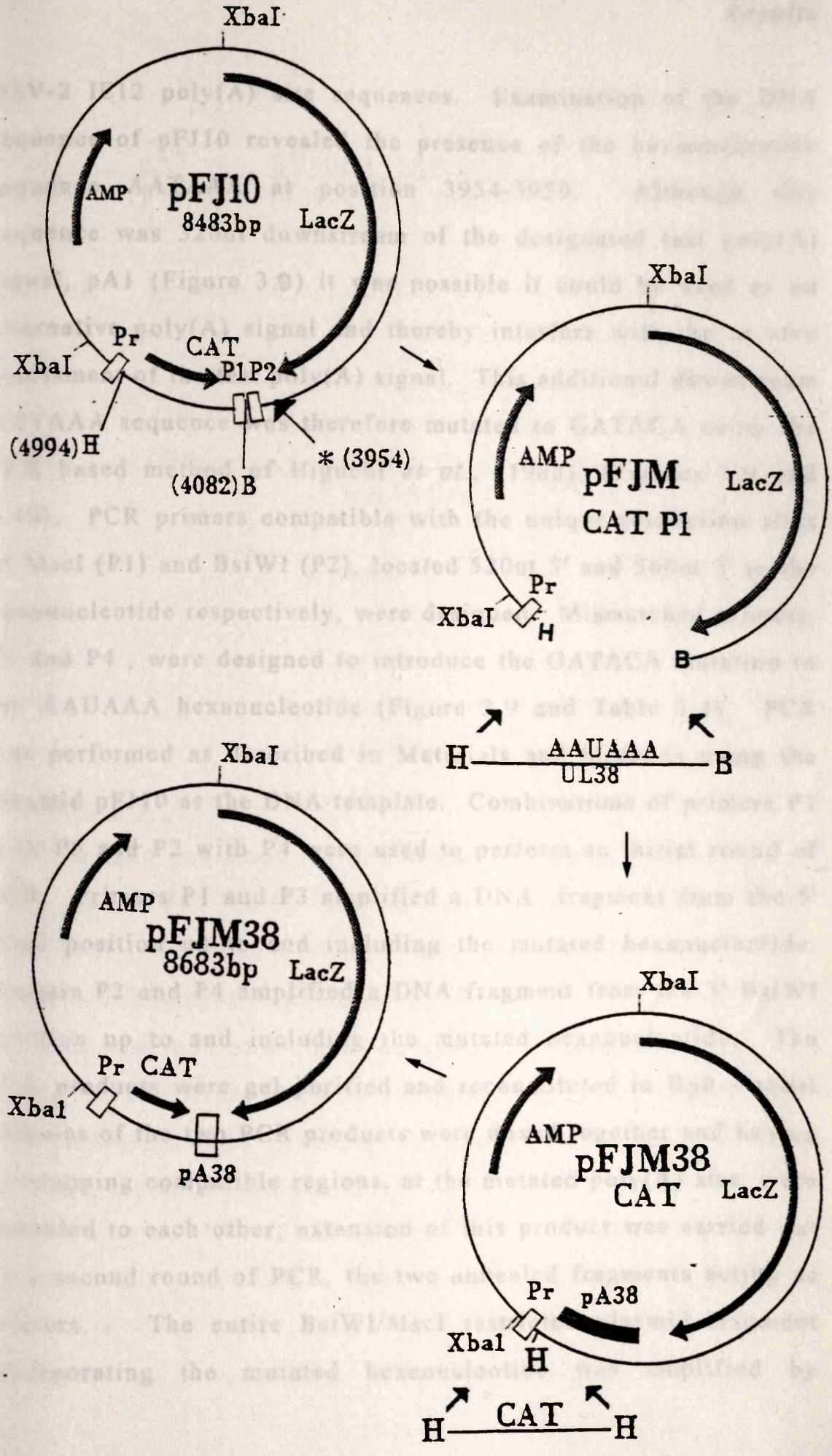
3.2.2 Construction of the expression plasmid pFJM38

(a) Mutation of pFJ10

The expression plasmid pFJM38 which contains the HSV-1 UL38 late poly(A) site was constructed from the plasmid pFJ10 (Figure 3.8). Plasmid pFJ10 had previously been used in the production of the vFJ10 virus recombinant (Rixon and McLauchlan, 1990), and contains the HSV-2 IE68/12 promoter fused upstream of the CAT reporter gene with the downstream

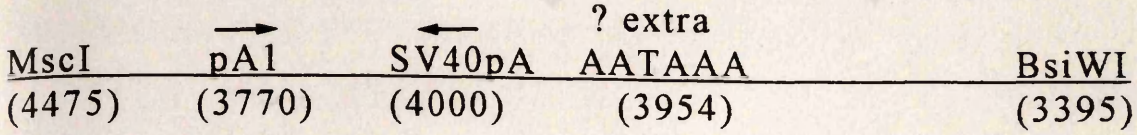
FIGURE 3.8 :

The CAT expression plasmid pFJM38 was constructed from the plasmid pFJ10 which contains the HSV-2 IE68/12 promoter (Pr) sequences fused upstream of the CAT coding region, which is upstream of the HSV-2 IE12 poly(A) site (P1). The LacZ cassette contains an SV40 early promoter fused upstream of the β-galactosidase coding region, and the SV40 early poly(A) site (P2) (McLauchlan *et al.*, 1990). After the initial mutation of the extra AATAAA sequence at map position 3954 to GATACA, the plasmid was further modified by removal of the HSV-2 IE 12 poly(A) site and the CAT coding region as a HindIII/BamHI restricted fragment. pFJM38 was generated by insertion of the HindIII/BamHI restricted poly(A) site of the HSV-1 late UL38 gene followed by insertion of the HindIII restricted CAT coding region of the plasmid pCAT into the now unique HindIII site. B : BamHI restriction site, H : HindIII restriction site, pA38 : HSV-1 late UL38 poly(A) site, * : Position of extra AATAAA sequence. Positions given in bp given in parenthesis are relative to the pFJ10 plasmid map.


XbaI
AMP
pFJ10
8483bp
LacZ
Pr
CAT
XbaI
P1P2
(4994)H
(4082)B
* (3954)
XbaI
AMP
pFJM
CAT P1
LacZ
Pr
XbaI
H
B
H
AAUAAA
UL38
B
XbaI
AMP
pFJM38
8683bp
LacZ
Pr CAT
XbaI
pA38
XbaI
AMP
pFJM38
CAT
LacZ
Pr
pA38
XbaI
H
H
CAT
H

HSV-2 IE12 poly(A) site sequences. Examination of the DNA sequence of pFJ10 revealed the presence of the hexanucleotide sequence AATAAA at position 3954-3959. Although this sequence was 320nt downstream of the designated test poly(A) signal, pA1 (Figure 3.9) it was possible it could be used as an alternative poly(A) signal and thereby interfere with the *in vivo* assessment of the test poly(A) signal. This additional downstream AATAAA sequence was therefore mutated to GATACA using the PCR based method of Higuchi *et al.*, (1988), (Figures 3.9 and 3.10). PCR primers compatible with the unique restriction sites of MscI (P1) and BsiWI (P2), located 520nt 5' and 560nt 3' to the hexanucleotide respectively, were designed. Mismatched primers, P3 and P4 , were designed to introduce the GATACA mutation to the AAUAAA hexanucleotide (Figure 3.9 and Table 3.4). PCR was performed as described in Materials and Methods using the plasmid pFJ10 as the DNA template. Combinations of primers P1 with P3 and P2 with P4 were used to perform an initial round of PCR. Primers P1 and P3 amplified a DNA fragment from the 5' MscI position up to and including the mutated hexanucleotide. Primers P2 and P4 amplified a DNA fragment from the 3' BsiWI position up to and including the mutated hexanucleotide. The PCR products were gel purified and reconstituted in H_20 - equal volumes of the two PCR products were mixed together and having overlapping compatible regions, at the mutated poly(A) site, were annealed to each other; extension of this product was carried out by a second round of PCR, the two annealed fragments acting as primers. The entire BsiWI/MscI restricted plasmid fragment incorporating the mutated hexanucleotide was amplified by

A.



B.

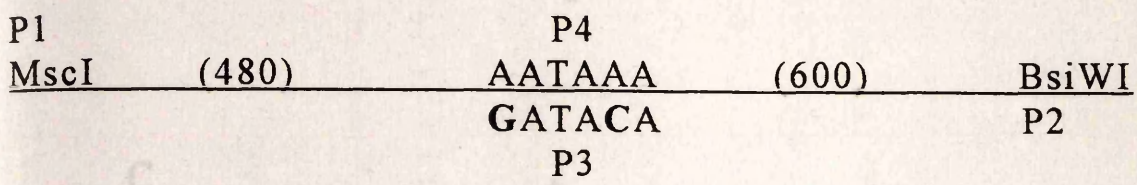


FIGURE 3.9 :

A : The map positions (in bp) of the test poly(A) site pA1, the SV40 early poly(A) site and the extra AATAAA sequence located on the MscI/BsiWI restricted fragment of plasmid pFJ10.

B : The positions of the primers used in the generation of the AATAAA to GATACA mutation. The predicted fragment sizes (bp) generated by the initial round of PCR are given in parenthesis.

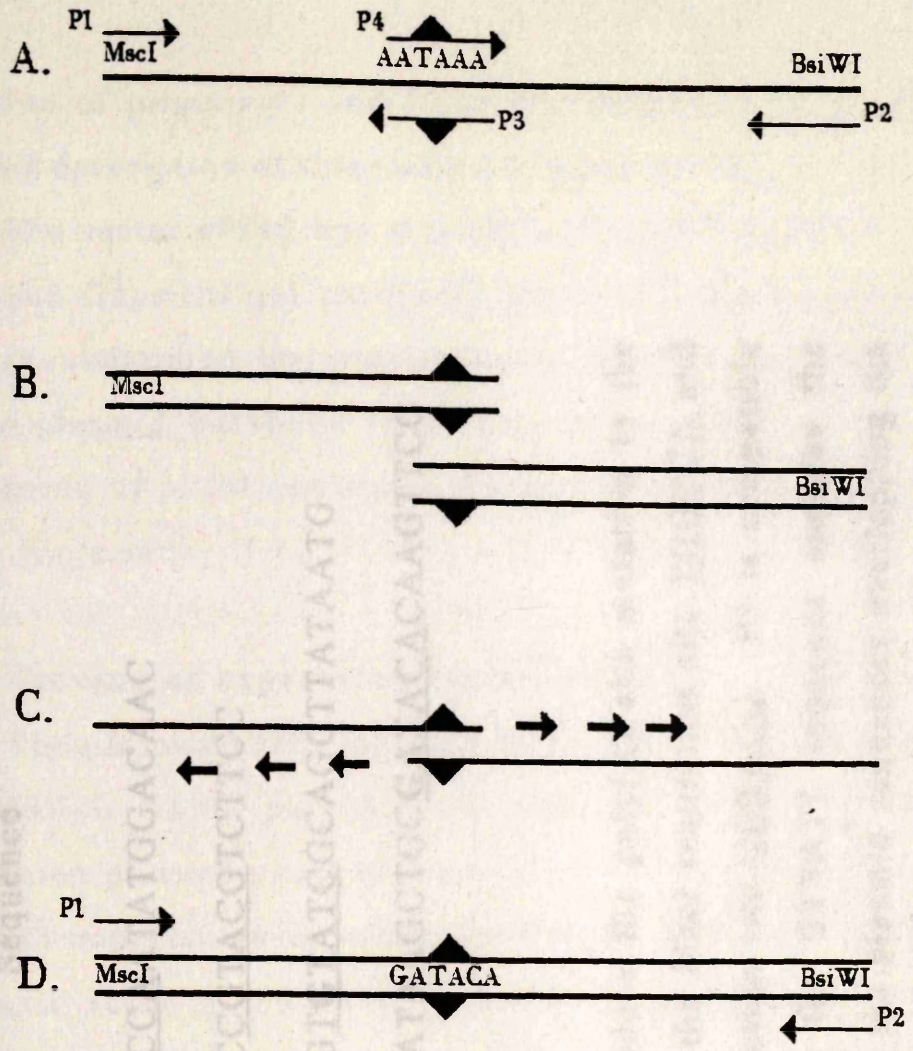


FIGURE 3.10:

Diagram of the method used to introduce the GATACA mutation to the extra AATAAA sequence in the plasmid pFJ10. A: using primer P1 in conjunction with primer P3 (containing the mutation) and primer P2 in conjunction with primer P4 (containing the mutation) the two halves of the DNA fragment were amplified in an initial round of PCR (B). C: the two overlapping ends of the fragments (containing the mutation) produced in B were allowed to anneal. D: Extension and amplification of this product was carried out by a second round of PCR using the P1 and P2 primers flanking the DNA fragment.

OLIGONUCLEOTIDE PRIMERS FOR PCR MUTATION OF pFJ10.

Primer	Position on pFJ10 (bp)	Sequence
P1	4482-4462	AACGTGGCCAATATGGACAAC
P2	3387-3406	GTATACCCCGTACGTCTTCC
P3	3946-3972	GGAACTTGTGTATCGCAGCTTATAATG
P4	3972-3946	CATTATAAGCTGCGATACACAAGTTCC

TABLE 3.4:

The oligonucleotide primers used to introduce the poly(A) site mutation to the plasmid pFJ10. P1 the 5' primer encodes the MscI restriction site TGGCCA and P2 the 3' primer encodes the BsiWI restriction site CGTACG. P3 is compatible with the plasmid sequences overlapping the TTTATT sequence encodes the mutation TGTATC. P4 is compatible with the plasmid sequences overlapping the AATAAA sequence encodes the mutation GATACA.

inclusion of primers P1 and P2 in this second round of PCR. A detailed description of this method is given in Figure 3.10.

The vector pFJ10 was digested with BsiWI and MscI and the backbone fragment gel purified. The BsiWI/MscI digested PCR product, containing the mutated hexanucleotide, was then ligated to the plasmid backbone to produce the plasmid pFJM. DNA sequencing of pFJM confirmed the mutation and correct insertion of the fragment.

(b) Cloning of expression vector pFJM38

The plasmid vector pFJM38 was constructed by insertion of the HSV-1 UL38 poly(A) site fragment of pG38 into the expression plasmid pFJM (Figure 3.8). The plasmid pFJM carries the CAT reporter gene, under the control of the HSV-2 IE68/12 promoter, fused to the HSV-2 poly(A) site fragment of IE12. The β–galactosidase coding sequence under the control of an SV40 early promoter, fused to an SV40 early poly(A) site fragment, lies in a non overlapping and opposite orientation immediately adjacent to the CAT gene. Expression from the β-galactosidase gene was used as a marker for successful insertion of the plasmid fragment into the viral DNA during the production of recombinant virus. The CAT coding sequences and the HSV-2 IE12 poly(A) site sequences were removed from pFJM by cleavage with HindIII and BamHI restriction endonucleases. The HindIII/BamHI fragment from pG38 was then ligated into the pFJM vector backbone. The unique HindIII site produced by this procedure was used for insertion of the HindIII restricted CAT coding sequences from the plasmid pCAT, resulting in production of the plasmid pFJM38. Restriction endonuclease mapping confirmed

the correct insertion and orientation of the pG38 and pCAT fragments.

3.2.3 Production of recombinant virus vFJM38

The Xba-1 restricted fragment of pFJM38 containing the CAT gene and β–galactosidase gene was ligated to Xba-1 digested HSV-1 1802 viral DNA. The unique Xba-1 site of HSV-1 1802 is located in the BamHI z fragment of the viral genome (Rixon and McLauchlan, 1990), this allows insertion of the plasmid fragment into a non-essential region of the viral genome. The recombinant virus was put through three rounds of plaque purification and the titre and purity of the virus stock determined. Southern blot analysis of BamHI digested viral DNA was used to confirm the presence of the inserted and wt UL38 poly(A) sites (Figure 3.11)..

HSV DNA exists as four equimolar forms due to inversion of the short and long genome segments (Hayward *et al.*, 1975 ; Delius and Clements, 1976). The fragments produced by Xba-1 digestion of HSV-1 1802 would therefore arise from all four isomeric forms. As ligations were performed with unseparated DNA fragments all possible recombinations of fragments would occur. No recombinant viruses generated by this procedure with genome structures different from wt virus were detected in the original studies (Rixon and McLauchlan, 1990).

3.2.4 CAT activity of the recombinant viruses

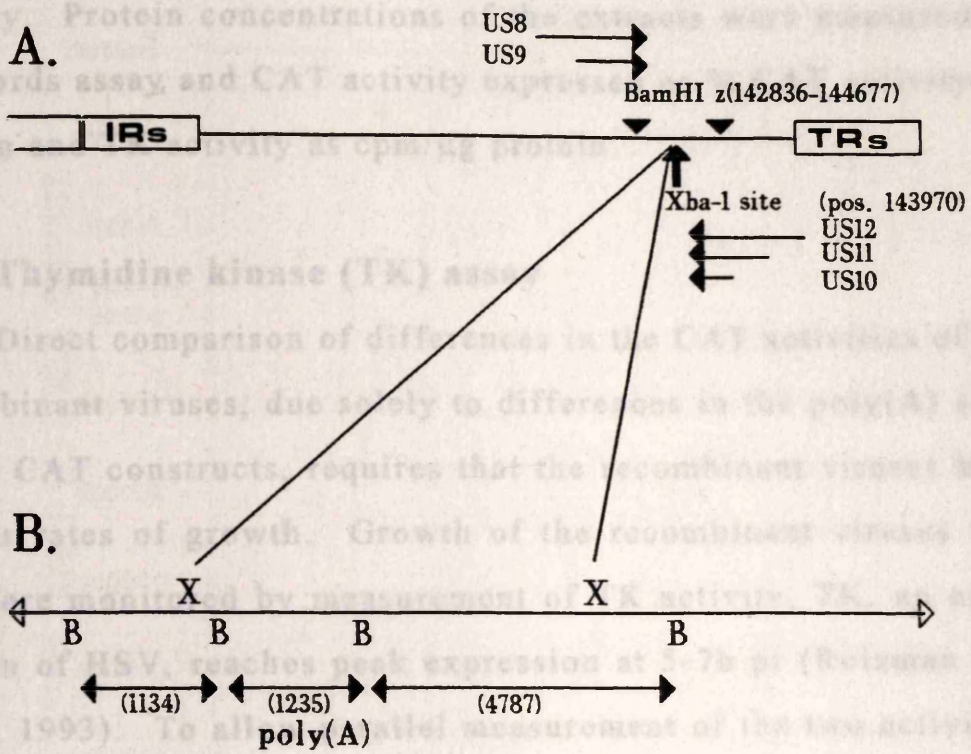
The four recombinant viruses vFJ7, vSAU3, vFJ10 and vFJM38 were used to infect BHK cells at a moi of 10pfu/cell. Cell extracts were prepared in CAT buffer at 2, 4, 8, 12, 18 and

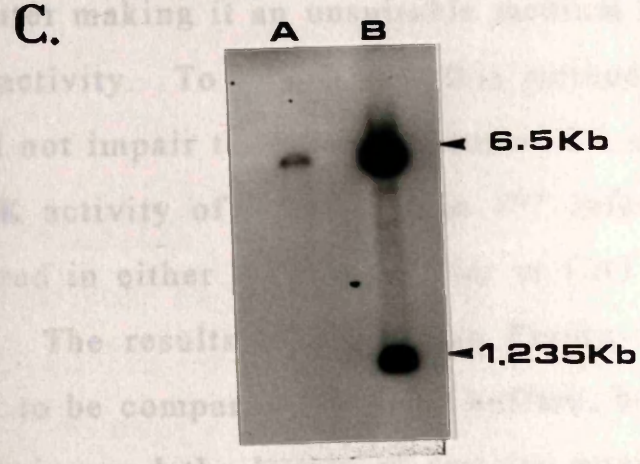
FIGURE 3.11:

A : The position in basepairs (bp) of the unique Xba-1 site of HSV-1 1802 which is located within the BamHI z fragment of the US genome region, in the intergenic region between the poly(A) sites of the US10, US11 and US12 genes and the US8 and US9 genes. IRs internal repeat sequences, TRs terminal repeat sequences.

B : Diagram of the relative positioning of the pFJM Xba-1 restricted insert within the BamHI z fragment of the virus genome, B-BamHI restriction sites, X-Xba-1 restriction sites. The inserted UL38 poly(A) sequences are located on a 1.235Kb fragment of the inserted DNA.

C : Southern blot analysis of the BamHI digest of the virus recombinant vFJM38 and wt 1802 HSV-1 DNA. BamHI digestion of the viral DNA produces a 6.5Kb fragment containing the wild type UL38 poly(A) sequences and a 1.235Kb fragment containing the UL38 poly(A) sequences from the plasmid pFJM. Lane A wild type virus, lane B vFJM38 virus.


A.
US8
US9
BamHI z(142836-144677)
IRs
TRs
Xba-1 site
(pos. 143970)
US12
US11
US10
B.
X
X
B
B
B
B
(1134)
(1235)
(4787)
poly(A)


C.
A
B
6.5Kb
1.235Kb

24h pi and each extract assayed for both CAT activity and TK activity. Protein concentrations of the extracts were measured by Bradfords assay and CAT activity expressed as % CAT activity/μg protein and TK activity as cpm/μg protein.

(a) Thymidine kinase (TK) assay

Direct comparison of differences in the CAT activities of the recombinant viruses, due solely to differences in the poly(A) sites of the CAT constructs, requires that the recombinant viruses have similar rates of growth. Growth of the recombinant viruses was therefore monitored by measurement of TK activity, TK, an early protein of HSV, reaches peak expression at 5-7h pi (Roizman and Sears, 1993). To allow parallel measurement of the two activites, virus infected cell extracts were prepared in CAT assay buffer. CAT extraction buffer was chosen in preference to TK lysis buffer, the high salt concentration and presence of detergent in the latter making it an unsuitable medium for the measurement of CAT activity. To ensure that this method of extract preparation would not impair the measurement of TK activity, comparisons of the TK activity of HSV-1 strain 17^+ infected BHK cell extracts prepared in either TK lysis buffer or CAT extraction buffer were made. The results are shown in Figure 3.12. TK activity was found to be comparable in both buffers, both in the timing of TK expression and the levels of activity attained. In the following experiments, TK activity was therefore measured from extracts produced in CAT extraction buffer.

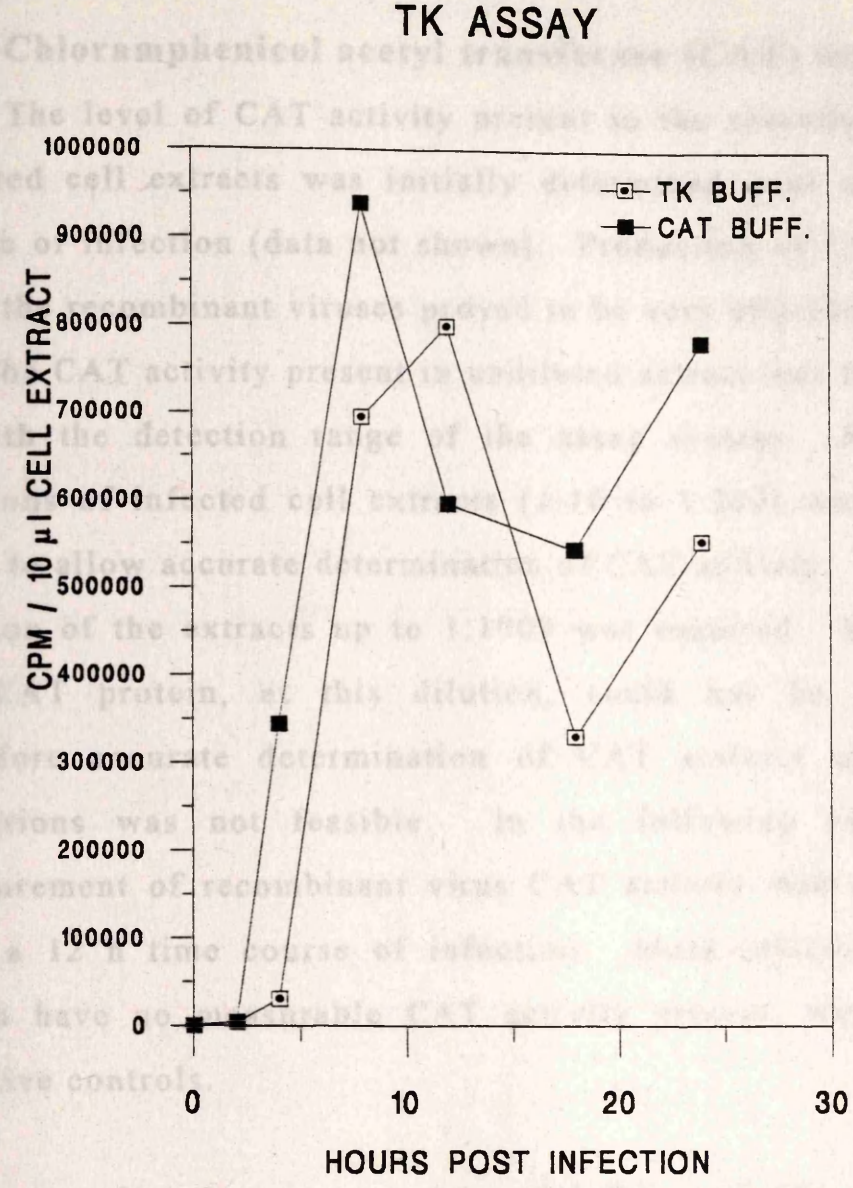


FIGURE 3.12:

Comparison of the viral TK activites of cell extracts prepared in either TK lysis buffer (TK) -⊡- or CAT buffer -■- over a 24h time course of infection. TK activity is expressed as counts per minute per 10 µl of cell extract.

(b) Chloramphenicol acetyl transferase (CAT) assay

The level of CAT activity present in the recombinant virus infected cell extracts was initially determined over a 24h time course of infection (data not shown). Production of CAT protein from the recombinant viruses proved to be very efficient and by 4 h pi the CAT activity present in undiluted extract was found to be outwith the detection range of the assay system. A range of dilutions of infected cell extracts (1:10 to 1:200) was therefore made to allow accurate determination of CAT activity. By 18h pi, dilution of the extracts up to 1:1000 was required. Stability of the CAT protein, at this dilution, could not be guaranteed therefore accurate determination of CAT activity under these conditions was not feasible. In the following experiments, measurement of recombinant virus CAT activity was carried out over a 12 h time course of infection. Mock-infected extracts, which have no measurable CAT activity present, were used as negative controls.

3.2.5 *In vivo* demonstration of LPF activity

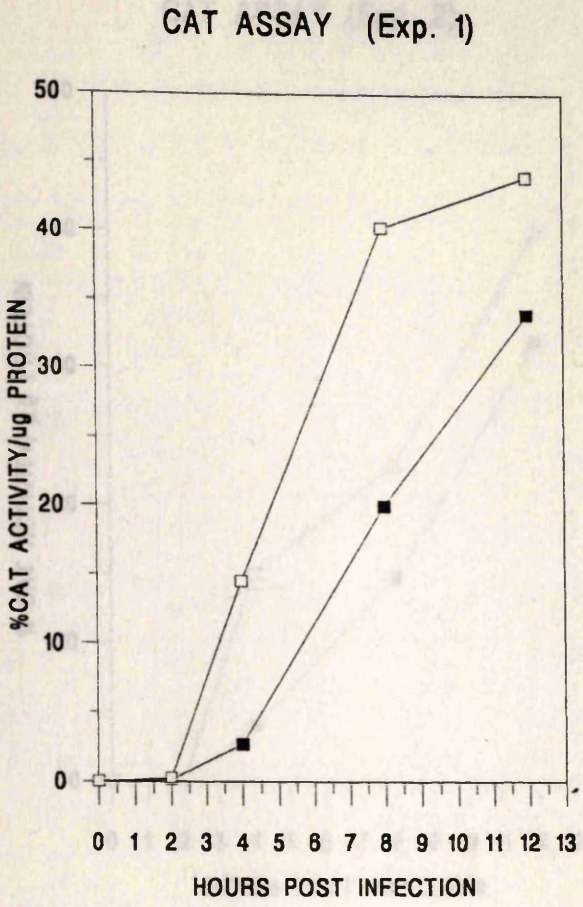
Comparison of the TK activities of the recombinant viruses, vFJ7 and vSAU3, confirmed that the two viruses had similar growth rates. The results of three separate experiments are shown in Figures 3.13 A,B,C (lower panels). Determination of CAT activity produced from these two viruses showed that vSAU3, containing the HSV-2 UL38 late poly(A) site, consistently produced higher levels of CAT activity than vFJ7, which contained the HSV-2 IE12 poly(A) site. This difference in CAT activity was apparent from early in infection (2-4h pi) (Table 3.5 and Figures 3.13 A,B,C upper panels). This finding was in

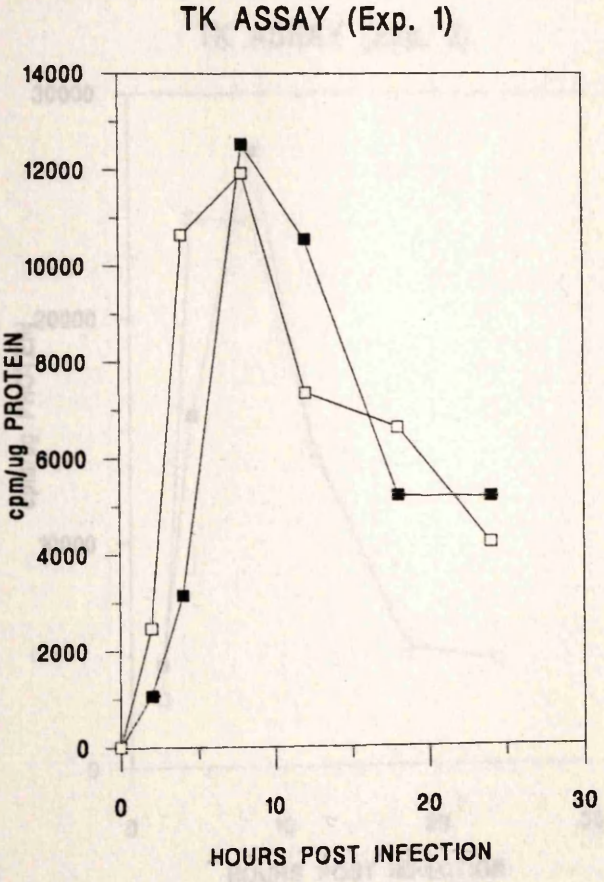
FIGURES 3.13 A, B AND C :

Upper panels : graphical representation of the CAT activities of the two recombinant viruses vSAU3 □ containing the HSV-2 late UL38 poly(A) site and vFJ7 ■ containing the HSV-2 IE12 poly(A) site, compared during three separate experiments.

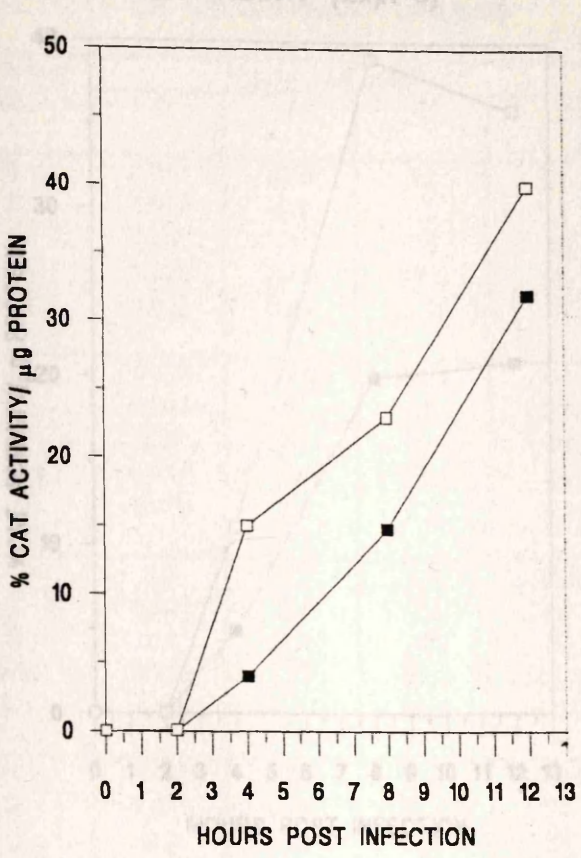
Lower panels : Graphical representation of the TK activity of these viruses compared during the above CAT activity experiments.

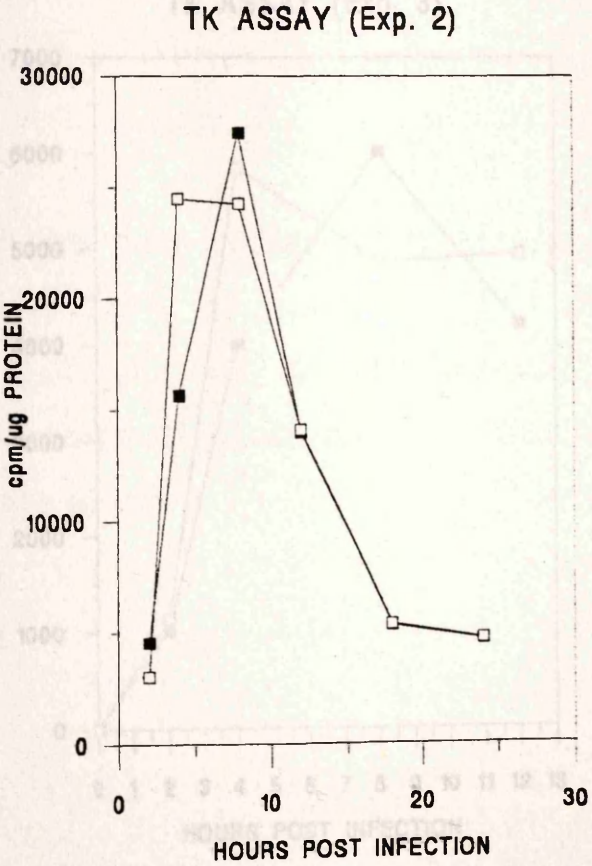
A


CAT ASSAY (Exp. 1)
%CAT ACTIVITY/ug PROTEIN
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
HOURS POST INFECTION

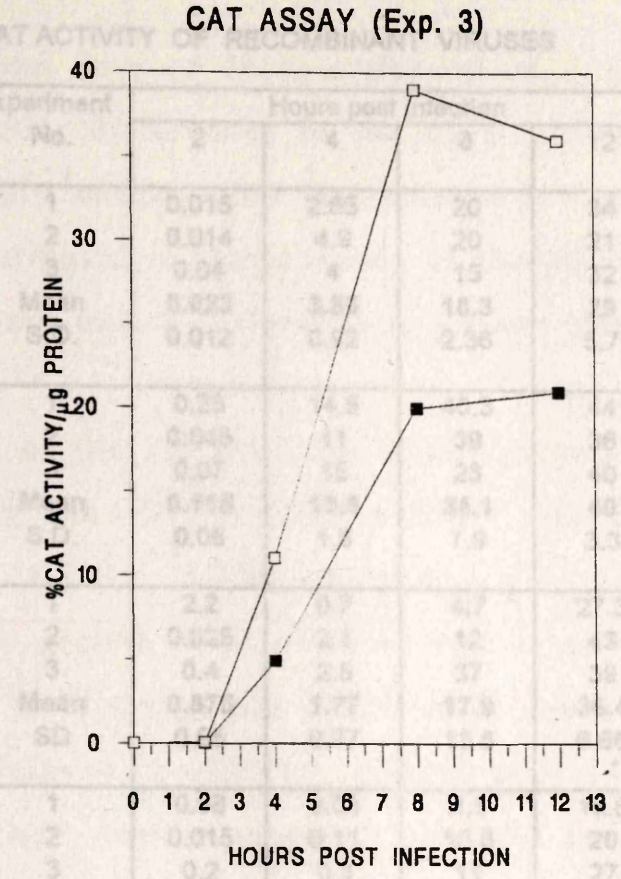

TK ASSAY (Exp. 1)
cpm/ug PROTEIN
14000
12000
10000
8000
6000
4000
2000
0
0
10
20
30
HOURS POST INFECTION

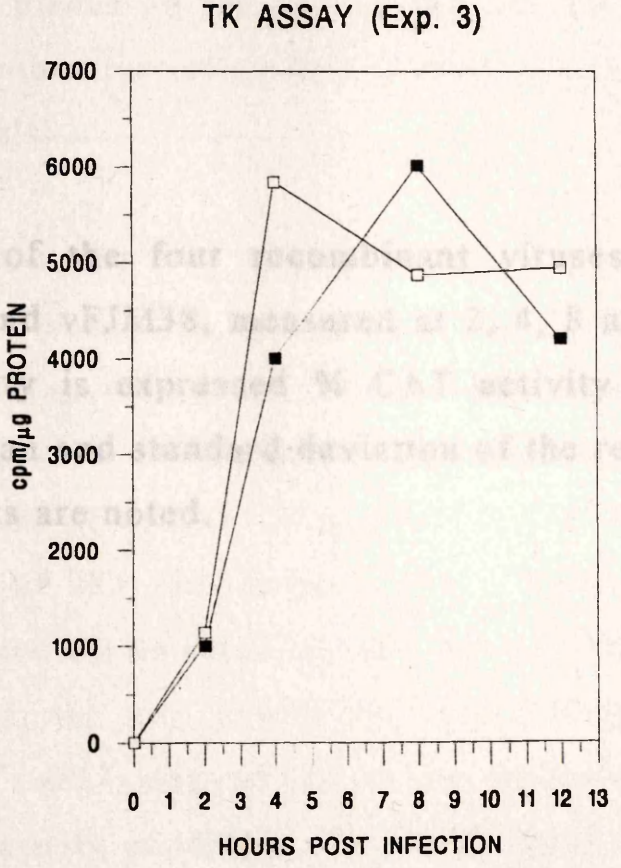
B


CAT ASSAY (Exp. 2)
% CAT ACTIVITY/ µg PROTEIN
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
HOURS POST INFECTION


TK ASSAY (Exp. 2)
cpm/ug PROTEIN
30000
20000
10000
0
0
10
20
30
HOURS POST INFECTION

C


CAT ASSAY (Exp. 3)
%CAT ACTIVITY/µg PROTEIN
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
HOURS POST INFECTION


TK ASSAY (Exp. 3)
cpm/µg PROTEIN
7000
6000
5000
4000
3000
2000
1000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
HOURS POST INFECTION

CAT ACTIVITY OF RECOMBINANT VIRUSES

Viruses	Experiment No.	Hours post infection			
		2	4	8	12
vFJ7	1	0.015	2.65	20	34
	2	0.014	4.9	20	21
	3	0.04	4	15	32
	Mean	**0.023**	**3.85**	**18.3**	**29**
	S.D.	0.012	0.92	2.36	5.7
vSAU3	1	0.23	14.5	40.3	44
	2	0.045	11	39	36
	3	0.07	15	23	40
	Mean	**0.115**	**13.5**	**34.1**	**40**
	S.D.	0.08	1.8	7.9	3.3
vFJ10	1	2.2	0.7	4.7	27.3
	2	0.025	2.1	12	43
	3	0.4	2.5	37	39
	Mean	**0.875**	**1.77**	**17.9**	**36.4**
	SD	0.95	0.77	13.8	6.66
vFJM38	1	0.08	0.03	2.5	15.6
	2	0.015	0.11	10.5	20
	3	0.2	0.3	11	27
	Mean	**0.098**	**0.15**	**8**	**20.9**
	S.D.	0.077	0.11	3.9	4.7

TABLE 3.5:

CAT activities of the four recombinant viruses, vFJ7, vSAU3, vFJ10 and vFJM38, measured at 2, 4, 8 and 12 h pi. CAT activity is expressed % CAT activity per µg protein. The mean and standard deviation of the results of three experiments are noted.

agreement with the data of McLauchlan *et al.*, (1992b) which demonstrated similar differences in the CAT activities of these viruses. The results confirm that the increase in CAT activity of vSAU3 can be attributed to the presence of the HSV-2 UL38 late poly(A) site and not to any difference in growth rates of the two viruses.

Comparison of the TK activities of the recombinant viruses vFJ10, containing the HSV-2 IE12 poly(A) site and vFJM38, containing the HSV-1 UL38 poly(A) site, demonstrated differences in the growth rates of these two viruses. For some unknown reason TK activity was consistently lower and enzyme production was delayed in vFJM38 infected cells (Table 3.5 and Figures 3.14 A,B,C lower panels). Hence, because of the differences in growth rates a valid comparison of the CAT activities of these two viruses could not be made. Therefore no significance was placed on the finding that the CAT activity of vFJ10 was higher than that of vFJM38 (Table 3.5 and Figures 3.14 A,B,C upper panels).

3.2.6 Discussion

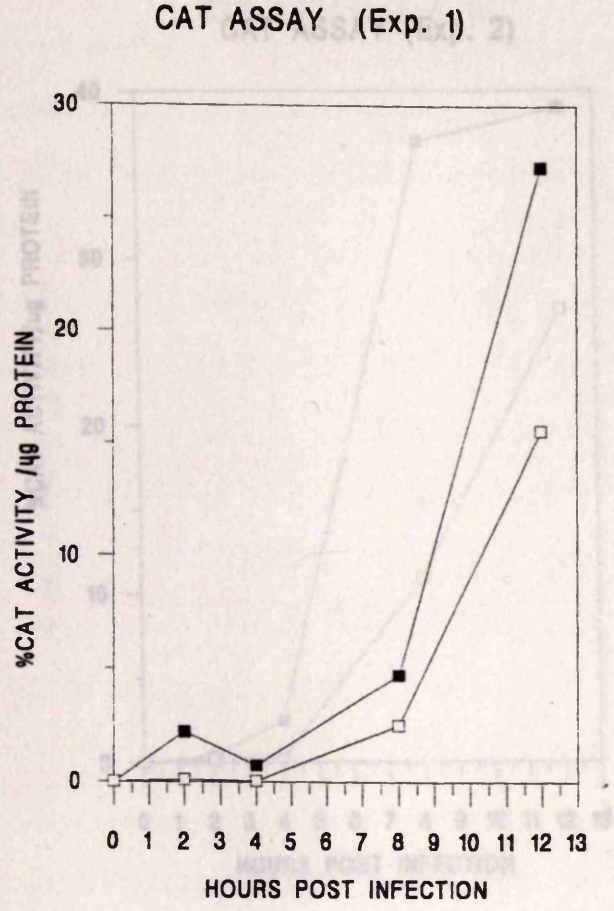
The results presented in this section confirm the original *in vivo* studies of McLauchlan *et al.*, (1989; 1992b), and demonstrate that LPF functions *in vivo*. We have in addition demonstrated that the increased level of CAT activity found in vSAU3 is due to increased usage of the late poly(A) site. Monitoring of the growth rates of the recombinant viruses, by measurement of TK expression, ruled out any possibility that differences in the growth rates of vFJ7 and vSAU3 were responsible for the increased CAT activity of vSAU3. The LPF effect was therefore

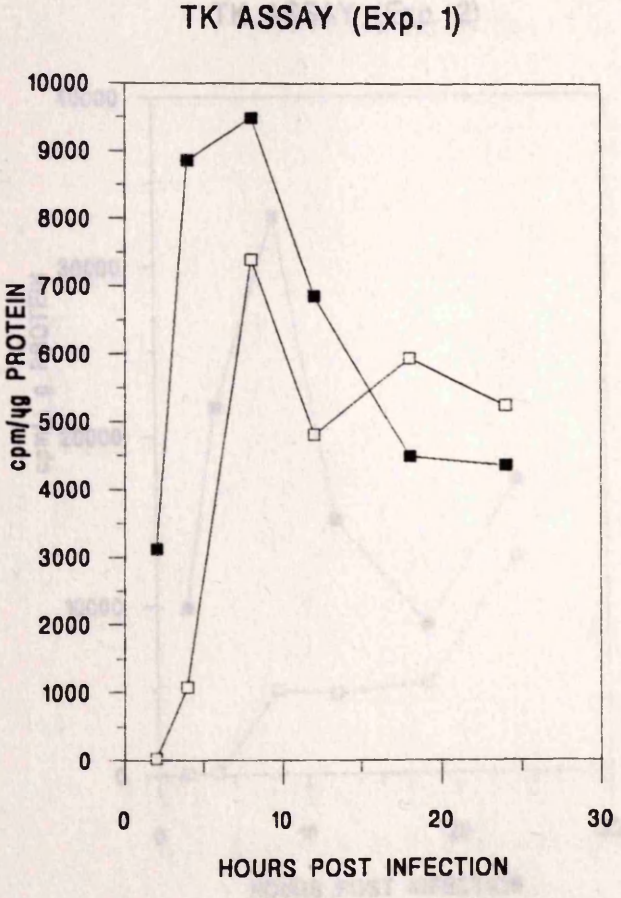
FIGURES 3.14 A. B and C :

Upper panels : Graphical representation of the CAT activities of the two recombinant viruses vFJ10 ■ containing the HSV-2 IE12 poly(A) site and vFJM38 □ containing the HSV-1 UL38 poly(A) site compared during three separate experiments.

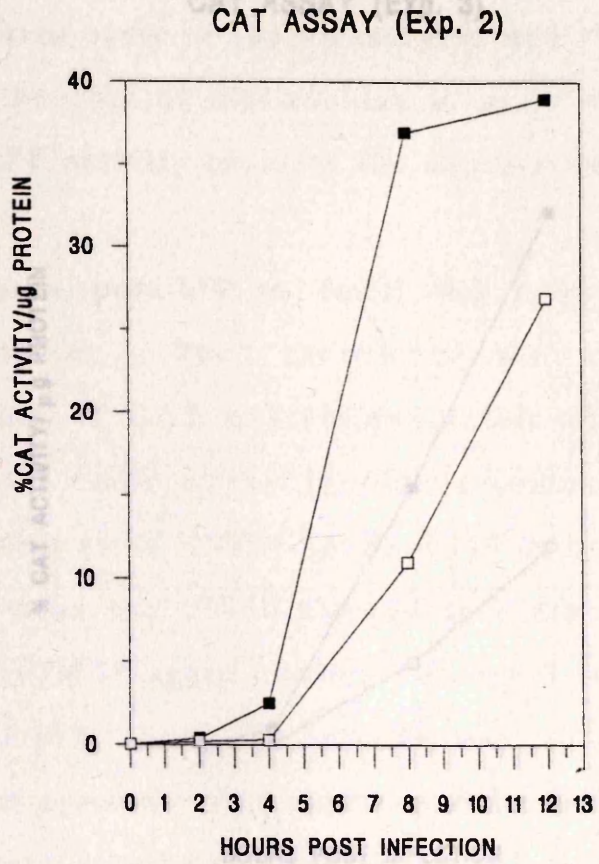
Lower panels : Graphical representation of the TK activity of these viruses compared during the above CAT activity experiments.

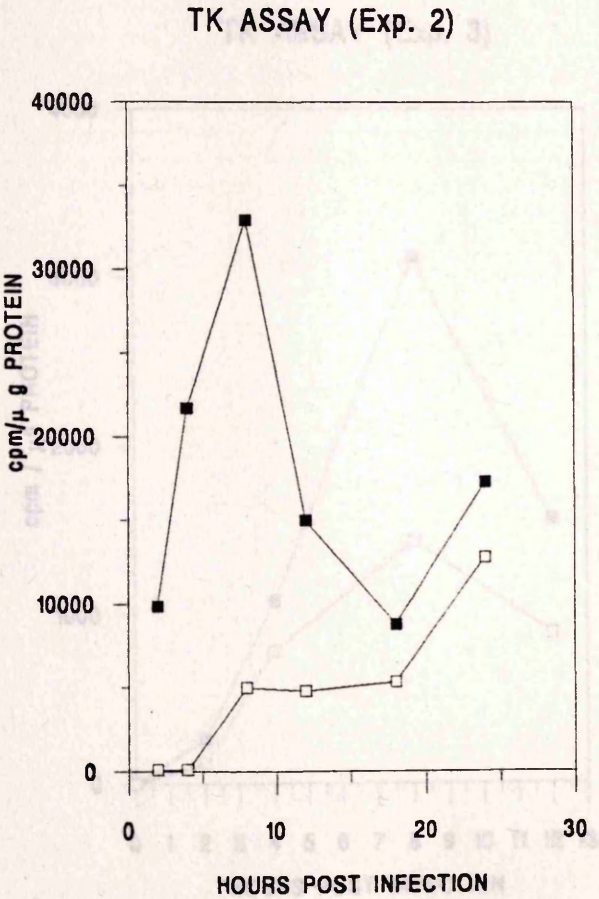
A


CAT ASSAY (Exp. 1)
%CAT ACTIVITY /µg PROTEIN
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
HOURS POST INFECTION

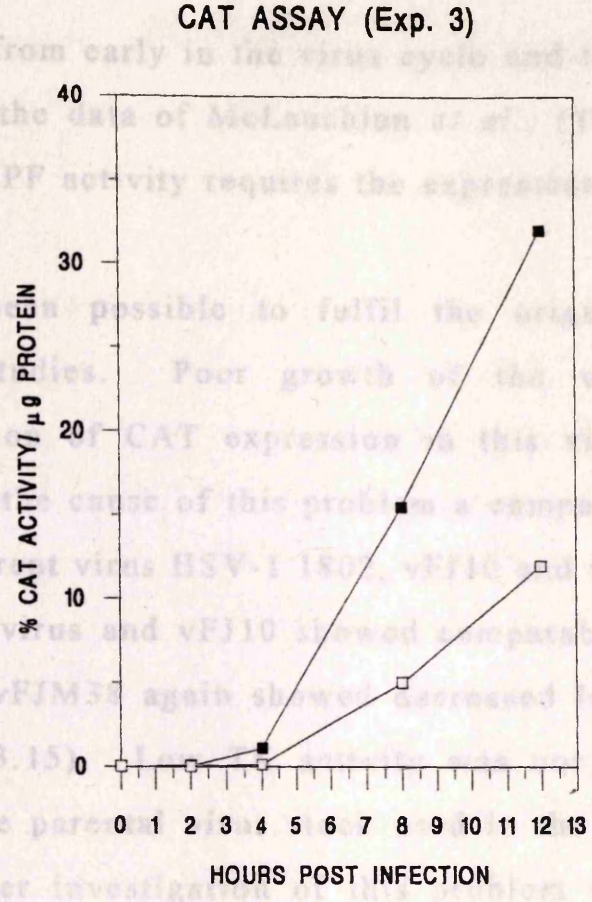

TK ASSAY (Exp. 1)
cpm/µg PROTEIN
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
HOURS POST INFECTION

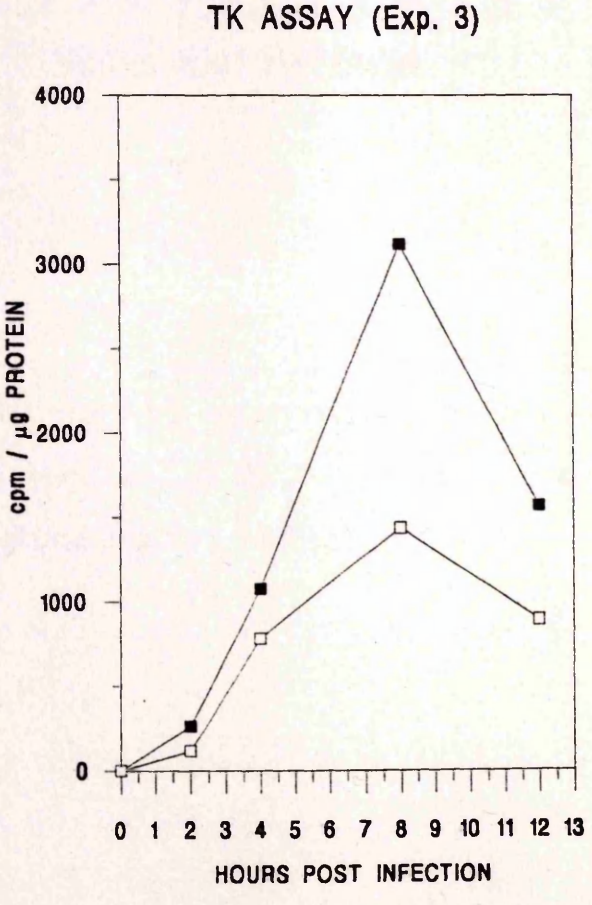
B


CAT ASSAY (Exp. 2)
%CAT ACTIVITY/ug PROTEIN
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
HOURS POST INFECTION


TK ASSAY (Exp. 2)
cpm/μ g PROTEIN
40000
30000
20000
10000
0
0
10
20
30
HOURS POST INFECTION

c


CAT ASSAY (Exp. 3)
% CAT ACTIVITY/ µg PROTEIN
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
HOURS POST INFECTION


TK ASSAY (Exp. 3)
cpm / µg PROTEIN
4000
3000
2000
1000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
HOURS POST INFECTION

detectable *in vivo* from early in the virus cycle and this result is in agreement with the data of McLauchlan *et al.*, (1989; 1992b) which shows that LPF activity requires the expression of an HSV IE gene (IE63).

It has not been possible to fulfil the original aim of extending these studies. Poor growth of the vFJM38 has prevented comparison of CAT expression in this virus. In an attempt to identify the cause of this problem a comparison of the TK levels of the parent virus HSV-1 1802, vFJ10 and vFJM38 was made. The parent virus and vFJ10 showed comparable levels of TK activity while vFJM38 again showed decreased levels of TK activity. (Figure 3.15). Low TK activity was not therefore a characteristic of the parental virus stock used in the preparation of vFJM38. Further investigation of this problem was deemed impractical due to the time constraints of the project.

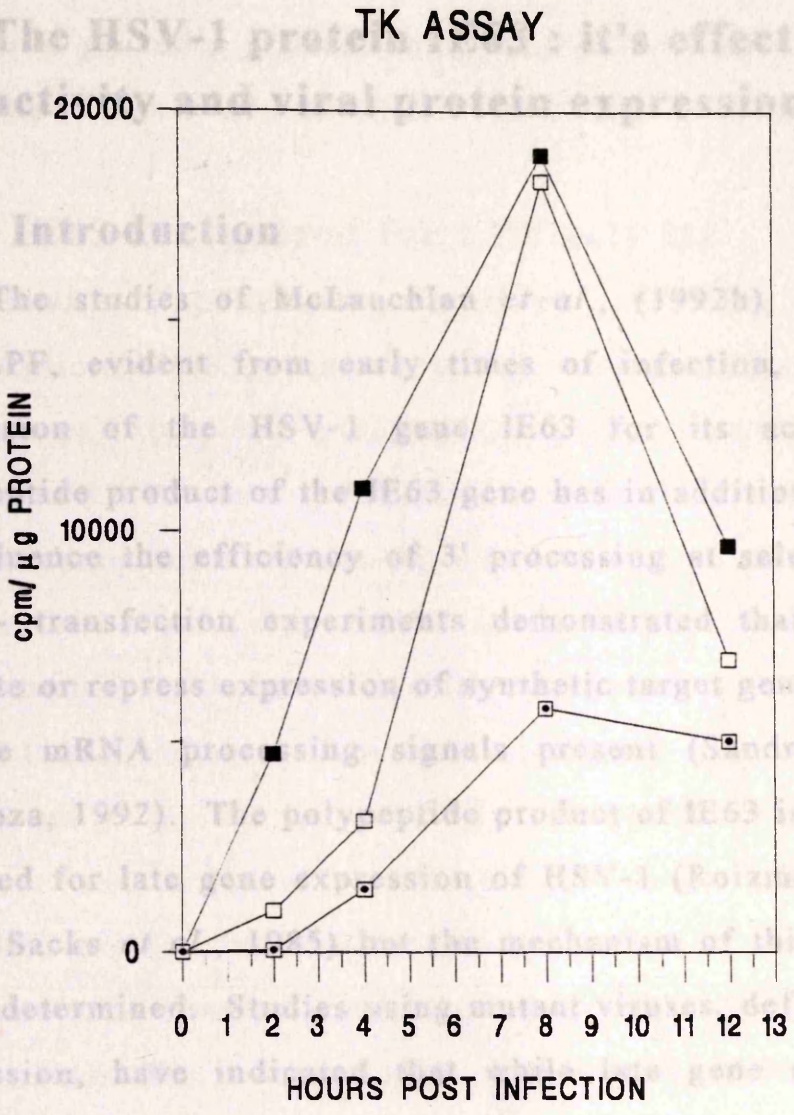


FIGURE 3.15:

Comparison of the TK activities of the two recombinant viruses vFJ10 ■ , vFJM38 □ and the parental virus stock HSV-1 (1802) ⊡ .

3.3 The HSV-1 protein IE63 : it's effect on LPF activity and viral protein expression

3.3.1 Introduction

The studies of McLauchlan *et al.*, (1992b), demonstrated that LPF, evident from early times of infection, required the expression of the HSV-1 gene IE63 for its activity. The polypeptide product of the IE63 gene has in addition been shown to influence the efficiency of 3' processing at selected poly(A) sites - transfection experiments demonstrated that IE63 could activate or repress expression of synthetic target genes, dependant on the mRNA processing signals present (Sandri-Goldin and Mendoza, 1992). The polypeptide product of IE63 is known to be required for late gene expression of HSV-1 (Roizman and Sears, 1993; Sacks *et al.*, 1985) but the mechanism of this effect is as yet undetermined. Studies using mutant viruses, deficient in IE63 expression, have indicated that while late gene expression of these viruses was reduced or absent, the defect was not at the level of de novo transcription of mRNA (Smith *et al.*, 1992). This suggests that IE63 acts, at least in part, post-transcriptionally to regulate late gene expression.

The following experiments examine firstly the influence of IE63 on LPF activity and secondly the effect of IE63 on viral protein expression. The relative 3' processing efficiencies of the previously defined range of poly(A) sites and viral protein expression in the presence and absence of the IE63 polypeptide have been examined using the IE63 insertion mutant virus 27-LacZ. An insertion in the leader sequence of the IE63 gene of

this virus prevents production of the polypeptide product from this gene (Smith *et al.*, 1992).

3.3.2 IE63 is required for LPF activity

(a) *In vitro* 3' processing reactions

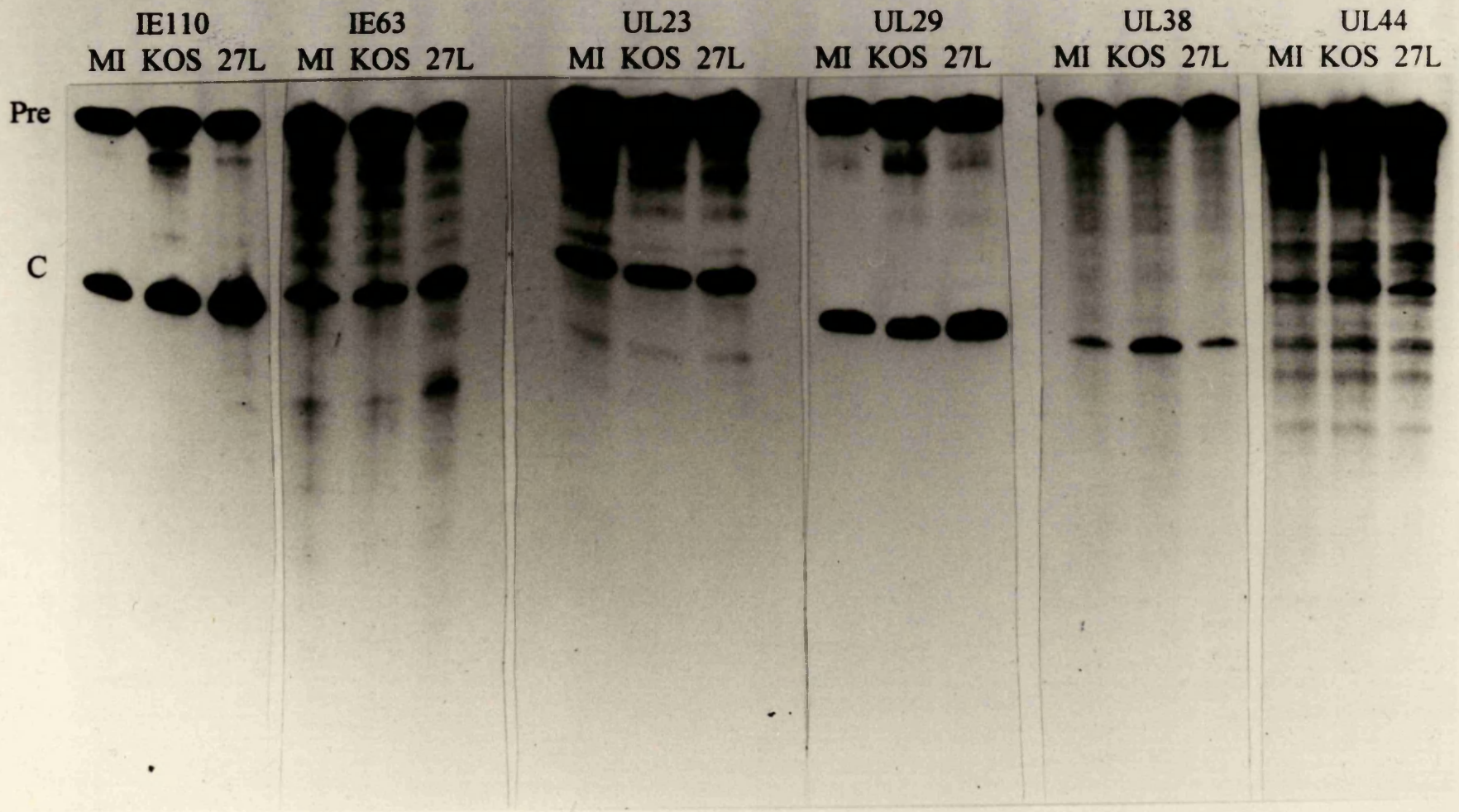
Radiolabelled precursor mRNA was synthesised from the plasmids pG110, pG63, pG23, pG29, pG38 and pG44 as detailed in Section 3.1.4. Sets of MI, HSV-1 strain KOS infected and 27-LacZ infected HeLa cell nuclear extracts were prepared in parallel as described previously. HSV-1 strain KOS is the parental virus strain used in the production of the mutant virus, 27-LacZ (Smith *et al.*, 1992). In total, 2 or 3 *in vitro* processing reactions were carried out on the six test poly(A) sites, quantitative analysis was therefore not practical. The extracts were standardised as described previously, each extract being used to process as many of the poly(A) sites as possible (a minimum of five poly(A) sites per extract). The products of these reactions, which consisted of a mixture of uncleaved and cleaved radiolabelled precursor mRNA, were separated as before on 6% polyacrylamide gels and the bands visualised by autoradiography.

(b) The IE63 protein can affect the 3' processing efficiencies of selected HSV-1 genes

Figure 3.16 shows a typical autoradiograph of the *in vitro* 3' processing efficiencies of the IE110, IE63, UL23, UL29, UL38 and UL44 poly(A) sites. The late poly(A) sites of UL38 and UL44 were processed three times and in each case the level of 3' processing efficiency was increased using the KOS infected nuclear extract, above that of the MI extract. The level of 3'

FIGURE 3.16 :

Autoradiograph of typical 3' processing reactions using mock-infected (MI), HSV-1 strain KOS infected (KOS) and 27-LacZ infected (27L) nuclear extracts. Pre : uncleaved precursor RNA (approximately 400nt), C : cleaved RNA species (200-250nt) obtained by cleavage of the test poly(A) sequences from the HSV-1 genes IE110, IE63, UL23, UL29, UL38 and UL44.


IE110
MI KOS 27L
IE63
MI KOS 27L
UL23
MI KOS 27L
UL29
MI KOS 27L
UL38
MI KOS 27L
UL44
MI KOS 27L
Pre
C

processing efficiency of these two late poly(A) sites was decreased in the 27-LacZ infected extract to a level below that obtained with the KOS infected extract and was similar to the MI processing levels. Little change was noted in the 3' processing efficiencies of the IE63, IE110, UL23 and UL29 poly(A) sites when processing was performed with either MI, KOS infected or 27-LacZ infected nuclear extracts. The results of the *in vitro* 3' processing reactions demonstrated that expression of the HSV-1 gene IE63 is required for the LPF associated increase in processing efficiencies of the late poly(A) sites of UL38 and UL44. The processing efficiencies of the IE63, IE110, UL23 and UL29 poly(A) sites, which have previously been shown to be unresponsive to LPF, are unaffected by the presence or absence of the polypeptide product of the IE63 gene.

3.3.3 Examination of viral protein expression in cells infected with the IE63 insertion mutant virus 27-LacZ

(a) Protein extracts

Protein extracts were made from Vero cells infected with the IE63 insertion mutant 27-LacZ. This virus mutant does not express IE63 enabling the examination of viral protein expression in its absence. Protein extracts of control infections were prepared in parallel. Vero cells infected with HSV-1 strain KOS, the parental strain of the mutant virus, provided the postive control, showing the protein profile expected of a wt infection. A second control of 2.2 cells infected with 27-LacZ, was also prepared - 2.2 cells are the complementing cell line for the 27-LacZ insertion mutant virus, they carry the IE63 gene under the

control of an HSV-1 IE promoter and expression of IE63 in this cell line requires transactivation by Vmw65 either by infection or transfection. Protein expression differing from wt, which is not due to the IE63 mutation in 27-LacZ virus, would be evident using this control.

Infections were carried out over a 24h time course and cells harvested at 2, 4, 8, 12, 18 and 24h pi. Mock infected controls were harvested with the 24h time point. Viral and cellular proteins were extracted from the cells by lysis and separated on 11% or 12.5% SDS denaturing polyacrylamide mini-gels. Protein size was estimated by comparison with protein molecular weight markers.

(b) Antibodies

Primary antibodies to the polypeptide products of the HSV genes IE110, IE63, IE175, UL29, UL38, UL42, UL44, US6, US8 and US11, were used for identification of the viral proteins produced in the extracts described above. Antibody dilutions, optimal for use in Western blot analysis, were those recommended by the antibody donors (see Materials). Calibration of the antibodies to allow absolute protein quantification was not carried out. Using the enhanced chemiluminescence detection system, changes in protein levels would be undetectable once saturation of the system was reached. Taking into account these limitations, use of this system would allow gross changes in protein expression to be detected and compared under the three infection conditions outlined.

(c) Western Blot

Western blot was performed on the protein mini-gels as described in Methods. Secondary antibodies conjugated to HRP were used for visualisation of the protein-antibody complexes, using the ECL detection system. Reactive protein bands were visualised by exposure of these membranes to X-ray film for 1-5min.

3.3.4 IE63 expression is required for the synthesis of selected late viral proteins

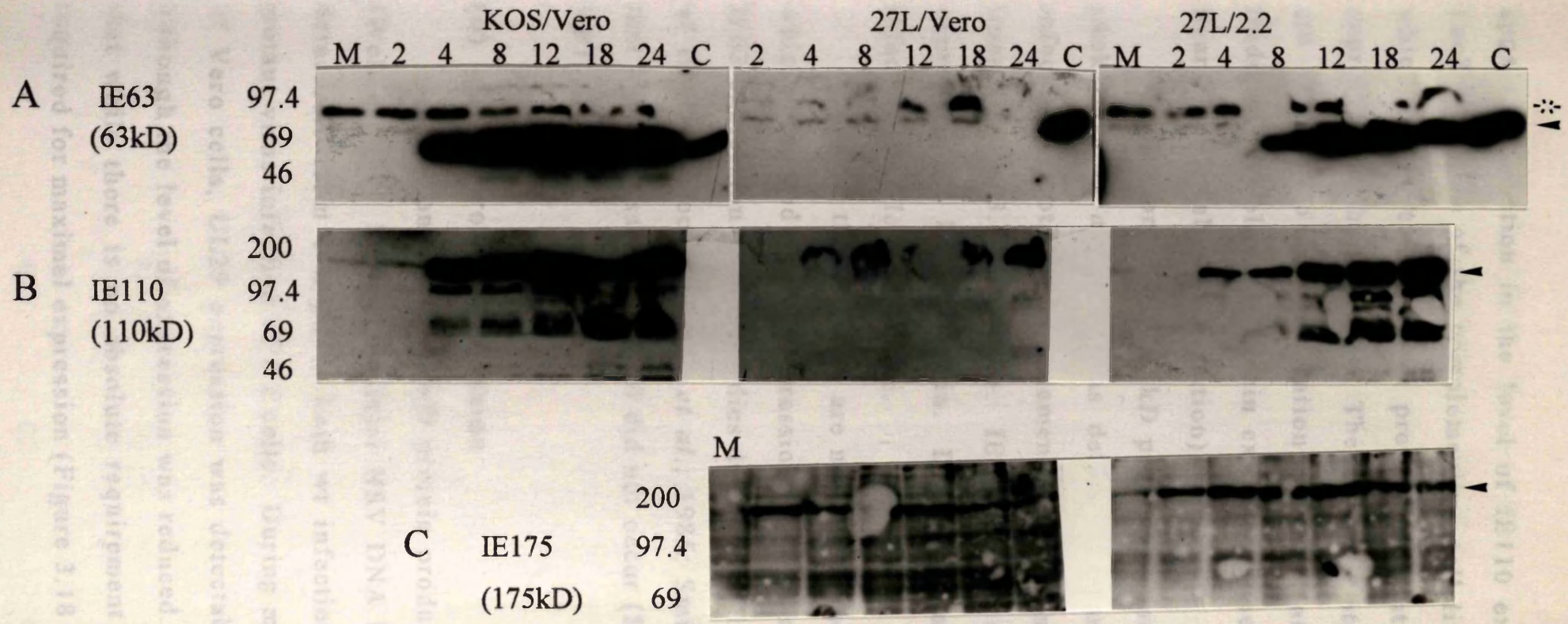
(a) Immediate-early protein expression

The 63kD protein product of the IE63 gene (Ackerman *et al.*, 1984) was detectable during wt infection from 4h pi. During 27-LacZ infection of the complementing cell line, IE63 expression was detectable from 8h pi. Virus transactivators are required to switch on the IE63 gene of the complementing 2.2 cells, however there is a delay in IE63 expression under these conditions, which is perhaps due to the integration of the IE63 gene into the host cell genome. No IE63 expression was seen in the non-complementing Vero cell line, confirming the mutant phenotype of 27-LacZ (Figure 3.17 panel A).

Expression of the 110kD protein product of the IE110 gene (Ackerman *et al.*, 1984) was detectable from 4h pi under all three infection conditions (Figure 3.17 panel B). While IE110 protein was detectable, from 4h pi, in the absence of IE63, there was a marked reduction in the level of expression. It is possible that whilst IE110 expression has no absolute requirement for IE63, its absence leads to a reduction in the efficiency of IE110 expression. In support of this finding was the observation of a slight but

FIGURE 3.17 :

Western blot analysis of protein expression from the HSV-1 genes IE63 (A), IE110 (B) and IE175 (C). Protein extracts were prepared from HSV-1 strain KOS infected Vero cells (KOS/Vero), 27-LacZ infected Vero cells (27L/Vero) and 27-LacZ infected 2.2 cells (27L/2.2) at 2, 4, 8, 12, 18 and 24h pi. The position of protein molecular weight markers run alongside the extracts are given to the left of the figure. The predicted molecular weight of each test protein is given in parenthseis. Protein band ➤ , cross-reacting cellular protein ✲ , IE63 positive control C, mock infected control M.


KOS/Vero
27L/Vero
27L/2.2
M 2 4 8 12 18 24 C
2 4 8 12 18 24 C
M 2 4 8 12 18 24 C
A
IE63
(63kD)
97.4
69
46
B
IE110
(110kD)
200
97.4
69
46
M
C
IE175
(175kD)
200
97.4
69

apparent reduction in the level of IE110 expression during 27-LacZ infection of the complementing cell line, at 4h and 8h pi, which could be due to the previously mentioned delay in IE63 expression in this cell line. The smaller proteins seen in this blot are thought to be degradation products of IE110, a common finding in whole cell protein extracts of the kind used here (R. Everett personal communication).

Expression of the 175kD protein product of the IE175 gene (Ackerman *et al.*,1984) was detectable from 2h pi in 27-LacZ infection of both the complementing and non-complementing cell lines (Figure 3.17 panel C). IE63 appears to have no effect on expression the IE175 protein. Due to technical problems no wt infection profile is available.

However these results are not compatible with other studies which examined protein expression in ts virus mutants deficient in IE63 expression. These studies demonstrated that over expression of IE175 did occur (Sacks *et al.*, 1985; Smith *et al.*, 1992) and that overexpression of IE110 did not occur (Sacks *et al.*, 1985) at NPT.

(b) Early protein expression

Expression of the 130kD protein product of the UL29 gene (Weller *et al.*, 1983), the major HSV DNA binding protein, was detectable from 8 h pi, in both wt infection of Vero cells and mutant virus infection of 2.2 cells. During mutant virus infection of Vero cells, UL29 expression was detectable at 8h and 12h pi although the level of expression was reduced. This would suggest that while there is no absolute requirement for IE63 it may be required for maximal expression (Figure 3.18 panel A).

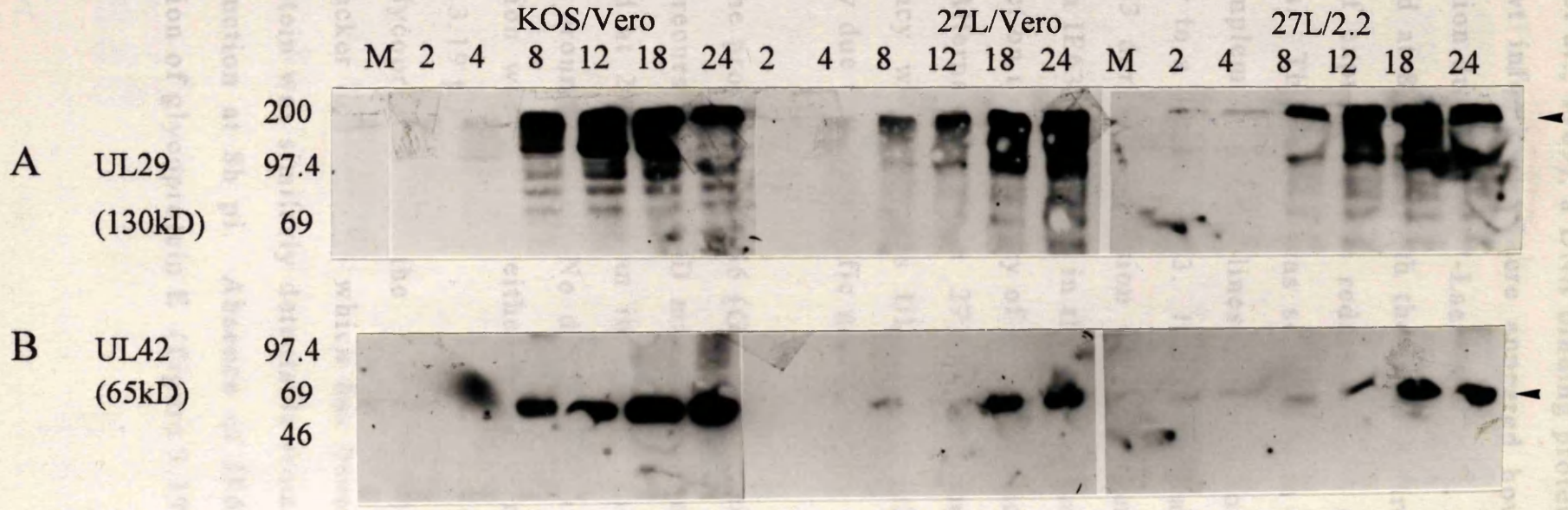


FIGURE: 3.18

Western blot analysis of protein expression from the HSV-1 genes UL29 and UL42.

Expression of the 65kD protein product of the UL42 gene (Gallo *et al.*, 1989), a DNA binding protein, was detected from 8h pi in wt infection. There appeared however to be a delay in expression during the 27-LacZ infection, while UL42 protein was detected at 8h pi in both the mutant virus infected extracts the level of expression was reduced below that of the wt infected extracts. This effect was seen in both the complementing and non-complementing cell lines and may not therefore be attributed entirely to a lack of IE63. It may be that there is a requirement for IE63 during expression of UL42, and the previously noted delay in IE63 expression in the complementing cell line may have an effect on the efficiency of UL42 production (Figure 3.18 panel B). Alternatively, the 27-LacZ mutant could have another deficiency which affects UL42 production - this was thought unlikely due to the specific nature of the mutation induced in 27-LacZ.

The products of US6 (Glorioso *et al.*, 1983), present as the 50kD precursor and 58kD mature form of glycoprotein D, were detected at 2h pi with an increase in protein expression being evident around 8h pi. No difference in this pattern of protein expression was seen in either the presence or absence of IE63 (Figure 3.19 panel A).

Glycoprotein E the 59kD protein product of US8 (Longnecker *et al.*,1987) which has been classified as an early-late protein was similarly detectable from 2h pi, with an increase in production at 8h pi. Absence of IE63 had no effect on the expression of glycoprotein E (Figure 3.19 panel B).

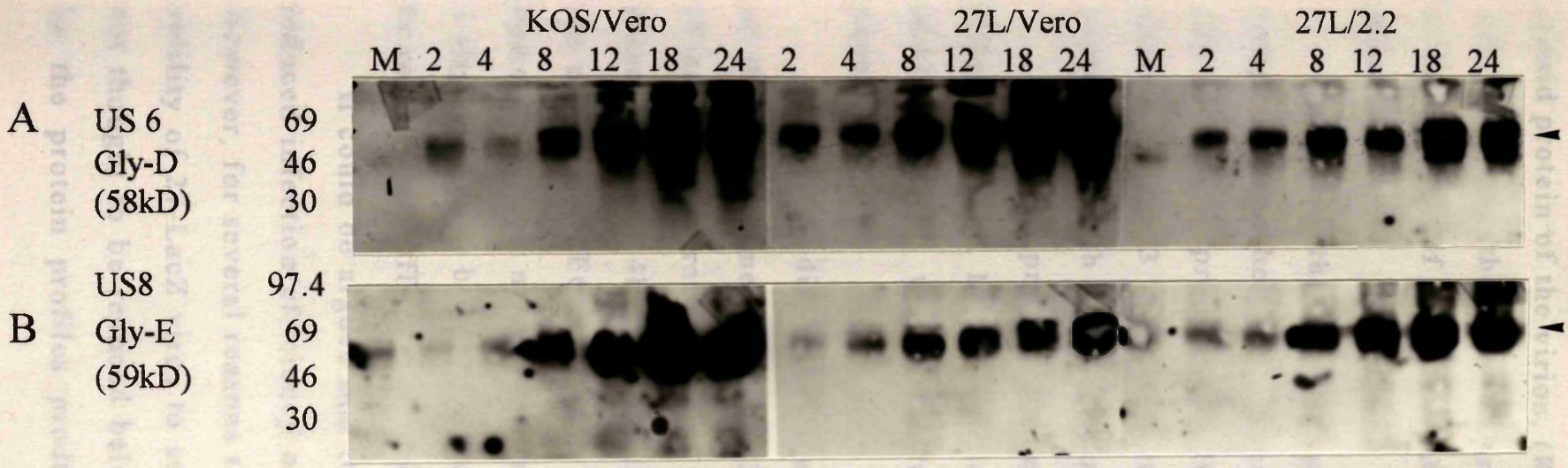


FIGURE 3.19 :

Western blot analysis of protein expression from the HSV-1 genes US6 (glycoprotein D) and US8 (glycoprotein E).

(c) Late protein expression

Expression of the 54kD protein product of the UL38 gene, a capsid protein of the virion, (Rixon *et al.*,1990) was detected from 12h pi in both the wt and complemented viral mutant infections. In the absence of IE63 no UL38 protein was detected (Figure 3.20 panel A).

Similarly the 21/22kD protein products of the US11 gene, components of the virion, (MacLean *et al.*, 1987) were detected at 12h pi in the presence of IE63 and were undetectable in the absence of IE63 (Figure 3.20 panel B). With a reduction in expression at 12h pi in the complementing cell line.

The 92kD protein product of the UL44 gene, glycoprotein C (Glorioso *et al.*, 1983), was detected at 12h pi in the presence of IE63 and was undectable in the absence of IE63 (Figure 3.20 panel A).

A slightly different picture appeared for the protein product of the UL45 gene (Figure 3.21 panel B), an 18kD late protein (Visalli and Brandt, 1991; 1993). The 18kD protein was detectable from 4h pi with expression increasing from 8h pi. In the absence of IE63 the 18kD protein was detectable from 4h pi, however little or no increase in expression was seen at 8h pi, only a slight increase being noted at 24h pi. IE63 appears therefore to be required for efficient expression of the UL45 protein.

It could be argued that the effects seen here could be due to reduced infection efficiency of Vero cells with the mutant virus. However, for several reasons this was not felt to be the case. The ablility of 27-LacZ virus to adsorb to and penetrate into cells, is not thought to be reduced below wt levels. This is corroborated by the protein profiles produced by 27-LacZ infection of the

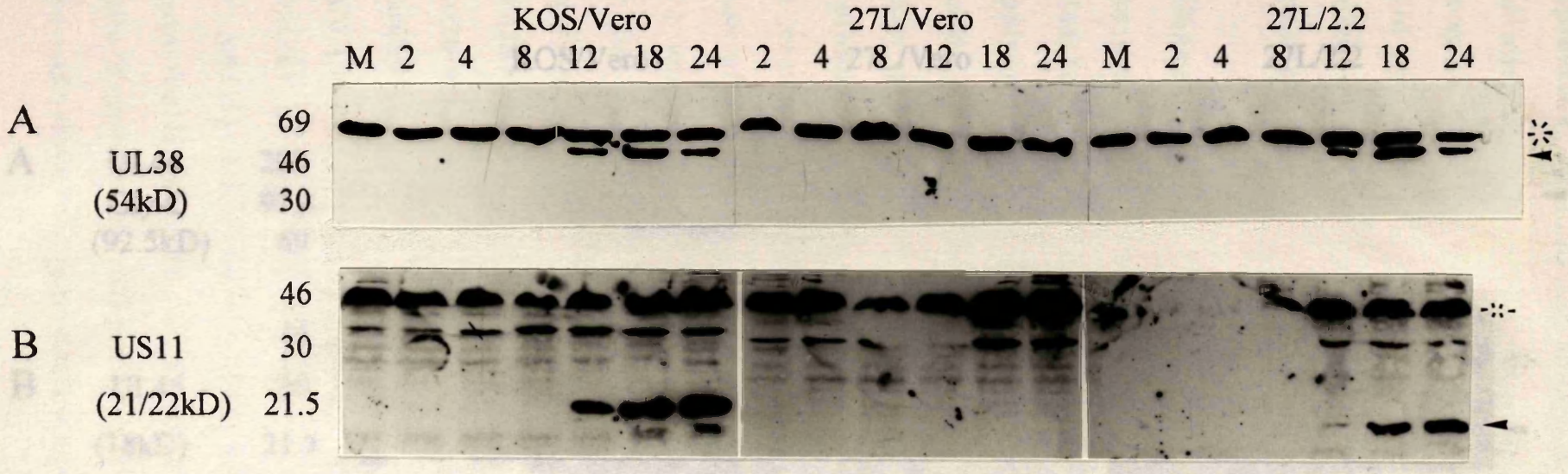


FIGURE 3.20 :

Western blot analysis of protein expression from the HSV-1 genes UL38 and US11.

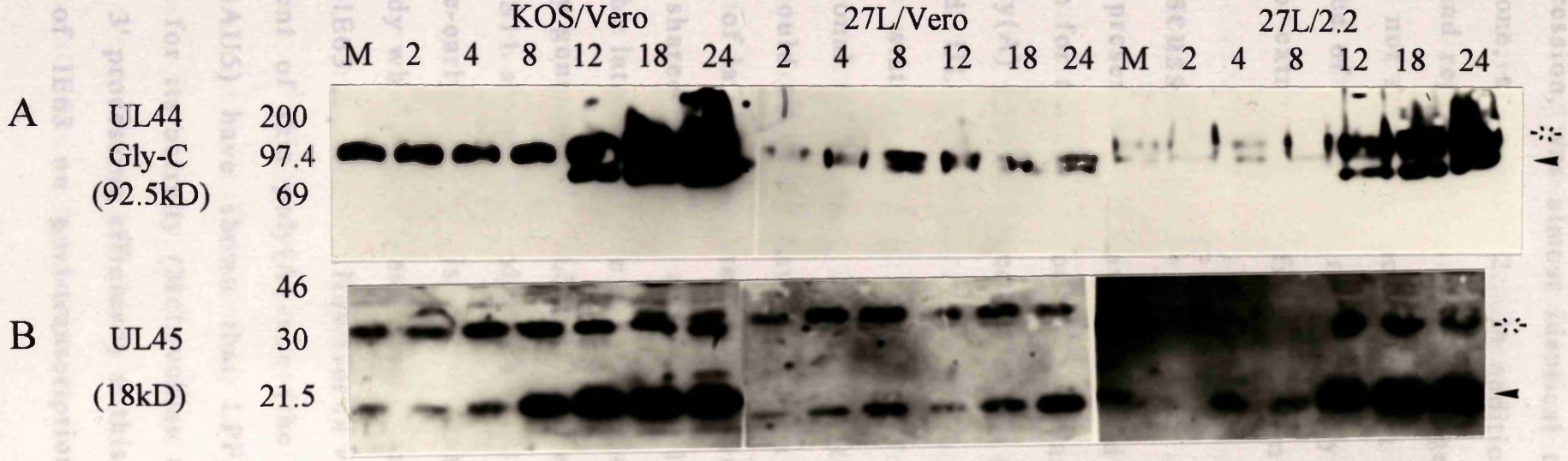


FIGURE 3.21 :

Western blot analysis of protein expression from the HSV-1 genes UL44 (glycoprotein C) and UL45

complementing cell line, which taking account of the delay in IE63 expression, was almost identical to wt infection in every case but one, that of UL42. In addition, each protein gel was stripped and reprobed with antibodies generated against proteins which did not show a reduction or lack of protein expression in the absence of IE63, thus removing any variation between blots due to poor extracts or inefficient protein transfer.

3.3.5 Discussion

The present studies have confirmed the requirement of IE63 expression for the LPF associated increase in 3' RNA processing at the poly(A) sites of selected HSV-1 genes, the late genes of UL38 and UL44. This increase in processing occurs, as previously stated, in the absence of any influence due to transcriptional regulation. IE63 mediated increases in poly(A) site use could therefore have a role in the post-transcriptional regulation of late gene expression. However, some HSV poly(A) sites are shared by genes of both early and late classes, for example the late UL35 gene shares the same poly(A) site as the UL33 early gene and the UL34 gene, of unknown class. The late gene of US11 shares the poly(A) site of US10 (early) and US12 (immediate-early). Only this latter situation has been considered in this study where protein expression from US11 has been shown to require IE63 expression. However *in vitro* studies on the HSV-2 equivalent of this poly(A) site (the IE site on the control plasmid SAU5) have shown that LPF, which requires IE63 expression for its activity (McLauchlan *et al.*, 1992b), does not upregulate 3' processing efficiency at this poly(A) site. Thus, the influence of IE63 on post-transcriptional mechanisms cannot

therefore be the sole means of regulation of L gene expression. It may be that posttranscriptional regulation is additional to other means of regulation such as transcriptional control.

Analysis of viral protein expression during infection with the IE63 insertion mutant, 27-LacZ, has shown that IE63 is required for the expression of selected late viral proteins, namely US11, UL38 and UL44. Expression of the late protein product of UL45, also appears to be reduced in the absence of IE63. The gene encoding UL45 is co-terminal with that of UL44, sharing the same poly(A) site, the reduction rather than absence of UL45 protein indicates that IE63 is required for efficient expression, low levels of protein being produced in the absence of IE63. These results are in agreement with the general pattern of expression observed in other studies which showed a reduction in late gene expression (UL48, glycoprotein B) and in particular true late gene expression (glycoprotein C, UL38), in the absence of IE63 (Sacks *et al.*, 1985; Smith *et al.*, 1992; Rice and Knipe, 1988).

IE63 is known to be involved in the switch from early to late gene expression, but the exact mechanism of this regulation has not been determined. The function of the IE63 gene product has been investigated by a number of research groups, and the results produced have provided conflicting evidence regarding the role of IE63 in the regulation of viral gene expression. The variability of these results is likely to be due to differences in the analysis systems used and the multifunctional nature of this protein. There is evidence, provided by transient transfection studies, for IE63 mediated regulation of gene expression being at the transcriptional level (McCarthy *et al.*, 1989) and at the

posttranscriptional level (Sandri-Goldin and Mendoza, 1992; Smith *et al.*, 1989). There is evidence both for (McCarthy *et al.*, 1989; Rice *et al.*, 1993) and against (Sacks *et al.*, 1985) the involvement of IE63 in viral DNA synthesis. Late gene expression in HSV is associated with the onset of viral DNA replication (Roizman and Sears, 1993), IE63 could therefore affect late gene expression by an indirect effect on DNA synthesis. In the absence of IE63, the major DNA binding protein UL29 (ICP8) has been shown to be conformationaly altered and it has been suggested that this prevents formation of active DNA replication complexes (Curtin and Knipe, 1993). However detection of conformational changes in the protein would depend on the specificity of the antibody and may not be detected by the UL29 antibody used here. The study of Curtin and Knipe, (1993) in addition showed that low levels of DNA replication occurred even in the absence of IE63 and this was dependent on the cell cycle of the infected cell. It is possible that the low level of expression of three of the proteins studied here (UL42, UL29 and UL45) was a result of low levels of DNA synthesis occurring in a small population of cells.

Our results suggest that IE63 may be required for maximal expression of UL42 (part of the DNA polymerase) and UL29. It is possible that IE63 could influence DNA synthesis by this route. If there is no absolute requirement for IE63 in UL42 and UL29 expression there may be a subtle regulation, dependent on the timing of expression of the three proteins.

Other studies (Sacks *et al.*, 1985), have noted an accumulation of early proteins in the absence of IE63. However, due to the limitations of the Western blot analysis carried out

here, accumulation of proteins above the saturation level of the detection system would not be detected.

3.4 Sequence, structure and protein binding properties of HSV-1 poly(A) sites

3.4.1 Introduction

The formation of the 3' end of mRNA requires specific endonucleolytic cleavage of the precursor mRNA and the subsequent addition of approximately 200 adenylate residues. RNA sequence requirements for efficient cleavage and polyadenylation include the conserved AAUAAA hexanucleotide poly(A) signal located 10-30nt upstream of the cleavage site, and a downstream U-or GU-rich element (McDevitt *et al.*, 1986; Sadofsky and Alwine, 1984; Sadofsky *et al.*, 1985; Manley, 1988; Conway and Wickens, 1987; Gil and Proudfoot, 1987). Consensus sequences have been proposed for these downstream elements YGTGTTYY (McLauchlan *et al.*, 1985), TTG/ANNNTTTTTT (Renan, 1987), and RGUUUUYRR (Sadofsky *et al.*, 1985). Recent studies have indicated an additional requirement for elements upstream of the poly(A) signal for efficient cleavage and polyadenylation of mRNA from yeast (Hou *et al.*, 1994), HIV (Valsamakis *et al.*, 1992; Gilmartin *et al.*, 1992; Cherrington and Ganem, 1992), ground squirrel hepatitis virus (GHSV) (Cherrington *et al.*, 1992) and the SV40 late poly(A) signal (Schek *et al.*, 1992), consisting of amorphous GU- or U-rich regions. Indeed pyrimidine rich sequences, which are present in the functional regions of many mRNA molecules are emerging as important motifs in a variety of aspects of RNA metabolism (review by Morris *et al.*, 1993), including regulation of translation of mRNA (Shaw and Kamen, 1986; Bohjanen *et al.*, 1991; Vakalopolou *et al.*, 1991), pre-mRNA splicing (Frendeway and Keller, 1985; Ruskin and Greene, 1985), cytoplasmic

degradation and polyadenylation of mRNA (Fox *et al.*, 1992; Vasalli *et al.*, 1989; Paris and Richter, 1990).

The spatial arrangement of these elements is also critical for efficient poly(A) site usage. Increasing the distance between the AAUAAA signal and the downstream element, beyond 40nt, decreases the efficiency of poly(A) site usage (McDevitt *et al.*, 1986; Gil and Proudfoot, 1987). Recently several studies have shown that there is a requirement for secondary structure features in the 3' end formation of certain mRNAs. For example, 3' end formation of the major histone mRNAs requires the presence of a conserved stem-loop structure and a 3' purine rich region for efficient endonucleolytic cleavage (Pandey *et al.*, 1994). In HTLV-1, polyadenylation occurs >250nt 3' to the poly(A) signal, the formation of a stable stem-loop structure from the intervening sequence bringing the AAUAAA and GU-rich element into close proximity, allowing polyadenylation to occur (Seiki *et al.*, 1983). Using synthetic poly(A) site constructs Brown *et al.*, (1991) demonstrated that insertion of sequences, which increase the distance between the AAUAAA and GU-rich element of the poly(A) site, do not inhibit polyadenylation provided they form a stable stem-loop structure which brings the two elements together. The efficiency of poly(A) site usage could therefore be influenced by the presence or absence of essential sequence elements, the spacing of these elements in relation to the AAUAAA hexanucleotide and the overall two dimensional conformation of the poly(A) site RNA.

Components of the protein complexes required for 3' end processing of mRNA have recently been identified (reviewed by Manley, 1988) and complex formation involves the association of

at least nine proteins. The CPSF complex, consisting of four proteins of 160kD, 100kD, 73kD, and 35kD, is thought to bind to the AAUAAA hexanucleotide poly(A) signal (Keller *et al.*, 1991; Wahle and Keller, 1992; Gilmartin and Nevins, 1989), CPSF-RNA binding appears to be stabilised by the subsequent binding of the cleavage stimulation factor (CstF or CF1), a complex of three 77kD, 64kD and 50kD proteins. Binding of CstF appears to be dependent on the hexanucleotide motif and the presence of a downstream sequence element (Wahle and Keller, 1992; Gilmartin and Nevins, 1989; Weiss *et al.*, 1991; Zarkower and Wickens, 1988). UV cross-linking analysis has shown that both the 160kD and 30kD components of the CPSF and the 64kD component of CstF are directly bound to pre-mRNA poly(A) site sequences. The ability of the pre-mRNA to bind these proteins, which is related to the efficiency of complex formation, could therefore be used as an indication of the efficiency of poly(A) site usage.

In the following experiments a comparison of the RNA-protein binding capacities of the six test HSV poly(A) sites, in MI and INF nuclear extracts, was determined using the technique of UV RNA-protein cross-linking. In addition the poly(A) RNA sequences of all HSV-1 genes were examined for the presence of possible GU- or U-rich elements. Finally a study was made of the positioning of the sequence elements of the six test poly(A) sites, within the predicted two dimensional RNA structure.

3.4.2 Examination of the protein binding properties of selected HSV-1 poly(A) sites

(a) Nuclear extracts

Mock infected and HSV infected HeLa cell nuclear extracts were prepared in parallel as described previously. The extracts were dialysed against the binding buffer to be used in the cross-linking assay thus lowering the salt concentration and facilitating binding of protein to RNA. Protein concentrations of the paired extracts were determined by Bradford's assay and normalised before performing the binding assay, adding 10-15μg of protein to each reaction. To allow a valid comparison of the protein-RNA binding patterns of the poly(A) sites, each pair of MI and INF extracts was used in binding reactions with as many of the test poly(A) sites as possble (at least four per extract).

(b) UV cross-linking of RNA-protein complexes

UV irradiation covalently cross-links proteins to nucleic acid at their point of contact, the complexes formed are resistant to heat, detergent and alkali treatments and therefore highly stable (Pelle' and Murphy, 1993).

(c) Protein-RNA complex formation

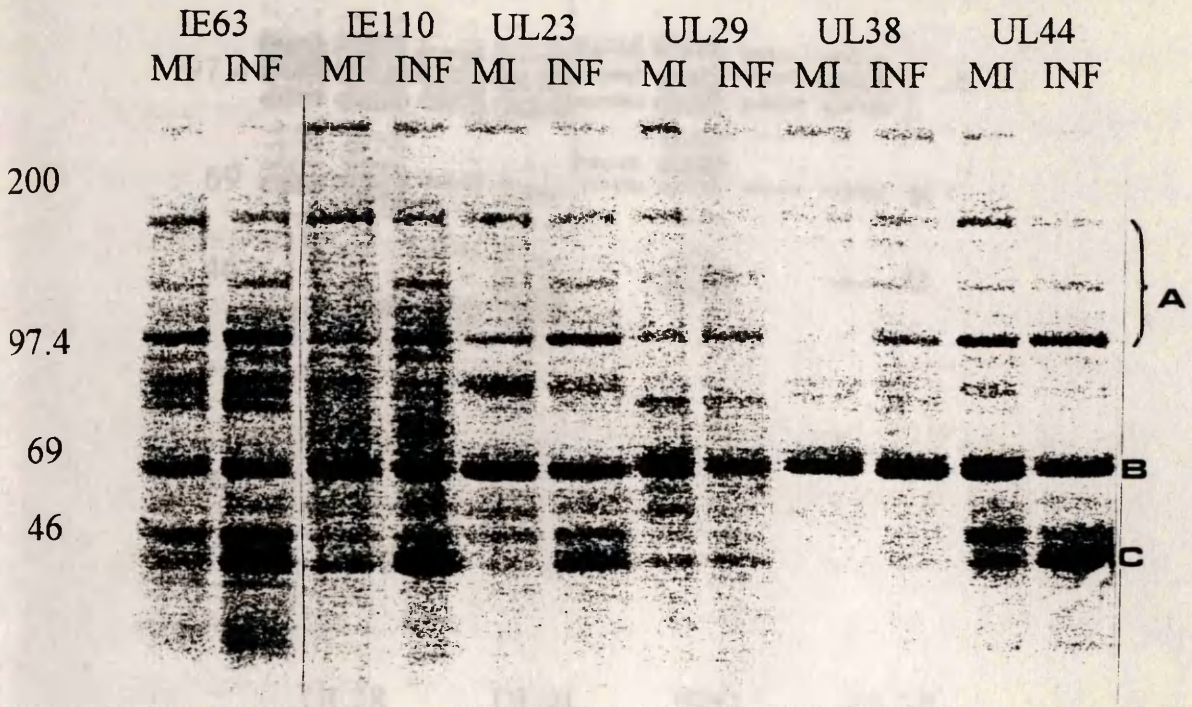
MI and INF nuclear extracts were mixed at RT with radiolabelled precursor mRNA transcribed from the range of pGEM1 plasmids, containing the HSV-1 poly(A) sites, described in Section 3.1. All soluble components required for cleavage and polyadenylation of the precursor mRNAs were supplied in the nuclear extracts. UV irradiation of the mixture covalently cross-linked any proteins closely bound to the RNA and any RNA

unprotected by bound protein was removed by RNaseA digestion. The protein-RNA complexes were separated on denaturing polyacrylamide gels and the protein bands thus radiolabelled with RNA, visualised using a Molecular Dynamics Phosphorimager. The molecular weights of the protein bands were estimated by comparison with protein molecular weight markers.

3.4.3 The protein-RNA binding patterns of selected HSV-1 poly(A) sites

Typical phosphorimages of the protein-RNA complexes produced by UV cross-linking are shown in Figures 3.22, 3.23 and 3.24. Variability of extract activity has already been noted in Section 3.1.5 and was again evident in these studies. However a consistent pattern of protein-RNA binding did emerge from the experiments carried out.

Three groups of proteins were consistently observed on the protein gels. Firstly, a triplet of protein bands (A) of approximately 90-150kD which are most clearly demonstrated in Figure 3.23. Secondly, a protein band (B) of just under 69kD most clearly seen in Figure 3.22, and finally a protein band (C) of just under 46kD most clearly demonstrated in Figure 3.22. Very similar binding patterns were observed for each precursor with a general overall increase in protein-RNA binding noted in the infected nuclear extracts. Different extracts showed this differential binding to a greater or lesser extent but the increased binding was always seen and was particularly apparent in the case of band C the <46kD protein. Since the experiments were standardised as much as possible by the addition of equal concentrations of MI and INF extract proteins to a constant



FIGURES : 3.22, 3.23 and 3.24

Typical phosphorimages obtained from RNA-protein UV cross-linking studies. Proteins present in mock infected (MI) and HSV-1 infected (INF) nuclear extracts were UV cross-linked to the radiolabelled pre-mRNAs of the six test poly(A) sites. The sizes of protein molecular weight markers run alongside the gel are noted on the left of the image. A - triplet of 90-150kD proteins, B - <69kD protein, C- <46kD protein.

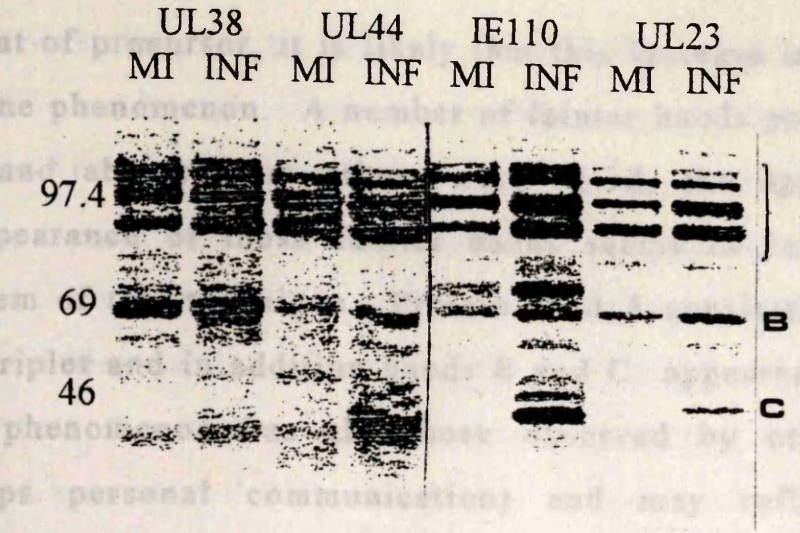


FIGURE : 3.23

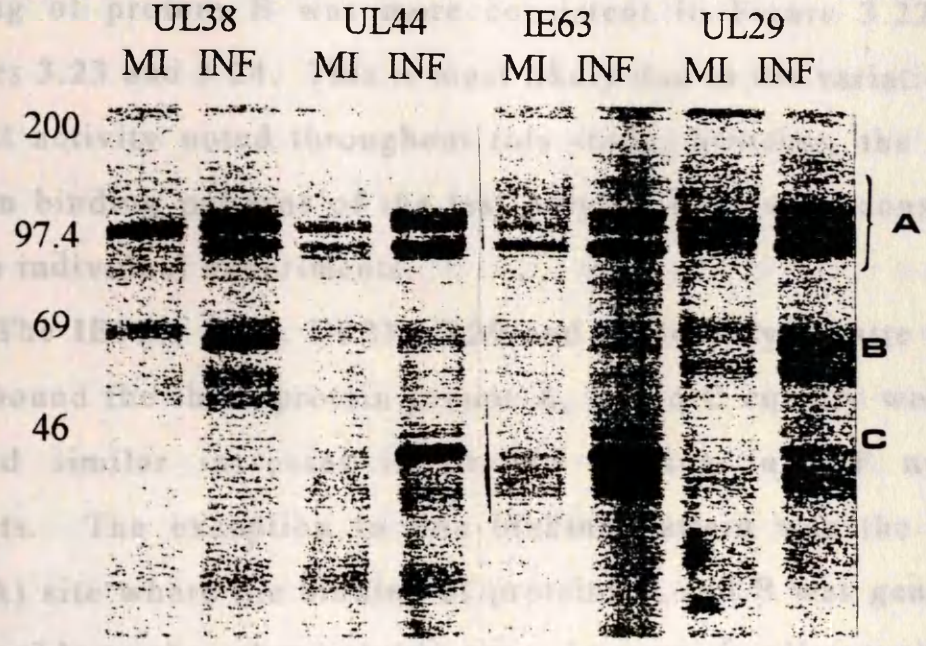


FIGURE : 3.24

amount of precursor, it is likely that this increase in binding is a genuine phenomenon. A number of fainter bands present in some gels and absent from others were noted, the appearance and disappearance of these fainter bands seems to be an accepted problem of this technique. Protein band A consistently appeared as a triplet and in addition bands B and C appeared as doublets. This phenomenon has also been observed by others (Dr. C. Phillips personal communication) and may reflect different phosphorylated or processed forms of the same protein. The RNA binding capacities varied between sets of paired extracts, for example in Figure 3.22 the binding of the protein A triplet was very weak compared to that seen in Figures 2.23 and 3.24, and binding of protein B was more consistent in Figure 3.22 than Figures 3.23 and 3.24. This is most likely due to the variations in extract activity noted throughout this study, however, the RNA-protein binding patterns of the test poly(A) sites were consistent within individual experiments.

The IE110, IE63, UL23 UL29 and UL44 poly(A) site RNAs each bound the three protein groups A, B and C equally well and showed similar increases in protein binding in INF nuclear extracts. The exception to this binding pattern was the UL38 poly(A) site where the binding of proteins A and B was generally comparable to the other poly(A) sites, however in all experiments carried out the binding of protein C was either very weak or absent.

3.4.4 Comparison of HSV-1 poly(A) site sequences

A comparison of the RNA sequences of all HSV poly(A) site regions was carried out using the vax programme Lineup. Each

region was searched by eye for the presence of sequences such as the GU- or U-rich elements described by McLauchlan *et al.*, (1985); Renan, (1987) and Sadofsky *et al.*, (1985), or indeed any GU- or U-rich tracts. The sequences were aligned with respect to the AAUAAA hexanucleotide and were grouped according to the temporal class of the gene to which they belong, Figures 3.25 and 3.26 show the RNA sequences downstream and upstream of the AAUAAA respectively. Included in these alignments are the HSV-2 IE12 and UL38 poly(A) site sequences which form the IE and late poly(A) site constructs from the studies of McLauchlan *et al.*, (1989).

(a) Poly(A) site downstream elements (DSEs)

Examination of the HSV RNA sequences downstream of the poly(A) signals revealed a number of possible GU- or U-rich elements (DSEs). Of the 47 poly(A) site sequences examined all but two contained GU-rich elements, with 19 (40%) of poly(A) sites containing one or more element which conformed to the consensus YGUGUUYY of McLauchlan *et al.*, (1985), and 21 (47%) containing one or more element conforming to the core of the consensus, RRGUUUYRR, identified by Sadofsky *et al.*, (1985) (Figure 3.25 and Table 3.6). The poly(A) site sequences of the UL19 and UL31/32 genes which have no GU-rich element contain instead a U-rich element. Because of the overall nature of the sequences and locations of the GU-elements it would be unwise to place too much emphasis on these consensus sequences. These GU- or U-rich regions are present in a variety of forms either as single elements of approximately 7nt, in tandem with other GU- or U-rich elements or as extended tracts of up to 26nt.

FIGURE 3.25:

Downstream elements. Shows the alignment of the RNA sequences of all known functional HSV-1 poly(A) sites (McGeoch *et al.*, 1988) and the poly(A) sites of the HSV-2 genes IE12 and UL38. Alignments are of the 100nt region downstream of the AAUAAA poly(A) signal hexanucleotide, GU-rich and U-rich regions are underlined and sequences corresponding to the consensus, YGUGUUYY, identified by McLauchlan *et al.*, (1985), are in bold type. The positions downstream of the U of the AAUAAA are given on the top line of the figure. α : IE genes, β : early genes, γ1 : early-late genes, γ2 : true-late genes, UN : class unknown.

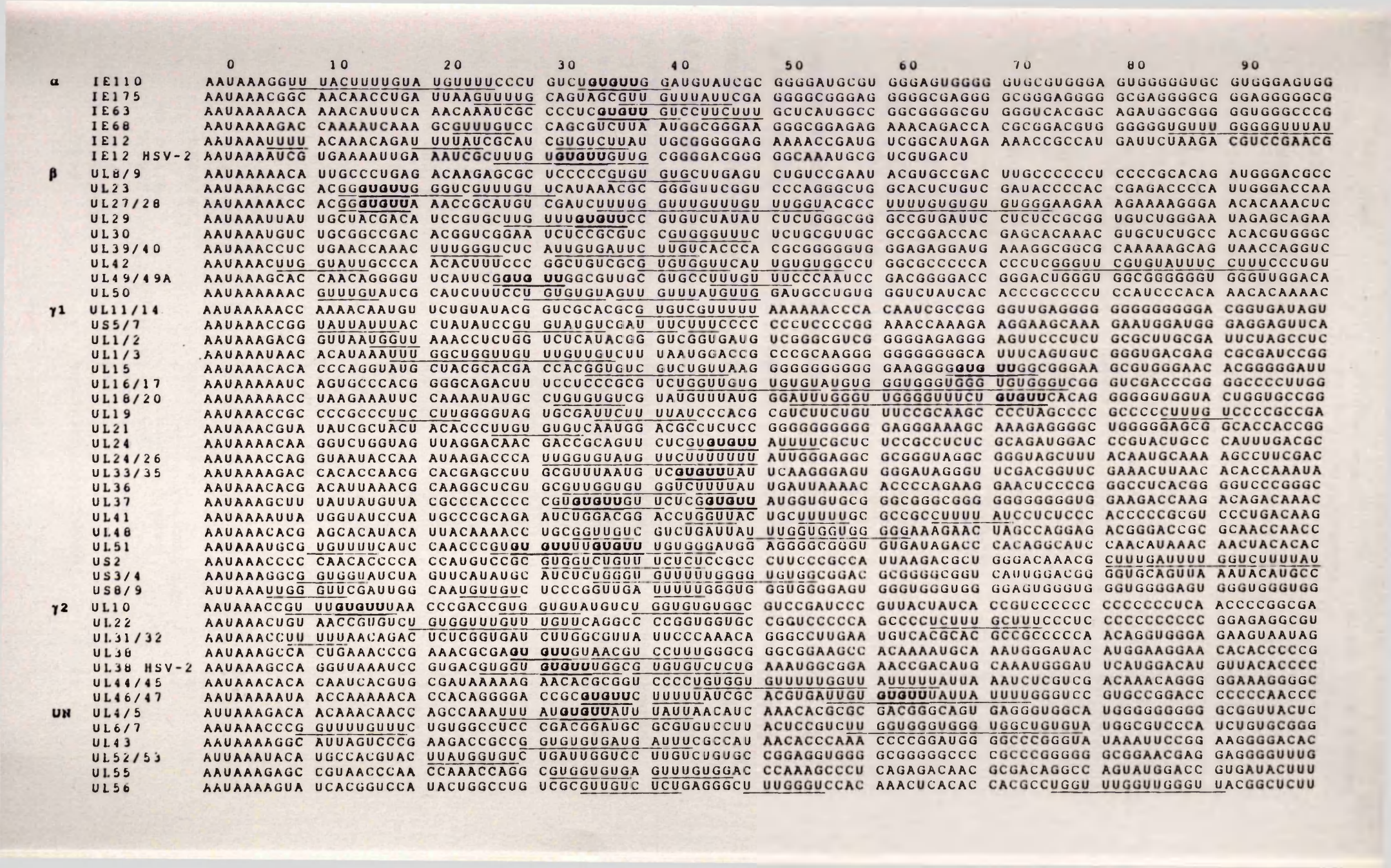

0 10 20 30 40 50 60 70 80 90
α IE110 AAUAAAGGUU UACUUUUGUA UGUUUUCCCU GUCUGUGUUG GAUGUAUCGC GGGGAUGCGU GGGAGUGGGG GUGCGUGGGA GUGGGGGUGC GUGGGAGUGG
IE175 AAUAAACGGC AACAACCUGA UUAAGUUUUG CAGUAGCGUU GUUUAUUCGA GGGGCGGGAG GGGGCGAGGG GCGGGAGGGG GCGAGGGGCG GGAGGGGGCG
IE63 AAUAAAAACA AAACAUUUCA AACAAAUCGC CCCUCGUGUU GUCCUUCUUU GCUCAUGGCC GGCGGGGCGU GGGUCACGGC AGAUGGCGGG GGUGGGCCCG
IE68 AAUAAAAGAC CAAAAUCAAA GCGUUUGUCC CAGCGUCUUA AUGGCGGGAA GGGCGGAGAG AAACAGACCA CGCGGACGUG GGGGGUGUUU GGGGGUUUAU
IE12 AAUAAAUUUU ACAAACAGAU UUUAUCGCAU CGUGUCUUAU UGCGGGGGAG AAAACCGAUG UCGGCAUAGA AAACCGCCAU GAUUCUAAGA CGUCCGAACG
IE12 HSV-2 AAUAAAAUCG UGAAAAUUGA AAUCGCUUUG UGUGUUGUUG CGGGGACGGG GGCAAAUGCG UCGUGACU
β UL8/9 AAUAAAAACA UUGCCCUGAG ACAAGAGCGC UCCCCCGUGU GUGCUUGAGU CUGUCCGAAU ACGUGCCGAC UUGCCCCCCU CCCCGCACAG AUGGGACGCC
UL23 AAUAAAACGC ACGGGUGUUG GGUCGUUUGU UCAUAAACGC GGGGUUCGGU CCCAGGGCUG GCACUCUGUC GAUACCCCAC CGAGACCCCA UUGGGACCAA
UL27/28 AAUAAAAACC ACGGGUGUUA AACCGCAUGU CGAUCUUUUG GUUUGUUUGU UUGGUACGCC UUUUGUGUGU GUGGGAAGAA AGAAAAGGGA ACACAAACUC
UL29 AAUAAAUUAU UGCUACGACA UCCGUGCUUG UUUGUGUUCC GUGUCUAUAU CUCUGGGCGG GCCGUGAUUC CUCUCCGCGG UGUCUGGGAA UAGAGCAGAA
UL30 AAUAAAUGUC UGCGGCCGAC ACGGUCGGAA UCUCCGCGUC CGUGGGUUUC UCUGCGUUGC GCCGGACCAC GAGCACAAAC GUGCUCUGCC ACACGUGGGC
UL39/40 AAUAAACCUC UGAACCAAAC UUUGGGUCUC AUUGUGAUUC UUGUCACCCA CGCGGGGGUG GGAGAGGAUG AAAGGCGGCG CAAAAAGCAG UAACCAGGUC
UL42 AAUAAACUUG GUAUUGCCCA ACACUUUCCC GGCUGUCGCG UGUGGUUCAU UGUGUGGCCU GGCGCCCCCA CCCUCGGGUU CGUGUAUUUC CUUUCCCUGU
UL49/49A AAUAAAGCAC CAACAGGGGU UCAUUCGGUG UUGGCGUUGC GUGCCUUUGU UUCCCAAUCC GACGGGGACC GGGACUGGGU GGCGGGGGGU GGGUUGGACA
UL50 AAUAAAAAAC GUUUGUAUCG CAUCUUUCCU GUGUGUAGUU GUUUAUGUUG GAUGCCUGUG GGUCUAUCAC ACCCGCCCCU CCAUCCCACA AACACAAAAC
γ1 UL11/14 AAUAAAAACC AAAACAAUGU UCUGUAUACG GUCGCACGCG UGUCGUUUUU AAAAAACCCA CAAUCGCCGG GGUUGAGGGG GGGGGGGGGA CGGUGAUAGU
US5/7 AAUAAACCGG UAUUAUUUAC CUAUAUCCGU GUAUGUCGAU UUCUUUCCCC CCCUCCCCGG AAACCAAAGA AGGAAGCAAA GAAUGGAUGG GAGGAGUUCA
UL1/2 AAUAAAGACG GUUAAUGGUU AAACCUCUGG UCUCAUACGG GUCGGUGAUG UCGGGCGUCG GGGGAGAGGG AGUUCCCUCU GCGCUUGCGA UUCUAGCCUC
UL1/3 AAUAAAUAAC ACAUAAAUUU GGCUGGUUGU UUGUUGUCUU UAAUGGACCG CCCGCAAGGG GGGGGGGGCA UUUCAGUGUC GGGUGACGAG CGCGAUCCGG
UL15 AAUAAACACA CCCAGGUAUG CUACGCACGA CCACGGUGUC GUCUGUUAAG GGGGGGGGGG GAAGGGGGUG UUGGCGGGAA GCGUGGGAAC ACGGGGGAUU
UL16/17 AAUAAAAAUC AGUGCCCACG GGGCAGACUU UCCUCCCGCG UCUGGUUGUG UGUGUAUGUG GGUGGGUGGG UGUGGGUCGG GUCGACCCGG GGCCCCUUGG
UL18/20 AAUAAAAACC UAAGAAAUUC CAAAAUAUGC CUGUGUGUCG UAUGUUUAUG GGAUUUGGGU UGGGGUUUCU GUGUUCACAG GGGGGUGGUA CUGGUGCCGG
UL19 AAUAAACCGC CCCGCCCUUC CUUGGGGUAG UGCGAUUCUU UUAUCCCACG CGUCUUCUGU UUCCGCAAGC CCCUAGCCCC GCCCCCUUUG UCCCCGCCGA
UL21 AAUAAACGUA UAUCGCUACU ACACCCUUGU GUGUCAAUGG ACGCCUCUCC GGGGGGGGGG GAGGGAAAGC AAAGAGGGGC UGGGGGAGCG GCACCACCGG
UL24 AAUAAAACAA GGUCUGGUAG UUAGGACAAC GACCGCAGUU CUCGUGUGUU AUUUUCGCUC UCCGCCUCUC GCAGAUGGAC CCGUACUGCC CAUUUGACGC
UL24/26 AAUAAACCAG GUAAUACCAA AUAAGACCCA UUGGUGUAUG UUCUUUUUUU AUUGGGAGGC GCGGGUAGCC GGGUAGCUUU ACAAUGCAAA AGCCUUCGAC
UL33/35 AAUAAAAGAC CACACCAACG CACGAGCCUU GCGUUUAAUG UCGUGUUUAU UCAAGGGAGU GGGAUAGGGU UCGACGGUUC GAAACUUAAC ACACCAAAUA
UL36 AAUAAACACG ACAUUAAACG CAAGGCUCGU GCGUUGGUGU GGUCUUUUAU UGAUUAAAAC ACCCCAGAAG GAACUCCCCG GGCCUCACGG GGUCCCGGGC
UL37 AAUAAAGCUU UAUUAUGUUA CGCCCACCCC CGUGUGUUGU UCUCGGUGUU AUGGUGUGCG GGCGGGCGGG GGGGGGGGUG GAAGACCAAG ACAGACAAAC
UL41 AAUAAAAUUA UGGUAUCCUA UGCCCGCAGA AUCUGGACGG ACCUGGUUAC UGCUUUUUGC GCCGCCUUUU AUCCUCUCCC ACCCCCGCGU CCCUGACAAG
UL48 AAUAAACACG AGCACAUACA UUACAAAACC UGCGGUUGUC GUCUGAUUAU UUGGUGGUGG GGGAAAGAAC UAGCCAGGAG ACGGGACCGC GCAACCAACC
UL51 AAUAAAUGCG UGUUUUCAUC CAACCCGUGU GUUUUGUGUU UGUGGGAUGG AGGGGCGGGU GUGAUAGACC CACAGGCAUC CAACAUAAAC AACUACACAC
US2 AAUAAACCCC CAACACCCCA CCAUGUCCGC GUGGUCUGUU UCUCUCCGCC CUUCCCGCCA UUAAGACGCU GGGACAAACG CUUUGAUUUU GGUCUUUUAU
US3/4 AAUAAAGGCG GUGGUAUCUA GUUCAUAUGC AUCUCUGGGU GUUUUUGGGG UGUGGCGGAC GCGGGGCGGU CAUUGGACGG GGUGCAGUUA AAUACAUGCC
US8/9 AUUAAAUUGG GUUCGAUUGG CAAUGUUGUC UCCCGGUUGA UUUUUGGGUG GGUGGGGAGU GGGUGGGUGG GGAGUGGGUG GGUGGGGAGU GGGUGGGUGG
γ2 UL10 AAUAAACCGU UUGUGUUUAA CCCGACCGUG GUGUAUGUCU GGUGUGUGGC GUCCGAUCCC GUUACUAUCA CCGUCCCCCC CCCCCCCUCA ACCCCGGCGA
UL22 AAUAAACUGU AACCGUGUCU GUGGUUUGUU UGUUCAGGCC CCGGUGGUGC CGGUCCCCCA GCCCCUCUUU GCUUUCCCUC CCCCCCCCCC GGAGAGGCGU
UL31/32 AAUAAACCUU UUUAACAGAC UCUCGGUGAU CUUGGCGUUA UUCCCAAACA GGGCCUUGAA UGUCACGCAC GCCGCCCCCA ACAGGUGGGA GAAGUAAUAG
UL30 AAUAAAGCCA CUGAAACCCG AAACGCGAGU GUUGUAACGU CCUUUGGGCG GGCGGAAGCC ACAAAAUGCA AAUGGGAUAC AUGGAAGGAA CACACCCCCG
UL38 HSV-2 AAUAAAGCCA GGUUAAAUCC GUGACGUGGU GUGUUUGGCG UGUGUCUCUG AAAUGGCGGA AACCGACAUG CAAAUGGGAU UCAUGGACAU GUUACACCCC
UL44/45 AAUAAACACA CAAUCACGUG CGAUAAAAAG AACACGCGGU CCCCUGUGGU GUUUUUGGUU AUUUUUAUUA AAUCUCGUCG ACAAACAGGG GGAAAGGGGC
UL46/47 AAUAAAAAUA ACCAAAAACA CCACAGGGGA CCGCGUGUUC UUUUUAUCGC ACGUGAUUGU GUGUUUAUUA UUUUGGGUCC GUGCCGGACC CCCCCAACCC
UN UL4/5 AUUAAAGACA ACAAACAACC AGCCAAAUUU AUGUGUUAUU UAUUAACAUC AAACACGCGC GACGGGCAGU GAGGGUGGCA UGGGGGGGGG GCGGUUACUC
UL6/7 AAUAAACCCG GUUUUGUUUC UGUGGCCUCC CGACGGAUGC GCGUGUCCUU ACUCCGUCUU GGUGGGUGGG UGGCGUGCCA UCUGUGCGGG
UL43 AAUAAAAGGC AUUAGUCCCG AAGACCGCCG GUGUGUGAUG AUUUCGCCAU AACACCCAAA CCCCGGAUGG GGCCCGGGUA UAAAUUCCGG AAGGGGACAC
UL52/53 AUUAAAUACA UGCCACGUAC UUAUGGUGUC UGAUUGGUCC UUGUCUGUGC CGGAGGUGGG GCGGGGGCCC CGCCCGGGGG GCGGAACGAG GAGGGGUUUG
UL55 AAUAAAGAGC CGUAACCCAA CCAAACCAGG CGUGGUGUGA GUUUGUGGAC CCAAAGCCCU CAGAGACAAC GCGACAGGCC AGUAUGGACC GUGAUACUUU
UL56 AAUAAAAGUA UCACGGUCCA UACUGGCCUG UCGCGUUGUC UCUGAGGGCU UUGGGUCCAC AAACUCACAC CACGCCUGGU UUGGUUGGGU UACGGCUCUU

Table 3.6 :

Shows the sequence composition of the GU- and U-rich downstream elements identified within the poly(A) sites of all known HSV-1 genes (McGeoch *et al.*, 1988) and the HSV-2 poly(A) sites of IE12 and UL38. Showing the idstance (in nt) of the most 5' nucleotide of each region from the U of the AAUAAA hexanucleotide. Sequences corresponding to the consensus YGUGUUYY of McLauchlan *et al.*, (1985) are underlined.

	HSV gene	GU/U-element	dist.3'of AAUAAA
α	E110	UUUACUUUUUGUAUGUUUU	6
		UGUGUUGG	31
	IE175	GUUUUG	22
		GUUGUUUA	35
	IE63	GUGUUGUCCUUCUUU	33
	IE68	GUUUGU	21
		GUGUUUGGGGGUUU	82
	IE12	UUUU	4
		UUUUAU	17
		GUGUCUU	29
	IE12 HSV-2	UUUGUGUGUUGUU	24
β	UL8/9	GUGUGUG	34
	UL23	GGGUGUUGGGUCGUUUGUU	11
	UL27/28	GGGGUGUU	10
		UUUUGGUUUGUUUGUUUGGU	33
		UUUUGUGUGUGUGGG	57
	UL29	UUGUUUGUGUU	25
	UL30	GUGGGUUU	39
	UL39/40	UUUGGGU	18
		UUGUAUUCUUGU	29
	UL42	UUGUAUUG	5
		GUGUGGUUCAUUGUGUGG	37
		GGGUUCGUGUAUUUCCUUU	73
	UL49/49A	GGUGUUGG	25
		UUUGUUU	43
	UL50	GUUUGU	8
		UGUGUGUAGUUGUUUAUGUUGG	27
γ1	UL11/14	GUGUCGUUUUU	37
	US5/7	UAUUAUUU	8
		GUGUAUGU	26
		UUUCUUU	37
	UL1/2	UGGUU	13
		GGUGAUG	41
	UL1/3	UUUGGCUGGUUGUUGUUG	15
	UL15	GGUGUCGUCUGUU	33
		GUGUUG	65
	UL16/17	UGGUUGUGUGGUAUGUGCGUGGGUGG	40
	UL18/20	UGUGUGU	30
		UUUGGGUUGGGUUUCUGUGUU	51
	UL19	UUCUUUAU	33
	UL21	UUGUGUGU	24
	UL24	GUGUGUUAUUUU	41
	UL24/25	UUGGUGUAUGUUCUUUUUUUAUU	28
	UL33/35	GUGUUUAUU	41
	UL36	GUUGGUGUGUUGGUCUUUUAUU	30
	UL37	GUGUGUUGUUCUCGGUGUU	29
	UL41	UGGUU	41
		CUUUUUG	51
		CUUUUAU	63
	UL48	GGUUGU	31
		UUUGGUGGUGGUGGGGG	47
	UL51	GUGUUUU	7
		GUGUGUUUUGUGUUUGUGGG	24
	US2	GUGGUCUGUUUCUCU	28
		UUUGAUUUUGGUCUUUU	79
	US3/4	GGUGGU	7
		UGGGUGUUUUUGGGGUGU	33
	US8/9	UUGGGUU	4
		UGUUGU	21
		AUUUUUG	38
γ2	UL10	GUUUGUGUUU	6
		GUGGUGU	25
		UGGUGUGUGG	37
	UL22	UGUGGUUUGUUUGUU	15
		UUUGCUUU	65
	UL31/32	UUUUU	6
	UL38	GUGUUGUAACGUCCUUU	26
	UL38 HSV-2	GUGGUGUGUUUGGCGUGUGUCUCUG	23
	UL44/45	UGUGGUGUUUUUUGUUUAUUUUU	42
	UL46/47	GUGUUCUUUUU	32
		UUGUGUGUUUAUUAUUUUGGGU	54
un	UL4/5	UUUAUGUGUUAUUUAUU	25
	UL6/7	GGUUUUGUUU	7
		UUGGUGGGUGGGUGGCUGUGU	56
	UL43	GGUGUGUG	27
	UL52/53	UUAUGGUGU	18
	UL55	GUGGUGUGAGUUUUGUGG	29

The distance from the U of the hexanucleotide to the first nt of the nearest downstream element varies considerably, ranging from 6-42nt, with the majority of elements falling within the 20-35nt group (Table 3.6). Two of the test poly(A) sites fall outwith this group, the extensive U-rich element of the efficient IE110 poly(A) site lying only 6nt 3' of the AAUAAA and the extensive GU element of the less efficient UL44 poly(A) site lying 42nt 3' of the AAUAAA. There is however no definitive pattern either in the nt composition of the element or the distance of the element from the AAUAAA among the poly(A) sites studied. The general summary of the data shown in Table 3.6 indicates that each of the poly(A) sites contains a GU- or U-rich element approximately 20-35nt 5' of the hexanucleotide and further no relationship between poly(A) sites from the same temporal class of gene is apparent.

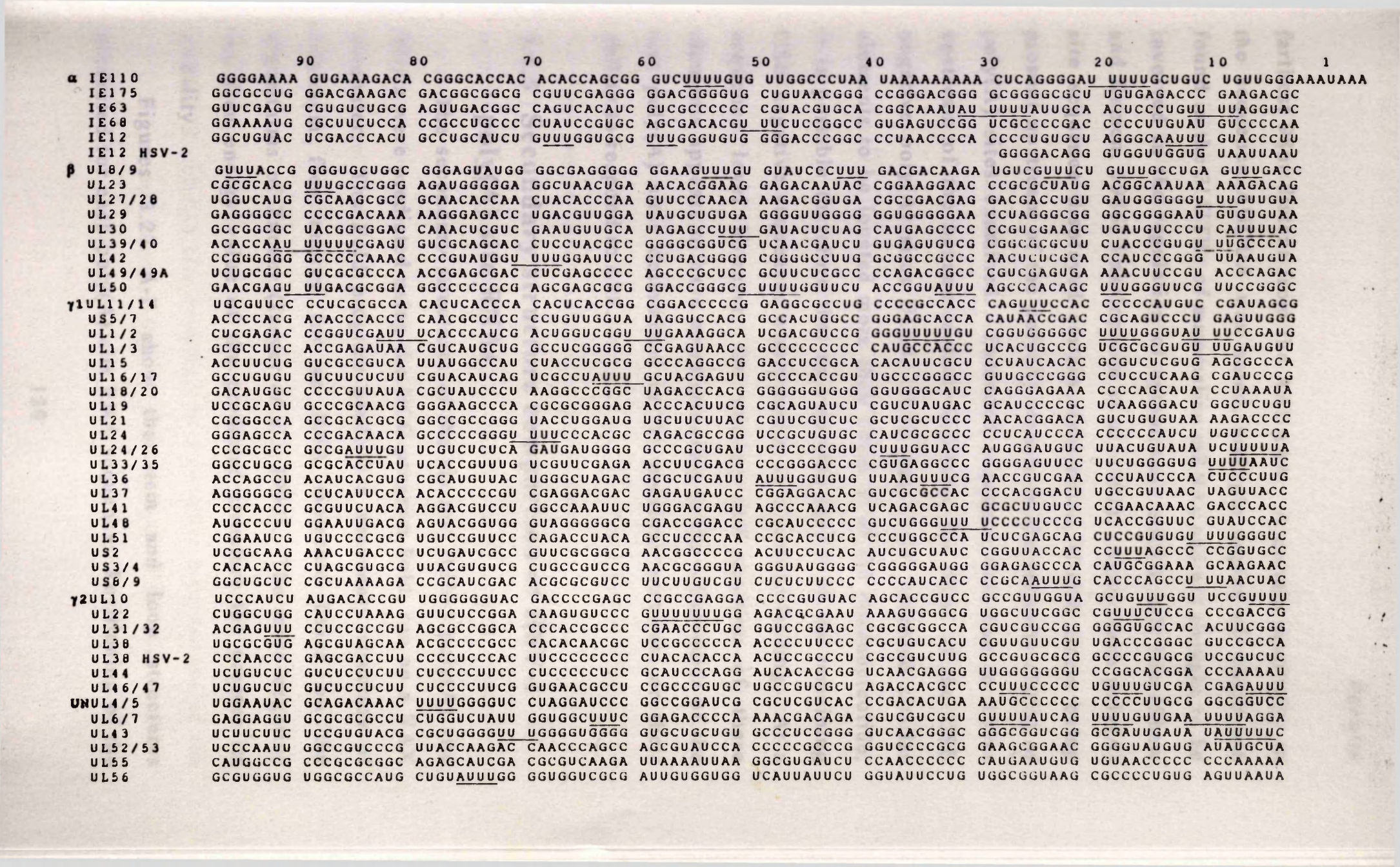
The point mutation which occurred during the construction of the UL23 poly(A) site pGEM1 plasmid is located within a U-rich tract 111nt downstream of the AAUAAA (data not shown). These extended U-rich tracts were observed in a minority of poly(A) sites of all temporal classes, the variable nature of these tracts and the position of the mutation make it unlikely that the G for U substitution would affect processing of the RNA.

(b) Poly(A) site upstream elements (USEs)

A similar examination of the upstream regions of the HSV poly(A) sites revealed the presence of a number of U- rich elements (Figure 3.26). Each element consisting of a tract of 3-4 Us is present either singly or in multiples of 3-4 copies. The distance of the most 3' nt of these elements from the U of the hexanucleotide varied widely, the closest located 3nt and the

FIGURE 3.26 :

Upstream el ements. Alignment of the RNA sequences of all known HSV-1 poly(A) sites (McGeoch *et al.*, 1988), and the poly(A) sites of the HSV-2 genes IE12 and UL38. Alignments are of the 100nt region upstream of the AAUAAA hexanucleotide poly(A) signal, U-rich regions are underlined. The position in nucleotides upstream of the U of the AAUAAA sequence is given on the top line of the figure. α : IE genes, β : early genes, γ1 : early-late genes. γ2 : true-late genes, UN : class unknown.


90 80 70 60 50 40 30 20 10 1
α IE110 GGGGAAAA GUGAAAGACA CGGGCACCAC ACACCAGCGG GUCUUUUGUG UUGGCCCUAA UAAAAAAAAA CUCAGGGGAU UUUUGCUGUC UGUUGGGAAAUAAA
IE175 GGCGCCUG GGACGAAGAC GACGGCGGCG CGUUCGAGGG GGACGGGGUG CUGUAACGGG CGGGGCGCGU UGUGAGACCC GAAGACGC
IE63 GUUCGAGU CGUGUCUGCG AGUUGACGGC CAGUCACAUC GUCGCCCCCC CGUACGUGCA CGGCAAAUAU UUUUAUUGCA ACUCCCUGUU UUAGGUAC
IE68 GGAAAAUG CGCUUCUCCA CCGCCUGCCC CUAUCCGUGC AGCGACACGU UUCUCCGGCC GUGAGUCCGG UCGCCCCGAC CCCCUUGUAU GUCCCCAA
IE12 GGCUGUAC UCGACCCACU GCCUGCAUCU GUUUGGUGCG UUUGGGUGUG GGGACCCGGC CCUAACCCCA CCCCUGUGCU AGGGCAAUUU GUACCCUU
IE12 HSV-2 GGGGACAGG GUGGUUGGUG UAAUUAAU
β UL8/9 GUUUACCG GGGUGCUGGC GGGAGUAUGG GGCGAGGGGG GGAAGUUUGU GUAUCCCUUU GACGACAAGA UGUCGUUUCU GUUUGCCUGA GUUUGACC
UL23 CGCGCACG UUUGCCCGGG AGAUGGGGGA GGCUAACUGA AACACGGAAG GAGACAAUAC CGGAAGGAAC CCGCGCUAUG ACGGCAAUAA AAAGACAG
UL27/28 UGGUCAUG CGCAAGCGCC GCAACACCAA CUACACCCAA GUUCCCAACA AAGACGGUGA CGCCGACGAG GACGACCUGU GAUGGGGGGU UUGUUGUA
UL29 GAGGGGCC CCCCGACAAA AAGGGAGACC UGACGUUGGA UAUGCUGUGA GGGGUUGGGG GGUGGGGGAA CCUAGGGCGG GGCGGGGAAU GUGUGUAA
UL30 GCCGGCGC UACGGCGGAC CAAACUCGUC GAAUGUUGCA UAGAGCCUUU GAUACUCUAG CAUGAGCCCC CCGUCGAAGC UGAUGUCCCU CAUUUUAC
UL39/40 ACACCAAU UUUUUCGAGU GUCGCAGCAC CUCCUACGCC GGGGCGGUCG UCAACGAUCU GUGAGUGUCG CGGCGCGCUU CUACCCGUGU UUGCCCAU
UL42 CCGGGGGG GCCCCCAAAC CCCGUAUGGU UUUGGAUUCC CCUGACGGGG CGGGGCCUUG GCGGCCGCCC AACUCUCGCA CCAUCCCGGG UUAAUGUA
UL49/49A UCUGCGGC GUCGCGCCCA ACCGAGCGAC CUCGAGCCCC AGCCCGCUCC GCUUCUCGCC CCAGACGGCC CGUCGAGUGA AAACUUCCGU ACCCAGAC
UL50 GAACGAGU UUGACGCGGA GGCCCCCCCG AGCGAGCGCG GGACCGGGCG UUUUGGUUCU ACCGGUAUUU AGCCCACAGC UUUGGGUUCG UUCCGGGC
γ1UL11/14 UGCGUUCC CCUCGCGCCA CACUCACCCA CACUCACCGG CGGACCCCCG GAGGCGCCUG CCCCGCCACC CAGUUUCCAC CCCCCAUGUC CGAUAGCG
US5/7 ACCCCACG ACACCCACCC CAACGCCUCC CCUGUUGGUA UAGGUCCACG GCCACUGGCC GGGAGCACCA CAUAACCGAC CGCAGUCCCU GAGUUGGG
UL1/2 CUCGAGAC CCGGUCGAUU UCACCCAUCG ACUGGUCGGU UUGAAAGGCA UCGACGUCCG GGGUUUUUGU CGGUGGGGGC UUUUGGGUAU UUCCGAUG
UL1/3 GCGCCUCC ACCGAGAUAA CGUCAUGCUG GCCUCGGGGG CCGAGUAACC GCCCCCCCCC CAUGCCACCC UCACUGCCCG UCGCGCGUGU UUGAUGUU
UL15 ACCUUCUG GUCGCCGUCA UUAUGGCCAU CUACCUCGCG GCCCAGGCCG GACCUCCGCA CACAUUCGCU CCUAUCACAC GCGUCUCGUG AGCGCCCA
UL16/17 GCCUGUGU GUCUUCUCUU CGUACAUCAG UCGCCUAUUU GCUACGAGUU GCCCCACCGU UGCCCGGGCC GUUGCCCGGG CCUCCUCAAG CGAUCCCG
UL18/20 GACAUGGC CCCCGUUAUA CGCUAUCCCU AAGGCCCGGC UAGACCCACG GGGGGGUGGG GGUGGGCAUC CAGGGAGAAA CCCCAGCAUA CCUAAAUA
UL19 UCCGCAGU GCCCGCAACG GGGAAGCCCA CGCGCGGGAG ACCCACUUCG CGCAGUAUCU CGUCUAUGAC GCAUCCCCGC UCAAGGGACU GGCUCUGU
UL21 CGCGGCCA GCCGCACGCG GGCCGCCGGG UACCUGGAUG UGCUUCUUAC CGUUCGUCUC GCUCGCUCCC AACACGGACA GUCUGUGUAA AAGACCCC
UL24 GGGAGCCA CCCGACAACA GCCCCCGGGU UUUCCCACGC CAGACGCCGG UCCGCUGUGC CAUCGCGCCC CCUCAUCCCA CCCCCCAUCU UGUCCCCA
UL24/26 CCCGCGCC GCCGAUUUGU UCGUCUCUCA GAUGAUGGGG GCCCGCUGAU UCGCCCCGGU CUUUGGUACC AUGGGAUGUC UUACUGUAUA UCUUUUUA
UL33/35 GGCCUGCG GCGCACCUAU UCACCGUUUG UCGUUCGAGA ACCUUCGACG CCCGGGACCC CGUGAGGCCC GGGGAGUUCC UUCUGGGGUG UUUUAAUC
UL36 ACCAGCCU ACAUCACGUG CGCAUGUUAC UGGGCUAGAC GCGCUCGAUU AUUUGGUGUG UUAAGUUUCG AACCGUCGAA CCCUAUCCCA CUCCCUUG
UL37 AGGGGGCG CCUCAUUCCA ACACCCCCGU CGAGGACGAC GAGAUGAUCC CGGAGGACAC GUCGCGCCAC CCCACGGACU UGCCGUUAAC UAGUUACC
UL41 CCCCACCC GCGUUCUACA AGGACGUCCU GGCCAAAUUC UGGGACGAGU AGCCCAAACG UCAGACGAGC GCGCUUGUCC CCGAACAAAC GACCCACC
UL48 AUGCCCUU GGAAUUGACG AGUACGGUGG GUAGGGGGCG CGACCGGACC CGCAUCCCCC GUCUGGGUUU UCCCCUCCCG UCACCGGUUC GUAUCCAC
UL51 CGGAAUCG UGUCCCCGCG UGUCCGUUCC CAGACCUACA GCCUCCCCAA CCGCACCUCG CCCUGGCCCA UCUCGAGCAG CUCCGUGUGU UUUGGGUC
US2 UCCGCAAG AAACUGACCC UCUGAUCGCC GUUCGCGGCG AACGGCCCCG ACUUCCUCAC AUCUGCUAUC CGGUUACCAC CCUUUAGCCC CCGGUGCC
US3/4 CACACACC CUAGCGUGCG UUACGUGUCG CUGCCGUCCG AACGCGGGUA GGGUAUGGGG CGGGGGAUGG GGAGAGCCCA CAUGCGGAAA GCAAGAAC
US8/9 GGCUGCUC CGCUAAAAGA CCGCAUCGAC ACGCGCGUCC UUCUUGUCGU CUCUCUUCCC CCCCAUCACC CCGCAAUUUG CACCCAGCCU UUAACUAC
γ2UL10 UCCCAUCU AUGACACCGU UGGGGGGUAC GACCCCGAGC CCGCCGAGGA CCCCGUGUAC AGCACCGUCC GCCGUUGGUA GCUGUUUGGU UCCGUUUU
UL22 CUGGCUGG CAUCCUAAAG GUUCUCCGGA CAAGUGUCCC GUUUUUUUGG AGACGCGAAU AAAGUGGGCG UGGCUUCGGC CGUUUCUCCG CCCGACCG
UL31/32 ACGAGUUU CCUCCGCCGU AGCGCCGGCA CCCACCGCCC CGAACCCUGC GGUCCGGAGC CGCGCGGCCA CGUCGUCCGG GGGGUGCCAC ACUUCGGG
UL38 UGCGCGUG AGCGUAGCAA ACGCCCCGCC CACACAACGC UCCGCCCCCA ACCCCUUCCC CGCUGUCACU CGUUGUUCGU UGACCCGGGC GUCCGCCA
UL38 HSV-2 CCCAACCC GAGCGACCUU CCCCUCCCAC UUCCCCCCCC CUACACACCA ACUCCGCCCU CGCCGUCUUG GCCGUGCGCG GCCCCGUGCG UCCGUCUC
UL44 UCUGUCUC GUCUCCUCUU CUCCCCUUCC CUCCCCCUCC GCAUCCCAGG AUCACACCGG UCAACGAGGG UUGGGGGGGU CCGGCACGGA CCCAAAAU
UL46/47 UCUGUCUC GUCUCCUCUU CUCCCCUUCG GUGAACGCCU CCGCCCGUGC UGCCGUCGCU AGACCACGCC CCUUUCCCCC UGUUUGUCGA CGAGAUUU
UNUL4/5 UGGAAUAC GCAGACAAAC UUUUGGGGUC CUAGGAUCCC GGCCGGAUCG CGCUCGUCAC CCGACACUGA AAUGCCCCCC CCCCCUUGCG GGCGGUCC
UL6/7 GAGGAGGU GCGCGCGCCU CUGGUCUAUU GGUGGCUUUC GGAGACCCCA AAACGACAGA CGUCGUCGCU GUUUUAUCAG UUUUGUUGAA UUUUAGGA
UL43 UCUUCUUC UCCGUGUACG CGCUGGGGUU UGGGGUGGGG GUGCUGCUGU GCCCUCCGGG GUCAACGGGC GGGCGGUCGG GCGAUUGAUA UAUUUUUC
UL52/53 UCCCAAUU GGCCGUCCCG UUACCAAGAC CAACCCAGCC AGCGUAUCCA CCCCCGCCCG GGUCCCCGCG GAAGCGGAAC GGGGUAUGUG AUAUGCUA
UL55 CAUGGCCG CCCGCGCGGC AGAGCAUCGA CGCGUCAAGA UUAAAAUUAA GGCGUGAUCU CCAACCCCCC CAUGAAUGUG UGUAACCCCC CCCAAAAA
UL56 GCGUGGUG UGGCGCCAUG CUGUAUUUGG GGUGGUCGCG AUUGUGGUGG UCAUUAUUCU GGUAUUCCUG UGGCGGUAAG CGCCCCUGUG AGUUAAUA

farthest 98nt distant from the AAUAAA. A certain similarity to the sequence AUUUA was noted, multiple copies of which are found in the 3' UTRs of mammalian mRNAs and are thought to be involved in the regulation of cytoplasmic mRNA half life (Shaw and Kamen, 1986; Vakalopolou *et al.*,1991). Again each poly(A) site sequence displayed certain similarities, U-rich tracts being present upstream of the poly(A) signals in the majority (70%) of poly(A) sites examined. Extended sequence data (up to 150nt upstream of the hexanucleotide) available for three of the USE negative poly(A) sites UL29, UL38 and UL44, revealed similar elements to be located more than 100nt 5' of the hexanucleotide, it is possible therefore that an extended search of the remaining USE negative sequences would result in a similar finding. At a sequence level however, the number and position of these elements appears to have no relationship to the efficiencies of the test poly(A) sites determined in the previous sections, or their ability to respond to LPF.

3.4.5 Secondary structure comparison of HSV-1 poly(A) site RNAs

The secondary structures of the six test HSV-1 poly(A) site RNAs were predicted using the GCG program Fold, run on a VAX computer. The Fold program predicts a secondary structure of minimum free energy for an RNA molecule based on the algorithims of Zucker (Jaeger *et al.*, 1990). The more negative the free energy value of the predicted structure the greater its stability.

Figures 3.27 A-E show the stem and loop structures predicted for each poly(A) site RNA sequence. Positioning of the

poly(A) signal and the DSEs within the two dimensional structure was examined. In five of the six poly(A) site RNAs (UL38, UL23, UL29, IE110, UL44) the poly(A) signal is found on a loop or bulge of RNA, this correlates well with the studies of Woychik, *et al.*, (1984) which identified a similar structure for the poly(A) site of the bovine growth hormone. Examination of the RNA secondary structures of a selection of HSV poly(A) sites (22 in total), revealed that in all but one site the poly(A) signal was located on a loop or bulge of RNA (data not shown). The length of the stems supporting these loops are variable, ranging from the short four base paired stem of UL38 to the long forty-four base paired stem of IE110. The exception to this is the two dimensional structure of the IE63 poly(A) site RNA where the poly(A) signal is located on the stem of the stem loop structure. However it must be emphasized that these two dimensional structures are only predictions of the possible conformations of the RNA and do not take into account the effect proteins bound to the RNA would have on the overall structure.

The downstream sequence elements described above (GU- and U-rich DSEs) were found either on the same stem-loop structure as the poly(A) signal (IE110, UL23, UL44) or on an adjacent stem structure (IE63, UL38, UL29).

Some unique features were noted in the predicted structures of the UL38 and UL44 poly(A) sites : the UL44 poly(A) signal was located on the opposite side of the stem structure overlapping the GU-rich DSE, and the predicted two dimensional structure of the UL38 poly(A) site appeared as a complex arrangement of ten stem loops, compared to the 5-6 stem loop structures of the other poly(A) sites. The possible relationship of these observations to

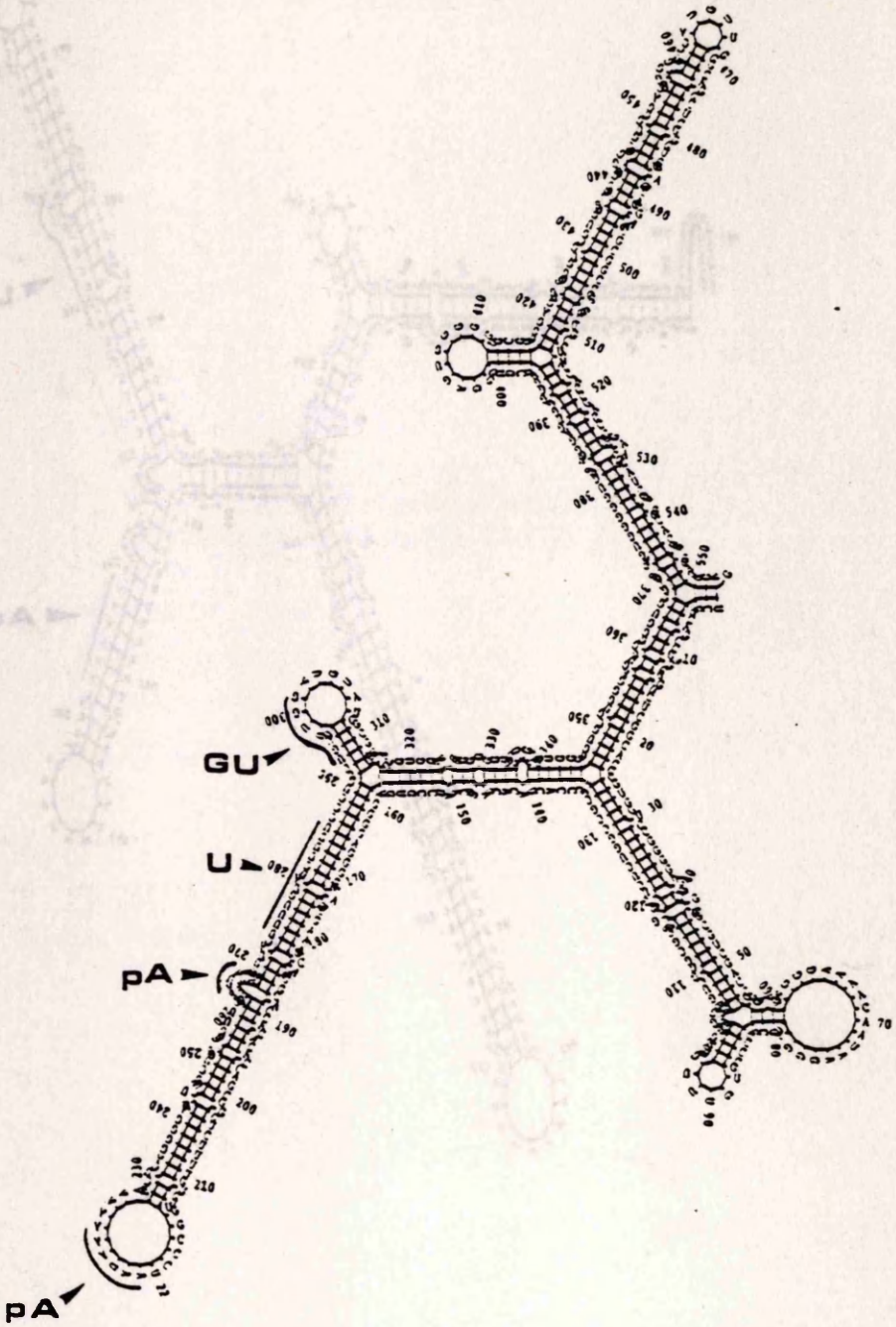


FIGURE 3.27 A:

The predicted secondary structure of the IE110 poly(A) site RNA, predicted using the VAX Fold program.

pA : poly(A) signal AAUAAA. GU: GU-rich downstream element. U : U-rich downstream elements.

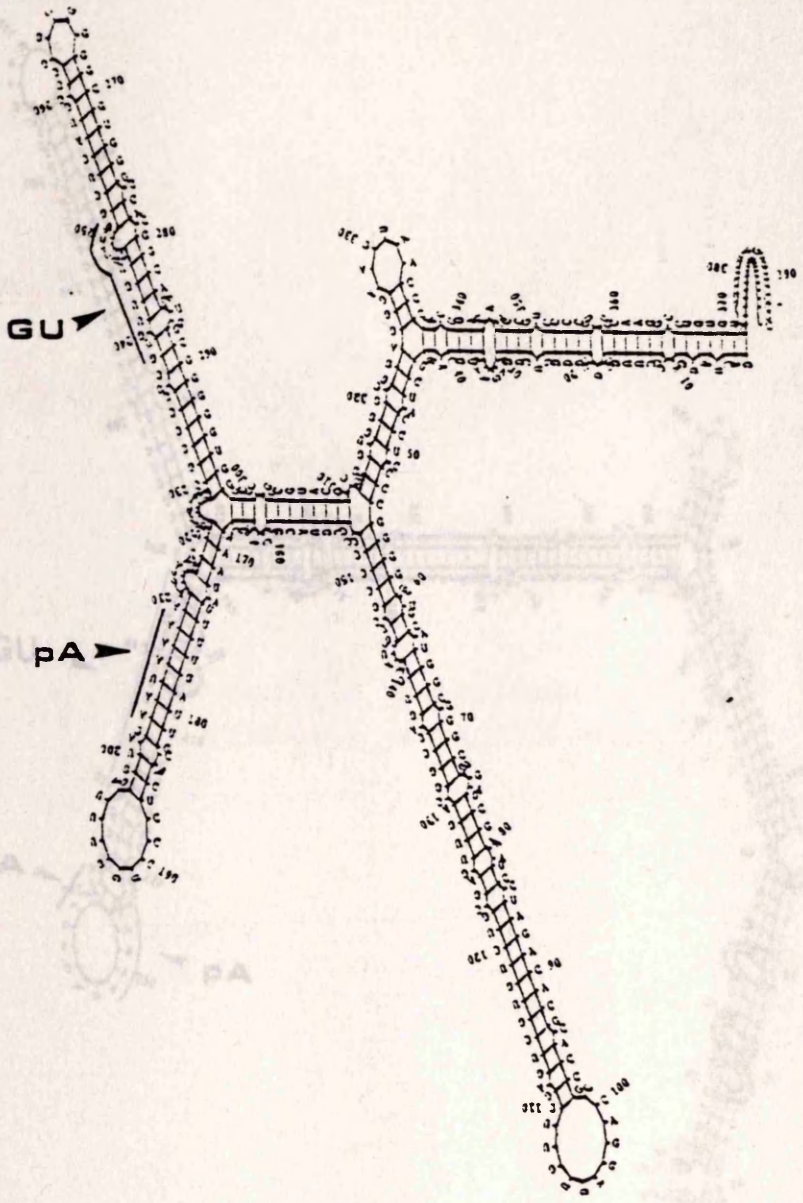


FIGURE 3.27 B:

The predicted secondary structure of the IE63 poly(A) site RNA, predicted using the VAX Fold program.

pA : poly(A) signal AAUAAA. GU: GU-rich downstream element. U : U-rich downstream elements.

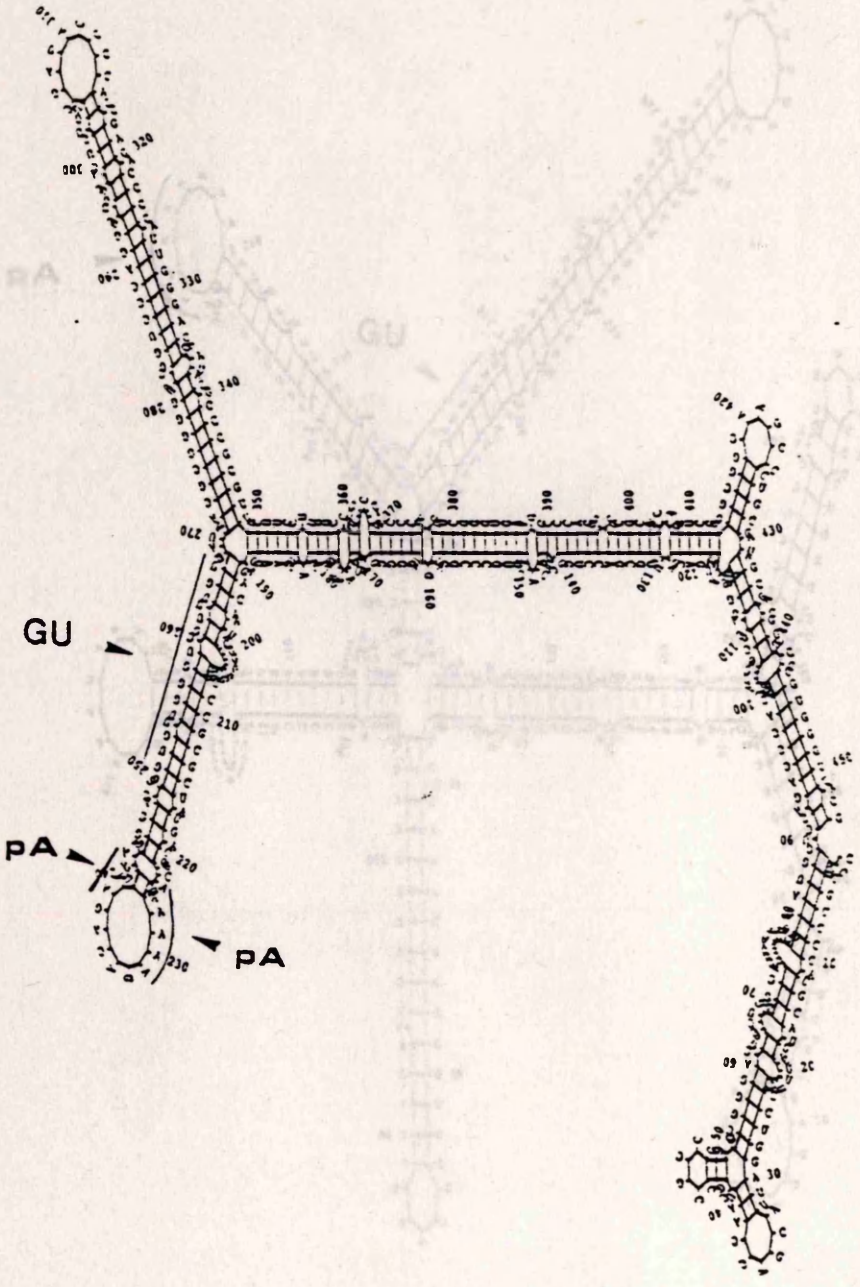


FIGURE 3.27 C:

The predicted secondary structure of the UL23 poly(A) site RNA, predicted using the VAX Fold program.

pA : poly(A) signal AAUAAA. GU: GU-rich downstream element. U : U-rich downstream elements.

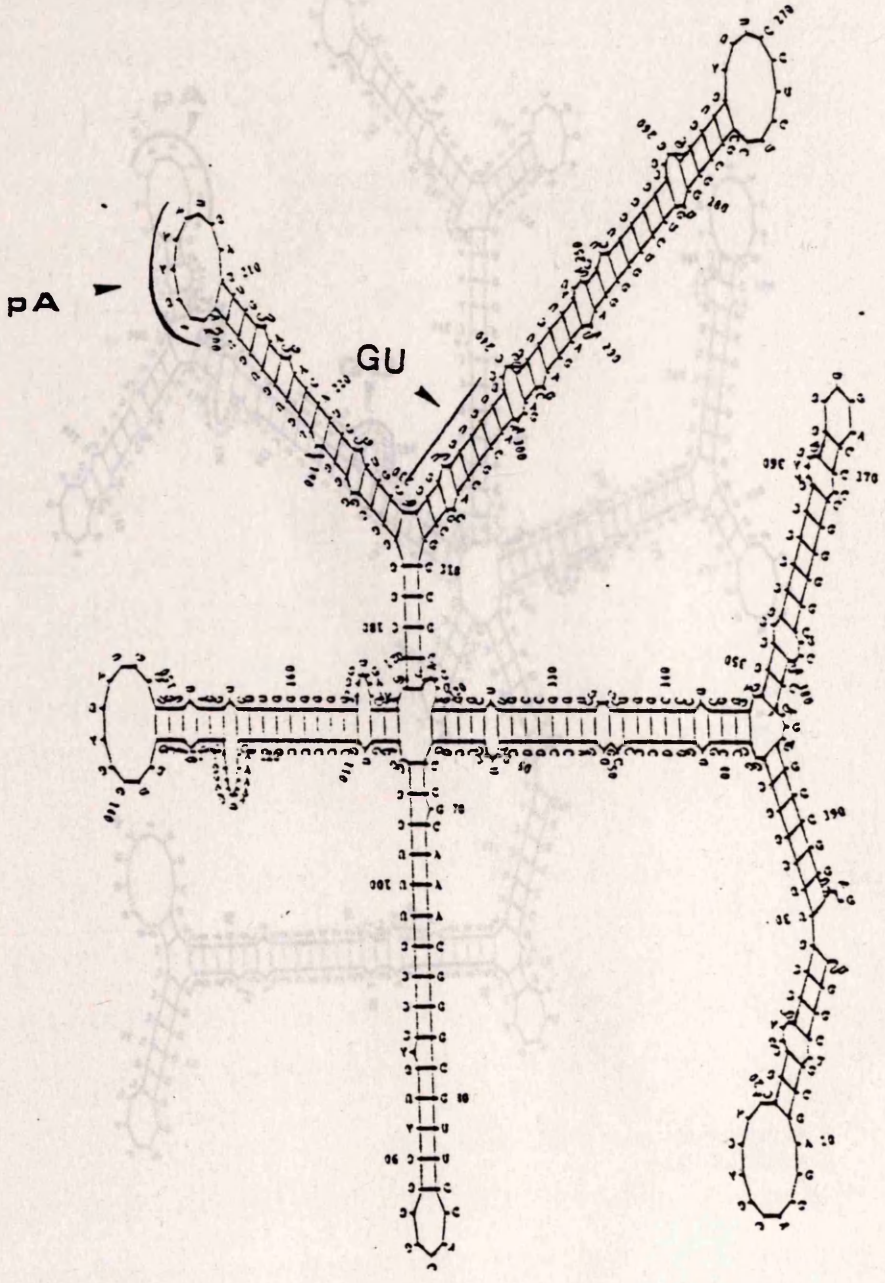


FIGURE 3.27 D:

The predicted secondary structure of the UL29 poly(A) site RNA, predicted using the VAX Fold program.

pA : poly(A) signal AAUAAA. GU: GU-rich downstream element. U : U-rich downstream elements.

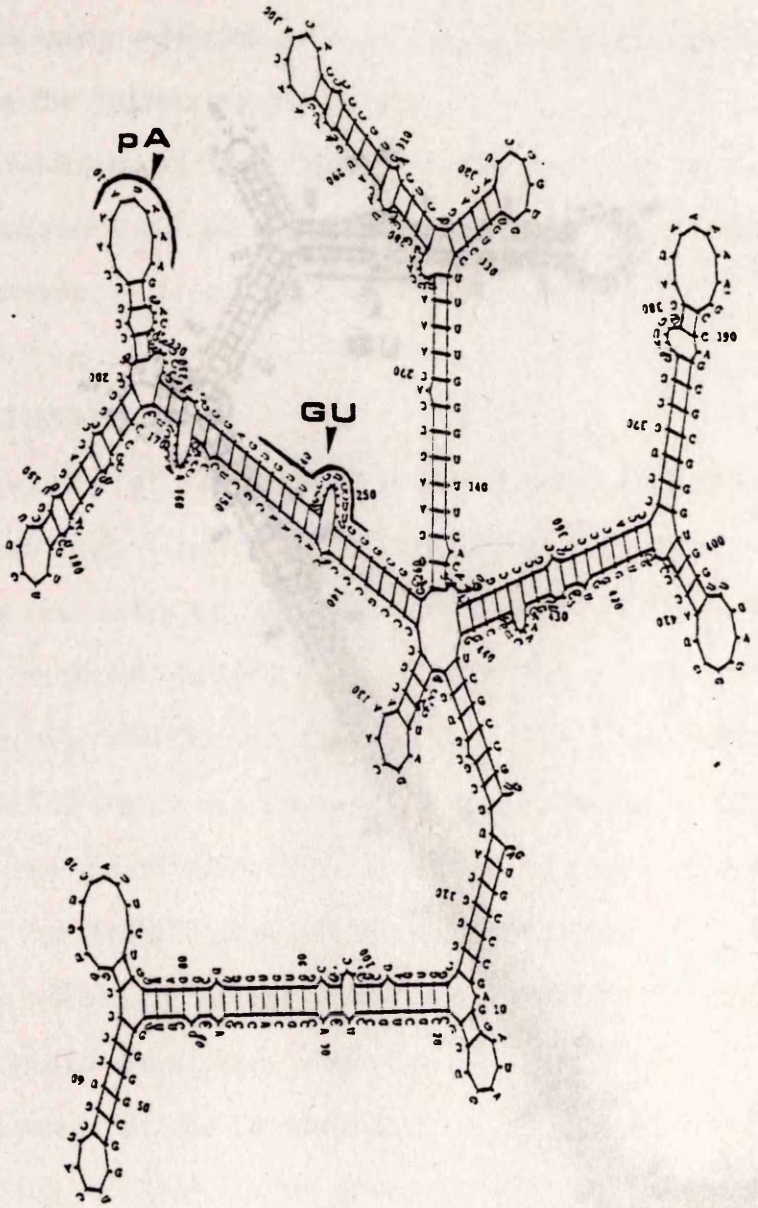


FIGURE 3.27 E:

The predicted secondary structure of the UL38 poly(A) site RNA, predicted using the VAX Fold program.

pA : poly(A) signal AAUAAA. GU: GU-rich downstream element. U : U-rich downstream elements.

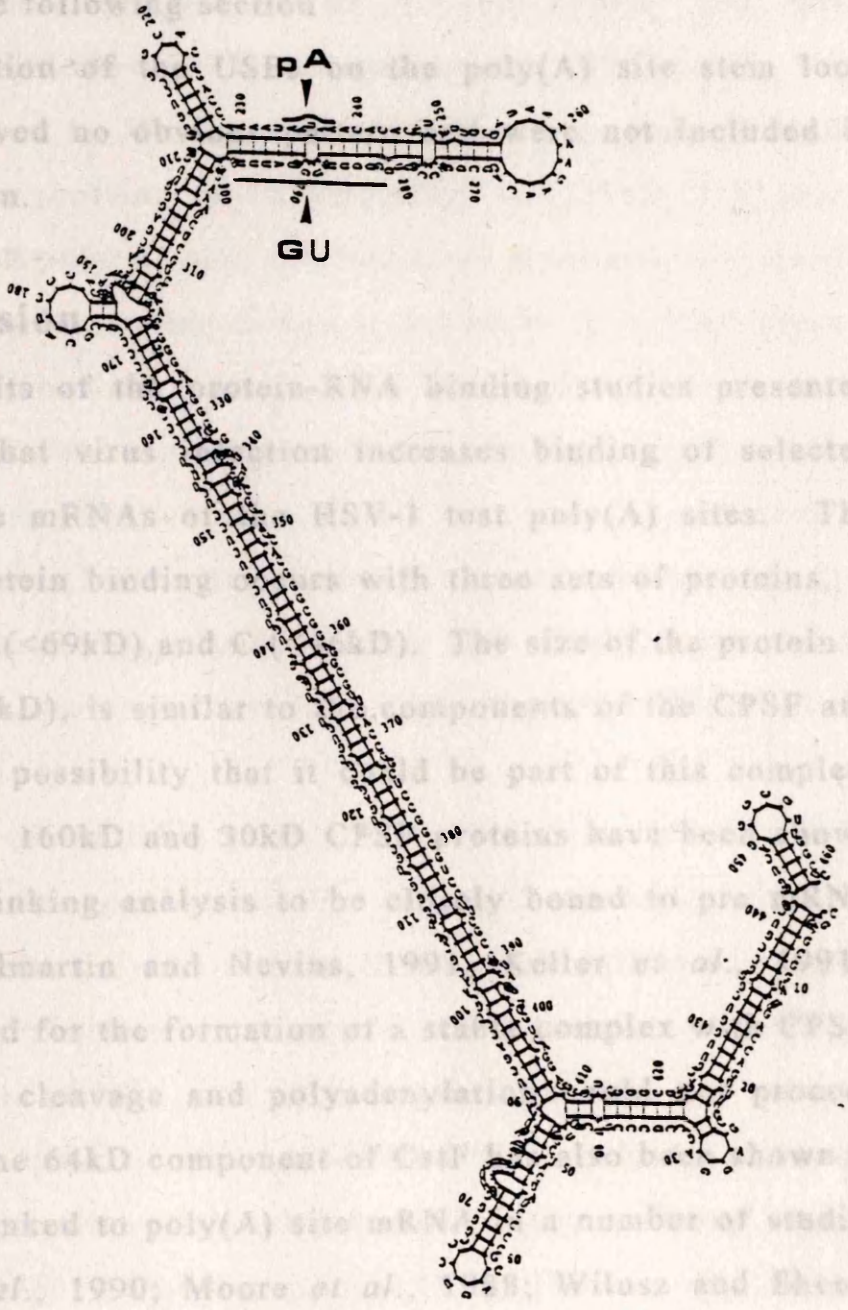


FIGURE 3.27 F:

The predicted secondary structure of the UL44 poly(A) site RNA, predicted using the VAX Fold program.
pA : poly(A) signal AAUAAA. GU: GU-rich downstream element. U : U-rich downstream elements.

the 3' processing efficiencies of these two sequences will be discussed in the following section

The location of the USEs on the poly(A) site stem loop structures showed no obvious pattern and were not included in this examination.

3.4.6 Discussion

The results of the protein-RNA binding studies presented here suggest that virus infection increases binding of selected proteins to the mRNAs of the HSV-1 test poly(A) sites. The increase in protein binding occurs with three sets of proteins, A (90-150kD), B (<69kD),and C (<46kD). The size of the protein A triplet (90-150kD), is similar to the components of the CPSF and this raises the possibility that it could be part of this complex, and indeed the 160kD and 30kD CPSF proteins have been shown by UV cross-linking analysis to be closely bound to pre mRNA substrates (Gilmartin and Nevins, 1991; Keller *et al.*, 1991). CstF is required for the formation of a stable complex with CPSF, without which cleavage and polyadenylation could not proceed efficiently. The 64kD component of CstF has also been shown to be UV cross-linked to poly(A) site mRNA in a number of studies (Takagaki *et al.*, 1990; Moore *et al.*, 1988; Wilusz and Shenk, 1988) and contains a ribonucleoprotein type (RNP) RNA binding domain (Manley *et al.*, 1993). The size of protein B also suggests it could be the 64kD component of CstF. Binding of the 64kD component of CstF appears essential for complex formation, and indeed protein B of these studies was bound consistently by all poly(A) site mRNAs examined. Protein C showed the most obvious increase in binding in the INF extracts. The size of this

protein (<46kD) suggests it could be related to either the PAP which has been reported to be between 40kD and 60kD (Christofori and Keller, 1988; Takagaki *et al.*, 1990) or the 38kD protein identified by Moore *et al.*, (1988). The study of Moore identified three proteins of 155kD, 68kD and 38kD, UV cross-linked to the L3 poly(A) site of adenovirus a pattern very similar to the RNA-protein binding demonstrated here. The 38kD protein of Moore's studies was identified by immunoprecipitation to be the C protein of the heterogeneous ribonucleoprotein particles. Although we can only speculate at this time as to the identity of proteins A, B and C, the observations presented here suggest they could be those of the polyadenylation and cleavage complexes. However these proteins remain to be formally identified by Western blot analysis.

Analysis of the HSV poly(A) site RNAs has shown that the majority contain predominantly GU-rich elements 20-35nt downstream of the poly(A) signal. However no pattern of element composition or positioning is evident to demarcate the different temporal classes of HSV genes. A number of studies have carried out similar analysis of mammalian and viral poly(A) sites and identified comparable GU-rich elements (McLauchlan, *et al.*, 1985; Renan, 1987; Sadofsky, *et al.*, 1985). The presence of such elements would therefore seem a general feature of both viral and mammalian poly(A) sites.

The upstream elements identified from analysis of the poly(A) site RNA sequences showed no pattern in either the location relative to the poly(A) signal or copy number within the sequence, indeed a number of the poly(A) sites contained no such elements within the 100nt region studied. These U-rich elements

resembled sequences identified in some mammalian mRNAs, GM-CSF, c-fos and c-myc, which confer instability on the mRNA and promote their rapid turnover (Shaw and Kamen, 1986; Vakalopolou *et al.*, 1991). It is possible that these elements perform a similar role in the HSV transcripts. This study however was designed to examine the processing efficiencies of the poly(A) sites and the relationship of LPF in the promotion of poly(A) site usage and in addition it has previously been shown that mRNA stability does not influence the LPF mediated increase in poly(A) site usage (McLauchlan *et al.*, 1989). Therefore no importance has been placed on the presence of these elements in relation to the current investigation.

The features that make the UL38 and UL44 poly(A) sites inefficient 3' processing sites in MI extracts and able to respond to LPF remain to be identified. The observations presented here have produced some suggestions for further investigation. UL38 does not appear to bind protein C which could be one aspect of its inefficiency. There could be several reasons for this lack of binding - the UL38 poly(A) site, like other poly(A) sites, contains a GU-rich DSE which is positioned within the normal distance downstream of the hexanucleotide, if these elements are required for efficient 3' mRNA processing, the proteins involved must either bind to an as yet unidentified sequence absent in UL38, or binding to the GU-rich DSE must be inhibited in some way. The complex nature of the secondary structure, the proximity and number of stem loops present in the UL38 RNA may result in an inhibition of binding of protein C.

The protein binding pattern of the UL44 poly(A) site displays no obvious dissimilarity to that of the other poly(A)

sites. However, sequence comparisons have shown that the extended GU-rich DSE overlaps the poly(A) signal on the stem loop structure. It is possible therefore that the UL44 poly(A) site can bind all the necessary proteins but the correct arrangement of proteins within the polyadenylation complex is prevented. In addition, the distance of the DSE, from the AAUAAA, was greatest in this site which may affect the efficiency of poly(A) site use. A number of HSV-1 poly(A) sites were identified from the sequence anlaysis which contained similar distance constraints of the GU-DSE and AAUAAA, it would be of interest to measure the processing efficiency of these sites to determine the significance of this observation.

Chapter 4

General discussion

During HSV infection viral genes are expressed in a tightly regulated manner and a number of viral gene products act as regulators of the temporal expression of the three classes of viral genes, IE, early and late. Expression of the IE class of genes is stimulated by a component of the virus tegument, Vmw65, the product of a late HSV gene, UL48. Expression of the early and late gene classes requires synthesis of the IE proteins IE175, IE110 and IE63 with perhaps IE68, late gene expression has an additional requirement for viral DNA synthesis to be ongoing. The roles of the IE proteins in the induction of early and late gene expression are not fully understood. However it has been noted that the co-operative interaction of IE175 and IE110 is required for the most efficient induction of a number of viral genes, and IE63 has also been shown to co-operate with IE175 and IE110 in the regulation of early and late gene expression, repressing early genes activated by the IE175/IE110 combination and enhancing activation of late gene expression. These IE proteins may also be capable of autoregulating their own expression, IE175 and IE63 deficient virus mutants both show increased levels of IE proteins, suggesting that both proteins are involved in the downregulation of the IE protein pool. Recent interest has focused on the influence that post-transcriptional events may have on the regulation of viral gene expression. McLauchlan *et al.*, (1989) identified a factor, termed LPF, which was capable of increasing

the 3' processing efficiency at the poly(A) site of a late gene of HSV-2, while having no effect on another HSV-2 poly(A) site. Post-transcriptional events which influence and regulate gene expression have been described in a number of systems, two of which, the adenovirus and IgM heavy chain systems, involving the differential usage of poly(A) sites have already been discussed in detail. It would seem likely that LPF, which is capable of selectively increasing HSV poly(A) site usage could by analogy be able to regulate viral gene expression.

LPF can influence gene expression at a post-transcriptional level. Using an *in vitro* assay designed to measure the efficiency of mRNA 3' cleavage, which was taken as an indication of 3' processing efficiency of the poly(A) site, the processing efficiencies of a selection of HSV poly(A) sites were measured. Our studies showed that the HSV induced activity, LPF, could significantly increase the *in vitro* 3' processing efficiencies of two HSV-1 poly(A) sites and one HSV-2 poly(A) site while having no significant effect on the *in vitro* 3' processing efficiencies of another four HSV-1 and one HSV-2 poly(A) site. The poly(A) sites which responded to LPF belong to the late class of HSV genes and the poly(A) sites which did not respond belong to the IE and early classes of genes. LPF therefore appears to be acting selectively to increase 3' processing of the late class of HSV genes and it is possible that a function of LPF is the post-transcriptional regulation of late gene expression in HSV infection.

There is however a caveat to this hypothesis, the HSV-2 poly(A) site which did not respond to LPF is utilised by US12 an

IE gene, US10 an early gene and US11 a late gene of HSV-2. It is therefore not simply that LPF can increase the 3' processing of all late poly(A) sites, thereby enhancing late gene expression, additional factors must also play a part. In this study the 3' processing efficiencies of the selected HSV poly(A) sites were examined in the absence of any influence that transcriptional control might exert. It is possible therefore that enhancement of late viral gene expression requires additional factors which complement control mediated post-transcriptionaly via selective enhancement of poly(A) site processing efficiency.

One interesting feature of the poly(A) sites examined was the inefficient basal processing activity of the poly(A) sites which responded to LPF. If weak poly(A) sites were a general, although obviously not exclusive feature of late poly(A) sites, this could be a means of regulating gene expression. It is possible that the role of LPF is to increase the processing efficiencies of inherently weak poly(A) sites or LPF may effect a general increase in the processing efficiency of all poly(A) sites. It may be that *in vitro*, the only sites at which this is apparent are those which are weak processing sites. In this way the role of LPF could be to somehow give a boost to viral gene expression as the cellular proteins required for these 3' processing reactions decline as viral infection progresses.

The *in vivo* activity of LPF, originally demonstrated by McLauchlan, *et al.*, (1989; 1992b), has been confirmed, showing that LPF is active from early times of infection and that the *in vitro* increase is mirrored *in vivo*. *In vivo* the activity of the LPF responsive HSV-2 poly(A) site was increased above the level of the non-responsive poly(A) site. However *in vitro* the enhanced

processing efficiency of the responsive poly(A) site did not attain the level of the non-responsive site, while this could be due to the constraints of the *in vitro* assay, it is also possible that additional factors influence processing efficiency *in vivo*. Regardless of the explanation it is obvious that LPF can function *in vivo* to selectively enhance gene expression.

The 3' processing efficiencies of HSV poly(A) sites vary. It is clear from the *in vitro* data presented here that the poly(A) sites of HSV genes have different 3' processing efficiencies, with the UL38 and UL44 poly(A) sites appearing inherently weak. Analysis of the sequences surrounding the poly(A) signal of all functional HSV-1 poly(A) sites has given no clear evidence of sequence variations which could account for these differences in 3' processing efficiencies. All poly(A) sites examined contain GU- or U-rich elements downstream of the poly(A) signal, with the majority located 20-35nt downstream of the poly (A) signal. It has been shown in other systems that increasing the distance between the poly(A) signal and the downstream elements can drastically reduce processing at the poly(A) site. It was noted that the UL44 GU-element lies 41nt downstream of the poly(A) signal, it is possible therefore that this could be responsible for its inefficiency. The downstream elements of a small number of other HSV-1 early and early-late poly(A) sites are also located around 41nt downstream and it is therefore by no means an exclusive property of the poly(A) sites of true late genes. It would be interesting to determine the processing efficiency of these other sites and to see whether a reduction of the GU-element - poly(A) signal distance would result in an increase in processing

efficiency at the UL44 site. The GU-element of the UL38 poly(A) site lies within the normal distance range, this proposed distance effect therefore cannot be the sole factor determining processing efficiency. LPF could therefore function by either stabilising what might be an inefficiently formed processing complex or by bringing the two elements (GU and AAUAAA) to within a functional distance of each other.

Analysis of the predicted secondary structures of HSV-1 poly(A) site RNAs showed that the majority of poly(A) sites (26 out of the 28 examined) are located on single stranded regions of RNA, present as loops or bulges within stem-loop structures. While there is no evidence that these structures exist *in vivo* it is interesting that the poly(A) signal is consistently located on a region of single stranded RNA. Perhaps recognition by the processing complex is facilitated by the prominent positioning of the AAUAAA or the proteins involved bind ssRNA preferentially. However it should be kept in mind that binding of any proteins to the RNA, not just those involved in 3' processing, would have an effect on the overall conformation of the structure, and this has not been taken into account in these predictions. Again no obvious features of the secondary structures single the UL38 and UL44 poly(A) sites out to be different from the other poly(A) sites examined. There is a slight possibility that the UL38 poly(A) site secondary structure, which appears more complicated than the other poly(A) sites, forming a greater number of stem-loops, prevents the ready access of the processing complex. It is possible therefore that LPF could function by changing the conformation of the structure to a more favourable or accessible form or enhance binding of the processing complex either by

direct of indirect interaction with the RNA or cellular processing proteins.

The protein-RNA UV cross-linking studies showed a distinctive pattern of protein binding. The sizes of the protein bands correlate well with the sizes of proteins known to be part of the CPSF and CstF components required for formation of the mRNA processing complex. These proteins have been shown by a number of studies to be UV cross-linked to pre mRNA substrates. However formal identification of the proteins by Western blot analysis remains to be carried out. HSV infection produced a general increase in the binding of these proteins to all the test poly(A) site RNAs examined, with the exception of the smaller <46kD protein which did not bind to the UL38 poly(A) site. Moore *et al.*, (1988) identified a similarly sized protein, UV cross-linked to the adenovirus L_3 poly(A) site, as an hnRNP C protein. It has recently been proposed that hnRNP proteins may play an active role in the splicing and transport of mRNA from the nucleus to the cytoplasm in addition to packaging pre-mRNA molecules. However the significance of the binding or lack of binding of such a protein in determination of the processing efficiency of a particular poly(A) site is unclear.

The evidence presented in these studies suggests that the efficiency of poly(A) site usage is determined by the individual differences of particular poly(A) sites. The role of LPF could be the induction of a generalised increase of processing components, either by stimulating their synthesis or by increasing their stability, thereby increasing the relative concentrations of the processing components in the cell extracts. While the general picture of host cell protein synthesis during HSV infection is one

of declining levels, exceptions to this rule have been demonstrated. During HSV-1 and HSV-2 infection in cell culture, an accumulation of stress response proteins has been shown (Notarianni and Preston, 1982; La Thangue *et al.*, 1984). It is possible therefore for virus infection to induce the accumulation of certain cell proteins. Alternatively LPF may stabilise the processing complex either directly by protein-protein interaction or indirectly by post-translational modification of the components to a more stable form, thus increasing the likelihood of complex formation, which could produce the observed increase in protein binding without an actual increase in synthesis of the protein components.

IE63 : a central role in the virus life cycle? The studies of McLauchlan *et al.*, (1992b), demonstrated that the IE63 gene product was required for LPF activity and the current studies have shown that IE63 is required for the increase in processing efficiency at both the LPF responsive HSV-1 late poly(A) sites. A lack of IE63 had no effect on the processing efficiencies of the four remaining non-responsive HSV-1 poly(A) sites. IE63 is known to be required for late gene expression and there is evidence that IE63 can enhance late gene expression both transcriptionally and post-transcriptionally. Further, there is evidence both for and against viral DNA synthesis requiring IE63 expression, which could have a knock on effect on late gene expression. The processing data presented herein supports the theory that IE63 exerts a post-transcriptional influence on late gene expression. Analysis of the profile of viral protein expression from an IE63 deficient virus mutant, showed that IE63

was required for the expression of the true-late US11, UL38, UL44 and UL45 HSV genes. While expression of the early-late gene US8, the early gene US6 and IE gene IE175 was unaltered. Interestingly the other genes affected by the lack of IE63 expression are either involved in DNA synthesis (UL29 and UL42) or known to have a regulatory role (IE110). This latter group of proteins did not display an absolute requirement for IE63 expression but rather the level of protein expressed was reduced or delayed in the absence IE63. It is possible therefore that IE63, via an effect on these proteins, could disrupt the normal sequence of events required for efficient DNA synthesis and activation of gene expression. Indeed other studies have shown that IE63 can alter the electrophoretic mobility of the UL29 and the IE175 proteins, presumably by some post-translational modification. This proposed role for IE63 in the efficient induction of viral DNA synthesis could help to explain the finding that IE63 is required for US11 expression while having no effect on the 3' processing efficiency of the US11/US12/US10 poly(A) site. IE63 may therefore have a dual role in gene expression.

Recent studies (Phelan *et al.*, 1993) have shown that during HSV infection the components required for splicing (snRNPs) form distinct foci within the infected cell and that IE63 is required for this localisation. In addition transfection studies have shown that IE63 represses the 3' processing of a poly(A) site immediately downstream of an intron. If the processes of splicing and polyadenylation are linked, which remains a possibility if unproven at this time, IE63 could thus affect the 3' processing of intron containing transcripts. During HSV infection however there is little need for splicing to occur, it would make sense

therefore for these foci to be inactive sequestrations of snRNPs. It is not known however if these snRNP foci are active or inactive.

It is difficult to believe that one protein can exert so many different influences on viral replication. Although it has been shown that the IE63 protein has a number of different functional domains, including activation, repression, a putative DNA binding region and nuclear localisation signal. It is more likely that IE63 influences the different aspects of viral replication by one central means, for example an ability to regulate other viral proteins by some post-translational mechanism. A mechanism which could in the case of 3' processing serve to enhance production of or stablise the protein components of the processing complex, and could enhance production of or activate the proteins required for viral DNA replication or affect the intracellular transport and correct localisation of these proteins as appears to be the case with the splicing components. Recent studies have demonstrated that IE63 and IE175 affect the cellular localisation pattern of IE110, with IE175 promoting and IE63 inhibiting nuclear localisation (Zhu *et al.*, 1994). In addition the correct intracellular localisation of UL29 has been shown to require IE63 expression (Curtin and Knipe, 1993). One post-translational event which has been shown in other systems to have such a universal effect is the phosphorylation and dephosphorylation of proteins (Hunter and Karin, 1992; Kwon and Hecht, 1993). In fact the aberrant phosphorylation of the cellular RNA polymerase II during HSV infection has been shown to alter the localisation and activity of this protein to the advantage of the virus (Rice *et al.*, 1993). IE63 is itself phosphorylated with the phosphate

cycling on and off during the virus replication cycle (Wilcox *et al.*, 1980). However no relationship between the different phosphorylated forms and the activities of IE63 has been established, and no data is available which shows whether IE63 has itself any phosphokinase or phosphatase activity. Alternatively IE63 could affect the activity of viral or cellular kinases or phosphatases.

In summary IE63 appears to play a central role in the regulation of viral gene expression, however the exact means whereby IE63 exerts its influence on gene expression is unknown. The evidence presented here suggests that at least part of the mechanism is an ability to increase the processing efficiencies of selected poly(A) sites, perhaps by generally increasing the concentration or stability of the protein components required for efficient 3' processing, with specific deficiencies in individual poly(A) sites making them targets for regulation. It is clear that this is not the whole story and that IE63 may have an additional role in regulation of viral DNA synthesis.

Future work

1. Determine whether IE63 alone is directly responsible for LPF activity by using purified IE63 protein in the *in vitro* 3' processing reactions.

2. Carry out Western blot analysis on the protein bands A, B and C, which were shown to bind to the test poly(A) sites, using antibodies to the components of the 3' processing complex.

3. Determine whether inefficient 3' processing is a general feature of the poly(A) sites of the class of true late HSV-1 genes.

4. Perform deletion analysis to determine if the GU-rich elements are required for efficient 3' processing and/or the protein binding properties of the test poly(A) sites.

5. Determine whether the inefficiency of 3' processing at the UL44 poly(A) site is due to the distance between the poly(A) signal and GU-rich element and further compare the 3' processing efficiencies of those additional poly(A) sites which have GU-rich elements >41nt downstream of the poly(A) signal.

Chapter 5

The mutagenic properties of HSV

5.1.1 Introduction

The association of HSV-1 with head and neck cancers and HSV-2 infection with an increased risk of cervical cancer has been based on serological and epidemiological studies. It was found that a greater number of women with cervical cancer had circulating antibodies against HSV-2 than women without the disease (Naib *et al.*, 1969) and in addition HSV-2 has been isolated from cervical cancer cells and HSV-2 antigen demonstrated in these cells by immunofluorescence (Royston and Aurelian, 1970; Aurelian *et al.*, 1970). Patients with head and neck cancers have also been shown to have higher levels of antibody to HSV-1 than control subjects (Larsson *et al.*, 1991; Kumari *et al.*, 1982; Shillitoe *et al.*, 1982). These serological studies have not identified any antibody response that is unique to cancer patients, this coupled with the widespread nature of the disease makes it difficult to provide direct evidence of a causative role for HSV in the aetiology of cancer. However it remains a strong possibility that HSV is involved at some point in the multistage process of oncogenic transformation. HSV infection may therefore have a role in the initiation of pre-cancerous changes or progression of pre-cancerous cells to a more tumourigenic phenotype induced by other factors such as exposure to carcinogens, UV light, smoking (Dokko *et al.*, 1991; Johnson, 1982; Park *et al.*, 1990), or other infectious agents such as human

papillomavirus (HPV). While there is strong evidence of an association between certain types of HPV (types 16 and 18) and cervical cancer it is known that infection by itself is not sufficient for malignant development (reviewed by Zur Hausen, 1991) and other co-factors, of which HSV infection may be one, are required (Di Paolo *et al.*, 1990; Guis and Laimonis, 1989; Wymer and Aurelian, 1990; Iwasaka *et al.*, 1988). The relationship of these factors to initiation or progression of neoplastic transformation is unknown.

HSV proteins have been shown to be expressed in some tumour cell lines (Jariwalla *et al.*, 1986; Lewis *et al.*, 1982) and viral RNA (Collard *et al.*, 1973; Copple and McDougall, 1976) and DNA (Frenkel *et al.*, 1976; Galloway *et al.*, 1980) have been detected in some tumours. In addition HSV-1 and HSV-2 have been shown to be capable of transforming rodent cells to a malignant phenotype (Rapp and Duff, 1973; Takahashi and Yamanishi, 1974). There is no evidence however that maintenance of the transformed state requires either the continued expression of a particular HSV protein or that viral DNA is retained or expressed. Indeed HSV infection almost always results in cell lysis and death, for cellular transformation to result from HSV infection therefore this cycle of events would have to be aborted. This obviously rare event may result in the alteration of the infected cell in such a way as to contribute to the progression towards malignancy. This has led to the hypothesis that HSV acts in a hit-and-run manner to induce the changes which result in transformation of the cell (reviewed by Galloway and McDougall, 1983; Macnab, 1987).

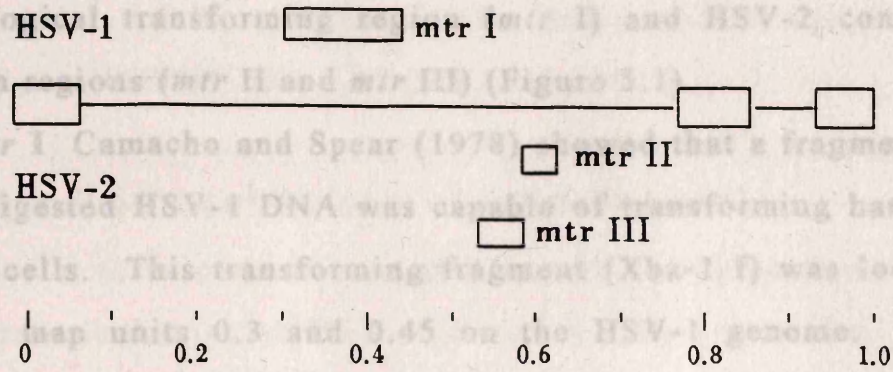


FIGURE 5.1 :

Shows the locations of the morphological transforming regions within the HSV genome (given in map units). The blocks indicate the positions of *mtr*I, *mtr*II and *mtr*III along the prototype arrangement of the HSV genome.

5.1.2 Morphological transformation regions of HSV

Transfection studies have identified three separate regions of the HSV genome which are associated with the morphological transformation of cultured cells. HSV-1 contains one morphological transforming region (*mtr* I) and HSV-2 contains two such regions (*mtr* II and *mtr* III) (Figure 5.1).

***mtr* I** Camacho and Spear (1978) showed that a fragment of Xba-1 digested HSV-1 DNA was capable of transforming hamster embryo cells. This transforming fragment (Xba-1 f) was located between map units 0.3 and 0.45 on the HSV-1 genome. This finding was subsequently confirmed by Reyes *et al.*, (1979) who identified the BglII i fragment, map units 0.311-0.45, as the transforming region.

***mtr* II** The *mtr* II of HSV-2 has been mapped to the BglII n fragment, map units 0.58-0.625 (Reyes *et al.*, 1979; Macnab and McDougall, 1980; Galloway and McDougall, 1981). The coding sequences of the UL41 (VHS) gene lie within this region, however expression of this protein was not detected in cells transformed by this fragment (Galloway *et al.*, 1984). Analysis of this DNA fragment to determine the minimal region required for transformation has produced conflicting results, with both the left hand (Galloway *et al.*, 1984) and right hand regions of the *mtr* II implicated (Pilon *et al.*, 1989).

***mtr* III** The second morphological transforming region of HSV-2 confusingly called mtr III, maps within the BglII c fragment, map units 0.54-0.58, of the HSV-2 genome (Peden *et al.*, 1982; Jariwalla *et al.*, 1983). The left hand 64% of the Bgl IIc fragment has been shown to immortalise rat cells (Jariwalla *et al.*, 1983) and the right hand 30% can induce neoplastic

transformation of immortalised rat cells (Jariwalla *et al.*, 1986). Leading to the hypothesis that the BglII c fragment contains independent immortalising and transforming functions (Jariwalla *et al.*, 1986). The minimum region that is capable of neoplastic transformation of immortalised cells maps within the PstI c fragment, map units 0.562-0.57 (Ali *et al.*, 1991).

5.1.3 Possible mechanisms of transformation

Several theories have been put forward in explanation of the hit-and-run mechanism of HSV mediated cell transformation.

(a) Activation of cellular genes

Transformation could result from the constitutive induction of cellular genes involved in growth regulation. While HSV infection results in the shut off of host macromolecular synthesis the expression of a limited number of proteins is stimulated. Cellular heat shock proteins have been shown to accumulate in HSV infected cells (La Thangue, 1984; Notarianni and Preston, 1982) and a specific set of three polypeptides of 90kD, 40kD and 32kD are expressed in transformed cells and are identical to ones induced by HSV infection (Macnab *et al.*, 1985). It is possible therefore for the virus to induce the aberrant and perhaps unregulated expression of cellular genes which could result in transformation of the cell.

(b) Activation of endogenous virus

The early studies of Hampar (Hampar *et al.*, 1976; Boyd *et al.*, 1978), showed that HSV infection of cells transformed by Maloney murine sarcoma virus led to activation of the endogenous

virus. The sequences of HSV-1 which are capable of inducing retroviruses have been localised to DNA regions which correspond to map units 0.29-0.32, 0.46-0.49 and 0.92-0.97, the first of which overlaps with *mtr* I (Boyd *et al.*, 1980). It is not clear however if the two functions coincide. Endonuclease digestion of the TK gene (UL23), which overlaps the 0.29-0.32 region, was shown to inactivate the induction of retroviruses. However other regions (0.46-0.49 and 0.92-0.97) of the HSV-1 genome can also reactivate the endogenous virus and the role of TK in induction is therefore unclear.

(c) Amplification of cellular sequences

HSV infection has been shown to induce the amplification of integrated SV40 genes present in transformed hamster (Schelhofer *et al.*, 1983;) and human cell lines (Schelhofer *et al.*, 1986) in a manner similar to chemical carcinogens (Lavi, 1981). HSV can also amplify human papillomavirus sequences resident in cervical carcinoma cell lines (Brandt *et al.*, 1987). This observation could explain the proposed synergistic role of HPV and HSV in the initiation and/or progression of cervical cancer. While it is not clear how HSV mediates amplification of these integrated viral sequences it has been shown to be dependent on continued viral replication and in particular requiring the HSV genes involved in viral DNA replication (UL30/UL42, UL29 and UL5/UL8,/UL52) (Danovich and Frenkel, 1988).

(d) Mutagenesis of cellular genes

One way in which a hit-and-run mechanism might operate is by increasing the frequency of mutations in cellular DNA, and

there is strong evidence that mutations play a fundamental role in cellular transformation and carcinogenesis (reviewed by Bishop, 1983). The hypothesis that HSV may act as a mutagen was first proposed when chromasomal aberrations were observed in HSV infected cells (Stich *et al.*, 1964; Hampar and Ellison, 1961). Inactivated HSV-1 (UV irradiated or neutral-red inactivated) or infection of non-premissive cell lines with wt HSV-2 has been shown to induce mutations in the cellular hypoxanthine guanine phosphoribosyl transferase (HGPRT) gene (Schelhofer and Zur Hausen, 1982; Pilon *et al.*, 1985). A cloned fragment of DNA corresponding to *mtr* I of HSV-1 has in addition been shown to produce an increase in the frequency of histidine reversion mutants in E. coli (Shillitoe *et al.*, 1986).

Analysis of the mutagenic properties of HSV has been facilitated by the use of the shuttle vector system of Seidman (Seidman *et al.*, 1985), which had previously been used to examine the mutagenic effects of UV-irradiation and chemical carcinogens on mammalian cells (Hauser *et al.*, 1986; Munson *et al.*, 1987; Dixon *et al.*, 1989; Seidman *et al.*, 1987; Moraes *et al.*, 1989). It was shown that HSV-1 infection induced a significant increase in the mutation frequency of the target plasmid above the level of mutations arising spontaneously in uninfected cells and that this increase was evident from 2h pi, peaking between 4h-8h pi (Hwang and Shillitoe, 1990; Clarke and Clements., 1991). Analysis of a series of HSV-1 ts, insertion and deletion mutants and UV irradiated virus, demonstrated that neither viral replication or gene expression were required for the mutagenic effect of the virus. The mutagenic activity of the virus was therefore likely to be a component of the incoming virion or the

viral DNA itself (Clarke and Clements., 1991). It was also shown that lytic infection was not required for mutagenesis, lytic infection with vaccinia virus did not increase the mutation frequency and non-lytic infection with adenovirus did (Hwang and Shillitoe, 1990). Analysis of the types of mutations induced showed that mutations arising spontaneously were different from those arising from HSV infection. A greater proportion of spontaneous mutations were point mutations (64% vs 40%) the majority of virus induced mutations arising from deletion of plasmid DNA or insertion of regions of cellular or plasmid DNA, with the proportion of more complex mutations increasing as infection proceeded (Hwang and Shillitoe, 1990). There was good correlation reported between the types of mutations induced by HSV infection (Hwang and Shillitoe, 1991) and those seen in chromasomal DNA resulting from exposure to UV light (Hauser *et al.*, 1987; 1988) and also those occurring in patients with a DNA repair deficiency (Seidman *et al.*, 1987; Bredberg *et al.*, 1986).

Recent studies have ruled out the involvement of a number of HSV-1 genes, namely IE175, UL41 (VHS), UL23 (TK), UL30/UL42 (DNA polymerase) (Shillitoe *et al.*, 1993) and UL39/UL40 (ribonucleotide reductase) (Clarke *et al.*, 1991). In addition Shillitoe *et al.*, (1993) propose that a small viral peptide, encoded within the HSV-1 *mtr* I, which was previously shown to increase the mutation frequency of bacteria (Shillitoe *et al.*, 1986), is responsible for the mutagenic effects of HSV-1.

The original aim of this part of my thesis was to confirm and extend the work carried out in this Institute by P. Clarke (Clarke *et al.*, 1991). To identify the exact component of HSV responsible for the observed mutagenic properties of the virus,

with the short-term aim of increasing the efficiency of the shuttle vector assay system used in the initial studies of Clarke and Clements, (1991).

5.2 Materials and Methods

In this section the additional methods and materials required for this part of the work are described. The majority of materials and methods used are as described in Chapter 2.

5.2.1 Materials

(a) Cells

CV-1 cells, derived from African Green monkey kidney fibroblasts, were used for transfection studies.

FG-293 cells, derived from human embryonal kidney, transformed by adenovirus type 5, were used for transfection studies and the pZ189 mutagenesis assay.

(b) Bacterial cells

E. coli strain MBM7070 (F-, lacZamCA7020, lacY1, hsdR-, hsdM+, ara-D139, (araABC-leu)7679, galU, galK, rpsL, thi), was used for propagation of the pZ189 plasmid in the mutagenesis assay. Bacteria were propagated in either L-broth or LM broth (L-broth supplemented with 10mM $MgSO_4$ and 10mM Mg Cl_2).

(c) Viruses

HSV-1 strain 17G41, a recombinant virus generated from HSV-1 strain 17^+ containing the UL41 (VHS) gene from HSV-2 strain G (Fenwick and Everett, 1990) was supplied by Dr. J. McLauchlan. HSV-1 strain 14-012 was a gift from Prof; E. Shillitoe, Houston, Texas.

(d) Plasmid

The pZ189 plasmid designed by Seidman *et al.*, (1985) was used for the mutagenesis assay.

(e) Tissue culture media

FG-293 cells were grown in Eagle's A+B (with phenol red), supplemented with 4mM glutamine, 100units/ml pencillin, 100units/ml streptomycin and 10% foetal calf serum. Cells were grown and harvested as described in Chapter 2.

5.2.2 Methods

(a) Virus growth curve

35mm tissue culture plates were seeded with either BHK or FG-293 cells to give a confluent monolayer after overnight incubation at 37°C in 5% CO_2. The cells were then infected with virus at 5pfu/cell in 200µl of PBS supplemented with 10% NBCS. Virus was left to adsorb for 1h at 37°C, the cells were then washed twice in serum free medium and overlayed with growth medium. Plates were harvested at appropriate times by scraping the cells into the medium and sonicating the suspension to disperse any cell clumps. Each sample was then titrated on BHK monolayers.

(b) Preparation of competent MBM7070 cells

Method A: a 10ml overnight culture of MBM7070 bacteria was grown in LM broth, 1ml of this culture was transferred to 100mls of LM and incubated at 37°C in a shaking incubator until an OD_{600} of 0.45 was reached. The cells were pelleted by centrifugation (4000rpm, 5min, 4°C) and the pellet resuspended in

40ml ice cold buffer (100mM $CaCl_2$, 10mM DTT and 10mM MES) and held on ice for 20min. The cells were pelleted as before and resuspended in a 4ml final volume of buffer. Cells were stored at 4°C for up to 5 days.

Method B: 2ml of an overnight culture of bacteria was transferred to 100mls of L-broth and incubated in a shaking incubator at 37°C until an OD_{600} of 0.2 was reached. The cells were pelleted and resuspended in 40 ml of ice cold 0.1M $CaCl_2$ and held on ice for 40min pelleted once more and resuspended in 2ml of 0.1M $CaCl_2$. Cells were stored at 4°C for up to 5 days.

Method C: 1ml of an overnight culture of bacteria was transferred to 100ml of L broth and incubated at 37°C in a shaking incubator until an OD_{600} of 0.45 was reached. The cells were pelleted at 2500rpm for 10min at 4°C and resuspended in 10ml ice cold L broth containing 10% PEG, 5% DMSO, 10mM $MgCl_2$ and 10mM $MgSO_4$ and held on ice for 10min. Cells were aliquoted in 100µl volumes and either used immediately or stored at -70°C.

Method D: The preparation of competent cells for electroporation has been described in Materials and Methods Section 2.2.10 (a).

(c) Transformation of bacterial cells

Competent cells prepared by methods A, B and C were transformed in essentially the same manner. 100pg of DNA was mixed with 100µl of competent cells and incubated on ice for 45min. The cells were then heat shocked at 42°C for 2min after which 900µl of L broth was added and the mixture incubated in a shaking incubator for 1h at 37°C. The cells were plated onto

solid medium (1.5% Bacto agar in L broth) containing ampicillin (1μg/ml) and incubated at 37°C overnight to allow the formation of bacterial colonies. Heat shock was omitted when the competent cells were prepared by method C. Transformation by electroporation has been described in Materials and Methods Section 2.2.10 (b).

(d) Transfection of DNA into tissue culture cells

Three transfection methods were used two of which, calcium phosphate precipitation with a DMSO boost and Lipofection, were described in Materials and Methods Section 2.2.6 (a) and (b). The third method a simple calcium phosphate precipitation is essentially as described by Graham and Van der EB, (1973). 10μg of plasmid DNA was mixed with 420μl of TE and 60μl of 2M $CaCl_2$ was added dropwise whilst vortexing. The DNA/$CaCl_2$ solution was then added dropwise, whilst vortexing, to 480μl of 2xHBS (280mM NaCl, 1.5mM Na_2HPO_4, 50mM hepes pH 7.1). The mixture was left at RT for 20min to allow the precipitate to form, and was then added dropwise to the 70-80% confluent cell monolayer (50mm tissue culture plates) by swirl mixing into the GM. After overnight incubation at 37°C the GM was removed and the cell monolayer washed twice in serum free medium, the cells were then overlayed with fresh GM before further incubation.

(e) Hirt extraction of plasmid DNA from eukaryotic cells

As described by Hirt (1967), GM was removed from the cells and replaced with 0.5ml Hirt extraction buffer (0.5% w:v SDS, 10mM EDTA, pH 7.5) followed by incubation at RT for 15-20min. The viscous lysate was scraped from the plates and poured into a

vial, 5M NaCl was then added giving a final concentration of 1M NaCl and the solution mixed gently by inversion. The mixture was stored for at least 8h at 4°C, then centrifuged (17,000rpm, 30min, 4°C) to pellet the genomic DNA. The supernatant, containing the plasmid DNA, was treated with RNaseA (100µg/ml) for 3h at 37°C, phenol/chloroform extracted 3 times, chloroform extracted once and the DNA ethanol precipitated. After lyophilisation the DNA was resuspended in 30µl H_2O.

(f) pZ189 mutagenesis assay

The method is essentially as described by Shillitoe *et al.*, (1990). Using the simple calcium phosphate precipitation method subconfluent 50mm plates of FG-293 cells were transfected with 10µg of the plasmid pZ189. Transfection medium was subsequently replaced with fresh GM at 18h. Mock infected cells were incubated for a further 24h to allow replication of the plasmid. Cells to be infected with virus were left for 8h, 18h or 20h after replacing the transfection medium before being infected with virus at a moi of 10pfu/cell (approximately $4x10^6$ cells/plate) for 18h, 6h or 4h respectively. Mock infected and infected plates were therefore harvested after a total incubation period of 42h. Progeny plasmids were extracted from the cell lysate using the extraction method of Hirt (1967), followed by treatment with the DpnI restriction endonuclease to remove any unreplicated forms. The enzyme DpnI cuts only DNA that has been methylated by the E.coli dam methylase, all plasmids which have undergone replication in a eukaryotic system escape digestion, whereas incoming plasmid DNA of bacterial origin is digested. Progeny plasmids were then used to transform MBM7070 bacteria and the

resultant colonies grown on LB agar plates supplemented with 50μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 45μg/ml isopropylthio-β-D galactoside (IPTG). This strain of E. coli contains a suppressible (amber) mutation in the β-galactosidase gene, when transformed with pZ189 encoding a suppressor tRNA, blue colonies are produced when grown in the presence of X-gal and IPTG and white colonies are produced when the suppressor tRNA is mutated. Any white colonies isolated were replated twice to confirm their mutant phenotype. The percentage frequency of mutagenesis was calculated using the following formula.

$$\text{Mutation frequency} = \frac{\text{No. of white colonies}}{\text{total No. of colonies}} \times 100$$

(h) Hydroxylamine mutation of plasmid DNA

24μg of plasmid DNA was added to 1ml of 400mM hydroxylamine solution (400mM hydroxylamine, 1mM EDTA, 50mM Na pyrophosphate). The mixture was incubated on ice for 45min then incubated at 75°C for 1h. The action of hydroxylamine was quenched by the addition of Tris buffer and the mutated DNA was recovered by ethanol precipitation.

(i) Chloramphenicol acetyl transferase assay

Thin layer chromatography method : Cells extracts were produced by several cycles of freeze thawing in Tris buffer as described previously. 25μl of cell extract was added to the 35μl reaction mix (0.5μCi ^{14}C chloramphenicol, 1mM acetyl co-enzyme A, 200mM Tris pH 7.8) and incubated at 37°C for 1h, 200μl of ethyl acetate was then added and mixed thoroughly. The mixture

was centrifuged (13,000rpm for 2h) and the ethyl acetate fraction, containing the chloramphenicol and its acetyl derivatives removed to a fresh tube and evaporated to dryness. The residue was redissolved in 30µl of ethyl acetate and applied to a thin layer chromatography plate (tlc). Ascending chromatography was performed using a 95% chloroform and 5% methanol (v:v) solvent. Finally the tlc plate was air dried and the bands visualised by autoradiography.

(i) Assay of virion host shut off (VHS)

Virus particle counts were performed in this Institute by J. Aiken on HSV-1 strain 17^+, HSV-1 strain 17G41 virions and L-particles. Virion particle and L-particle dilutions were prepared in Glasgow's modified Eagle's medium containing 2.5µg/ml actinomycin D. Confluent 35mm tissue culture plates containing BHK or FG-293 cell monolayers were inoculated with either virion or L-particle dilutions at $5x10^9$particles/cell; $1x10^9$particles/cell; $2x10^8$particles/cell $4x10^7$particles/cell and $4x10^6$particles/cell. After 1h the inoculum was replaced with GMEM containing 1/5th the normal concentration of methionine, supplemented with 2% NBCS and 2.5µg/ml actinomycin D. At 2.5h after inoculation with the virus particles the cells were labelled for 1h with 180µCi/ml [^{35}S]-L-methionine in GMEM containing 5% NBCS and actinomycin D. After incubation the medium was discarded and the cells harvested by scraping into 200µl protein gel boiling mix and heated to 100°C for 5min.. 1/10th of each sample was precipitated with trichloroacetic acid and the amount of radiolabelled protein present determined by measurement of the radioactive counts present using a

scintillation counter. The efficiency of protein synthesis in the infected samples was expressed as a percentage of the protein synthesis found in the mock infected control.

5.3 Results

5.3.1 The pZ189 mutagenesis assay

Developed by Seidman *et al.*, (1985), the pZ189 mutagenesis assay has been used extensively to study the effects of mutagens on mammalian cells. The plasmid, pZ189 (Figure 5.2), contains both the origin of replication and early gene region from the SV40 virus and the pBR327 plasmid origin and as such can replicate in both SV40 permissive cell lines and bacterial cells, replicating as efficiently as wt SV40 viral DNA (Seidman *et al.*, 1985). Several features of the plasmid make it particularly useful for the study of mutagenesis. The mutagenesis marker, the tRNA *supF* gene, is located between two essential functions of the plasmid, the ampicillin resistance gene and the pBR327 origin of replication, producing a vector with a greatly reduced frequency of deletion/insertion mutations following passage through mammalian cells and a spontaneous mutation frequency which is 10- to 20-fold lower than other similar shuttle vector plasmids (Seidman *et al.*, 1985). The tRNA suppresses an amber mutation in the lacZ gene of the E. coli strain MBM7070. Transformation with intact *sup F* allows the lacZ gene to function normally and form blue colonies on medium supplemented with X-gal and IPTG. Mutation of the tRNA results in inactivation of the lacZ gene with the production of white colonies. The mutagenic frequency of any mutagen can therefore be measured simply and expressed quantitatively as the percentage of white colonies formed in the total population of transformants. In addition the marker gene is only 150bp in size, the entire length of the gene can be readily sequenced and determination of the types of mutations which have been induced is therefore simple.

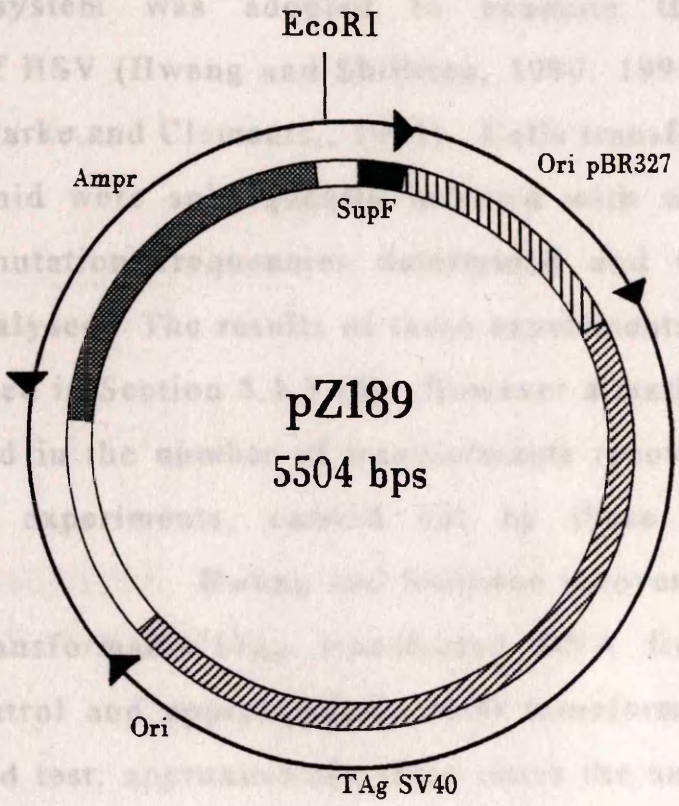


FIGURE 5.2 :

The pZ189 shuttle vector. The 5504bp vector contains the ampicillin resistance gene (Ampr), the pBR327 origin of replication (Ori pBR327), the E.Coli *sup*F suppressor tRNA and the origin of replication and TAg coding region from SV40. Arrows indicate direction of transcription.

This system was adopted to examine the mutagenic properties of HSV (Hwang and Shillitoe, 1990; 1991; Shillitoe *et al.*, 1993; Clarke and Clements., 1991). Cells transfected with the pZ189 plasmid were subsequently infected with wt and mutant HSV, the mutation frequencies determined and the mutations produced analysed. The results of these experiments have already been discussed in Section 5.1.3 (d). However a marked difference was observed in the number of transformants recovered from the transfection experiments, carried out by these two research groups. Hwang and Shillitoe recovered an average of 9000 transformants/10μg transfected DNA from the mock infected control and approximately 8000 transformants from the HSV infected test: approximately three times the number of mock infected transformants and five times the number of HSV infected transformants obtained by Clarke and Clements, (1991). The level of recovery attained by Hwang and Shillitoe was in keeping with that attained by other research groups, using the assay to study the effect of chemical mutagens on mammalian cells. It was essential therefore that the efficiency of transformant recovery was increased to allow confirmation and expansion of the initial data of Clarke and Clements, (1991). Each aspect of the assay - plasmid preparation, transfection, infection and transformation has therefore been studied in turn and optimised.

5.3.2 Plasmid preparation

Different batches of pZ189 plasmid DNA were tested for the frequency of occurrence of spontaneous mutations (Table 5.1). Large scale preparation of plasmid DNA was carried out as described in Materials and Methods Section 2.2.2 (b). Three of

Batch Date	Total colonies	Mutant colonies	Mutation frequency	Chloramphenicol W/WO
15/08/91	29888	53	0.18	with
08/08/91	11968	0	0	with
21/08/91	27680	0	0	with
14/10/91	422218	42	0.099	without
21/10/91	14562	0	0	without

TABLE 5.1:

The spontaneous mutation frequencies of different batches of pZ189 plasmid DNA, prepared with and without chloramphenicol. Showing the pooled results from two experiments.

the five batches received an additional boost of chloramphenicol (100μg/ml). Chloramphenicol is known to enhance replication of plasmid DNA by preventing bacterial protein synthesis, however there is also a concomitant increase in misincorporation of ribonucleotides, instead of deoxyribonucleotides. This is thought to stimulate the DNA repair mechanism and due to the poor proof reading ability of this mechanism can result in point mutations within the DNA. DNA from each plasmid batch was used to transform E. coli strain MBM7070 (prepared by method A Section 5.2.2 (b)). The total number of transformants recovered and the number of white (mutant) colonies were counted and the mutation frequencies determined (Table 5.1). Two of the plasmid batches had unacceptably high levels of spontaneous mutations and were therefore not used in this study. Addition of chloramphenicol did not appear to influence the occurrence of these spontaneous mutations, two out of three batches which received the chloramphenicol boost produced no mutants, while one of the two untreated batches had a mutation frequency of 0.099 which is higher than the average recorded spontaneous mutation frequency (0.05%) of the mutagenesis assay (Seidman *et al.*, 1985).

5.3.3 Transfection of mammalian cells

A comparison of three transfection methods was carried out using three cell lines FG-293, CV-1 and HeLa. The transfection methods used were : A - lipofection described in Materials and Methods Section 2.2.6 (b), B - the simple calcium phosphate precipitation method described in Section 5.2.2 (d) with and without the addition of 1μg of calf-thymus carrier DNA/μg of plasmid DNA and C - calcium phosphate precipitation with a

boost of 20% DMSO or 20% glycerol. The plasmid pLW2, which contains a CAT reporter gene, was transfected into the three cell lines and CAT activity, determined by the *tlc* method, was used as a measure of transfection efficency. Figure 5.3 shows the autoradiographs obtained from these transfection studies. Of the three cell lines tested the FG-293 cells, which were recommended by Seidman because they have a low spontaneous mutation frequency, gave the best results with all transfection methods. FG-293 cell extracts, from cells transfected using method B (without carrier DNA), when diluted 1:300 in CAT buffer, were capable of completely converting chloramphenicol to its acetyl derivatives. While the CV-1 and HeLa cell extracts were positive for CAT activity when undiluted, no activity was detected at the 1:300 dilution of extract (Figure 5.3 panels A and B). Using method C the FG-293 cell line was similarly the most readily transfected (Figure 5.3 panel C) with neither the glycerol or DMSO boost confering any advantage. All transfections were therefore carried out by the simple calcium phosphate precipitation method using the FG-293 cell line.

5.3.4 Infection with HSV-1

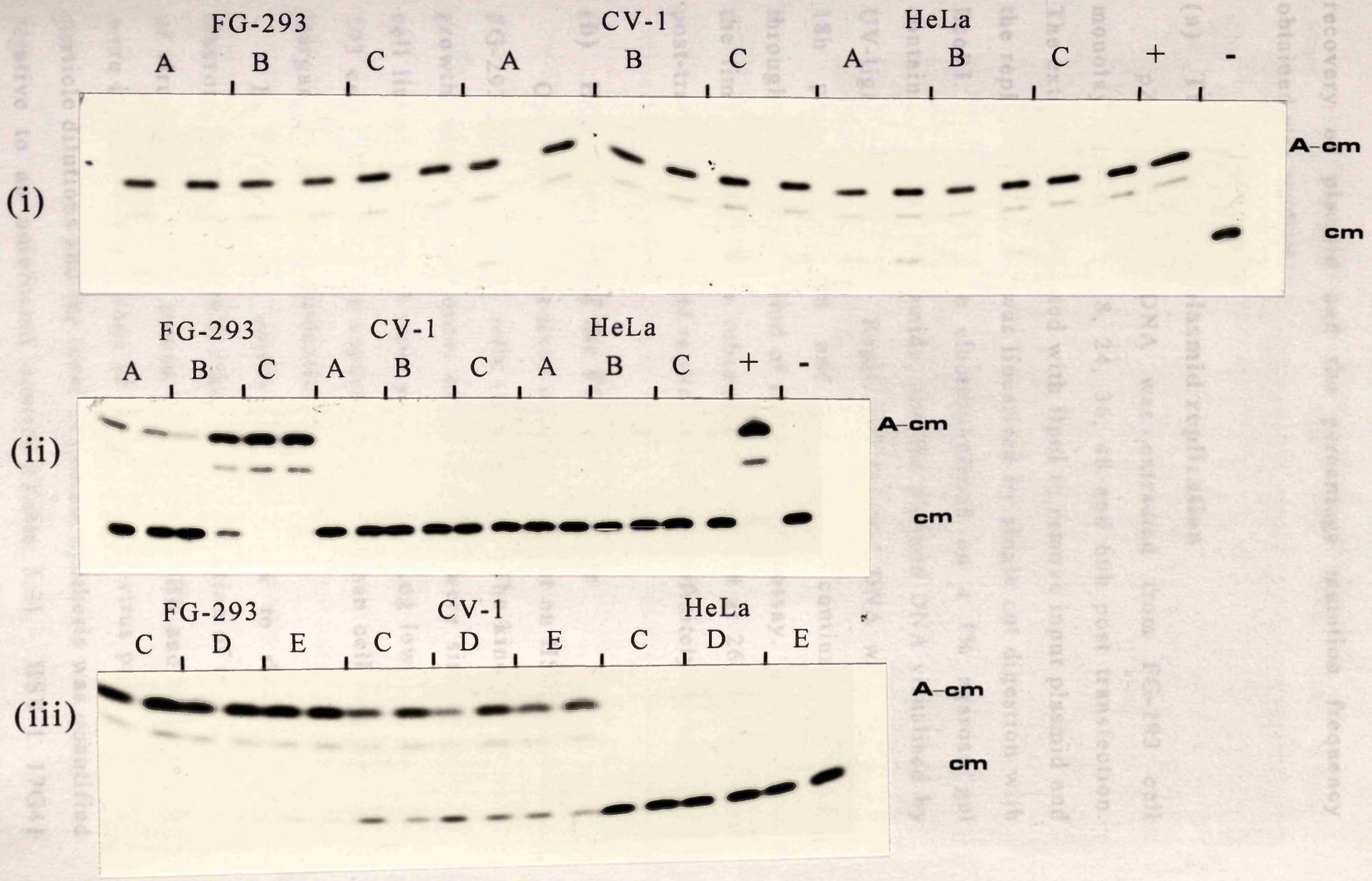
Several aspects of this part of the mutagenesis assay were studied. Firstly to ensure that sufficient time was allowed for the tranfected plasmid to replicate in the FG-293 cell line before infecting with HSV-1, a time course of plasmid replication was carried out. Secondly the ability of HSV-1 to infect the human derived cell line FG-293 compared to the hamster cell line (BHK), which was used for characterisation and propagation of the virus, was examined. Thirdly the effect of different infection times on

FIGURE 5.3:

Chloramphenicol acetyl transferase activities produced by the CAT plasmid pLW2 during transfection of FG-293 cells, CV-1 cells and HeLa cells. The positions of the acetylated (A-cm) and non-acetylated (cm) forms of ^{14}C chloramphenicol are indicated by arrows.

Transfection methods : A - lipofection, B - calcium phosphate precipitation with calf thymus carrier DNA, C - calcium phosphate precipitation, D - calcium phosphate precipitation with a 20% glycerol boost, E - calcium phosphate precipitation with a 20% DMSO boost.

Panel (i) and (iii) neat extracts, panel (ii) extracts diluted 1:300 in CAT buffer.


FG-293
CV-1
HeLa
A
B
C
A
B
C
A
B
C
+
-
(i)
A-cm
cm
FG-293
CV-1
HeLa
A
B
C
A
B
C
A
B
C
+
-
(ii)
A-cm
cm
FG-293
CV-1
HeLa
C
D
E
C
D
E
C
D
E
(iii)
A-cm
cm

recovery of plasmid and the percentage mutation frequency obtained was studied.

(a) Time course of plasmid replication

pZ189 plasmid DNA was extracted from FG-293 cell monolayers at 6, 12, 18, 24, 36, 48 and 60h post transfection. The extracts were digested with DpnI to remove input plasmid and the replicated plasmid was linearised by single cut digestion with EcoRI. Aliquots were electrophoresed on a 1% agarose gel containing ethidium bromide, and the plasmid DNA visualised by UV-light (Figure 5.4). Replicated plasmid DNA was visible by 18h post transfection and replication continued steadily throughout the time period of the replication assay. Therefore by the time the cells were subsequently infected at 26, 36 and 38h post-transfection plasmid replication would definitely be ongoing.

(b) HSV infection of the FG-293 cell line

One step growth curves were carried out on HSV-1 infected FG-293 cells and BHK cells (Figure 5.5). The kinetics of virus growth, lag time and onset of replication, were similar in both cell lines. The overall virus yield was one Log lower in the FG-293 cells which was as expected in this human cell line (Dr. D. Dargan personal communication).

In addition the ability of the virus to shut off host macromolecular synthesis, taken as an indication of the efficiency of virus infection, was measured. Both the BHK and FG-293 cells were infected with a range of HSV-1 17G41 virus particle and L-particle dilutions and the level of protein synthesis was quantified relative to an uninfected control (Table 5.2). HSV-1 17G41

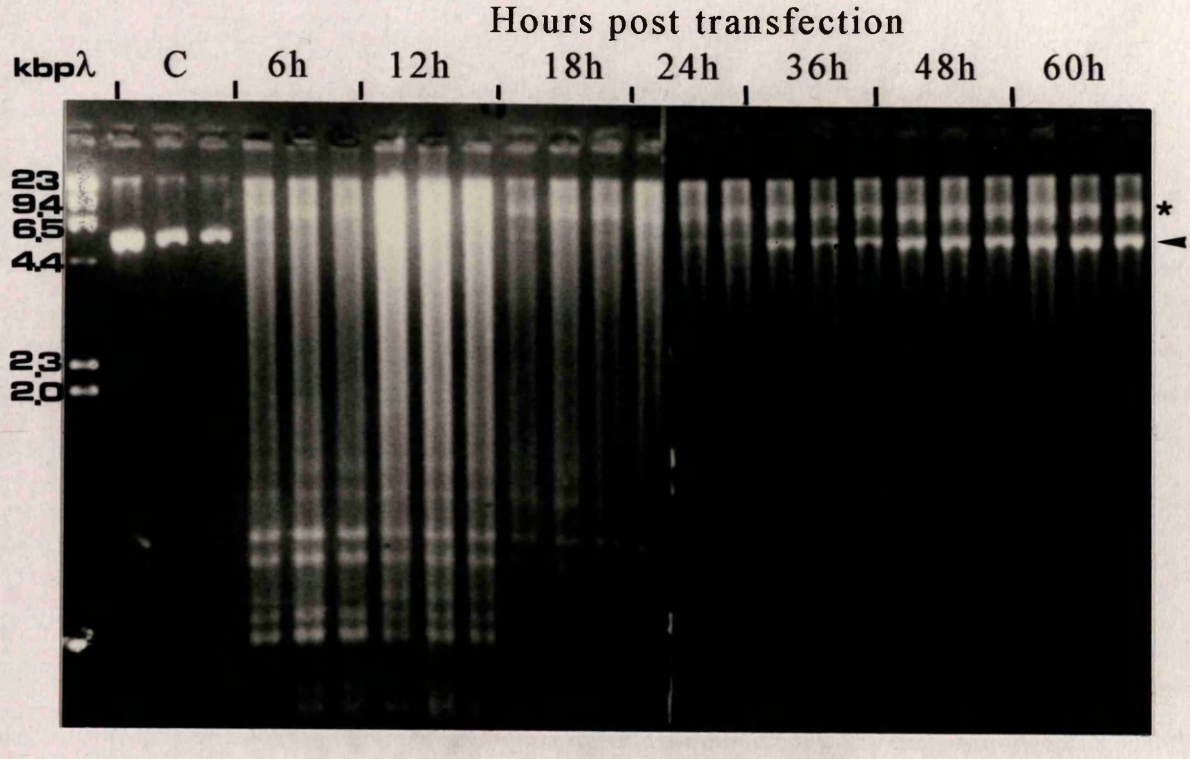


FIGURE 5.4:

Time course for replication of the pZ189 plasmid in FG-293 cells. Aliquots of linearised pZ189 DNA extracted from FG-293 cells at 6, 12, 18, 24, 36, 48 and 60h post-transfection were electrophoresed on an agarose gel containing ethidium bromide and the DNA bands visualised by UV light. Linearised pZ189 is indicated by an arrow. * - cellular contaminant, C - 500ng, 200ng and 100ng of linearised pZ189, λ - Lambda DNA size marker, the sizes of DNA fragments (bp) generated by HindIII digestion are indicated on the left of the gel. Transfections were performed in triplicate. Small bands of <2,000bp are fragments of pZ189 input DNA generated by DpnI digestion.

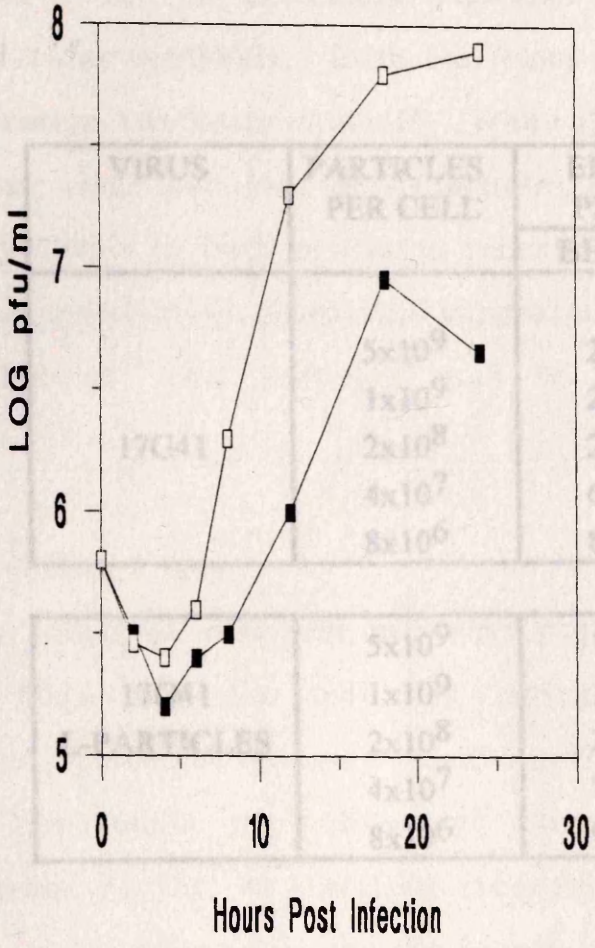


FIGURE 5.5 :

Single step growth curve of HSV-1 in FG-293 cells ■ and BHK cells □ infected at 5pfu/cell. Virus yield is given as LOG pfu/ml.

VIRUS	PARTICLES PER CELL	EFFICIENCY OF HOST PROTEIN SYNTHESIS	
		BHK-C13	FG-293
17G41	$5x10^9$	23.3%	18.7%
	$1x10^9$	26.3%	17.6%
	$2x10^8$	24.9%	44.3%
	$4x10^7$	63.8%	85.4%
	$8x10^6$	89.6%	100%
17G41 L-PARTICLES	$5x10^9$	17.0%	8.4%
	$1x10^9$	16.5%	14.8%
	$2x10^8$	24.5%	47.6%
	$4x10^7$	76.0%	56.6%
	$8x10^6$	90.5%	105.7%

TABLE 5.2:

Shows the level of host protein synthesis occurring in FG-293 and BHK cells following infection with either HSV-1 virus particles or L-particles, expressed as a % of the protein synthesis occurring in an uninfected control.

contains both the HSV-1 strain 17^+ and HSV-2 strain G UL41 (VHS) genes and is extremely efficient in shut off of host macromolecular synthesis. Both cell lines showed similar levels of host protein synthesis shut off. When the cells were infected with either virus particles or L-particles at $5x10^9$particles/cell, protein synthesis in both cell lines was reduced to approximately 20% of the uninfected control and suppression reduced steadily as the number of virus particles used to infect the cells was decreased.

(c) Infection times

The effect of different infection times on the recovery of plasmid DNA and the level of mutagenesis produced was determined. pZ189 transfected FG-293 cells were infected for 4h, 6h and 16h before the subsequent extraction of plasmid and measurement of the % mutation frequency. Recovery of the plasmid was unaffected by the the length of the infection period, averaging approximately 9000 transformants/transfection. The virus induced mutation frequency (0.05%) was also unaffected by the length of the infection period and was 2.5-fold higher that the mock infected level (Table 5.3). It was noted however that the virus induced increase in mutation frequency was not as great as expected, producing an average 2.5-fold increase instead of the 5-fold increase observed in the previous studies (Clarke and Clements, 1991; Hwang and Shillitoe, 1990).

Thus the FG-293 cell line proved suitable for use in the mutagenesis assay and the timing of transfection and infection appeared to have little effect on the overall efficiency of transformant recovery and the % mutagenesis frequency.

Infection conditions	Infection period	Total No. colonies	No. mutant colonies	% mutation frequency	Fold increase
Mock infected	4 h	8684 (1)	2	0.02	
Mock infected	6 h	9247 (1)	2	0.02	
HSV-1 infected	4 h	15860 (2)	9	0.057	2.8
HSV-1 infected	6 h	12554 (1)	6	0.048	2.4
HSV-1 infected	16 h	7736 (1)	4	0.05	2.6

TABLE 5.3:

Showing the number of transformants recovered and the % mutation frequency induced over different time periods of infection with wt HSV-1. The number of experiments carried out is given in parenthesis.

5.3.5 Transformation of E. coli strain MBM7070

Two methods of transformation were compared, heat shock described in Section 5.2.2 (d), using competent cells prepared by three calcium chloride derived methods A, B and C described in Section 5.2.2 (c) and electroporation described in Materials and Methods 2.2.10 (a) and (b). 100pg of either pZ189 or pUC18 DNA was used to transform MBM7070 cells by the methods detailed above, the results are shown in Table 5.4. Electroporation proved to be the method of choice producing $1.8x10^8$ transformants/μg of pZ189 DNA. The other methods producing 10^6 transformants/μg of pZ189 DNA.

5.3.6 HSV induced mutagenesis

The pZ189 mutagenesis assay was used to determine the mutation frequency induced by infection with a range of HSV-1 virus stocks. Cells were infected at a moi of 10 pfu/cell for 6h, the results of these experiments are shown in Table 5.5. Two of the experiments were carried out in Prof. E. Shillitoe's laboratory under the supervision of his research assistant S. Zhang. The virus induced mutation frequency was determined on average to be 0.043%, 2.5-fold above the spontaneous mutation frequency of 0.02%. These results are in agreement with those presented in Table 5.3. No difference in the ability to increase the mutagenic frequency was observed between the three HSV-1 strains or the four HSV-1 (17^+) stocks used. Included as a positive control for the assay are the results of transfection studies using hydroxylamine mutated pZ189 DNA. The resulting mutation frequency was extremely high 0.62% demonstrating that there is no deficiency in the detection of mutations which could account

Transformation method	Plasmid pZ189		pUC18
A	1.2×10^6 (7)		5.36×10^6 (5)
B	1.41×10^6 (6)		5.9×10^6 (6)
C	2.62×10^5 (3)		4.49×10^6 (3)
D	1.8×10^8 (6)		4.4×10^8 (2)

TABLE 5.4:

Comparison of four transformation methods. Values represent the mean number of transformants produced from 1μg of plasmid DNA. The number of experiments from which the mean was derived is given in parenthesis. Transformation was carried by heat shock using competent cells prepared by :-

A - calcium chloride precipitation with DTT and MES,

B - calcium chloride precipitation

C - calcium chloride precipitation with a DMSO boost.

Method D - transformation by electroporation.

Virus stock	Total No. colonies	No. of mutant colonies	% Mutation frequency	Fold increase
(mock infected)	49654 (6)	11	0.022	
A	12554 (2)	6	0.048	2.2
B	9188 (1)	1	0.01	nil
C	7636 (2)	3	0.039	1.77
D*	9664 (1)	6	0.06	2.73
KOS	15168 (2)	8	0.053	2.4
14-012 *	6196 (1)	3	0.048	2.2
hydroxylamine mutated pZ189	5648 (2)	35	0.62	28

TABLE 5.5:

Shows the % mutation frequency induced by infection with HSV-1 (17^+) stocks A ($1.1x10^{10}$pfu/ml), B ($2x10^{10}$pfu/ml), C ($1x10^{10}$ pfu/ml) D ($2.55x10^6$ pfu/ml) HSV-1 KOS ($2.8x10^8$ pfu/ml) and HSV-1 14-012 ($8x10^6$ pfu/ml) The number of experiments carried out is given in parenthesis. * Experiments carried out in Prof. E. Shillitoe's laboratory, using Cos-1 cells. Shown in the table is % mutation frequency produced by hydroxylamine treatment of the pZ189 plasmid.

for the reduced levels of HSV induced mutation frequency observed.

5.4 Discussion

The short term aim of increasing the efficiency of the mutagenesis assay has been achieved. Recovery of transformants from the mock infected control and virus infected test averaging 8448 and 7427 transformants/10μg transfected DNA respectively. This compares favourably with the data of Hwang and Shillitoe (1990). The virus induced mutation frequency was shown consistently to be around 2.5-fold higher than the spontaneous mutation frequency, which demonstrates that HSV-1 infection can increase the level of mutations occurring in target DNA and by inference cellular DNA. However this observed increase in the mutation frequency induced by HSV infection was lower than that obtained by Clarke and Clements, (1991) and Hwang and Shillitoe (1990). Statistical analysis of this data using Fisher's exact test showed that the virus induced mutation frequency was not significantly different from the spontaneous mutation frequency ($p>0.5$).

Fulfilment of the original aim of this study, identification of the mutagenic property of the virus, requires that individual properties of viral proteins and/or viral DNA be examined separately from the virus as a whole. Any mutation frequency produced would be likely under these circumstances to be lower than that produced by the whole virus. Using this assay at its current level of sensitivity it would seem unlikely that any changes in mutation frequency could be unequivocally linked to a particular property of the virus. Considerable time had already

been spent on optimisation of transformant recovery in the mutagenesis assay and further optimisation was deemed impractical. In view of this and the fact that Prof. E. Shillitoe had intimated that he was ready to publish a detailed analysis of the mutagenic properties of HSV (Shillitoe *et al.*, 1993), it was decided that further work on the project would not be carried out.

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