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ANALYSIS OF THE HSV-1 UL2 AND UL1 GENES BY INSERTIONAL MUTAGENESIS

by

James Mullaney

A thesis presented for the degree of
Doctor of Philosophy

in

The Faculty of Science,
University of Glasgow

Institute of Virology
Church Street
Glasgow G11 5JR

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SUMMARY

Insertional mutagenesis has been used to investigate the functional role of the HSV-1 UL1 and UL2 gene products in the lytic cycle of the virus. The genes were mutagenised by disruption of the protein coding sequences, which was achieved by inserting a segment of foreign DNA into a plasmid-cloned copy of the target gene. The insert contained a marker gene, the *Escherichia coli LacZ* gene under the direction of the SV40 early promoter, and was tranferred into the *wt* genome by homologous recombination of the flanking sequences.

Recombinant progeny were isolated by the addition of X-gal, a chromogenic substrate for B-galactosidase (the product of the *LacZ* gene) to an agar overlay. Plaques of recombinant virus, which appear blue using this screening technique, were subjected to plaque purification.

This technique was used in attempts to construct three different recombinants. In all three cases, the co-transfection gave rise to blue plaques, and in two cases the recombinants were purified. The first of these (designated in1601) carried the insert in the coding region of the gene UL2 as verified by restriction endonuclease analysis. Earlier mapping studies had indicated a possible function for the product of this gene, namely uracil-DNA glycosylase, a DNA repair enzyme which appears to be ubiquitous. Enzyme assays performed on extracts of tissue culture cells infected with in1601 were consistent with this assignation, as they demonstrated that in1601-infected extracts had only mock-infected levels of uracil-DNA glycosylase activity, but *wt* levels of three other virus-induced enzymes. Final confirmation came with comparison between the amino acid sequence of the UL2 gene product and the amino acid sequences translated from other known uracil-DNA glycosylase genes (*E. coli*, *Saccharomyces cerevisiae* and human). Immunoblotting analysis, using

antisera raised against a synthetic oligopeptide representing the carboxy terminus of the protein, showed a reaction with a protein of approximate M_r 39,000, which is similar to enzymes from other sources.

The mutant was readily isolated and possesses growth characteristics very similar to *wt*, indicating that the enzyme does not appear to be required for lytic growth, at least in the tissue culture system employed for growth and maintenance of the recombinant. This is in contrast with the observations that a high degree of amino acid conservation exists between the enzymes from different sources, and that the gene is found in all of the herpesviruses sequenced to date. The latter would tend to imply an important role *in vivo* for this enzyme. It is accepted that this *in vivo* function is the removal of uracil from DNA, brought about by deamination of cytosine. This is a potentially mutagenic event, and if left uncorrected, would result in a G.C to A.T transition mutation.

The second recombinant which was purified was designated in1602 and was constructed by co-transfection of the mutated plasmid-borne copy of the UL2 gene with a virus containing an insertion in the gene encoding the virus induced dUTPase. This enzyme is thought to be responsible for minimising the misincorporation of dUTP into DNA during replication, by lowering the pool of dUTP. Although this also results in the presence of uracil in the DNA, it will not result in mutagenesis if left uncorrected, as subsequent replication will not alter the sequence of the DNA.

Misincorporation of dUTP into DNA and *in situ* deamination of cytosine are the two major mutagenic events which result in the presence of uracil in DNA. Therefore the recombinant in1602 is deficient in both of these genes, as confirmed by restriction analysis, and in both of these activities, as confirmed by enzyme assays.

The third recombinant, whose construction was attempted unsuccessfully, represented insertion of the *LacZ* sequences into the coding sequence of the gene UL1. Although the initial co-transfection also yielded blue plaques, these failed to grow through subsequent rounds of plaque purification. The co-transfection was repeated twice and repeated attempts were made to titrate the blue plaques obtained from the co-transfection, all without success. Control experiments done in tandem with these titrations indicate that the procedure was functioning normally. The two most likely explanations for this phenomenon are: (a) The recombinant was unstable due to other, unpredicted alterations to the genome. This has been observed in the construction of other recombinant viruses, but usually when the construction has involved duplication of virus DNA sequences. (b) There is a stringent requirement for the UL1 gene product in the replicative cycle of the virus. At present there is no direct evidence which would support either of these explanations at the expense of the other.

Searches of amino acid sequence databases failed to identify any sequences which could be homologues of this protein, so there is no further functional information about this protein.

NON-STANDARD ABBREVIATIONS

A	deoxyadenylic acid residue in DNA
AP	apurinic/apyrimidinic
BHK	baby hamster kidney cells
BSA	bovine serum albumin
bp	base pair(s)
C	deoxycytidylic acid residue in DNA
C terminus	carboxy terminus of a protein
CCV	channel catfish virus
dAMP	2'deoxyadenosine 5'-monophosphate
dATP	2'deoxyadenosine 5'-triphosphate
dCTP	2'deoxycytidine 5'-triphosphate
dGMP	2'deoxyguanosine 5'-monophosphate
dGTP	2'deoxyguanosine 5'-triphosphate
dTTP	2'deoxythymidine 5'-triphosphate
dUMP	2'deoxyuridine 5'-monophosphate
dUTP	2'deoxyuridine 5'-triphosphate
DNase	deoxyribonuclease
EBV	Epstein-Barr virus
EDTA	ethylene diaminetetra acetic acid
G	deoxyguanylic acid residue in DNA
HCMV	human cytomegalovirus
HHV-6	human herpes virus 6
HSV-1	herpes simplex virus type-1
HSV-2	herpes simplex virus type-2
HVS	herpesvirus saimiri
HVT	herpesvirus of turkeys
kb	kilobase(s)
kbp	kilobase pairs
MDV	Marek's disease virus
M_r	Relative molecular mass
mu	map units
ORF	open reading frame
pfu	plaque-forming units
pi	post-infection
PMSF	phenylmethylsulphonyl fluoride

RNase	ribonuclease
SDS	sodium dodecylsulphate
T	deoxythymidylic acid residue in DNA
TCA	trichloroacetic acid
TK	thymidine kinase
TEMED	N,N,N',N' tetramethyl ethylenediamine
tris	tris(hydroxymethyl)aminomethane
ts	temperature-sensitive
UV	ultra-violet radiation
V_{mw}	viral protein
VZV	varicella-zoster virus
X-gal	5-bromo-4-chloro-3-indolyl B-D-galactopyranoside

INTRODUCTION

1.1 OBJECTIVES

Now that the genome of herpes simplex virus type-1 (HSV-1) has been sequenced in its entirety, the way is open for a systematic investigation of the functions of the genes encoded therein. The objective of the work described in this thesis was to investigate the functions of certain HSV-1 genes which had been previously uncharacterised. The route chosen for this investigation was that of insertional mutagenesis by homologous recombination with a plasmid carrying a marker gene inserted into the protein coding sequence of the target gene. The resulting recombinant viruses have been subjected to functional analysis and their deduced or proposed functions are discussed. The results are also discussed with respect to the evolutionary significance of these viruses.

This introduction comprises a short overview of the biology of the *Herpesviridae* family in general and of HSV-1 in particular. This is followed by an account of the structure and organisation of the HSV-1 virion and the structure and regulation of the genome. Succeeding this is a brief account of some of the proteins involved in DNA metabolism and replication, after which is a brief discussion of the evolutionary relationships between HSV-1 and the other human herpesviruses. The final section begins with an introduction to mutagenesis in HSV-1, before moving on to a topic which is directly relevant to the work described later in this thesis, namely the occurrence of uracil in DNA and the role of uracil-DNA glycosylase in its removal.

The field of herpesvirus virology is a diverse and rapidly advancing one. There is currently a large body of information available about the biology of HSV-1 and much of this information is remote from the theory and experimental work described in this thesis. For this reason, several areas of herpesvirus virology are not mentioned in this introduction or are discussed in a superficial manner. These areas include latency, transformation, pathogenesis and, to large extent, replication and regulation of gene expression.

1.2 CLASSIFICATION OF THE *HERPESVIRIDAE*

The family *Herpesviridae* is a large and diverse group of viruses which have a wide variety of hosts, from fish (e.g. channel catfish virus; CCV), birds (Marek's disease virus; MDV) through many vertebrates to humans (HSV-1) (reviewed in Roizman, 1982). All members of this family share certain physical characteristics. The virus particle is large, typically 120-200 nm in diameter; the viral capsid has a characteristic structure and is composed of 150 hexameric capsomers and 12 pentameric capsomers arranged in an icosahedral fashion (Wildy *et al.*, 1960; Schrag *et al.*, 1989); the viruses replicate within the nuclei of infected cells. The genomes of herpesviruses are large linear duplex DNA molecules with M_r from 80 to 150 X 10^6 (Honess and Watson, 1977) and are composed of unique and reiterated sequences.

1.3 HUMAN HERPESVIRUSES

Man is known to be the natural host for six herpesviruses; these are HSV-1, HSV-2, varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV) and human herpes virus type 6 (HHV-6, formerly known as human B-lymphotropic virus). The first five are relatively well-characterised with respect to biology and pathogenesis (Roizman, 1982; 1983; 1985; Roizman and Lopez, 1985). In contrast, HHV-6 was only recently discovered (Salahuddin *et al.*, 1986) and was first isolated from persons suffering from AIDS or other lymphoproliferative disorders. It has only been associated with one disease condition, exanthem subitum (a transient childhood illness, presumed to represent the primary infection). There is growing evidence that the majority of adults have been exposed to it (Briggs *et al.*, 1988). Recently, there has been a report of a seventh human herpesvirus, designated HHV-7 (Frenkel *et al.*, 1990), but this is as yet uncorroborated.

Members of the family *Herpesviridae* are further classified according to certain biological characteristics such as site of latency and restriction of host cell. However, this classification is neither rigorous nor exhaustive. These subgroups are the *Alpha-, Beta-* and *Gammaherpesvirinae* (Roizman, 1982; Roizman *et al.*, 1981)

1.3.1 *ALPHAHERPESVIRINAE*

Members of this sub-family (including HSV-1, HSV-2 and VZV) are mostly neurotropic viruses and generally have a short replicative cycle, usually less than 24 hours. This sub-family is characterised by variable host cell range, both *in vitro* and *in vivo* and primary infection is usually restricted to a mild epidermal or mucosal infection (usually small mucocutaneous lesions with HSV-1 and -2, and chicken pox with VZV). These viruses can persist in the central nervous system as a latent asymptomatic infection. The individual may then remain free of any clinical manifestation of the infection for months or even years, although there is generally periodic spontaneous reactivation of the virus, leading to further vesicular mucocutaneous lesions in the case of HSV-1 and -2, and shingles in the case of VZV.

1.3.2 *BETAHERPESVIRINAE*

Members of the *Betaherpesvirinae* (including HCMV) exhibit a restricted host cell range. Infection is widespread but is usually asymptomatic. However, infection can have serious or even life-threatening consequences in neonates or immunocompromised persons. *In vitro*, these viruses replicate best in fibroblasts but have a long replicative cycle, greater than 24 hours and infection often results in the formation of enlarged infected cells (cytomegalia). The virus can establish a latent infection in secretory glands and lymphoreticular cells. HCMV appears to be aetiologically implicated in the development of the European form of Kaposi's sarcoma (Giraldo *et al.*, 1975)

1.3.3 *GAMMAHERPESVIRINAE*

This sub-group includes EBV. Its members, like those of the *Betaherpesvirinae*, exhibit a restricted host cell range and have a tropism for lymphocytes. Although they all replicate in lymphoblastoid cells, the length of the replicative cycle varies between members. They often establish persistent, latent infections, and are capable of transforming lymphoblastoid cells in the process. EBV has a strong association with nasopharyngeal carcinoma and Burkitt's lymphoma (Desgranges *et al.*, 1975; de The, 1979).

1.4 BIOLOGY AND PATHOLOGY OF HERPES SIMPLEX VIRUS

Herpes simplex virus infection is typified by characteristic, vesicular lesions of the facial and genital mucosae. It is usually the case that infections which result in facial lesions, particularly around the mucosae of the mouth and nose, are caused by infection with HSV-1, and that infections leading to lesions around the genital mucosae are caused by HSV-2. However the two sites are not mutually exclusive for the respective viruses and increasing numbers of genital infections caused by HSV-1, and facial lesions as a result of HSV-2 infection, are being reported (Whitley, 1985). Most adults have been exposed to HSV-1 infection. Primary infection by HSV-1 usually occurs in childhood but the clinical manifestations of this infection range from being asymptomatic to ulcerative, vesicular lesions and gingivostomatitis. HSV establishes a latent infection in the trigeminal ganglia and can spontaneously reactivate via sensory neurons to the skin, usually as the archetypal lip lesion - herpes labialis (Whitley, 1985).

Although all of the clinical conditions described so far have not been of life-threatening severity, HSV is also responsible for a number of serious conditions, although these do not occur with the same frequency as the less serious conditions described so far. The more hazardous complications include herpes simplex induced acute necrotising encephalitis, eczema herpeticum and keratoconjunctivitis, which can lead to corneal blindness (Whitley, 1985). Also, the implications of HSV infection in immunocompromised individuals and neonates are far more serious (Rawls, 1973). HSV-2 infection has also been implicated in the aetiology of at least a proportion of cases of cervical carcinoma (Naib *et al.*, 1966; Aurelian *et al.*, 1973; McDougall *et al.*, 1980; Eglin *et al.*, 1981; Park *et al.*, 1983), although its precise role in the transformation process is not clear (zur Hausen, 1982; Galloway and McDougall, 1983; Rawls, 1985).

1.5 STRUCTURE OF THE HSV VIRION

The herpesvirus virion is a complex structure which can be regarded as being composed of four concentric layers (Hay *et.al*, 1987). These layers are the core, capsid, tegument and envelope. The virus DNA is to be found in the core, which is at the centre of the virion (Hay *et al.*, 1987). Recently, Schrag *et al.* (1989) have examined the HSV-1 nucleocapsid by computer analysis of low dose cryo-electron images of ice-embedded capsids. Using this technique, the authors have resolved the three-dimensional structure of the nucleocapsid. This is the first analysis where the structure has been determined directly as opposed to being inferred. It is proposed that the nucleocapsid is organised into at least three distinct structural layers, designated the outer, intermediate and inner shells (Schrag *et al.*, 1989).

The outer shell is defined as the mass between the radii of 425 A and 625 A. The study by Schrag *et al.* (1989) indicates three major structural features associated with the outer shell. The first of these consists of six-coordinated capsomers which appear to be hexagonal and to interact with connecting proteins. Predictions of capsomer masses are consistent with the nomination of VP5 as the capsomer protein (Weller *et al.*, 1987) and confirm studies which demonstrated the six-fold symmetry of these capsomers (Furlong, 1978; Steven *et al.*, 1986). The second structural feature of the outer shell is the presence of twelve capsomers residing on the icosahedral five-fold positions, which Schrag *et al.* (1989) have postulated are also composed of the virion protein VP5. The third structural feature is the presence of a mass density connecting adjacent capsomers. Surface labelling studies by Braun *et al.* (1984) demonstrated that VP23, a protein of M_r 36,000, is located on the outer capsid surface and therefore may comprise the capsomer connections.

The intermediate shell is described as the mass between the radii of 310 A and 425 A. It is proposed that the density between these radii represents a single structural layer. There are two lines of evidence which suggest this layer is composed of protein. Studies by Cook and Stevens (1970) showed that this layer in VZV sections was unaffected by DNase treatment and remained adhered to the inner capsid surface. Secondly, it is known that HSV-1 nucleocapsid assembly is a multi-stage process, and there are several stages in which internal proteinaceous structures are seen within the capsid. These "partial cores" are heterogeneous in both size and appearance. *ts* mutants of HSV-1 which fail to package DNA at the non-permissive temperature also produce these partially cored structures and it has been been suggested that they are part of the normal assembly process (Schaffer *et al.*, 1974; Preston *et al.*, 1983). These capsids do not contain DNA and so it is thought that this layer must be composed of protein. The reconstruction data of Schrag *et al.* (1989) suggest that the underlying lattice of this layer has T=4 icosahedral symmetry, although the composition of this layer is at present a matter for debate. It has been suggested that VP21, a protein of M_r 45,000, is the major component of the core (Gibson and Roizman, 1972) but this has since been disputed (Braun *et al.*, 1984). Further studies are required to establish the composition of the T=4 protein shell.

The mass density below the radius of 310 A is proposed to contain the genomic DNA. Although the structural organisation of this region could not be resolved by Schrag *et al.* (1989), they were able to accurately calculate the average mass density. The calculated dry mass is 2.13 x 10^{-16} g, which is large enough to accommodate some protein as well as the DNA.

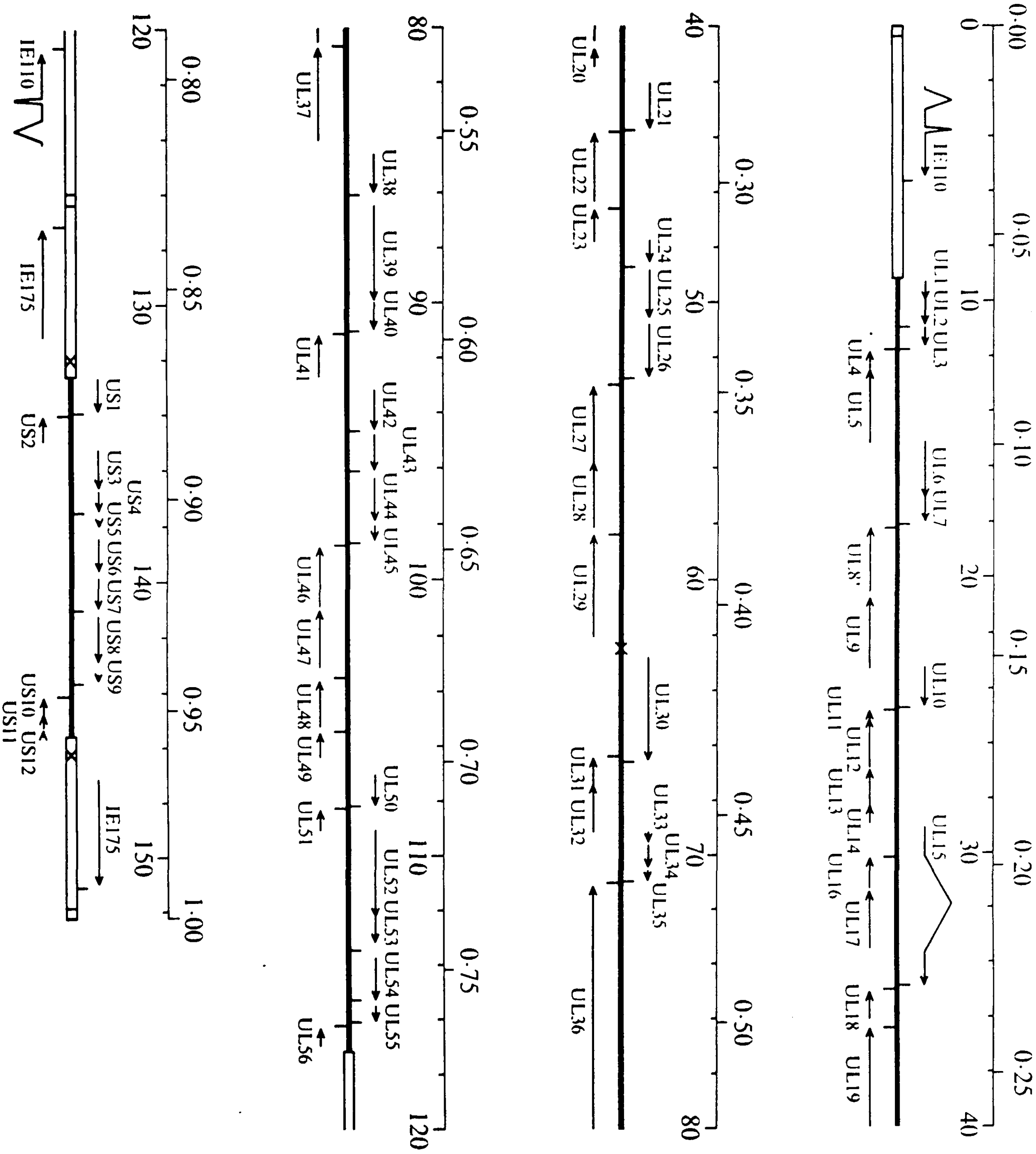
Surrounding the capsid is a layer of amorphous proteinaceous material known as the tegument (Roizman and Furlong, 1974). The diameter of this layer is variable and its function is not known at present.

The outermost layer of the virion is a lipid bilayer known as the envelope (Wildy *et al.*, 1960). The envelope is derived from the nuclear membrane and is studded with at least seven structural glycoproteins (namely gB, gC, gD, gE, gG, gH and gI) which are virus encoded (Buckmaster *et al.*, 1984; Spear, 1985; Frame *et al.*, 1986; McGeoch and Davison, 1986; Longnecker *et al.*, 1987).

1.6 STRUCTURE OF THE HSV GENOME

The genome of HSV-1 strain 17, as isolated from virions, is composed of a single, linear molecule of double stranded DNA, containing 152,260 bases in each strand (McGeoch *et al.*, 1985; McGeoch *et al.*, 1986, McGeoch *et al.*, 1988; Perry and McGeoch, 1988). However HSV-1, like all herpesviruses studied, exhibits heterogeneity between strains and so this number refers only to the isolate which has been sequenced. Genomic HSV DNA is unmethylated (Low *et al.*, 1969) and HSV-1 has a base composition of 68.3% G+C (McGeoch *et al.*, 1988). The G+C content is not uniform throughout the genome; the short repeat region, for example, has a base composition of 79.5% G+C (McGeoch *et al.*, 1986) and the short unique region is 64.3% G+C (McGeoch *et al.*, 1985). There is a single unpaired residue at each 3' terminus (Mocarski and Roizman, 1982). It is proposed that HSV-1 genome contains at least 72 distinct genes (McGeoch *et al.* 1988), although there are known to be two copies of two genes. The locations of all the proposed genes are depicted in figure 1.1.

HSV DNA is regarded as being composed of two segments, designated the long (L), and the short (S) regions, which are covalently joined (Delius and Clements, 1976; Roizman, 1979). Both of these segments are composed of unique sequences (U_L and U_S respectively) bounded by inverted repeat sequences. Thus, L is composed of U_L which is flanked by inverted repeat sequences at the terminus (TR_L) and the joint (IR_L). Similarly, S is composed of U_S, TR_S and IR_S. The sequences of R_L and R_S are distinct, with the exception of a 400 bp direct repeat, designated the *a* sequence, at the genome termini. There is one copy of this redundancy at the S terminus and variable numbers of it at the L terminus. It is also present, again in variable copy numbers and this time inverted with respect to the terminal *a* sequences, at the L\S joint (Wagner and Summers, 1978). The redundant sequence in TR_L and IR_L, excluding the *a* sequence, is known as the *b* sequence. Similarly, the short repeat regions are composed of an *a* and a *c* sequence (Roizman, 1979). The overall arrangement of the HSV-1 genome is shown diagrammatically in figure 1.2, and in figure 1.3 it is compared with the genomes of other human


0·00
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120
130
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IE110
UL1 UL2 UL3
UL4 UL5
UL6 UL7
UL8 UL9
UL10
UL11 UL12 UL13 UL14
UL15
UL16 UL17
UL18 UL19
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UL22 UL23
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UL41
UL42 UL43 UL44 UL45
UL46 UL47 UL48 UL49
UL50 UL51
UL52 UL53 UL54 UL55
UL56
IE110
IE175
US1 US2
US3 US4 US5 US6 US7 US8 US9
US10 US11 US12
IE175

Figure 1.2 : Structure of the HSV-1 genome

The figure depicts the genome of HSV-1, with the solid lines representing unique sequences, and the open boxes representing the repeated sequences. The positions of the *a*, *b* and *c* sequences are indicated, as are their respective inverted repeated units *a'*, *b'* and *c'*. The upper scale bar is in map units (mu).

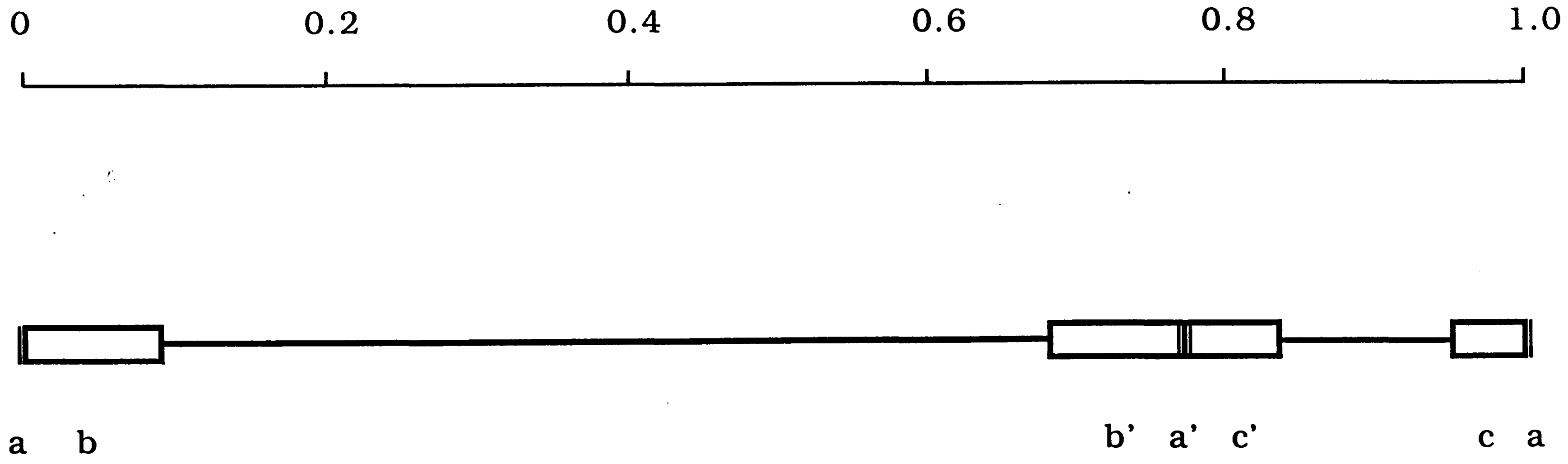
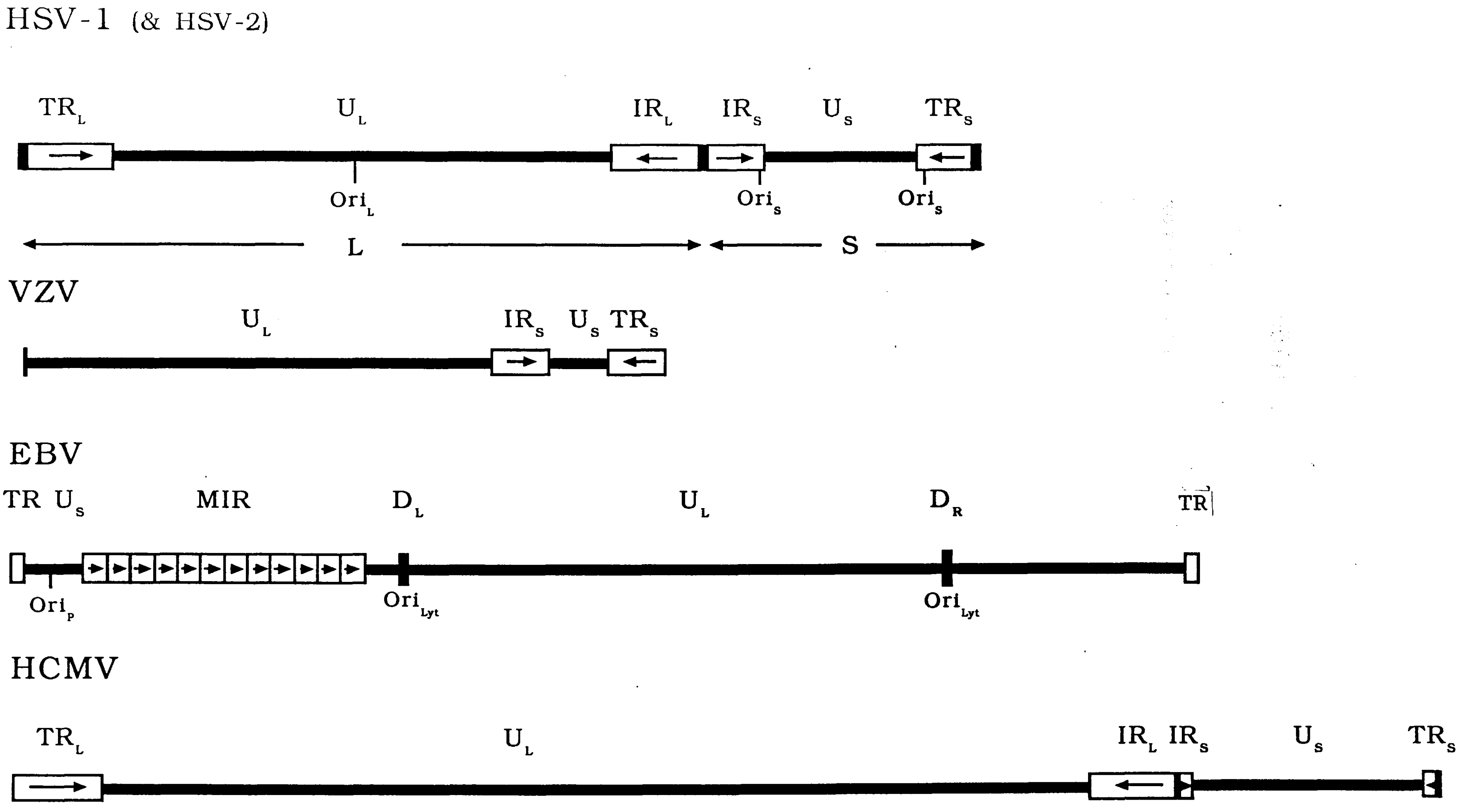

0
0.2
0.4
0.6
0.8
1.0
a
b
b’
a’
c’
c
a

Figure 1.3 : Layouts of human herpesvirus genomes

The genomic layouts of HSV-1, HSV-2, VZV, EBV and HCMV. As before, unique sequences are denoted by solid lines, and repeated sequences are denoted by open boxes. The arrows indicate the relative directions of reiterated sequences.

I refers to internal sequences
T refers to terminal sequences
U refers to unique sequences
R refers to repeat sequences
S refers to sequences in short segments
L refers to sequences in long segments

Ori refers to origins of DNA replication
MIR is the multiple internal repeat (of EBV)


HSV-1 (& HSV-2)
TR$_L$
U$_L$
IR$_L$
IR$_S$
U$_S$
TR$_S$
Ori$_L$
Ori$_S$
Ori$_S$
L
S
VZV
U$_L$
IR$_S$
U$_S$
TR$_S$
EBV
TR
U$_S$
MIR
D$_L$
U$_L$
D$_R$
TR
Ori$_P$
Ori$_{Lyt}$
Ori$_{Lyt}$
HCMV
TR$_L$
U$_L$
IR$_L$
IR$_S$
U$_S$
TR$_S$

herpesviruses.

1.6.1 ISOMERISATION OF THE HERPES SIMPLEX VIRUS GENOME

Because the sequences found at the termini are repeated internally, Sheldrick and Berthelot (1974) suggested that recombination between these sequences might be possible. The result of this would be inversion of the L or S segments with respect to each other, producing four sequence-orientation isomers. The existence of four such isomers was subsequently demonstrated, by restriction enzyme analysis (Hayward *et al.*, 1975; Clements *et al.*, 1976) and partial denaturing mapping of heteroduplex DNA (Delius and Clements, 1976). DNA extracted from *wt* virions is composed of all four isomers in equimolar amounts and all four are equally viable in terms of growth and replication (Davison and Wilkie, 1981). The isomers are termed P (Prototype), I_L (L inverted with respect to P), I_S (S inverted with respect to P) and I_{SL} (Both S and L inverted with respect to P) (Roizman *et al.*, 1979). The four isomers are depicted in figure 1.4. Studies on intertypic recombination between HSV-1 and -2 demonstrated that inversion has an absolute requirement for the *a* sequences, with those at the L terminus and the L\S joint being sufficient to mediate inversion (Davison and Wilkie, 1983b). Mutagenesis studies have shown that deletion of the L\S joint *a* sequence abolishes inversion, which can be restored by insertion of additional copies (Poffenberger *et al.*, 1983; Mocarski *et al.*, 1980).

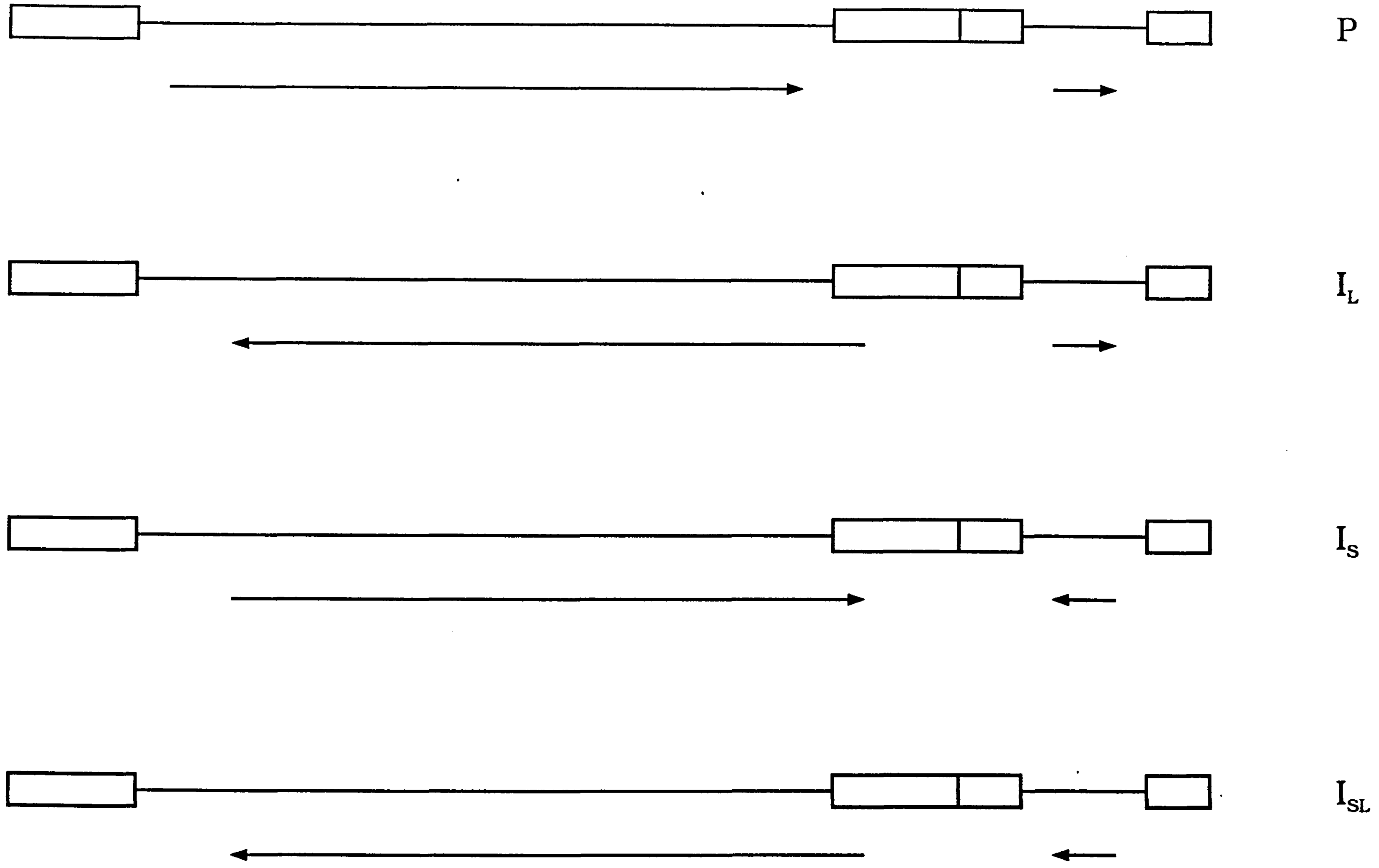
1.6.2 HERPES SIMPLEX VIRUS DNA REPLICATION

HSV DNA replication is one process in the infective cycle which at present is incompletely characterised. It is

Figure 1.4 : Sequence orientation isomers

The four sequence orientation isomers of HSV-1 are shown. The arrows refer to the relative orientations of the L and S segments in the isomers. The nomenclature is as follows;

P	Prototypic orientation
I_L	L inverted (with respect to P)
I_S	S inverted
I_{SL}	L and S inverted


P
I_L
I_S
I_SL

known that virus DNA accumulates in the nucleus of cells after infection, where it appears to circularise. This circularisation may be as a result of direct ligation of the termini (Poffenberger and Roizman, 1985), possibly mediated by the complementary unpaired residues present at the 3' termini of the genome (Mocarski and Roizman, 1982). Electron microscopic studies have revealed the presence of "large tangled masses" of replicating DNA (Hirsch *et al.*, 1977; Jacob and Roizman, 1977). It has been demonstrated that, late in infection, virus DNA is found in a form which sediments rapidly and has a low relative abundance of termini (Jacob *et al.*, 1979; Jongeneel and Bachenheimer, 1981). It is accepted that virus DNA replication is by a rolling circle mechanism, as long head to tail concatemers are found late in infection (Stow *et al.*, 1983; Stow, 1985). Cleavage of concatemeric DNA into unit lengths is possibly mediated by site-specific signals found in the *a* sequence (Davison and Wilkie, 1981; Varmuza *et al.*, 1985). Cleavage of the DNA is thought to be coupled to encapsidation (Deiss and Frenkel, 1986), and the *cis*-acting signals responsible for both events are all believed to be within the *a* sequence (Stow *et al.*, 1986).

Studies using defective virus DNA (Frenkel *et al.*, 1975; Kaerner *et al.*, 1979) and electron microscopy (Friedmann *et al.*, 1977; Hirsch *et al.*, 1977) suggested the presence of at least two distinct *cis*-acting signals which could mediate HSV DNA replication: one located near the centre of U_L (Ori_L) and another in both copies of R_S (Ori_S) and therefore diploid. Direct evidence that both of these sites contain a functional origin of DNA replication was tendered by demonstration that monomeric defective virus DNA could be amplified by cotransfection with *wt* helper virus, yielding head to tail concatemers of the defective virus DNA (Vlazny and Frenkel, 1982; Spaete and Frenkel, 1982; Spaete and Frenkel, 1985). The origins were more finely mapped using plasmid-borne helper DNA to rescue the defect (Mocarski and Roizman, 1982; Stow, 1982; Weller *et al.*, 1985). Both origins have subsequently been sequenced (Gray and Kaerner, 1984; Whitton and Clements, 1984; Quinn and McGeoch, 1985; Weller *et al.*, 1985). Genomic analysis of the origins has demonstrated that both lie between two divergently transcribed genes. Ori_L lies between the 5' ends of the early genes UL29 and UL30 which encode the major DNA-binding protein and the DNA polymerase, respectively. Each copy of Ori_S lies between the 5' ends of two IE genes, IE175 and US1 in the case of IR_S and between IE175 and US12 in the case of TR_S. Whether or not this has implications for replication or transcription is not known. The individual proteins involved in virus DNA replication (both directly and indirectly) are discussed separately.

1.7 STRUCTURE AND ORGANISATION OF HSV GENES

The synthesis and processing of virus-specific RNAs is similar to that of cellular RNA. HSV DNA is transcribed in the nucleus of the infected cell (Wagner and Roizman, 1969). Host encoded RNA polymerase II is utilised throughout infection (Alwine *et al.*, 1974; Ben-Zeeve and Becker, 1977; Costanzo *et al.*, 1977). The RNAs are capped at their 5' ends and internally methylated in a manner similar to host cell transcripts (Bartkoski and Roizman, 1976; Moss *et al.*, 1977). Most virus RNAs are polyadenylated at their 3' ends (Bachenheimer and Roizman, 1972).

It is, therefore, not surprising that most HSV genes possess upstream and downstream regulatory regions similar to those of the host cell genes (McKnight, 1980; Frink *et al.*, 1981; Everett, 1983; Preston *et al.*, 1984; Rixon *et al.*, 1984; Rixon and McGeoch, 1985). These include the 5' promoter sequences such as the 'TATA' box and 'CAAT' box motifs (Gannon *et al.*, 1979; Wasylyk *et al.*, 1980; Benoist *et al.*, 1980; Efstratiadis *et al.*, 1980) and the 3' pre-mRNA cleavage/polyadenylation signal, 'AATAAA' (Proudfoot and Brownlee, 1974, 1976; Fitzgerald and Shenk, 1981; Zarkower *et al.*, 1986). A number of HSV genes have a discernible 'TATA' box motif situated between 25-30 bp upstream of the translational start site of the gene (Corden *et al.*, 1980; McGeoch *et al.*, 1985). The sequence AATAAA or a close variant, ATTAAA has been found near the 3' termini of all HSV mRNAs mapped (McGeoch *et al.*, 1985). There is another motif, the 'YGTGTTYY' sequence, which is found downstream from the polyadenylation signal in a number of eukaryotic genes (Taya *et al.*, 1982; McLauchlan and Clements, 1983) and has subsequently been shown to be required for efficient processing of the 3' termini of mRNAs. This motif has been discovered in many HSV genes (McLauchlan *et al.*, 1985).

One aspect of gene structure which differs markedly from the situation in the host cell is the degree of splicing. Splicing is a relatively uncommon event in HSV gene organisation (Wagner, 1985) and only a few split genes are known (Watson *et al.*, 1981; Rixon and Clements, 1982; Frink *et al.*, 1983; Costa *et al.*, 1985; Perry *et al.*, 1986).

The genome of HSV-1 has been fully sequenced and the genetic organisation has been analysed (McGeoch *et al.*, 1985; 1986; 1988; Perry and McGeoch, 1988). It is proposed that there are 56 genes in U_L (designated UL1 to UL56), 12 in U_S (US1 to US12) and one in each copy of R_L and R_S, making a total of 72 genes encoding 70 distinct proteins (the genes present in the repeats being diploid). Although this analysis is extensive it is accepted as being open to augmentation. Indeed there has been a published report of another gene in R_L, upstream of IE110 (Chou and Roizman, 1986), but this assignment has been disputed (Perry and McGeoch, 1988). In general, the genetic organisation of HSV-1 is regarded as being compact but not unduly compressed. However, there is a region in both long repeat elements between the 3' end of IE110 and U_L, of approximately 3500 bp, whose functional potential is obscure. It has been demonstrated that part of this region is transcribed, most abundantly in latently infected neurons (Stevens *et al.*, 1987; Rock *et al.*, 1987; Spivack and Fraser, 1977). The transcript which initiates here is the latency associated transcript or LAT, and it is thought not to be translated into a protein product. Certainly no such protein has yet been elucidated and analysis of the proposed transcript does not support its existence (Perry and McGeoch, 1988; Wagner *et al.*, 1988).

1.8 REGULATION OF HSV GENE EXPRESSION

The expression of HSV genes is tightly regulated and systematically ordered in a sequential pattern. There are three broad groupings of genes. These are the immediate-early (IE) genes, the early (E) genes and the late (L) genes. Each of these is briefly discussed in turn.

1.8.1 IMMEDIATE-EARLY GENE EXPRESSION

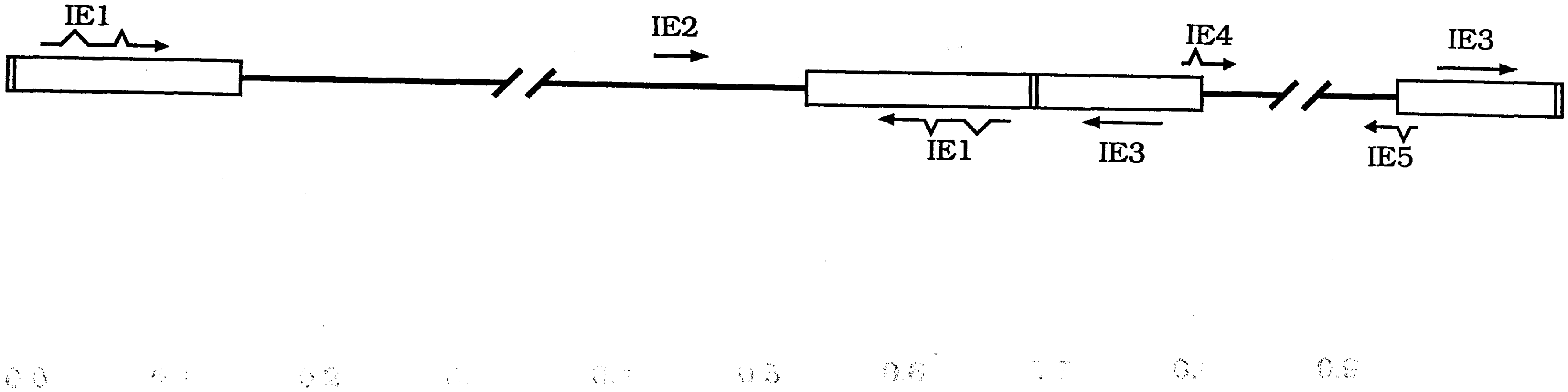
The first genes to be expressed are the five IE genes (Watson *et al.*, 1979). Expression of these genes is independent of *de novo* protein synthesis in newly-infected cells (Honess and Roizman, 1974; Harris-Hamilton and Bachenheimer, 1985). The five IE genes are located at or close to the inverted repeats, as shown in figure 1.5 (Clements *et al.*, 1979; Watson *et al.*, 1979; Anderson *et al.*, 1980; Watson *et al.*, 1981).

Although the IE genes are expressed immediately upon infection of the cell, their expression is subject to virion-mediated modulation (Batterson and Roizman, 1983). The product of gene UL48 (V_{mw}65, a major component of the tegument) has been to shown to augment IE gene expression from basal levels (Campbell *et al.*, 1984). This stimulation is specific to IE gene promoters (O'Hare and Hayward, 1985). IE gene expression is also controlled by a number of other virus genes. There is positive-feedback from expression of IE110, the product of IE gene 1 (O'Hare and Hayward, 1985) and down-regulation by IE175, the product of IE gene 3 (Preston, 1979). O'Hare and Hayward (1985) proposed that the overall level of IE gene expression may be controlled by the relative abundance of these two IE proteins.

It has been been known for some years that sequences upstream of the mRNA cap sites are important for control of IE gene expression (Post *et al.*, 1981; Mackem and Roizman, 1982a). There are sequence data available for the upstream regulatory regions of all five IE genes (Murchie and McGeoch, 1982; Mackem and Roizman, 1982a; 1982b; Whitton *et al.*, 1983). A highly conserved sequence motif is present in one or more copies, upstream of all IE genes (Mackem and Roizman, 1982b; Preston *et al.*, 1984). This sequence has the consensus 5'-TAATGARATTC-3' (where R = a purinic residue) and is conserved in HSV-2 (Whitton *et al.*, 1983;

Figure 1.5 : HSV-1 IE genes

The locations and orientations of the five IE genes are shown.


IE1
IE2
IE4
IE3
IE1
IE3
IE5
0.0
0.2
0.5
0.6
0.9

Whitton and Clements, 1984). Preston *et al.* (1984) demonstrated that this motif, which appears to be unique to IE genes, is crucial for stimulation of IE transcription by V_{mw}65. The conservation of this motif between all the IE genes and across the two serotypes is indicative of its importance in the regulation of IE gene expression.

1.8.2 EARLY GENE EXPRESSION

The next genes to be expressed are the E genes (Wagner *et al.*, 1972). A scheme of nomenclature has been proposed, whereby E genes are subdivided into two groups, β_1 and β_2, according to the time they are first expressed (Mavromara-Nazos *et al.*, 1986). Like the IE genes, the E genes are subject to regulatory control by a number of factors. IE175, as well as being responsible for repression of IE genes, also has a role in the activation of E gene expression (Watson and Clements, 1980). By itself, IE175 is capable of inducing E gene expression in short term transfection assays (Everett, 1984a). This induction appears to be specific and involves *cis*-acting sequences in the upstream regulatory region.

Short term transfection assays were also used to demonstrate that IE110 can independently stimulate E gene expression (O'Hare and Hayward, 1985). Transactivation by IE110 is less specific than that by V_{mw}65 or IE175 (Everett, 1984a) and is not present in all E genes. It is also the case that the level of transactivation of E genes by IE110 is variable (Gelman and Silverstein, 1985; O'Hare and Hayward, 1985).

Up-regulation of E gene expression is markedly enhanced in transfection assays where the genes encoding both IE110 and IE175 are co-transfected (Everett, 1984b; Quinlan and Knipe, 1985).

1.8.3 LATE GENE EXPRESSION

The last group of genes to expressed, at late times in infection, are the L genes (Wagner, 1972). Like the E genes, the L genes are divided into two groups; the leaky-late (γ_1) genes are expressed in the absence of virus DNA replication, and the true-late (γ_2) genes have a stringent requirement for virus DNA replication (Holland *et al.*, 1980).

Although the promoters of the L genes are similar in structure to those of E genes, the former have additional requirements for expression (Wagner, 1985). In transfection assays, the regulation of L gene expression appears mediated only by IE175 and IE110, although it is not clear if any other IE gene products are involved. Virus DNA replication also plays a significant part in L gene activation (Hall *et al.*, 1982; Johnson *et al.*, 1986). Watson and Clements (1980) proposed that IE175 is continuously required for transcription of L mRNAs. DeLuca *et al.* (1984) described a *ts* mutant of IE175 which, at the non-permissive temperature, expressed IE and E genes to the same level as *wt*, and initiated DNA replication, but which synthesised greatly reduced levels of L proteins.

In short term transfection assays, IE110 is capable of enhancing expression from L gene promoters to the same extent as from E gene promoters (Mavromara-Nazos *et al.*, 1986).

1.9 HSV ENCODED PROTEINS

The genome of HSV-1 is thought to encode at least 70 distinct polypeptides (McGeoch *et al.* 1988), although many more species can be detected by routine techniques. For example, Haarr and Marsden (1981) were able to detect at least 230 virus induced polypeptides by two dimensional electrophoretic analysis. It should be stated that among these are many processed species and so this impedes the enumeration of separate proteins. Amongst the first techniques employed in the functional analysis of distinct genes were the use of *ts* and drug resistant mutants. Marker rescue of thesemutationsfacilitated the assignment of genomic locations of specific genes (for example, see Stow *et al.*, 1978). At present, some sort of functional information is known for around 50 of the predicted genes but there are still aproximately 20 genes about which nothing is known (McGeoch, 1989). In general, HSV encoded proteins are loosely classified by many different criteria. Functional analysis organises the proteins into those involved in the structural integrity of the virion, those involved in processes of regulation and control and those associated with DNA replication and metabolism. Each of these groups is discussed in turn.

1.9.1 STRUCTURAL PROTEINS

The HSV virion is thought to contain 15-33 different structural polypeptides, including those of the nucleocapsid, the tegument and the glycoproteins located on the virion envelope (Spear and Roizman, 1980).

The rigid structure of the herpesvirus virion places architectural constraints on the size and number of proteins which make up the nucleocapsid. This is reflected in the striking similarity of the structural polypeptides from different herpesviruses (Dargan, 1986). The HSV-1 nucleocapsid is considered to be composed of seven strucural proteins. Gibson and Roizman (1972) identified six of these, with Heilman *et al.* (1979) and Cohen *et al.* (1980) subsequently identifying the seventh.

Very little is known about the nature of the tegument, and this scarcity of knowledge extends to the polypeptides which make up this structure. It is estimated that there are approximately 15 non-glycosylated polypeptides which the virion acquires during envelopment (Dargan, 1986). Most of these have yet to be functionally characterised, although it is known that at least some of them are phosphorylated *in vivo* (Gibson and Roizman, 1974). Spear and Roizman (1980) suggested that some of these are involved in envelopment, through interactions with the nucleocapsid and envelope proteins. One of the major tegument components is $V_{mw}65$, the product of gene UL48. This protein is involved in transactivation of IE genes (Campbell *et al.*, 1984), and has already been discussed.

The glycoproteins associated with the envelope are the most intensively studied of the structural proteins. In addition to their role as part of the envelope structure, HSV glycoproteins have been detected in both the nuclear and cytoplasmic membranes of infected cells (Spear *et al.*, 1970). HSV-1 is known to encode at least seven glycoproteins, namely, gB, gC, gD, gE, gG, gH and gI, which are the products of genes UL27, UL44, US6, US8, US4, UL22 and US7, respectively (Spear, 1985; Frame *et al*, 1986; McGeoch and Davison, 1986; Buckmaster *et al.*, 1984;

Longnecker *et al.*, 1987). All HSV-1 glycoproteins are known to have homologues in HSV-2 (Spear, 1985; Frame *et al.*, 1986; McGeoch *et al.*, 1987; D. McGeoch, personal communication). In addition to their structural role, the glycoproteins are thought to be involved in attachment and penetration of the host cell, in envelopment and budding of progeny virus particles and in the cell to cell spread of infection (Spear, 1985; Spear and Roizman, 1980).

1.9.2 PROTEINS INVOLVED IN CONTROL PROCESSES

Virus gene expression in HSV infected cells is subject to control at a variety of levels by a number of different proteins. These have already been discussed in section 1.8.

Many of the virus-encoded proteins are subject to post-translational modification, including phosphorylation, glycosylation and sulphation. These types of modification may alter the location or activity of proteins, both virus-encoded and host-derived. There are two HSV-1 genes, US3 and UL13, which encode amino acid sequences with motifs characteristic of the protein kinase family and are proposed to encode species of protein kinase (Chee *et al.*, 1989; McGeoch and Davison, 1986; Smith and Smith, 1989). The gene product of US3 has been identified as a protein kinase whose activity has been detected in infected cells, although its substrate has yet to be determined (Frame *et al.*, 1987; Purves *et al.*, 1987).

In addition to the proteins already mentioned, HSV also encodes a function which mediates the shutoff of host cell protein synthesis and degradation of host cell mRNA (Kwong *et al.*, 1988). This is known as the virion host shutoff (*vhs*) function and is encoded by gene UL41.

1.9.3 PROTEINS INVOLVED IN DNA REPLICATION

HSV encodes a number of proteins which are responsible for supplying the precursors for DNA replication (to be discussed separately) and for the replicative process itself. Among the latter are POL, the replicative DNA polymerase (Coen *et al.*, 1984), EXO, the alkaline exonuclease (Banks *et al.*, 1983) and mDBP, the major, single-stranded DNA binding protein (Weller *et al.*, 1983). It is known that these are not the only proteins required for replication of the viral genome and recently Challberg (1986) described a method for identifying those genes whose products are necessary for replication of HSV DNA. This was based on a short term transfection assay where a plasmid containing the *cis*-acting signals for replication was co-transfected with other plasmids, which supplied the required *trans*-acting genes. Employing this technique, Wu *et al.* (1988) were able to identify a total of seven genes whose products were necessary and sufficient to facilitate replication of the plasmid containing the origin of DNA synthesis. In addition to POL and mDBP, they demonstrated the requirement for the products of the genes UL5, UL8, UL9, UL42 and UL52. The products of all these genes are discussed below.

i DNA POLYMERASE

The replicative DNA polymerase is encoded by the gene designated as UL30. The HSV-induced polymerase activity is associated with a polypeptide with M_r of 136K (Powell and Purifoy, 1977). It is known that the activity is distinct from the host-cell DNA polymerase both biochemically and immunologically (Keir *et al.*, 1966b; Powell and Purifoy, 1977; O'Donnell *et al.*, 1987), that the protein is essential for virus replication (Purifoy *et al.*, 1977) and that it has both polymerase and 3' - 5' exonuclease activities (Weissbach *et al.*, 1973; Knopf, 1979). Recently, it has deen demonstrated that the POL gene product also contains an intrinsic ribonuclease H activity which specifically degrades duplex DNA or DNA/RNA heteroduplexes in the 5' to 3' direction (Crute and Lehman, 1989). It is proposed that this activity is analogous to the 5'-3' exonuclease activity associated with the DNA polymerase I of *E. coli* which is required for DNA repair and removal of RNA primers during replication (Kornberg, 1980). Although it has been shown to be active as a monomer *in vitro* (Knopf, 1979; Ostrander and Cheng, 1980), the HSV-2 DNA polymerase co-purifies with another protein, of M_r 55,000, now known to be the product of the gene UL42 and it is now considered that the holoenzyme is a heterodimer of the UL30 and UL42 gene products (Vaughan *et al.*, 1984; Parris *et al.*, 1988).

A number of different types of polymerase mutants have been isolated; *ts* (for example Coen *et al.*, 1974), drug resistance and drug hypersensitivity (Honess *et al.*,1984). The essentiality of the polymerase has made it a central target for antiviral agents, especially those which mimic dNTPs or pyrophosphate (for example acycloguanosine or phosphonoacetic acid), and resistance to such compounds can be due to a mutation in the polymerase gene (Hay and Subak-Sharpe, 1976; Chartrand *et al.*, 1979; Crumpacker *et al.*, 1980; Coen *et al.*, 1982; Honess *et al.*, 1984).

ii MAJOR DNA BINDING PROTEIN

After the polymerase, the major DNA binding protein (mDBP) is the best characterised protein which has a role in DNA replication. The protein, which is encoded by gene UL29, has M_r of 128K and has been shown to be essential for virus replication (Conley *et al.*, 1981; Weller *et al.*, 1983). Its precise role in virus replication has not yet been determined although it has been shown to denature poly-deoxyadenylic/poly-deoxythymidylic acid duplexes at 4°C (Powell *et al.*, 1981). mDBP mutants have been isolated which have altered sensitivities to inhibitors of virus DNA polymerase, suggesting a functional interaction between the two proteins (Chiou *et al.*, 1985). It has been postulated that the mDBP may be active in the form of a complex with exonuclease as well as the DNA polymerase (Littler *et al.*, 1983).

iii DS DNA BINDING PROTEIN

HSV-1 also encodes a double-stranded DNA binding protein (dsDBP), of M_r 65,000, which also co-purifies with the DNA polymerase and is regarded as being the homologue of the HSV-2 protein (M_r 55,000) described earlier (Vaughan *et al.*, 1984). This protein is encoded by the gene UL42 (Parris *et al.*, 1988), which distinguishes it from $V_{mw}65$, a protein of similar M_r (Marsden *et al.*, 1987). Although not required for DNA polymerase activity, dsDBP has a stimulatory effect on polymerase activity (Gallo *et al.*, 1989), supporting the suggestion that the *in vivo* polymerase holoenzyme is a heterodimer composed of polymerase and dsDBP subunits.

iv THE UL5, UL8, UL9 AND UL52 GENE PRODUCTS

There are four other genes which are known to be required for virus DNA replication; these are UL5, UL8, UL9 and UL52. Until recently the products of these genes could not be identified among the proteins of infected cells. They were only isolated when cloned into expression vectors (Olivo *et al.*, 1988; 1989; Calder and Stow, in press). Due to the complex nature of DNA replication, these proteins could be responsible for a number of accessory functions, such as origin recognition, primer synthesis (and degradation) and topoisomerase. It is now known that the origin binding protein first described by Elias *et al.* (1986) is the product of the UL9 gene (Olivo *et al.*, 1988; Weir and Stow, 1990).

Infection with HSV induces novel helicase and primase activities (Crute *et al.*, 1988; 1989; Holmes *et al.*, 1988), although the primase activity described by Holmes *et al.* (1988) was subsequently shown to be of cellular origin (Tsurumi and Lehman, 1990). However, the helicase activity described by Crute *et al.* (1988) has a tightly associated DNA primase activity (Crute *et al.*, 1989). It is thought that these two activities are components of a three subunit enzyme composed of the products of the UL5, UL8 and UL52 genes, although the activities have not been assigned to individual genes. Recently, Calder and Stow (in press) have demonstrated that a combination of the products of genes UL5 and UL52 has helicase activity.

v ALKALINE EXONUCLEASE

An increased alkaline exonuclease activity associated with infected cells was first reported by Keir and Gold (1963). Further studies with the purified protein showed it to be composed of a single polypeptide chain and to exhibit not only 3' and 5' exonuclease activities but also endonuclease activity (Morrison and Keir, 1968; Hoffman and

Cheng, 1978, 1979; Strobel-Fidler and Francke, 1980; Hoffman, 1981). The importance of this enzyme in DNA replication is a matter for debate. Studies involving *ts* mutants have not fully resolved the significance of the exonuclease for virus DNA synthesis (Moss *et al.*, 1979; Francke and Garrett, 1982; Moss, 1986); certainly, Wu *et al.* (1988) did not report it as being necessary for *trans*-stimulation of DNA synthesis. It has been suggested that a possible role for the enzyme is in the packaging of progeny virus DNA (Weller *et al.*, 1988).

1.10 PROTEINS INVOLVED IN NUCLEOTIDE METABOLISM

i THYMIDINE KINASE

As well as the proteins already mentioned, which are or appear to be directly involved in DNA replication, there are other proteins which impinge on the replicative process indirectly. These are the enzymes of DNA metabolism. Among the most-well characterised of these is the thymidine kinase enzyme (TK) (Kit and Dubbs, 1963; Dubbs and kit, 1964; Munyon *et al.*, 1971), the product of gene UL23. In addition to thymidine, TK also phosphorylates deoxycytidine and thymidylate (Jamieson and Subak-Sharpe, 1974; Jamieson *et al.*, 1974; Chen and Prusoff, 1978). However, deoxycytidine is not incorporated into the DNA, which indicates that the major route of dCDP (and therefore dCTP) synthesis is not the phosphorylation of dCMP, but is due to reduction of CDP (Jamieson and Subak-Sharpe, 1976). TK is not essential for virus growth in tissue culture, although the pathogenicity of these mutants is significantly reduced (Field and Wildy, 1978).

ii RIBONUCLEOTIDE REDUCTASE

Another HSV-encoded enzyme involved in nucleotide synthesis is ribonucleotide reductase (RR). RR catalyses the reduction of all four ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphates (Cohen, 1972; Dutia, 1983). The enzyme is composed of two pairs of two subunits (ie a $\alpha_2\beta_2$ arrangement), which are encoded by two distinct but adjoining genes, (Preston *et al.*, 1984; Bacchetti *et al.*, 1984; Frame *et al.*, 1985; McLauchlan and Clements, 1983), UL39 and UL40. The virus-encoded enzyme differs from its cellular homotype in that it is not sensitive to feedback inhibition by TTP (Cohen, 1972). It has been postulated that, in contrast to TK, RR is an essential enzyme for virus growth in tissue culture. This is thought to be due to it occupying such a crucial role in the metabolic pathways concerned with supplying precursors for DNA metabolism (Preston, *et al.*, 1974). However, it has subsequently been shown that under certain conditions, virus-infected cells can compensate for the loss of the large sub-unit of this enzyme, presumably by complementation of the activity by the host cell enzyme (Goldstein and Weller, 1988).

iii DEOXYURIDINE-5'-NUCLEOTIDOHYDROLASE

HSV also encodes a deoxyuridine triphosphatase (dUTPase) (Wohlrab and Francke, 1980; Preston and Fisher, 1984), encoded by the gene UL50. Wohlrab and Francke, (1980) showed this enzyme to catalyse the hydrolysis of deoxyribo-pyrimidine nucleoside triphosphates to monophosphates and pyrophosphate, with dUTP being by far the best substrate, followed by dCTP and dTTP. However, in a subsequent study Caradonna and Cheng (1981) showed that a partially-purified preparation of the enzyme was in fact specific for dUTP. Unlike its cellular counterpart, the virus-encoded enzyme activity is found in the nuclear fraction of infected cells and can be assayed at $4^{o}C$ (Wohlrab and Franke, 1980; Caradonna and Cheng, 1981). The cellular enzyme has two proposed functions. It has been suggested that in *E. coli*, the enzyme has an important role in the prevention of misincorporation of uracil into DNA (Shlomai and Kornberg, 1978), so a similar role for the HSV-encoded enzyme is possible. The enzyme has a very low K_m for dUTP ($<5 \times 10^{-8}$ M) and it is thought that this is sufficient to keep the intracellular dUTP concentration far below the K_m of the virus-encoded DNA polymerase (Wohlrab and Francke, 1980). The second proposed function of the enzyme is the supply of dUMP for *de novo* synthesis of dTMP, but Fisher and Preston (1986) considered this role to be secondary to the lowering of the concentration of the dUTP pool. The enzyme is dispensible for virus growth in tissue culture (Fisher and Preston, 1986).

iv URACIL-DNA GLYCOSYLASE

HSV also encodes its own uracil-DNA glycosylase. Work by Caradonna and Cheng (1981) showed that the activity of uracil-DNA glycosylase increased in cell extracts following infection with HSV. As this enzyme is the major topic discussed in this thesis, its role in the lytic cycle of the virus is discussed separately, in section 1.14.

1.11 EVOLUTIONARY COMPARISONS BETWEEN HERPESVIRUSES

It has already been stated that herpesviruses exhibit great diversity in their biological and morphological properties. This is reflected at the genomic level, and is exemplified by disparities in size, arrangement (both of which have already been discussed) and base composition. Base composition of herpesvirus DNAs has a wide variation, ranging from 75% G+C for B-virus down to 32% G+C for canine herpesvirus (Honess and Watson, 1977).

This diversity notwithstanding, it has long been postulated that there are evolutionary relationships between herpesviruses and this has led to comparisons being made. Until recently, these comparative analyses relied almost exclusively on techniques like DNA hybridisation (Davison and Wilkie, 1983) and on phenomena such as antigenic cross-reaction of proteins. However, these are only of use in studies within groups of closely related viruses. Before the advent of large scale sequencing protocols, there was no way of comparing relations between sub-families. Classification within the *Herpesviridae* in the past has been made according to general characteristics, such as virion morphology and biology, and not by any direct evaluation of genome relatedness. Although it is generally the case that realisation of DNA sequence data has led to corroboration of the sub-group assignations originally made on the bases of morphology and pathogenesis in the herpesviruses, this is not always the case. For example, Buckmaster *et al.* (1988) investigated the assignation of Marek's Disease Virus (MDV) and Herpesvirus of Turkeys (HVT) to the *Gammaherpesvirinae*. Using computer-aided comparisons of amino acid sequences deduced from DNA sequence data, they showed that both MDV and HVT are more closely related to VZV and HSV, members of the *Alphaherpesvirinae*, than to EBV which is a gammaherpesvirus. Not only that, but they also demonstrated a significant degree of colinearity in the genome organisation between both of the avian viruses and VZV (and

HSV).

Direct comparisons of DNA sequences often provide little information about homology, and so the method of choice for performing such analyses has been computer aided comparison of the predicted amino acid sequences. This has facilitated the study of the relationships between the herpesviruses. The relationships between HSV-1 and other human herpesviruses are discussed below.

1.11.1 HSV-1 AND OTHER ALPHAHERPESVIRUSES

i HSV-2

HSV-1 and HSV-2 are two very closely related members of the *Alphaherpesvirinae*. They share multiple antigens and will cross-neutralise (Honess *et al.*, 1974). The existence of a close relationship between the two serotypes was confirmed by the isolation of intertypic recombinants (Timbury and Subak-Sharpe, 1973; Davison and Wilkie, 1983b). There is extensive DNA sequence homology between the genomes of these viruses (Kieff *et al.*, 1972), and Southern blotting analysis demonstrated that this homology is present, at varying levels, throughout the length of the genomes, confirming that the genomes are colinear (Davison and Wilkie, 1983a).

ii VZV

HSV-1 also exhibits DNA sequence homology to VZV (Davison and Wilkie, 1983a). As a technical qualification, the L segment of the genome of VZV is inverted with respective to the prototypic arrangement of the HSV genome (Davison and Wilkie, 1983b). The first regions of the genomes to be compared were the S segments (McGeoch, 1984; Davison and McGeoch, 1986). The HSV-1 U_S region contains ten complete genes (US2-US11) and the major parts of two more (US1 and US12), with each copy of R_S containing one gene (IE175). The VZV U_S region contains two complete genes (US2 and US3) and the major parts of two more (US1 and US4), with each copy of R_S containing three complete genes (RS1, RS2 and RS3). These studies demonstrated that each gene present in VZV has a homologue in HSV-1, but that six of the HSV-1 genes have no counterparts in VZV. The two viruses have a different layout of genes in the S segment, and this has been interpreted as a result of large scale expansions and contractions of the unique and repeat regions, brought about by recombinational events in the evolution of the viruses.

With the publication of the sequence of HSV-1 U_L (McGeoch *et al.*, 1988), comparisons can be made between the two viruses for this region. The VZV L region is predicted to contain 60 genes (Davison and Scott, 1986) and HSV-1 is reckoned to have 57 different genes in the L segment (although there are two copies of IE110). On the basis of DNA sequence interpretation, there are five genes in the L region of VZV without counterparts in HSV-1 and two HSV-1 genes without homologues in VZV. In general, these comparisons have shown that, although the genomes differ widely in terms of base composition, they are closely comparable in the arrangement of their genes, especially in U_L.

1.11.2 HSV-1 AND BETAHERPESVIRUSES AND GAMMAHERPESVIRUSES

DNA sequence data have been published for some of these viruses and varying levels of similarity have been observed. HCMV (a betaherpesvirus) and EBV (a gammaherpesvirus) show essentially no homology to HSV at the DNA sequence level, as examined by DNA cross-hybridisation studies (Honess and Watson, 1977). Studies by Davison and Taylor (1977) compared amino acid sequences between VZV and EBV and found twenty nine pairs of genes which exhibited amino acid sequence homology, ranging from strongly conserved to weakly conserved examples. A further fourteen pairs were also designated as being probably homologous, on the basis of such properties as the distribution of hydrophobic amino acids. All of these related genes are present in U_L, with both viruses possessing genes not found in the other. No homologous pairs were detected in the short segments of the genomes. Sequence data for HCMV (Chee *et al.*, 1990), indicate that a similar relationship exists between it and the alpha-herpesviruses (and with EBV).

These comparisons provide evidence that all the human herpesviruses may be descended from a common ancestor. However, evolution has led to extensive variation and consequently the genomes of these viruses are widely diverged. This is reflected in the phenotypic variety exhibited by these viruses today.

1.12 HSV MUTAGENESIS

The last section of this introduction starts with an account of some of the functional analyses which have been performed on HSV by mutagenesis. This not only leads into the major technique used in the work described later, but also introduces the formation of uracil as a mutagenic event and the role of uracil-DNA glycosylase in its removal. This is central to the experiments described in the remainder of this thesis.

The biology of herpes viruses is very diverse and has been subject to a wide variety of studies. Perhaps the most fundamental objective in the field of virus molecular biology is the elucidation of the functions encoded in the genome. One tool which has been especially useful in this field has been that of mutagenesis. The many different techniques involved in these kinds of analyses have given rise to a wide variety of mutation classes, including syncytial plaque morphology (*syn*, for example see Brown *et al.*, 1973 and Little and Schaffer, 1981), temperature-sensitivity (*ts*, Preston *et al.*, 1988), insertion (Goldstein and Weller, 1988), virion host shut-off (*vhs*, Kwong *et al.*, 1988) and deletion (Smith *et al.*, 1989). Many of these mutations are spontaneous, or at least have been fortuitously isolated, with the other lesions being deliberately engineered.

1.12.1 ENGINEERED MUTATIONS

One of the major diagnostic aids in the analysis of those genes whose functional potential is obscure, is disruption of the protein coding sequence. The resulting mutants can then be subjected to phenotypic analysis. There are many published examples of this and some have been referred to above. The usual methods for achieving such disruption are deletion of all or part of the reading frame, or insertion of a segment of foreign DNA into the gene under investigation. These manipulations are usually performed on plasmid-cloned copies of the genes. The mutated plasmid-borne alleles are then transferred into the genome of the parent virus by homologous recombination of flanking sequences.

One of the major drawbacks of deletion or insertion mutagenesis is difficulty in recognising the recombinant progeny. If the function of the gene which has been mutated is not known then the phenotype of the progeny cannot be predicted. Homologous recombination works, but the efficiency is limited. In the work described in this thesis, the recombination efficiency is lower than observed elsewhere, perhaps because of the use of a large (4.1 kbp) insert. Even with smaller inserts it may be necessary to screen thousands of plaques in order to be confident of isolating a mutant. If the gene under investigation was required for growth in tissue culture then the likelihood of isolating a mutant would be reduced to zero. Also, recombinant viruses may be restricted in their growth properties and number of progeny, and so may be overgrown by the *wt* parent. There have been several attempts to circumvent this problem, most relying on the use of a selectable marker. Under selection pressure only those recombinants carrying this selectable marker are encouraged to grow and can be isolated by plaque-purification. One method involves using the HSV thymidine kinase (TK) gene (Post and Roizman, 1981). The HSV TK gene product is non-essential for virus growth in tissue culture and it has been possible to isolate mutant strains of HSV which are deficient in TK activity (Dubbs and Kit, 1964). In addition, there are cell/medium selections both for and against expression of the TK gene (Post and Roizman, 1981). Early mutagenesis experiments involved insertion of foreign DNA sequences into the TK locus in the HSV genome. The progeny would have a tk^- phenotype against a tk^+ background. This technique potentially allows for rapid selection of the progeny, but the site of mutagenesis is restricted to within the TK gene. This was overcome by a modification of the procedure. In the first stage, the HSV-

1 TK gene is inserted into a plasmid-borne copy of the target region of the genome. This construct is then inserted into the genome of a *tk-* virus at the required locus by homologous recombination of the flanking sequences. Recombinant progeny, which are *tk+*, can be screened for, against the parental virus which have a *tk-* phenotype. Once purified and confirmed, these insertion mutants can then be used to generate deletion mutants. The procedure for this is similar to the previous one. Deletions are engineered into plasmid-cloned copies of the gene. These are again recombined into the mutant virus. The recombination event this time removes the TK gene (and thus the *tk+* phenotype) as well as the target sequences which flank it. In this case progeny are screened for their *tk-* phenotype against a background of *tk+* parents.

In practice this technique apparently works well. There are however difficulties which make the construction and screening of these mutants complicated and technically demanding. Special *tk-* cell lines are required for selection and maintenance of these viruses. The isolation of the engineered viruses demands the use of different tissue culture media and these may not be optimal for growth of both the viruses and the cells which they infect. It is also the case that the mutation is not engineered into a *wt* virus but a mutant which is *tk-* and this may have ramifications for the genotype and/or phenotype of the progeny. Although the TK enzyme is not required for growth in tissue culture, little is known about how the enzyme interacts with other aspects of nucleotide metabolism and how mutations in other genes relate to the growth of *tk-* viruses in culture.

Recently, mutagenesis techniques which are without the drawbacks described so far have been sought. One method along these lines was the introduction of the *E. coli LacZ* gene (for example, see Hall *et al.*, 1983). This gene can be used as a phenotypic marker, visualised by the simple addition of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), a chromogenic substrate for β-galactosidase, the product of the *LacZ* gene. Once again, the mutation, in this case an insertion, is constructed in plasmid-cloned copies of the target gene which is then co-transfected into *wt* virus as a selectable marker. The detection of recombinant progeny virus is facilitated by addition of X-gal to the agar used to overlay the tissue culture plates. One limitation of this system is the efficiency of expression of the marker gene. In practice, this can be overcome by fusing the gene to a promiscuous promoter, eg the SV40 early promoter, as in the plasmid pFJ3 (see fig. 2.2). Recombinant viruses containing the *LacZ* gene, express it to a detectable level (work in this thesis). This has the advantage of using *wt* parent virus and dispenses with the need for *tk-* cell lines. This technique has been used in the generation of the mutant viruses discussed in this thesis.

1.12.2 SPONTANEOUS MUTATIONS

Amongst the challenges which the cell has to meet, one of the most important is the prevention of an unacceptably high mutation rate. Sophisticated mechanisms have evolved to ensure that DNA is replicated with high fidelity, and mismatch repair activities exist which remove misincorporated residues which have escaped proofreading during replication. Potentially mutagenic changes can also occur in nonreplicating DNA, both by exposure to mutagenic agents and under normal physiological conditions (Lindahl, 1982).

1.13 URACIL IN DNA

Spontaneous degeneration of DNA due to depurination and depyrimidination of DNA is a normal occurrence in mammalian cells (Lindahl, 1982). One type of spurious residue which has been found in DNA is uracil. The two pathways which result in the presence of uracil in DNA are:

1) Misincorporation of dUTP (as dUMP) into the DNA during replication (Bessman *et al.*, 1958).

and

2) *In situ* deamination of cytosine residues in the DNA (Shapiro *et al.*, 1973).

These are discussed in turn below.

1.13.1 MISINCORPORATION OF dUTP

As has already been stated, it is a physiological possibility that, during normal DNA replication, dUTP (as dUMP) could be incorporated instead of dTTP into the newly synthesised DNA (Tye *et al.*, 1977). This misincorporation is minimised, but perhaps not totally suppressed, by selective enzymatic degradation of dUTP in cells by the enzyme dUTPase (Bertani *et al.*, 1963). It has been demonstrated that HSV-1 encodes its own dUTPase (Preston and Fisher, 1984).

1.13.2. *IN SITU* DEAMINATION OF CYTOSINE

In vivo, cytosine is the most labile of the four DNA bases to heat-induced hydrolysis (Shapiro and Klein, 1966) and direct measurements of the rate of deamination of cytosine to uracil in DNA have indicated that this process occurs at a significant rate under physiological conditions (Lindahl and Nyberg, 1974). Cytosine deamination can occur spontaneously (Hyatsu, 1977) and it is estimated that there is premutagenic deamination of approximately one hundred cytosine residues per genome per day in a typical mammalian cell (Lindahl, 1982). Apart from its spontaneous occurrence, deamination can also be mediated by exposure to sodium bisulphite or to nitrous acid (Shapiro and Klein, 1966). Such lesions would, unless corrected, give rise to a G.C to A.T transition mutation upon subsequent rounds of replication (Lindahl, 1982).

1.14 THE HSV-ENCODED URACIL-DNA GLYCOSYLASE AND ITS ROLE IN REMOVING URACIL FROM DNA

Enzymatic cleavage of DNA occurs by hydrolysis of phosphodiester bonds by deoxyribonucleases. Lindahl (1974, 1976) described a group of DNA-cleaving enzymes which hydrolyse glycosidic base-sugar bonds as opposed to phosphodiester bonds and which are active in DNA repair. These are the DNA glycosylases (Lindahl, 1974). Although several of these have been isolated, for example hypoxanthine-DNA glycosylase, 3-methyladenine-DNA glycosylase I and II, the best studied is uracil-DNA glycosylase (Lindahl, 1976), which is also the most abundant (Varshney *et al.*, 1988). This enzyme has been isolated from a variety of sources, from bacteria (Cone *et al.*, 1974) to humans (Caradonna & Cheng, 1980). It acts on DNA which contains dUMP in single-stranded regions or base-paired to either dAMP or dGMP in double-stranded DNA, releasing free uracil and DNA of unaltered chain length containing apyrimidinic sites. Lindahl *et al.* (1977)

demonstrated that the mechanism of action of the enzyme was not by dephosphorylation or by a *trans*-N-deoxyribosylase type reaction but by a direct cleavage of the glycosidic base-sugar bond. The enzyme shows no activity on free dUMP, RNA or the four common deoxyribonucleotides in DNA (Lindahl *et al.*, 1977; Friedberg *et al.*, 1975). The enzyme, as isolated and purified from *E. coli*, has a K_m for uracil of approximately 4 X 10^{-8} and a turnover number of 800 uracil residues released per min (Lindahl *et al.*, 1977).

As was stated earlier, Caradonna and Cheng (1981) reported induction of a uracil-DNA glycosylase activity in cells infected with HSV-1 and HSV-2, and recently Caradonna *et al.* (1987) described the isolation of an HSV-2 cDNA which encoded uracil-DNA glycosylase activity. This was achieved by construction of a cDNA library with mRNA from HSV-2-infected cells. Pooled isolates from this library were used in hybrid-arrest and *in vitro* translation experiments to isolate a clone which apparently encoded uracil-DNA glycosylase activity. Subsequent analysis, by Southern blotting, mapped this cDNA to between 0.065 and 0.080 map units (mu) on the prototypic HSV genome.

It has already been stated that there are two principal pathways which could result in the presence of uracil in the DNA, namely misincorporation of dUTP during replication and *in situ* deamination of cytosine to uracil. It is possible that uracil-DNA glycosylase could have a role in the correction of either (or both) event(s). However, it has been suggested that minimising the misincorporation of uracil (from dUTP) into DNA during replication is not the role of uracil-DNA glycosylase, due to the presence of the dUTPase. The latter enzyme lowers the intracellular pool of dUTP, making misincorporation a dynamically unfavourable reaction (Preston and Fisher, 1984). The ramifications of misincorporation of dUTP are not as serious as those of deamination of cytosine as the former case does not result in a mutation. For these reasons, it is now generally accepted that the *in vivo* role of the uracil-DNA glycosylase enzyme is to initiate the repair of G.U base pairs, which have a mutating potential (Caradonna *et al.*, 1987; Caradonna and Cheng, 1980). The enzyme itself is involved at the earliest stage of this procedure. It recognises the presence of uracil in the DNA and it removes it by hydrolysis of the glycosidic base-sugar linkage. Removal of the uracil creates an apyrimidinic site in the DNA. Indeed, the collective action of DNA glycosylases is the major cause of the formation of apurinic or apyrimidinic (AP) sites in DNA (Lindahl, 1979). Although these lesions are repaired very efficiently *in vivo*, they occur with sufficient frequency to be considered as being occasionally responsible for inactivation or mutagenesis of cells (Kunkel *et al.*, 1981). However, it seems unlikely that the generation of such AP sites in the DNA plays a major role in spontaneous mutagenesis on its own. This conclusion is drawn because of the existence of *E. coli xth* mutants. These are deficient in the major AP endonuclease and presumably have a reduced ability to remove these sites. It has been demonstrated that these mutants do not exhibit an increased spontaneous mutation frequency (Weiss *et al.*, 1978; Rogers and Weiss, 1980).

MATERIALS AND METHODS

2.1 ENZYMES

Restriction endonucleases, T4 DNA polymerase and T4 DNA ligase were obtained from Bethesda Research Laboratories. The large fragment of *Escherichia coli* DNA polymerase I (Klenow) was obtained from Boehringer Corporation plc, as was calf intestinal phosphatase.

Lysozyme was purchased from Sigma Chemical Company Ltd.

2.2 CHEMICALS

5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) was obtained from Sigma Chemical Company Ltd.

Other laboratory chemicals were routinely purchased from BDH or Sigma and were generally of analytical grade or better.

Unless stated otherwise, all tissue culture growth media and supplements were purchased from Gibco-BRL plc.

2.3 RADIOCHEMICALS

All radiochemicals were obtained from the Radiochemical centre, Amersham.

2.4 BUFFERS AND SOLUTIONS

Boiling mix	30%	(v/v)	Stacking gel buffer
	6%	(v/v)	SDS
	30%	(v/v)	Glycerol
	15%		β-Mercaptoethanol
	1%		Bromophenol blue

Destain (for protein gels)		
	5%	Methanol
	7%	Acetic acid
	in deionised H_2O	
E buffer	20 mM	Tris base
	10 mM	$NaH_2PO_4.2H_2O$
	0.5 mM	EDTA
Fix (for protein gels)		
	47%	Methanol
	47%	Deionised H_2O
	6%	Glacial acetic acid
HEBS buffer	140 mM	NaCl
pH7.05	5 mM	KCl
	0.5 mM	Na_2HPO_4
	5 mM	D-glucose
	20 mM	Hepes
NTE	10 mM	Tris-HCl
	100 mM	NaCl, pH 7.4
	1 mM	EDTA
PBS A	170 mM	NaCl
	3.4 mM	KCl
	1 mM	Na_2HPO_4
	2 mM	KH_2PO_4
		pH 7.2
Complete PBS	PBS A	
	6.8 mM	$CaCl_2$
	4.9 mM	$MgCl_2$

Protein extract dialysis buffer

	50 mM	Tris-HCl, pH 7.5
	1 mM	Dithiothreitol
	1 mM	$MgCl_2$
	20% (v/v)	Glycerol

Protein extraction buffer	200 mM	K-phosphate, pH 8.0
	1 mM	EDTA
	2 mM	$MgCl_2$
	2 mM	Dithiothreitol
	1% (v/v)	Triton X-100
	1 mM	PMSF
	20% (v/v)	Glycerol

RNase mix

	100 ul	RNase A soln. (10 mg/ml)
	50 ul	RNase T1 (0.67% v/v)
	850 ul	H_2O

The solution was prepared fresh each time and boiled for 10 min before use.

RSB	10 mM	Tris-HCl
	10 mM	KCl, pH 7.4
	1.5 mM	$MgCl_2$

Stain (for protein gels)

Fix + 0.2% Coomassie Brilliant Blue R-250

STET	8% (w/v)	Sucrose
	0.5% (v/v)	Triton X-100
	50 mM	EDTA, pH 8.0
	50 mM	Tris-HCl, pH 8.0

TBE	90 mM	Tris-base
	90 mM	Boric acid
	2.5 mM	EDTA, pH 8.3
TE	10 mM	Tris-HCl, pH 8.0
	0.1 mM	EDTA, pH 8.0
Triton lysis Solution		
	2%	Triton X-100
	50 mM	Tris-HCl pH8.0
	6.25 mM	EDTA
		in deionised H_2O
Zweig's buffer	100 mM	Tris-HCl, pH 8.0
	10%	Glycerol
	0.5%	Nonidet P-40
	0.5%	Na deoxycholate
	0.2 mM	PMSF

2.5 BACTERIAL GROWTH MEDIA

2YT	85 mM	NaCl
	1.0% (w/v)	Bactopeptone
	1.0% (w/v)	Yeast extract.
L-Broth	177 mM	NaCl
	1.0% (w/v)	Bactopeptone.
SOC	2.0% (w/v)	Bactotryptone
	0.5% (w/v)	Yeast extract
	10 mM	NaCl
	2.5 mM	KCl
	10 mM	$MgCl_2$
	10 mM	$MgSO_4$

	20 mM	D-Glucose

L-Broth Agar	1.5% (w/v) agar in L-Broth.
Antibiotics	L-Broth Agar supplemented with either 50 ug/ml ampicillin (Penbritin) or 20 ug/ml tetracycline.

2.6 TISSUE CULTURE GROWTH MEDIA

ETC$_{10}$	300 ml	Sterile distilled H_2O
	36 ml	Glasgow Modified Eagle's Medium (GMEM) (Busby *et al.*, 1964)
	14 ml	$NaHCO_3$
	100 U/ml	Penicillin
	100 ug/ml	Streptomycin
	7.8 ml	L-Glutamine
	40 ml	Calf serum
	20 ml	Tryptose phosphate broth

EMC5

GMEM supplemented with

100 U/ml	penicillin
100 ug/ml	streptomycin
1.5%	methyl cellulose
5%	calf serum

Agar Overlay (for screening recombinant virus)

GMEM supplemented with

0.64%	Noble Agar
1.5 mM	X-gal
1%	Calf serum

PIC	Phosphate-free Eagle's medium supplemented with 1% newborn calf serum.

2.7 TISSUE CULTURE AND VIRUS STOCKS

Throughout this study baby hamster kidney clone 13 (BHK C13) cells were used (MacPherson and Stoker, 1962). These were incubated in 35 mm, 50 mm or 90 mm tissue culture plates as required or in 80 oz roller bottles, under ETC_{10} unless stated otherwise. Tissue culture plates were incubated at 37°C in a humidified atmosphere of air enriched with 5% CO_2 and roller bottles were gassed to 5% CO_2, capped and kept rotating at 37°C.

Throughout this study wild-type HSV-1 strain 17^+ (Brown *et al.*, 1973) was used. Two mutants derived from HSV-1 17^+ which were also used, were a dUTPase deficient mutant - dut-1218 (a gift from Dr V G Preston), and a phosphonoacetic acid (PAA) resistant mutant - PAA^r-1 (a gift from M Murphy). dut-1218 contains an 8-mer *Hind*III oligonucleotide linker inserted into the *Kpn*I site within the coding site of the dUTPase gene, UL50 (Fisher and Preston, 1986). PAA^r-1 (Hay and Subak-Sharpe, 1976) contains a mutation within the DNA polymerase gene UL30 (Crumpacker *et al.*, 1980).

BHK C13 cells were grown until 90% confluent under ETC_{10}. They were infected at the required multiplicity of infection (moi) as follows. The medium was aspirated from the cells and was replaced with the appropriate virus in ETC_{10}. The cells were returned to the incubator for 1 hr before the inoculum was removed, the cells were washed twice with ETC_{10} and overlaid with the appropriate medium.

Elite virus stocks (with low serial passage number) were used to infect BHK C13 monolayers (approximately 80% confluent) at a multiplicity of 1 pfu/300 cells in 40 ml of ETC_{10}. The infected cells were incubated at 31°C and harvested when the cpe was maximal (usually 4-6 days pi).

The infected cells were shaken into the medium and pelleted by centrifugation at 2,000 rpm for 10 min. The supernatant was decanted and centrifuged at 12,000 rpm for 2 h. The pellet was resuspended in 5 ml supernatant and disrupted ultrasonically in a bath sonicator (50 W at 4°C) until homogeneous. This was retained as the supernatant virus stock. The pellet from the first centrifugation was resuspended in 5 ml of supernatant and disrupted ultrasonically as before, freeze/thawed, re-sonicated and centrifuged at 2,000 rpm for 10 min. The supernatant was retained and the pellet was resuspended in 5 ml of ETC_{10} and treated as for the pellet from the first centrifugation. The two supernatants were pooled and retained as the cell-associated virus stock.

Virus stocks were stored at -70°C and were checked for sterility and titrated before use.

2.8 STERILITY CHECKS

Brain heart infusion (BHI) plates were prepared by dissolving 26 g of Difco Bacto BHI agar in 500 ml of deionised H_2O and autoclaving for 20 min. This was cooled to around 40°C and poured onto 50 mm petri dishes (BHI plates). Alternatively, 50 ml of horse blood (Biocult) was added immediately prior to pouring (BHI blood agar plates).

Virus stocks were tested for fungal contamination by streaking, in duplicate, onto BHI plates. The plates were sealed with Parafilm and incubated at room temperature for 7 days. Yeast or bacterial contamination was detected by streaking onto BHI blood agar plates, which were incubated at 37°C for 7 days. If after this time no contamination was detected the stocks were considered to be sterile.

2.9 TITRATION OF VIRUS STOCKS

Virus samples, whether from co-transfection or otherwise, were titrated at 31°C and/or 37°C onto confluent monolayers of BHK C13 cells on 50 mm plates as follows. Serial tenfold dilutions of virus were prepared by taking 0.1 ml of virus into 0.9 ml complete PBS supplemented with 5% calf serum. 0.1 ml of this dilution was taken into another 0.9 ml of PBS with calf serum, and so on. Generally speaking, the dilutions from 10^{-2} to 10^{-6} were used for titration. The plates were taken from the incubator, the medium was removed and 0.2 ml of virus from the respective dilution was added to the plates. The plates were returned to the incubator for 1 hr before the inoculum was removed and the plates overlaid with EMC5. The plates were inspected daily. After 2 days the plates were stained with Giemsa stain for 30 min at room temperature and the plaques counted under a dissection microscope.

2.10 PREPARATION OF VIRUS DNA

Confluent monolayers of BHK C13 cells in 80 oz roller bottles were infected at a moi of 1/300 pfu/cell and incubated until the cpe was extensive. The virus-infected cells were harvested by shaking into the medium. This was then centrifuged at 2,000 rpm for 10 min. The supernatant was retained (SN1) and the pellet was resuspended in 10 ml of RSB. Nonidet P-40 (NP40) was added to a final concentration of 0.5% (v/v) and was left on ice for 10 min. The mixture was centrifuged as before to pellet the nuclei. The supernatant (SP2) was added to SP1 and the pellet re-extracted with RSB/NP40 as before. After the centrifugation step the supernatant was added to SN1 and SN2. This was centrifuged at 12,000 rpm for 2 h at 4°C in a Ti50 rotor. The supernatant was discarded and the pellet resuspended in 8 ml of NTE by sonication. To this was added EDTA and SDS to a final concentration of 10 mM and 2% (w/v) respectively. This was mixed gently with an equal volume of buffer saturated phenol. The phases were left to separate for 5 min at room temperature followed by 5 min on ice. The aqueous phase was re-extracted twice more with buffer saturated phenol on a rotary mixer, followed by an extraction with chloroform:iso-amyl alcohol (24:1). The aqueous phase was decanted and to this was added 0.1 volumes of 3 M NaOAc and 2.5 volumes of ethanol. The DNA was recovered and resuspended in TE.

2.11 CO-TRANSFECTION OF VIRUS DNA WITH PLASMID DNA

Co-transfection of virus DNA with plasmid DNA was performed by the calcium phosphate technique followed by a dimethyl sulphoxide (DMSO) boost (Stow and Wilkie, 1976). BHK C13 cells were grown to 90% confluence under ETC_{10}. The medium was aspirated and replaced with the transfection mixture, which was composed of the following; 1 ml Hebs pH 7.05, 70 ul of 2M $CaCl_2$, 5 ul of calf thymus DNA (1 mg/ml), 200 pfu of virus DNA and plasmid DNA. The plasmid DNA was prepared by digesting 60 ug of plasmid DNA with 100 units of restriction enzyme. The number of cutting sites appeared not to be significant as long as the plasmid had approximately 1 kbp of sequence homologous to the virus DNA on either side of the lesion. The digestion fragments were separated by electrophoresis on a 1% agarose gel. The fragment of interest was excised from the gel with a scalpel and the DNA recovered by electroelution. This fragment was purified by "Geneclean" (BIO101, USA), a DNA-binding matrix based on powdered glass, used according to the suppliers' instructions. The co-transfections were performed in duplicate, adding 0.1, 0.5, 1 and 5 ug of purified fragment to different plates. The plates were returned to the incubator for 1 h. After this time, 4ml of ETC_{10} was added to each plate and the incubation was continued.

At 4 h pi the plates were removed from the incubator. The medium was aspirated and the plates washed twice with ETC_{10} without serum before addition of 1 ml of DMSO (25% in Hebs, pH 7.05). This was left on for 4 mins before being removed. The plates were then washed twice more with ETC_{10} without serum before being overlaid with 4 ml of ETC_{10}. The plates were returned to the incubator and checked daily for cpe. After extensive (>75%) cpe was observed, the virus was harvested into the medium by scraping with a rubber policeman. This was disrupted ultrasonically and titrated as described in section 2.9.

2.12 DETECTION AND PURIFICATION OF RECOMBINANT VIRUS PROGENY

Recombinant viruses (containing the *E. coli LacZ* gene) were co-transfected as described in section 2.11 and titrated as described in section 2.9 except that instead of overlaying with EMC5, the plates were overlaid with agar overlay containing X-gal, a chromogenic substrate for B-galactosidase. These plates were again returned to the incubator and left overnight. After this time, plaques formed by recombinant progeny virus expressing the β-galactosidase gene turned blue. The recombinant viruses were purified to homogeneity by four successive rounds of plaque purification, picking ten blue plaques at each round and passing these. An elite stock, made from a single, isolated blue plaque picked at the fourth round of purification was used in all subsequent experiments involving the virus in question.

2.13 RESTRICTION ANALYSIS OF MUTANT VIRUSES

The genotype of the mutants was confirmed by restriction analysis, essentially by the method of Lonsdale (1979). BHK C13 cells were seeded in 24 well tissue culture plates and after 12 hours the medium was removed and replaced by 0.45 ml of PIC. After a further 12 hours the cells were infected with virus at a moi of 10 pfu/cell and maintained again in PIC. At 2 h pi, 50 uCi of ^{32}P in 50 ul of PIC was added to each well. The plates were returned to the incubator. Once the cpe was extensive (usually 2-3 days), the cells were lysed by addition of 0.5 ml of 5% SDS. The cells were scraped off the plate with a micropipette tip and transferred to a sterile 10 ml conical tube. The lysate was extracted with an equal volume of buffer-saturated phenol, incubated at room temperature for 5 min and then on ice for 5 min. The aqueous phase was ransferred into a clean assay tube and the DNA was precipitated by addition of 2 volumes of ethanol and pelleted by centrifugation at 2,000 rpm for 10 mins at $4^{\circ}C$. The pellet was drained, dried and resuspended in 175 ul of sterile distilled H_2O and 25 ul RNase mix by gentle agitation at $37^{\circ}C$. The solution was stored at $-20^{\circ}C$ until use.

Samples (20ul) of virus DNA were digested with between 1 and 2 units of appropriate restriction enzymes according to the suppliers instructions. After 4 hours the DNA restriction fragments in the samples were separated by horizontal agarose electrophoresis overnight at 40V, with the agarose concentration being decided by the size of the restriction fragments generated by the digest. After electrophoresis, the gel was dried and subjected to autoradiography at -70°C.

2.14 ONE-STEP GROWTH CURVE

Confluent monolayers of BHK C13 cells on 35 mm plates were infected with virus at a moi of 5 pfu/cell and incubated for 1 h. After this time, the plates were washed twice with ETC_{10} without serum and overlaid with 1.5 ml of ETC_{10}. Plates were harvested at 1, 2, 3, 4, 6, 8, 10, 12, 21 and 25 h pi by scraping into the medium with a rubber policeman. The harvests were disrupted ultrasonically and titrated onto 50 mm plates of confluent BHK C13 cells as previously described.

2.15 PREPARATION OF "EARLY" EXTRACTS

To prepare extracts of proteins produced in the absence of DNA synthesis, monolayers of BHK C13 cells (80% confluent) were used. The medium was removed and replaced by ETC_{10} supplemented with PAA at 0.3 mg/ml. The cells were incubated for 1 h before being infected with virus at a moi of 20 pfu/cell. The virus was allowed to absorb for 1 h at 37°C before being removed and the cells overlaid with ETC_{10} supplemented with 0.3mg/ml PAA. At 10 h pi extracts were prepared from the cells as described in section 2.16.

2.16 PREPARATION OF INFECTED AND MOCK-INFECTED CELL EXTRACTS FOR ENZYME ASSAYS

Extracts from HSV-infected or mock-infected monolayers of BHK C13 cells were prepared as described by Caradonna and Cheng (1980). At the appropriate time-point, the medium was removed from the plates, which were then rinsed twice with PBS. The cells were then scraped into PBS and pelleted by centrifugation at 2,000 rpm for 10 min at 4°C. The pellet was resuspended in 5 volumes of protein extraction buffer and subjected to 4 cycles of freeze/thawing in dry-ice/ethanol. The suspension was then disrupted ultrasonically before standing on ice for 1 h. Following this, the suspension was centrifuged at 2,000 rpm for 10 min to pellet cell debris. The supernatant was dialysed against 100 volumes of protein extract dialysis buffer at 4°C overnight. The dialysate was centrifuged at 2,000 rpm for 10 min to pellet precipitated material and the supernatant removed and stored at -70°C until use.

2.17 GLYCEROL STOCKS OF BACTERIA

Bacterial stocks were prepared from 20 ml overnight shaking cultures at 37°C in L-Broth or 2YT. These were centrifuged at 5,000 rpm for 10 min at 4°C. The pellets were resuspended in 4 ml of 2% (w/v) bactopeptone before addition of 4 ml 80% (v/v) glycerol. The stocks were routinely stored at -70°C

2.18 HSV-1 RECOMBINANT PLASMIDS

Recombinant plasmids were grown in *E. coli* strains DH5 and DH5α, obtained from Bethesda Research Ltd. The following recombinant plasmids were used in this work.

pGX23, containing the HSV-1 *Bam*HI *e* fragment, was provided by Dr V G Preston. Another plasmid used extensively throughout this work was pFJ3, provided by Drs F J Rixon and J McLauchlan. The structures of these plasmids are shown in figures 2.1 and 2.2, respectively.

2.19 TRANSFORMATION OF BACTERIAL CELLS WITH PLASMID DNA

Host bacterial strains, either DH5 or DH5α were obtained from Bethesda Research Ltd and used according to the suppliers instructions. 20 ul of competent bacteria was transferred to a 1.5 ml Eppendorf tube on ice. 100-500 ng of plasmid DNA was added and the mixture was incubated on ice for 20 min. The bacteria were heat-shocked at 42°C for 40 s before being returned to ice. 80 ul of SOC medium was added to the bacteria which were then incubated at 37°C for 1 h in an orbital incubator. 50 ul aliquots of the bacterial suspension were then plated out onto L-broth agar plates containing selective antibiotics. Colonies were picked and restreaked in parallel onto L-broth agar plates containing an antibiotic which the plasmid would confer resistance to and one which the plasmid would not. i.e. bacteria transformed with the plasmid pGX23 would be ampicillin resistant and tetracycline sensitive. Colonies exhibiting the correct spectrum of antibiotic resistance and sensitivity were picked and small scale cultures were prepared as described in section 2.20.

2.20 SMALL SCALE PLASMID DNA PREPARATION

Colonies exhibiting the desired antibiotic spectrum were picked with sterile toothpicks into sterile universals containing 2 ml of L-broth containing the appropriate antibiotic and incubated overnight at 37°C in an orbital

Figure 2.1 : The plasmid pGX23

The structure of plasmid pGX23 is depicted in this figure. pAT153 sequences extend from position 1 to 3657, and the HSV-1 *Bam*HI *e* fragment (residue 2907 to 11820 in the virus genome, McGeoch *et al.*, 1989) is from position 3658 to 12576 on the plasmid.

This plasmid was a gift from Dr V G Preston.

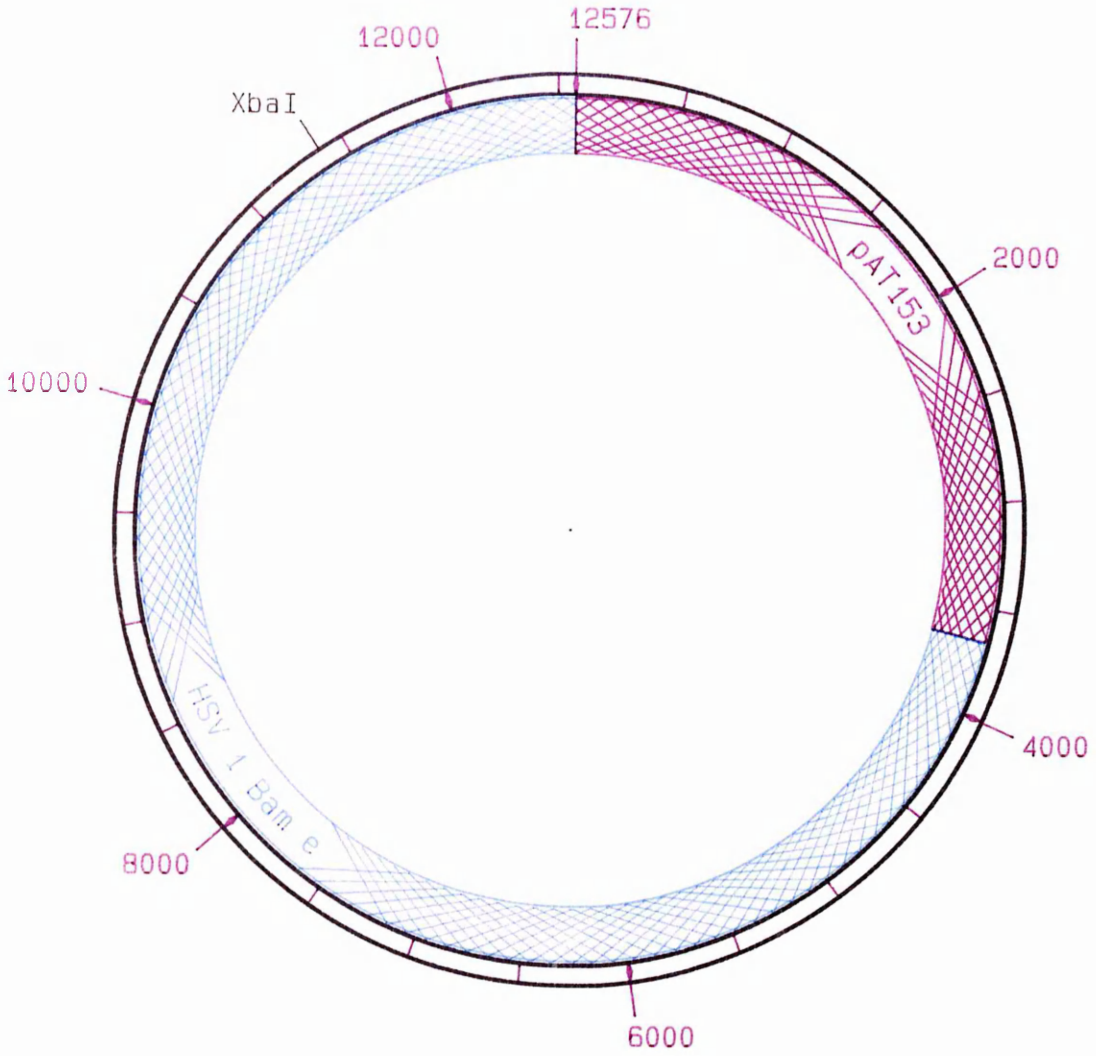
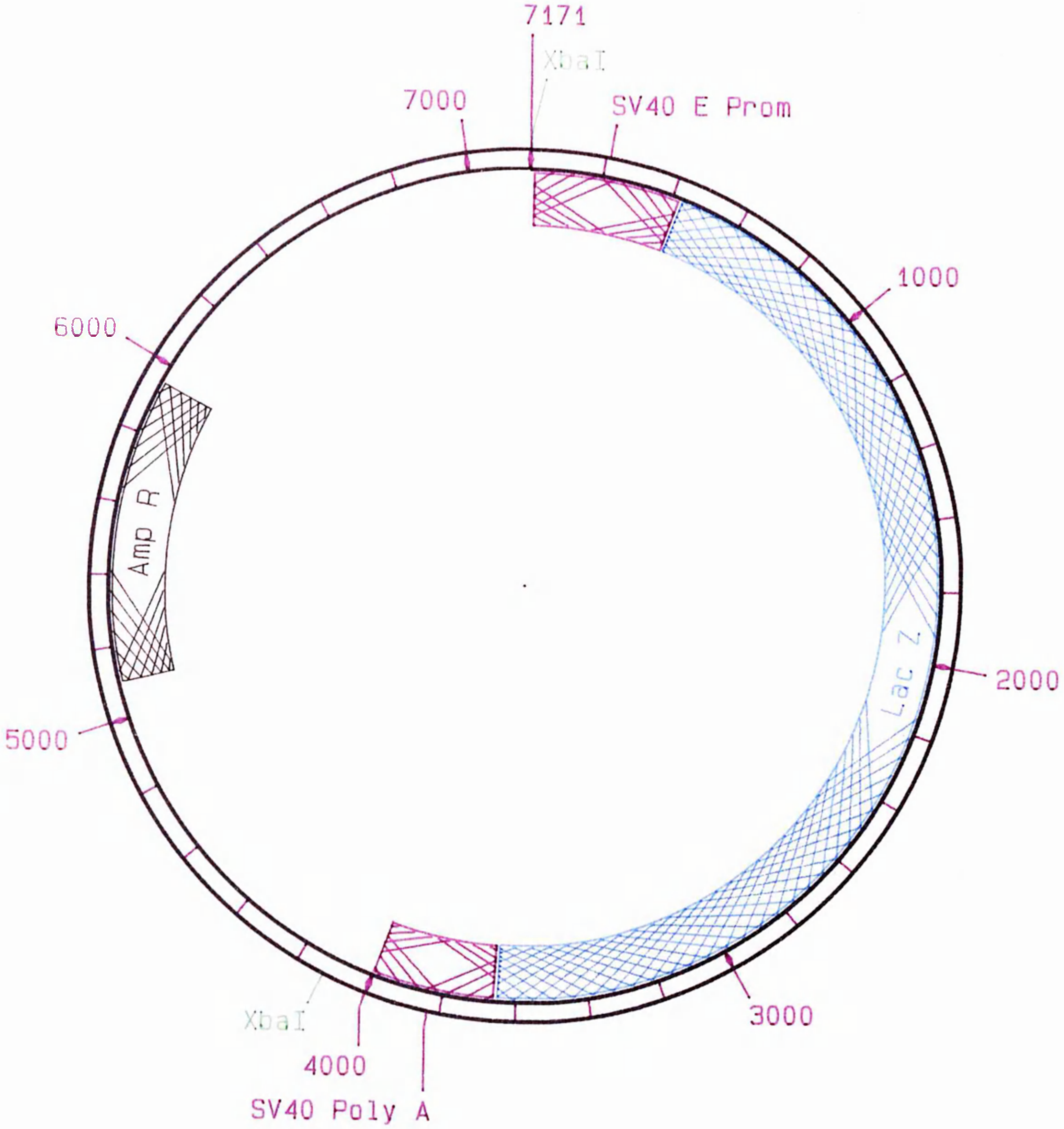

12576
12000
XbaI
2000
pAT153
10000
4000
HSV 1 Bam e
8000
6000

Figure 2.2 : The plasmid pFJ3

This plasmid was used in the mutagenesis experiments described. The fragment between the two *Xba*I sites at positions 5 and 4122 contains the *E. coli Lac Z* gene, with SV40 early promoter and polyadenylation signals, as indicated.

This plasmid was a gift from Drs F J Rixon and J McLauchlan.


7171
XbaI
7000
SV40 E Prom
1000
6000
Amp R
Lac Z
2000
5000
3000
XbaI
4000
SV40 Poly A

incubator. 1.5 ml of each culture was decanted into a sterile eppendorf tube. The tubes were centrifuged for 10 s in a bench-top microfuge. The supernatant was discarded and the pellet was resuspended in 0.1 ml of STET buffer. The tubes were vortexed before addition of 16 ul of lysozyme (10 mg/ml in 250 mM Tris-HCl, pH 8.0). The tubes were vortexed once more before being placed in a boiling water bath for 12 min. The tubes were then centrifuged for 15 min in a bench-top microfuge. The supernatant was decanted and the plasmid DNA precipitated by addition of 0.1 ml of isopropanol. After 10 min the tubes were centrifuged for 3 min in a bench-top microfuge. The tubes were drained, dried and the DNA was resuspended in 50 ul of TE. Clones with the expected restriction enzyme patterns were grown up to large scale cultures (2.21) and glycerol stocks were made (2.17).

2.21 LARGE SCALE PREPARATION OF PLASMID DNA

100 ml of 2YT broth supplemented with 100 ug/ml ampicillin was inoculated with 50 ul from the appropriate glycerol stock culture and shaken overnight at 37°C. This was added to 1.6 l of L-broth and incubated as before until the optical density at 550 nm was approximately 0.7 (usually 3-4 h). The plasmid was amplified by addition of chloramphenicol to a final concentration of 100 ug/ml (Clewell, 1972) and the culture was shaken overnight at 37°C.

The plasmid was isolated by soft lysis, essentially by the method of Katz *et al.* (1973), but with a few minor modifications. The amplified bacterial culture was centrifuged at 9,000 rpm for 15 min at 4°C. The pellet was resuspended in 16 ml of ice-cold 25% sucrose (w/v) in 50 mM

Tris-HCl pH 8.0. The suspension was distributed to 4 SS34 tubes on ice and all subsequent procedures were performed on ice. Lysozyme was added to each tube to a final concentration of 5 mg/ml. After 5 min incubation, 3 ml of 250 mM EDTA pH 8.0 was added to each tube, followed by a further 5 min on ice. Lysis of the bacterial cells was achieved by addition, to each tube, of 9 ml Triton lysis solution and incubation on ice for 20 min. After this time the lysate was centrifuged at 15,000 rpm at 4°C for 30 min to pellet cell debris and chromosomal DNA. The supernatant was extracted with an equal volume of TE-buffered phenol and the phases were separated by centrifugation at 2,000 rpm for 10 min. The aqueous phase was decanted into dialysis tubing and dialysed against 2 l of TE for 2 h at 4°C with 2 changes. After dialysis the DNA was precipitated by addition of 0.1 volumes of 3 M NaOAc and 0.5 volumes of isopropanol. The DNA was pelleted by centrifugation at 2,000 rpm for 10 min. The DNA pellet was resuspended in a total volume of 5 ml of TE.

The plasmid DNA was purified by isopycnic banding on CsCl gradients to remove residual host cell DNA and RNA. Gradients were prepared in TE with 1.55 g/ml CsCl and 0.5 mg/ml ethidium bromide and the centrifugation was performed at 40,000 rpm for 18 h at 15°C in a TV865B rotor. The band of supercoiled plasmid DNA was visualised under long-wave UV radiation (365 nm) and aspirated by needle and syringe. The ethidium bromide was removed by extraction with CsCl-saturated isopropanol. The plasmid was then dialysed against 2 l of TE at 4°C for 4 h with 2 changes to remove the CsCl. The DNA was precipitated by addition of 2.5 volumes of ethanol and 0.1 volumes of NaOAc and incubation at -20°C. The precipitate was centrifuged at 9,000 rpm for 15 min. The pellet was drained, dried and resuspended in TE to a final concentration of 0.5 mg/ml and stored at -20°C. The yield was generally between 0.2-0.8 mg.

2.22 RESTRICTION ENDONUCLEASE DIGESTION OF PLASMID DNA

Restriction enzymes were used in accordance with the suppliers' instructions, using the buffers provided and at the temperature specified.

2.23 ELECTROELUTION OF RESTRICTION FRAGMENTS FROM AGAROSE GELS

Restriction fragments of plasmid DNA were separated electrophoretically on agarose gels. The digests were visualised under long-wave ultra-violet light and the band(s) of interest excised in the minimum volume of agarose using a sterile scalpel. Each gel slice was placed into a dialysis bag, to which was added 200 ul of TBE. The bag was sealed, making sure to remove all air bubbles and the DNA electroeluted from the agarose at 100 V for 1 h. The polarity of the current was reversed for 30 s and the TBE (containing the DNA) decanted into a sterile tube. The solution was phenol extracted and the aqueous phase decanted. The DNA was precipitated under ethanol and resuspended in TE.

2.24 LIGATION OF DNA FRAGMENTS

If required, the DNA fragments were blunt-ended using T4 DNA polymerase, according to the suppliers' instructions. The DNA samples to be ligated were mixed in differing relative proportions, although the total amount of DNA was always approximately 0.1 ug. The ligation mixture contained; 0.1 ug DNA; 1 ul (2 U) of T4 DNA ligase;

* The specific activity of the [5-^{3}H]dUTP was 21 Ci/mmol.

5 ul of T4 DNA ligase buffer;200ng of oligonucleotide linker (if required) and H_2O to 20 ul. The ligation mixture was incubated at RT overnight.

2.25 PREPARATION OF DNA CONTAINING TRITIATED dUMP

The assay for uracil-DNA glycosylase depends on release of [5-^{3}H]uracil from DNA labelled with [5-^{3}H]dUTP. This DNA was prepared by the method of Caradonna and Cheng (1980) as follows. The reaction mixture (5 ml) consisted of 120 ug/ml activated calf thymus DNA, 50 mM Tris-HCl, pH7.5, 2 mM dithiothreitol, 8 mM $MgCl_2$, 100 ug/ml BSA, 100 uM each of dATP, dCTP, dGTP and 20 uM [5-^{3}H]dUTP*. The reaction was initiated by addition of *E. coli* polymerase I (fraction VIII) and was incubated at 37°C for 60 min. The reaction was terminated by addition of 20 mM EDTA and then heated to 70°C for 5 min. Unreacted nucleotides were eliminated from the sample by dialysis against buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 500 mM NaCl. Dialysis was then continued against the same buffer minus NaCl. The specific activity of the dUMP-containing DNA was generally 10-20,000 cpm/ug.

2.26 URACIL-DNA GLYCOSYLASE ASSAY

Assays for uracil-DNA glycosylase activity were performed, essentially by the method of Caradonna and Cheng (1980). The standard reaction mixture (100 ul) contained 50 mM Tris.HCl, pH 7.5, 2 mM dithiothreitol, 2-3 ug of DNA containing dUMP which had been radiolabelled with ^{3}H (10-20,000 cpm/ug, see 2.25 for preparation and labelling), and 10 ug of BSA. The reaction was initiated by addition of a tenfold dilution of the HSV infected (or mock-infected) cell extract (as described in 2.16) and was incubated at

37°C for 1 h. Termination of the reaction was achieved by chilling to 0°C followed by addition of 13 ul of sheared calf thymus DNA solution (1 mg/ml) and 13 ul of 4 M perchloric acid. After incubation at 0°C for 10 min the samples were centrifuged at 14000 x *g* for 10 min in a benchtop centrifuge. An aliquot of the supernatant (100 ul) was transferred to a vial containing Ecoscint scintillant and counted in a scintillation counter.

2.27 DNA POLYMERASE ASSAY

The infected and mock-infected cell extracts were also assayed for DNA polymerase activity, using the method of Keir *et al.* (1966). The assay mixture (150 ul) was composed of the following: 20 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 200 mM KCl (or 30 mM KCl if uninfected cellular DNA polymerase activity was being measured), 0.4 mM EDTA, 5 mM B-mercaptoethanol, 200 uM each deoxyribonucleoside triphosphate, 2 uCi ^{3}H-TTP and 60 ug denatured DNA (calf thymus or salmon sperm DNA, heated at 100°C for 10 min, then snap cooled in ice to prevent renaturation) or 60 ul activated DNA. 20 ul of enzyme preparation (ie extract) was added to the reaction mixture which was then incubated at 31°C for 1 h. The reaction was terminated by immersion in an ice-bath. Duplicate 50 ul aliquots were spotted onto Whatman No. 1 filter paper discs (2 cm) which were then subjected to four 5% TCA washes (5-10 min per wash) and two alcohol washes before drying under infra-red light and counting on a scintillation counter using Ecoscint as scintillant.

2.28 ALKALINE DEOXYRIBONUCLEASE ASSAY

Alkaline deoxyribonuclease assays were performed on the extracts, by the method of Morrison and Keir (1968). The assay mixture was incubated in a total volume of 100 ul and was comprised as follows: 50 mM Tris-HCl, pH 9.0 (for HSV-induced deoxyribonuclease activity), 2 mM $MgCl_2$, 10 mM B-mercaptoethanol, 12.5 ug native BHK C13 DNA and approximately 4 X 10^5 cpm/assay of ^{3}H-labelled BHK C13 DNA. 5 ul of a fivefold dilution of the extracts was added to the mixture which was then incubated at 31°C for 1 h. The reaction was terminated by immersion in an ice-bath. 200 ug of BSA (0.1 ml of a 2 mg/ml stock) was added to each tube, followed by 0.3 ml PCA/Hyflo Super Cel (12 mg Hyflo Super Cel per 0.6 ml 1 N PCA). The mixture was vortexed and left on ice for 10 min before centrifuging at 1,800 rpm for 10 min on a benchtop centrifuge. 250 ul aliquots of the supernatant were decanted into vials containing Ecoscint scintillant before being counted.

2.29 DEOXYURIDINE TRIPHOSPHATASE ASSAY

dUTPase was assayed by the method of Wohlrab *et al.* (1982). HSV-1 infected and mock-infected cell monolayers (50 mm plates containing 5 x 10^6 cells/plate) were washed with cold PBS before being hypotonically disrupted in buffer comprising 20 mM 4-(2-hydroxethyl)-1-piperazine ethanesulphonic acid buffer (HEPES), pH 7.8, 1 mM dithiothreitol, 1 mM $MgCl_2$. Hypotonic lysates obtained by this method, containing 2 x 10^7 cells in 150 ul, were diluted eightfold in the same buffer. The resulting suspension was treated with Nonidet P-40 (final concentration 0.2% v/v) on ice for 20 min and centrifuged at 500 x *g* for 10 min. The supernatant was made 80 mM in potassium acetate and clarified by centrifugation at 20,000 x *g* for 30 min; this constituted the soluble (cytoplasmic) fraction. The nuclear pellet was washed with 20 mM HEPES, pH 7.8, 1mM dithiothreitol, 1 mM $MgCl_2$ and suspended in the same buffer containing 80 mM potassium acetate at 2 x 10^7 nuclei per 300 ul. dUTPase assay mixture contained in a total volume of 50 ul: 40 ul of enzyme fraction, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ethylene glycol-bis(B-aminoethyl ether)-N,N,N',N',-tetraacetic acid (EGTA), 2 mM ATP, and 1 mM [^{3}H]dUTP (0.1 Ci/mM). The reaction was performed at 4^oC and was terminated by addition of 20 ul of 100 mM EDTA and 105 ul of methanol. Aliquots of the resulting mixture were applied to pre-washed polyethyleneimine-cellulose strips together with unlabelled marker nucleotides. The strips were developed at room temperature with 1 M HCOOH, 0.5 M LiCl and dried. The position of the deoxynucleotides was determined under UV light. Formation of ^{3}H-labelled dUMP was monitored by liquid scintillation counting of the corresponding spots cut from the chromatogram without prior elution (Wohlrab and Francke, 1980).

2.30 RADIOLABELLING OF HSV-1 INDUCED POLYPEPTIDES

Confluent monolayers of BHK C13 cells were either mock-infected or infected at a moi of 20 pfu/cell in 0.2 ml of ETC10. The cells were incubated at 37^oC for 1 h before the inoculum was removed. the cells were washed twice with Eagle's medium with reduced methionine before being overlaid with 2 ml of same. At 2.5 h pi, 100 uCi of ^{35}S-methionine was added directly to the medium. At 16 h post-infection the medium was aspirated and the cells were harvested by addition of boiling mix (see section 2.4) diluted 1:2 in H_2O. The extracts were heated to 80^oC for 5 min and stored at -20^oC until use. Immediately prior to use the extracts were boiled for 10 min.

2.31 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Infected cell extracts prepared as described in 2.30 were subjected to SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) in vertical, discontinuous, single concentration gels. Fresh stocks of 30% acrylamide were prepared in H_2O, with the ratio of acrylamide to N,N'-methylene bisacrylamide being 29.25:0.75 respectively. The stock was then filtered through Whatman No. 1 filter paper before use. The stock was used to give the desired acrylamide concentration in gels in resolving gel buffer giving the final concentration 375 mM Tris-HCl, pH8.9; 0.1% SDS. Polymerisation was initiated by addition of 200 ul of 10% (w/v) ammonium persulphate and 10 ul of TEMED, per 24 ml of gel solution,

The gel solutions were poured between two well-washed glass plates separated by 1.5 mm spacers and sealed with teflon tape to within 4 cm of the top of the plates. Butan-2-ol was layered on top of the gel solution until the latter polymerised. The butan-2-ol was removed and a 5% acrylamide stacking gel was added, into which was placed a teflon well-forming comb.

Samples (50 ul) were applied to the gel, which was run overnight at 15 mA in tank buffer.

Following electrophoresis gels were fixed for 1 h before being agitated through several successive changes of destain. They were then gently agitated in 3 volumes of En^{3}Hance (New England Nuclear) for 1 hr before being washed twice in 10 volumes of H_2O for 15-20 min. The gels were dried under vacuum onto Whatman grade 182 filter paper and exposed to Kodak X-Omat XS-1 film at -70°C.

2.32 AGAROSE GEL ELECTROPHORESIS

Masking tape was applied around all four edges of 163mm x 264mm glass plate. This was placed on a levelling board and made horizontal. Agarose was dissolved in TBE and cooled to 50°C. If required, ethidium bromide was added to a final concentration of 0.5 ug/ml. The gel was poured and allowed to set after insertion of a teflon comb. Once set, the masking tape was removed and the gel was placed into the horizontal gel electrophoresis apparatus. TBE was added up to the level of the top of the gel. Samples were mixed with 0.25 volumes of DF dye before loading into the wells and were electrophoresed at 40 V overnight.

For investigation of plasmid preparations, restriction digests, etc. a minigel apparatus was used. A gel comprising 50 ml of a 1% agarose in TBE with 0.5 ug/ml EtBr was cast in the apparatus, where it was electrophoresed for approximately 1 h at 50 V. In both cases the samples were visualised by exposure to uv light. For analytical gels short wave uv light was used. For preparative purposes, long wave uv was used.

2.33 IODINATION OF SYNTHETIC OLIGOPEPTIDES USING NaI^{125}

The iodination procedure is a modification of the procedure of Hunter and Greenwood (1962). 10 mg of each synthetic oligopeptide was dissolved in 100 ul of potassium phosphate buffer, pH 7.5. 100 uCi of NaI^{125} and 50 ul of chloramine T were then added. This was incubated at RT for 20 s, after which the reaction was stopped by the addition of 50 ul of sodium metabisulphite. Removal of the free NaI^{125} was achieved by passage through a Sephadex G10 column preblocked with 0.1% BSA in 0.16 M borate, pH 9.0

and equilibrated in 0.16 M borate, pH 9.0; 0.13 M NaCl. Iodinated oligopeptide was eluted with the equilibration buffer. 0.5ml fractions were collected and analysed in a gamma counter. The fractions containing the iodinated peptide were pooled and, unless used immediately, were stored at -70°C.

The iodination reactions were performed by Dr Christine MacLean.

2.34 PREPARATION OF BIS-DIAZOTISED BENZIDINE (DAB)

Bis-diazotised benzidine was prepared according to the method of Likhite and Sekar (1967). 0.23 g of benzidine hydrochloride was dissolved in 45 ml of 0.2 M HCl. 5 ml of 0.5 M sodium nitrite was added and the solution was stirred slowly for 1 h at 4°C. The DAB was then aliquoted in 2 ml lots and stored at -70°C until use.

Benzidine hydrochloride and its derivatives are highly toxic and therefore preparation of DAB was performed by Dr Howard Marsden.

2.35 LINKAGE REACTION

The coupling reaction was performed according to the method of Bassiri *et al.* (1979). 30 mg of BSA was dissolved in 1.5 ml of iodinated peptide (approximately 10 mg) in 0.16 M borate, pH 9.0; 0.13 M NaCl. 1 ml of DAB was added and the reaction was left to proceed at 4°C for 2 h. Free and coupled oligopeptides were separated by passage through a Sephadex G25 PD10 column. Samples were eluted with PBS and 10 drop fractions (approximately 0.5 ml) were collected and assayed in a gamma counter. BSA-coupled oligopeptide was eluted in the void volume and the relevant fractions were pooled, dialysed extensively against PBS A to remove residual DAB and stored at -70°C until use. The coupling efficiency is expressed as a percentage of the total radioactivity loaded.

2.36 PRODUCTION OF ANTISERA TO SYNTHETIC OLIGOPEPTIDES

Antisera to the synthetic oligopeptides were raised in New Zealand White rabbits. Immunisation, boosting and terminal bleeding of the animals was carried out by Serotec Ltd.

Antibodies to BSA were removed by adsorption against BSA coupled to Sepharose before the sera were tested by immunoblotting for reactivity against the appropriate protein.

2.37 IODINATION OF PROTEIN A

Protein A (Sigma) was reconstituted in PBS at a concentration of 0.5 mg/ml, aliquoted into 20 ul amounts and stored at -70°C until use. To 20 ul (10 ug) of protein A was added, consecutively, 10 ul of potassium phosphate buffer, pH7.5; 200 uCi of NaI^{125}; and 20 ul of chloramine T (0.5 mg/ml in PBS). The reaction was left for 20 s at RT before the reaction was stopped by the addition of 20 ul of sodium metabisulphite (1 mg/ml in PBS). Free NaI^{125} was removed by passage through a Sephadex G25 PD10 column. The iodinated protein A was eluted with PBS in 0.5 ml fractions. 10 ul samples of each fraction were assayed in a gamma counter and fractions containing the iodinated protein A were pooled and stored at 4°C in the presence of 0.01% sodium azide.

The iodination reactions were performed by Dr Christine MacLean.

2.38 IMMUNOBLOTTING

Immunoblotting was carried out essentially by the method of Towbin *et al.* (1979) but with several modifications. Infected cell extracts were prepared as described above and subjected to SDS-PAGE. Following electrophoresis, the proteins were transferred to nitrocellulose strips using a BioRad "Transblot" apparatus, used according to the manufacturer's instructions. Into a transfer cassette immersed in transfer buffer was placed a foam pad, followed by a sheet of Whatman grade 182 filter paper and then the gel. On top of this, numbered nitrocellulose strips, presoaked in transfer buffer, were placed face down on the gel, being careful to exclude air bubbles. Another sheet of filter paper and finally another foam pad were placed on top. The cassette was tightly closed and inserted into the transfer tank. The proteins were transfered onto the nitrocellulose by electrophoresis in transfer buffer at 250 mA for 3 h at RT.

Following transfer the nitrocellulose was blocked by agitation at 37oC for 2 x 30 min in small tubs containing TBS supplemented with 3% gelatin. After this time, the strips were rinsed twice in wash buffer containing 0.05% Tween 20. The strips were then ready to be incubated with the antisera. The antisera were first preabsorbed against BSA-Sepharose to remove anti-BSA antibodies before being diluted tenfold with wash buffer supplemented with 0.05% Tween 20 and 1% BSA. 3 ml of each antiserum was dispensed into channels cut into a perspex block. The strips were placed face up in the channels and covered with a glass plate before being incubated at RT overnight on a rotary shaker. The strips were removed and agitated in wash buffer with 0.05% Tween 20 for 4 periods of 30 min. The strips were then transferred into the channels of another perspex block into which had first been dispensed 3 ml of wash buffer with 0.05% Tween 20 and 500,000 cpm of I^{125}-protein A. A glass plate was placed over the block, which was then agitated for 2 h at RT. The strips were then washed for 2 periods of 30 min in wash buffer containing 1 M KI and then 2 x 15 min washes with PBS. The strips were placed on tissues to dry and then taped to cardboard sheets for autoradiography.

To determine which of the S^{35}-methionine labelled proteins the I^{125}-labelled protein A interacted with, the protocol of Haarr *et al.* (1985) was employed. Using this procedure the strips were used to simultaneously expose 3 films in an autoradiographic sandwich. One film was placed directly on top of the card to which the strips were taped. Over this was placed a sheet of black paper followed by a further two sheets of film and an image intensifying screen. Thus, the first film detects the S^{35}-methionine label which does not penetrate as far as the second film. The I^{125} label, which is of higher energy, passes through all three films with little absorption and interacts with the image intensifying screen to produce photons. These photons are detected mainly on the third film and partly on the second film. The photons are prevented from reaching the first film by the black paper. Alignment of the films was facilitated by spotting radioactive ink, containing C^{14} and I^{125}, onto the card.

RESULTS AND DISCUSSION

3.1 RECOMBINANT PLASMIDS

3.1.1 pFJ3

The DNA fragment which was used in the insertion mutagenesis work described in this thesis originated from the plasmid pFJ3. The structure of this plasmid, which was supplied by Drs Frazer Rixon and John McLauchlan, is shown in figure 3.1. The *LacZ* gene in this plasmid is under the direction of the SV40 early promoter (Hall *et al.*, 1983). The relevant moiety is the part numbered 5 to 4122, between the two *Xba*I sites. This fragment contains the *LacZ* indicator gene and was used to construct pJM3 and pJM4. pJM3 and pJM4 were used in the insertion mutagenesis experiments and their construction is described later. pFJ3 was digested with *Xba*I and the two resultant fragments were separated by electrophoresis on an agarose gel. The fragment containing the *LacZ* gene was purified by electroelution and used in the construction of pJM3.

3.1.2 pGX23

All manipulations involving the HSV-1 genes UL2 and UL1 were first performed on plasmid-borne copies of the genes. The plasmid pGX23 contains the HSV-1 *Bam*HI *e* fragment ligated into the *Bam*HI site in pAT153. This fragment of HSV-1 is from residues 2907 to 11820 of the virus genome (McGeoch *et al.*, 1988) and contains the genes UL1, UL2 and UL3. The plasmid is shown diagrammatically in figure 3.2.

3.1.3 pJM3

The plasmids pFJ3 and pGX23 were used in the construction of pJM3, which is shown in figure 3.3 and was

Figure 3.1 : The plasmid pFJ3

This plasmid was used in the mutagenesis experiments described. The fragment between the two *Xba*I sites at positions 5 and 4122 contains the *E. coli Lac Z* gene, with SV40 early promoter and polyadenylation signals, as indicated.

This plasmid was a gift from Drs F J Rixon and J McLauchlan.

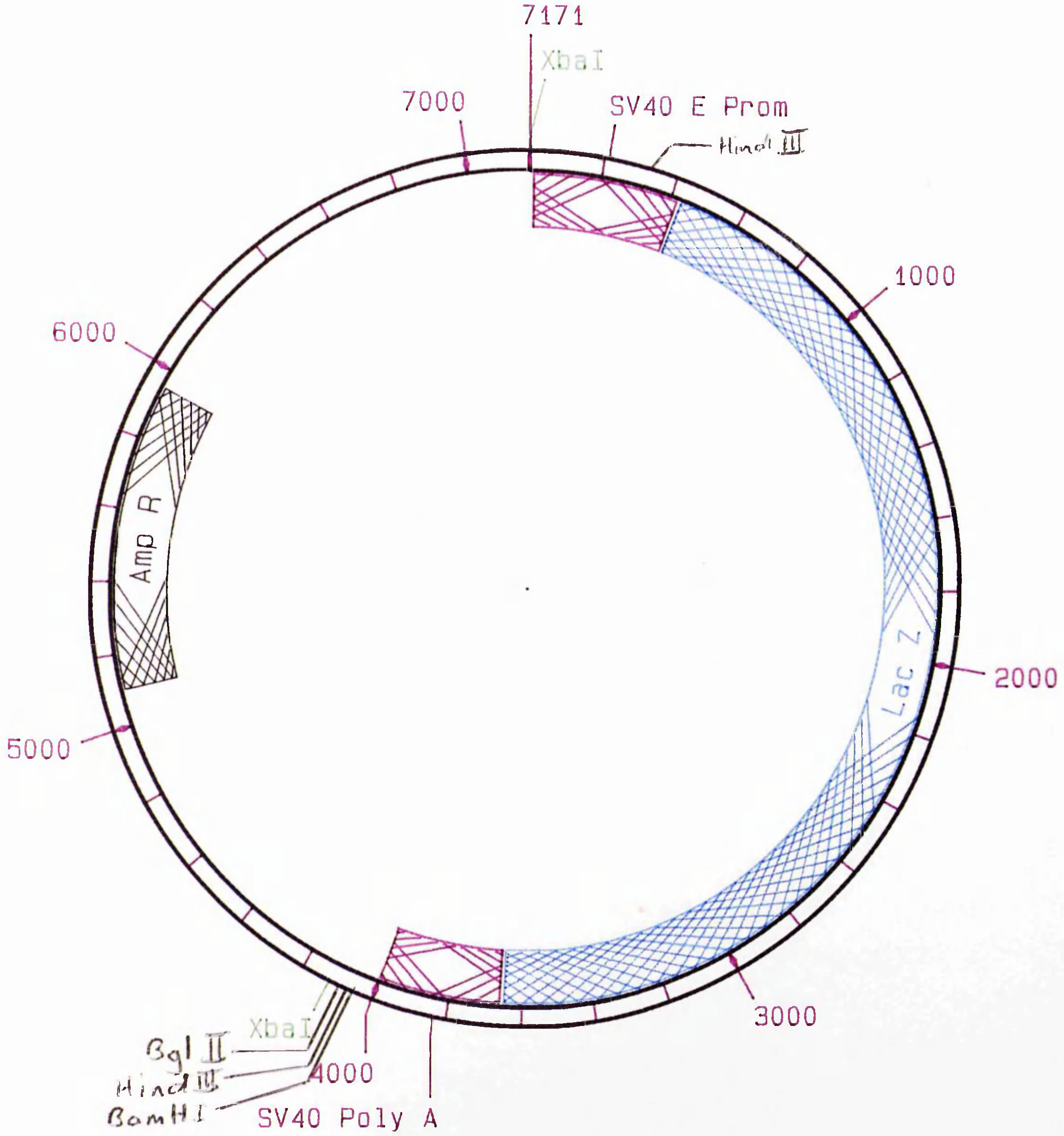
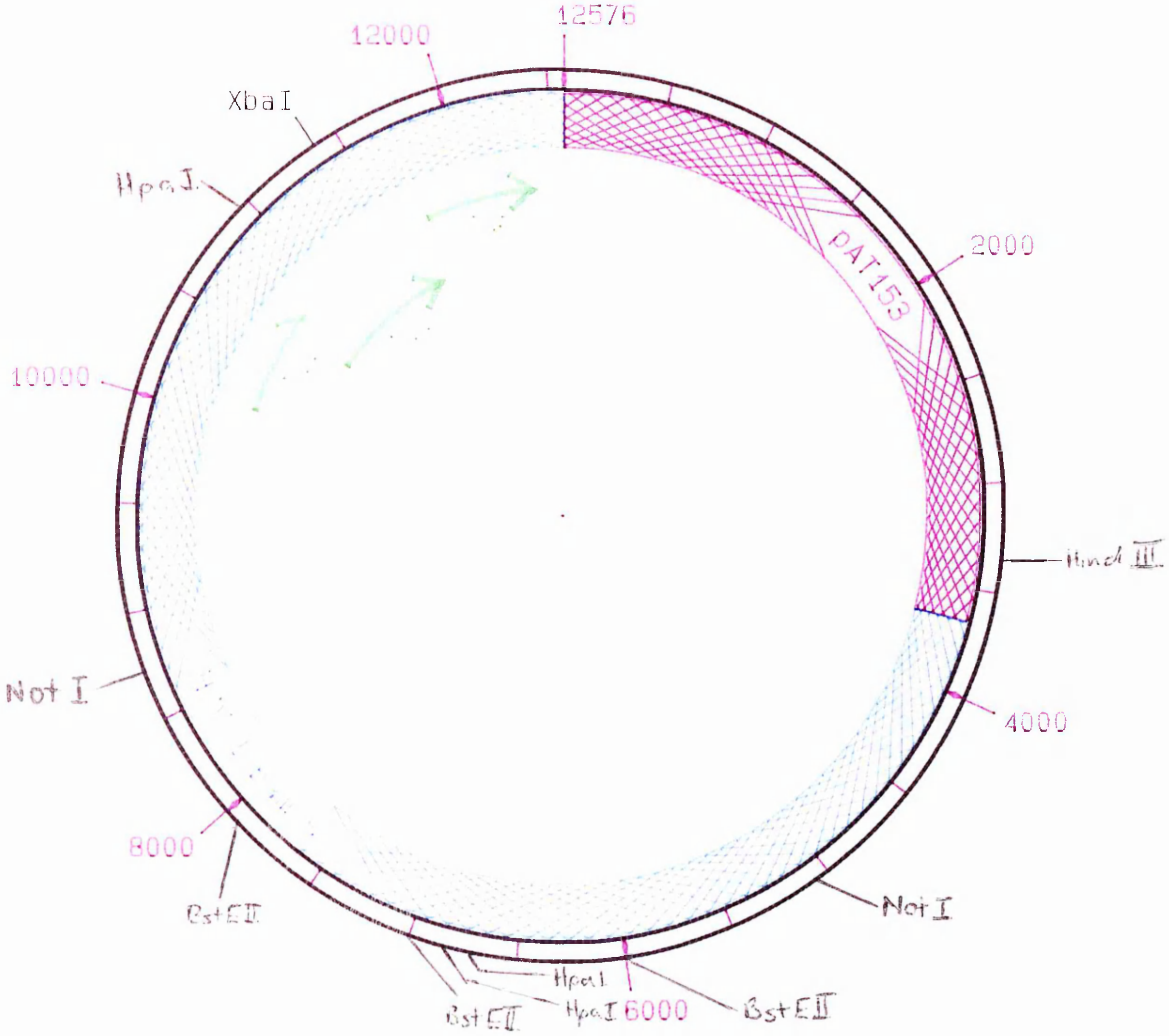

7171
XbaI
7000
SV40 E Prom
Hind III
1000
6000
Amp R
Lac Z
2000
5000
3000
XbaI
Bgl II
Hind III
BamHI
4000
SV40 Poly A

Figure 3.2 : The plasmid pGX23

The plasmid pGX23 was used to construct the mutations described in this section. The structure of the plasmid is depicted in this figure. pAT153 sequences extend from position 1 to 3657, and the HSV-1 *Bam*HI *e* fragment (residue 2907 to 11820 in the virus genome) is from position 3658 to 12576 on the plasmid. The positions of genes UL1, UL2 and UL3 are indicated.

This plasmid was a gift from Dr V G Preston.


12576
12000
Xba I
Hpa I
10000
Not I
8000
Bst E II
Bst E II
Hpa I
Hpa I
6000
Bst E II
Not I
4000
Hind III
2000
pAT153

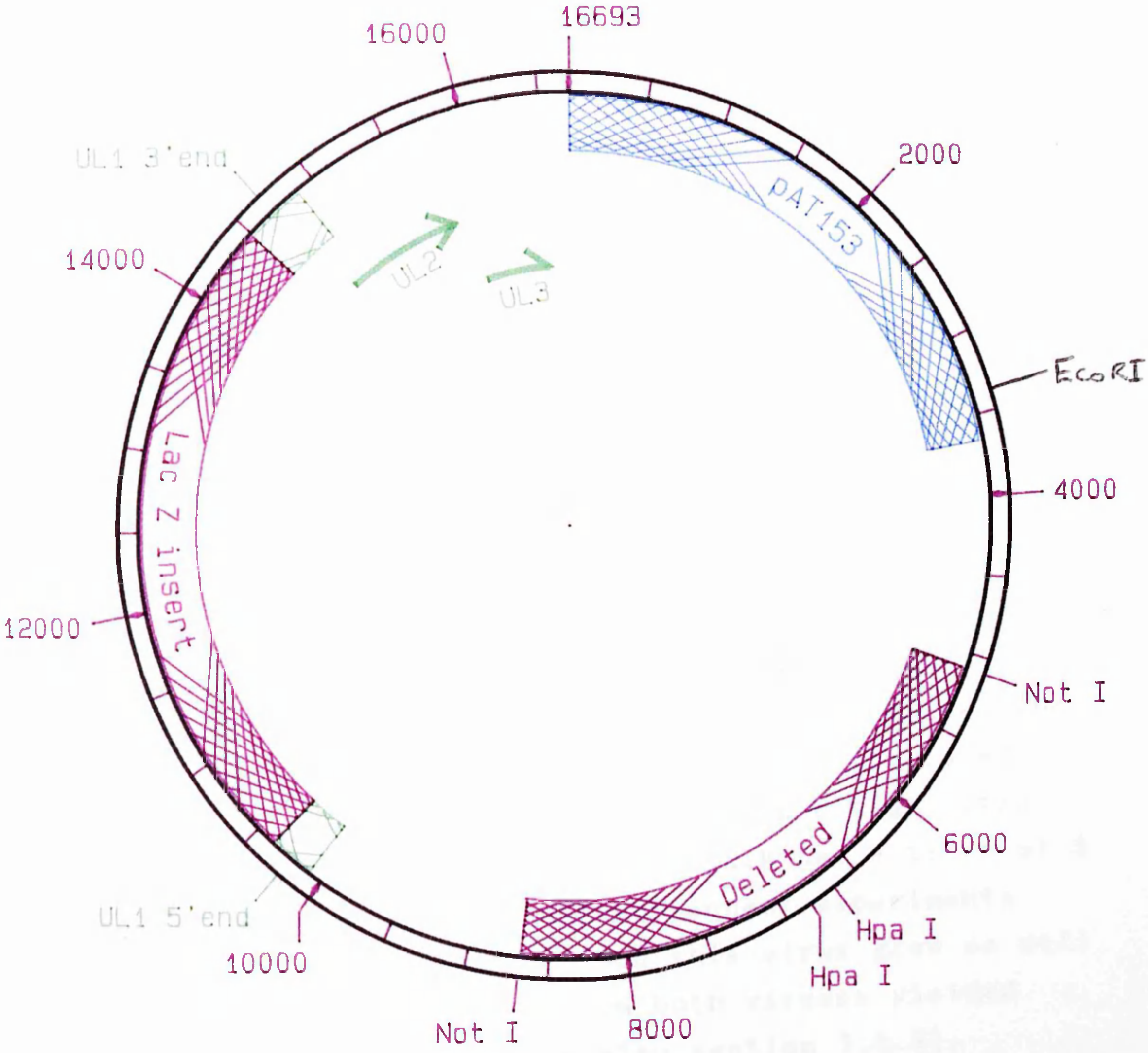
constructed as follows. The *Xba*I fragment from pFJ3 containing the *LacZ* gene was blunt-ended and ligated into the *Xba*I site in the plasmid pGX23. This site is within the coding sequence of the UL2 gene, (McGeoch *et al.*, 1988). The ligation mixture was used to transform competent *E. coli* DH5 on L-broth agar plates containing X-gal and ampicillin. Bacterial colonies transformed with a plasmid containing the *LacZ* gene were blue on these plates. Restriction analysis using the enzymes *Xba*I, *Bam*HI, *Eco*RI and *Pst*I confirmed the structure of pJM3 and indicated the orientation of the insert within the gene. pJM3 was subsequently used in the construction of the recombinant viruses in1601 and in1602.

3.1.4 pJM4

The plasmids pGX23 and pFJ3 were also used to construct the plasmid pJM4, which is shown in figure 3.4. pGX23 has a *Hpa*I restriction site within the coding sequence of UL1. There are two other *Hpa*I site in pGX23, which were removed by digestion with *Not*I and religation of the large fragment, containing the remaining *Hpa*I site. The *LacZ* fragment from pFJ3 was inserted into this unique *Hpa*I site and the ligation mixture was used to transform competent *E. coli* DH5 on L-broth agar plates containing X-gal and ampicillin. Colonies which had been transformed by the plasmid were blue on these plates. The structure of the plasmid was confirmed by restriction analysis, using the enzymes *Eco*RI, *Hpa*I, *Pst*I and *Hind*III. This plasmid was then used in the construction of the recombinant virus in1603.

Figure 3.3 : The plasmid pJM3

This plasmid used to engineer the mutations in UL2 present in the recombinant viruses in1601 and in1602, and was constructed by insertion of the appropriate *Xba*I fragment of pFJ3 into the unique *Xba*I site in the UL2 coding region. The positions of the HSV-1 genes and the insert are shown.


16693
16000
2000
pAT153
EcoRI
4000
Not I
6000
Deleted
Hpa I
Hpa I
8000
Not I
10000
UL1 5'end
12000
Lac Z insert
14000
UL1 3'end
UL2
UL3

3.2 RECOMBINANT VIRUS in1601

3.2.1 COTRANSFECTION AND PURIFICATION

The plasmid pJM3 was digested with the restriction enzyme *Bst*EII. The fragments were separated by electrophoresis on an agarose gel and the appropriate fragment was recoved by electroelution. This fragment was used with *wt* HSV-1 DNA in the cotransfection which gave rise to the mutant in1601.

The progeny from the initial cotransfection were titrated as described. All eleven blue plaques from the initial titration were picked and titrated. In subsequent rounds of plaque purification, ten blue plaques were picked and carried forward. The recombinant plaqued as well as *wt*. Plaque purification continued until all the progeny exhibited the recombinant phenotype. At this stage a single, isolated blue plaque was picked and used to generate a high-titre elite stock, designated in1601. This stock had a titre of 5 X 10^9 pfu/ml and was used in all subsequent experiments involving in1601. In tissue culture this virus grew as well as *wt*, in that plaques picked from both viruses yielded similar numbers of progeny (see also section 3.2.2). Plaques from in1601 are indistinguishable from those caused by infection with *wt*, with the exception that the former stain blue when grown under X-gal.

Isolation of the recombinant was facilitated by the phenotype conferred on it by the indicator gene.

The frequency of recombination in this case may be as low as 1 in 10^4 or less. This is lower (by at least two orders of magnitude) than the frequency of recombination reported by others. For example Ace *et al.* (1989) reported isolation of a mutant of HSV-1 which contains an insertion in UL48, the gene which encodes the virus trans-inducing factor, $V_{mw}65$. Their reported recombination efficiency was 1 mutant in 84 plaque isolates screened. However, in this case the insert was a 12 bp oligonucleotide. In the construction of dut-1218, a recombinant virus used later in this study, the recombination efficiency was estimated as approximately 1% (V G Preston, personal communication). Once again the insertion was of a small (8 bp) oligonucleotide. In the case of in1601, recombination may have been impeded by potential spatial constraints imposed by the relatively large (4.1 kbp) insert. Since the resulting mutant (in1601) subsequently showed no signs of impairment in its growth, it can be assumed that the low recombination frequency was not due to a secondary dysfunction (ie reduced viability of the mutants brought about by the mutation), but may be as a result of the low probability of a recombination event occurring on both sides of the insert. Addition of X-gal to the growth medium does not restrict the growth of *wt* virus and so there is no selection pressure which would preferentially encourage growth of the recombinant progeny. However, the ease with which recombinants can be recognised compensates for the low recombination frequencies obtained with such a large insert.

The genome of in1601 was verified by restriction endonuclease digestion, as described in section 2.13. The restriction enzymes used in this analysis were *Xba*I, *Bam*HI, *Bgl*II, and *Hind*III. The effects brought about by the mutation on the restriction profile of each of these enzymes is discussed in turn. In each case, the alterations are discussed first with respect to the prototypic orientation of the genome, followed by the effects due to orientation isomerisation.

a) *Xba*I

The insert is bounded by *Xba*I sites and has been inserted at the *Xba*I site between the *g* and *c* fragments. The predicted effect of the mutation in this case would be the appearance of a novel band (N), 4.1 kbp in size. All the other fragments should be identical to their *wt* counterparts. A partial *Xba*I restriction map of *wt* and in1601 (both in the prototypic orientation) is shown in figure 3.5.

The phenomenon of orientation isomerisation means that some restriction fragments only exist in certain orientations of the genome. Since *g* is a terminal fragment in the prototypic arrangement, it is only present in the prototype and I_S orientations. In the other two arrangements of the genome (I_L and I_{SL}), *g* is combined with the short segment of the genome (there are no *Xba*I sites in this segment) to become the *b* fragment. Therefore, any changes in the *g* fragment will also be reflected in the *b* fragment. However, in the case of in1601, the *g* fragment is still intact, as is the *Xba*I site between it and the *c* fragment, and so the mutation does not affect fragments which are present in orientations other than the prototype.

Figure 3.6 is an autoradiograph of the *Xba*I restriction profiles of *wt* and in1601 DNA. The figure demonstrates that the only genotypic change brought about by the insert

Figure 3.5 : Partial *Xba*I restriction maps of *wt* and in1601

The relative positions of *Xba*I restriction sites at the left end of the prototypic virus genome in both *wt* and in1601 are indicated. The *wt* genome has only one site in this region (the site of insertion). The genome of in1601 has an extra *Xba*I site, brought about by the insertion, therefore digestion with *Xba*I will give rise to a novel restriction fragment, N, corresponding to the insert, which is shown as a thick line.

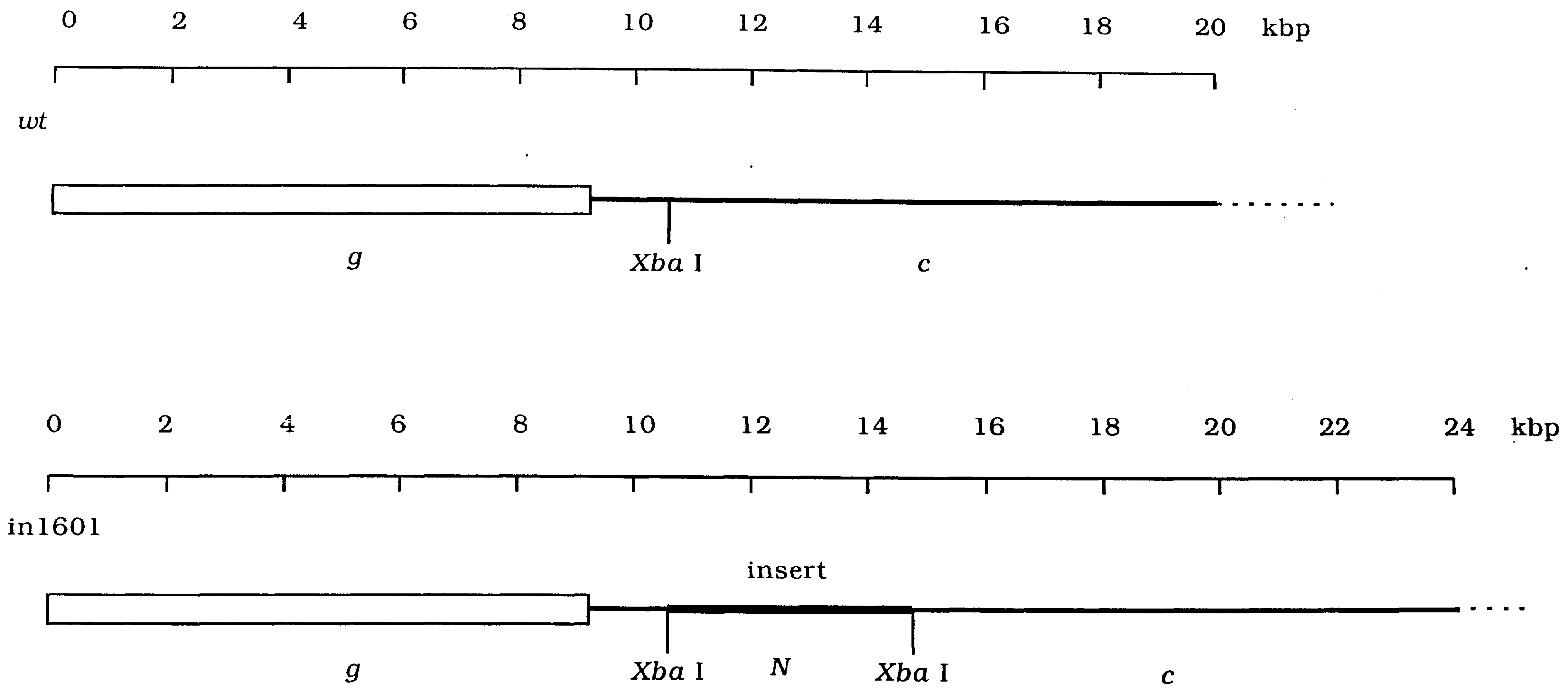
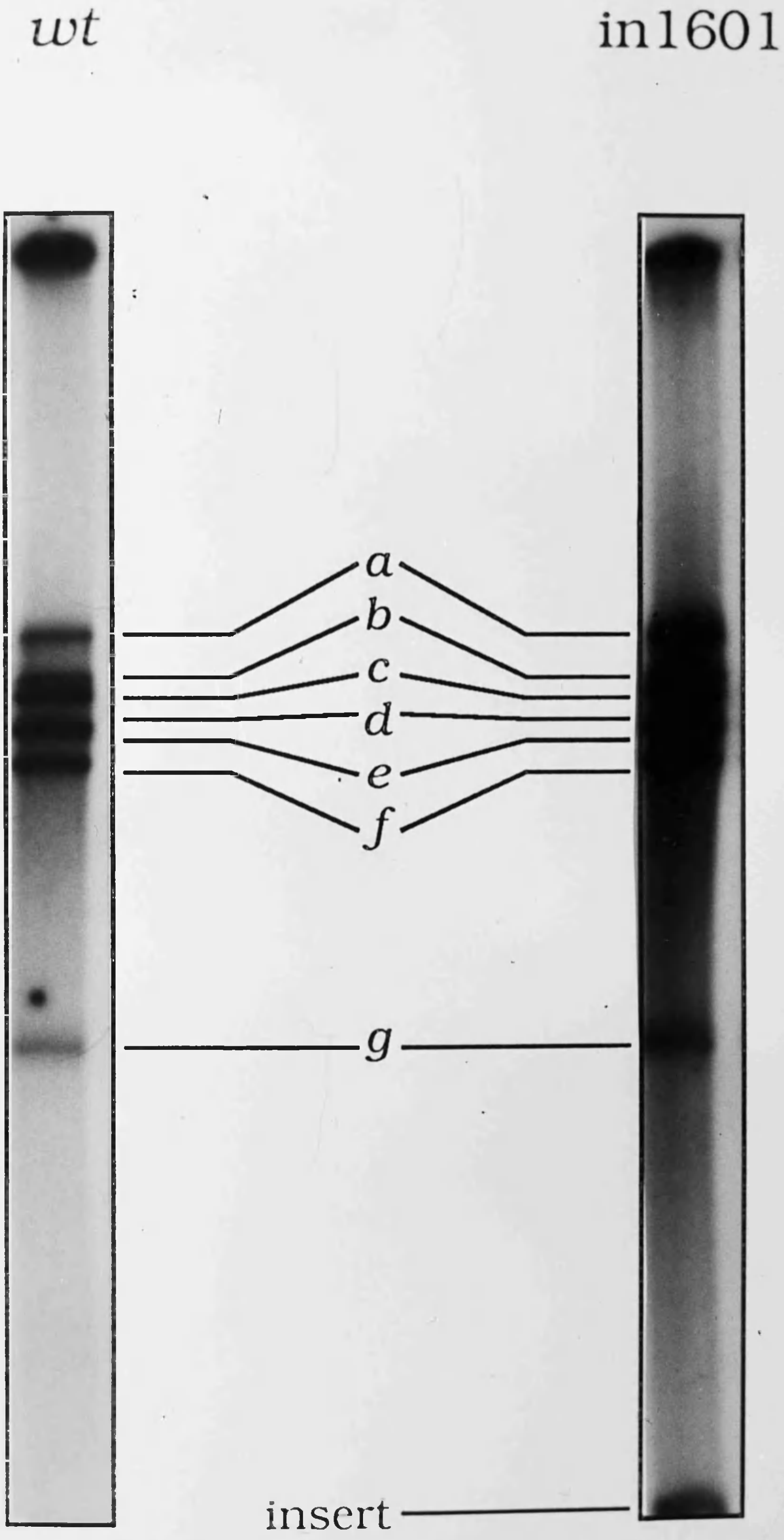

kbp
wt
g
Xba I
c
kbp
in1601
insert
g
Xba I
N
Xba I
c

Figure 3.6 : *Xba*I restriction profiles of *wt* and in1601

Figure 3.6 is an autoradiograph of *wt* and in1601 DNA digested with *Xba*I and separated on a 1.2% agarose gel. The *wt* has a normal restriction profile. The profile obtained from in1601 is the same as *wt*, with the exception of a novel band, N, which corresponds to the insert and migrates below *g*.


wt
in1601
a
b
c
d
e
f
g
insert

appears to be the presence of a novel restriction fragment (N) which migrates below *g*, and is of the predicted mobility.

b) *Bam*HI

The *LacZ* insert contains one *Bam*HI site and has been inserted into the HSV-1 *Bam*HI *e* fragment. The predicted changes from the *wt* genotype are :

The *e* band in *wt* HSV-1 is 8.9 kbp long. Insertion of the mutating DNA increases the length of *e* by 4.1 kbp. The insertion of this fragment introduces a novel *Bam* HI restriction site, such that cleavage by *Bam*HI should show the disappearance of the truncation of the *e* fragment to *e'*, and the appearance of a novel fragment, N. Changes in the *e* fragment should not be accompanied by changes in any of the other fragments, as the *e* fragment is not located at either of the termini or at the L-S junction. Figure 3.7 is a partial *Bam*HI restriction map of the prototype orientations of *wt* and in1601 DNA.

The autoradiograph (figure 3.8) displays the *Bam*HI restriction profiles of *wt* and in1601. The differences between the two profiles are as predicted ie the *e* fragment has been truncated to the fragment *e'* and a novel fragment, N, of the predicted mobility. This is observed in the in1601 track but not in the *wt* track.

c) *Bgl*II

The mutagenising fragment has been inserted into the *Bgl*II *j* fragment and contains one *Bgl*II site. The predicted alterations in the prototype isomer of the genome, due to the insertion are :

Figure 3.7 : Partial *Bam*HI restriction maps of *wt* and in1601

This figure depicts the *Bam*HI restriction sites found in this region of the *wt* and in1601 genomes. The insert, which is represented by a thick line, introduces a novel *Bam*HI site into the genome of in1601, which results in the appearence of a novel restriction fragment, N, into its restriction profile. This novel *Bam*HI site truncates the *e* fragment by 0.9 kbp, to *e*'.

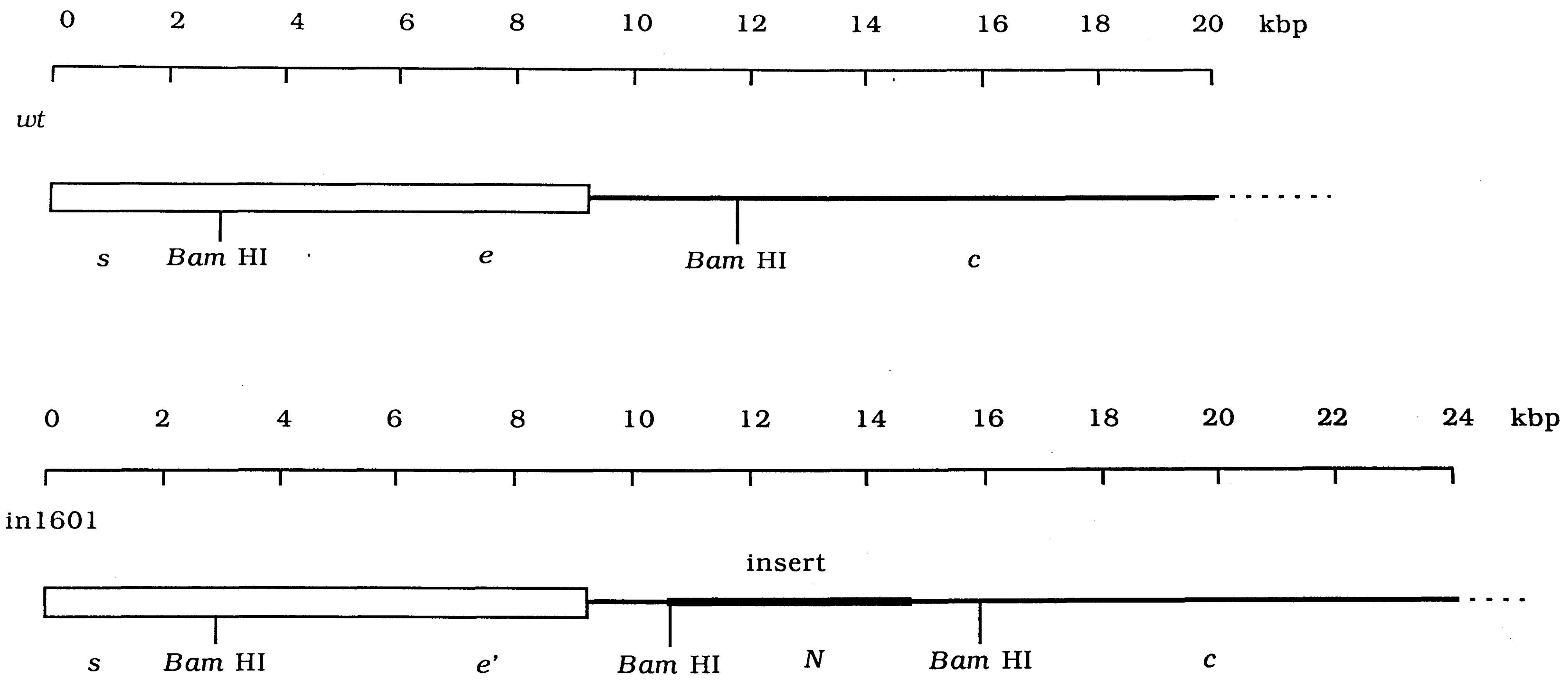
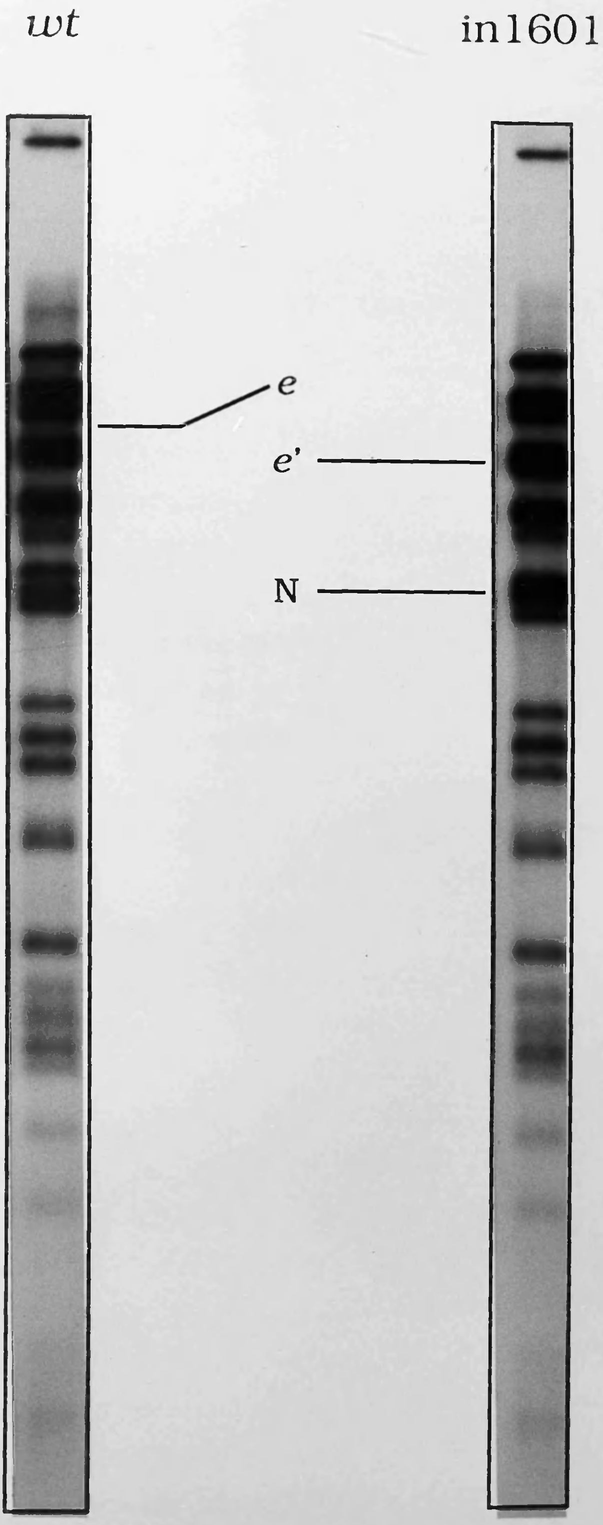

0
2
4
6
8
10
12
14
16
18
20
kbp
wt
s
Bam HI
e
Bam HI
c
0
2
4
6
8
10
12
14
16
18
20
22
24
kbp
in1601
insert
s
Bam HI
e'
Bam HI
N
Bam HI
c

Figure 3.8 : *Bam*HI restriction profiles of *wt* and in1601

The *Bam*HI restriction profiles of DNA derived from *wt* and in1601 virus is shown opposite. The *wt* genome exhibits a normal restriction profile. In the in1601 profile, the *e* fragment is replaced by the truncated *e*' fragment, and there is a novel fragment, N, not present in the *wt* track. These changes are as predicted. The restriction fragments were separated on a 0.7% agarose gel.


wt
in1601
e
e'
N

The *Bgl* II *j* fragment is 14.6 kbp in length and the insert increases the size of *j* by 4.1 kbp. The insert introduces one new *Bgl*II site into the *j* fragment. Digestion of in1601 with *Bgl*II should therefore give rise to the truncation of the *j* fragment to *j'*, and to the appearance of a novel band N. Figure 3.9 is a *Bgl*II restriction map of the two viruses in the prototypic orientation.

Because of the existence of the four sequence-orientation isomers, changes in the *j* fragment will also affect the *b* and *e* fragments, which include *j* and are seen only in the other genome arrangements. These two fragments will be replaced by the fragments *b'* and *e'*, respectively, and will also be accompanied by a novel band N, identical to the novel fragment accompanying the *j'* fragment.

The autoradiograph figure 3.10 shows the fragments obtained by digestion of *wt* and in1601 DNA by *Bgl*II. The band corresponding to the *j* fragment is absent in the in1601 profile. However, the predicted band of lower mobility, corresponding to the truncated *j* fragment cannot be discerned. This band, the *j'* fragment is predicted to be 10.6 kbp in length, which is approximately the same size as the *k* fragment, which is also 10.6 kbp. It is expected that these two bands would therefore comigrate. Because *j* is a terminal fragment, it is represented in half-molar amounts, with respect to *k*. This would limit the increase in the intensity of the band representing the *k* + *j'* fragments. The *b* and *e* fragments are also truncated in the mutant, but again the truncated bands (*b'* and *e'* respectively) are not visualised, due to their comigration with the *c* and *f* bands, respectively. Both *b* and *e* are quarter-molar bands and so the changes in the intensities of the *c* and *f* bands

Figure 3.9 : *Bgl*II restriction maps of *wt* and in1601

As can be seen from this figure, the insert is located within the *Bgl*II *j* fragment, and introduces a novel *Bgl*II restriction site into this fragment, truncating it to the fragment *j'*. The introduction of the novel *Bgl*II site also results in the presence of a novel restriction fragment, N. *j* is a terminal fragment and as such is only present in two arrangements of the genome (prototype and I_S). In the other two arrangements, *j* is incorporated into the *b* and the *e* fragments. Correspondingly, the mutation, which truncates *j*, will also truncate *b* and *e*, to *b'* and *e'*, respectively, and in both cases this will also be accompanied by the appearance of the novel fragment, N.

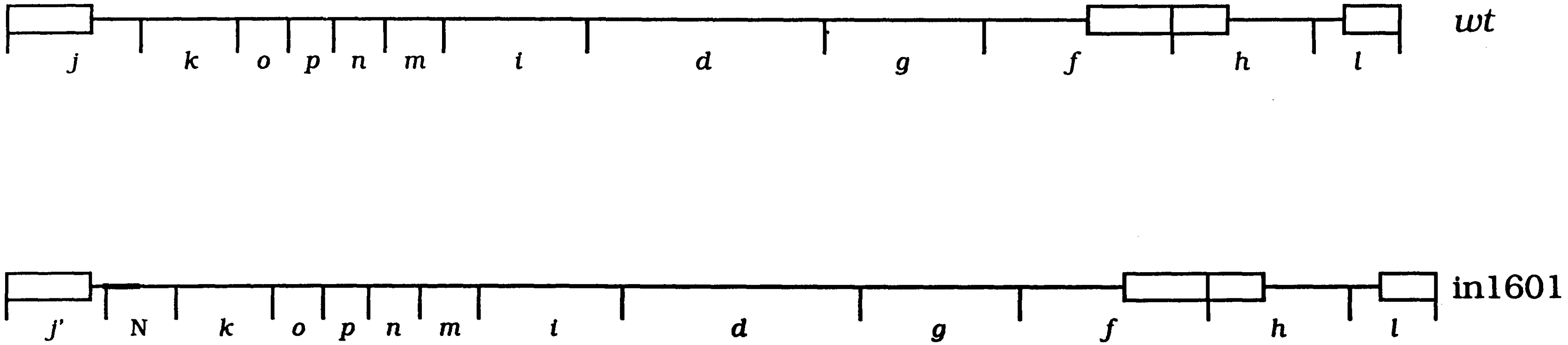
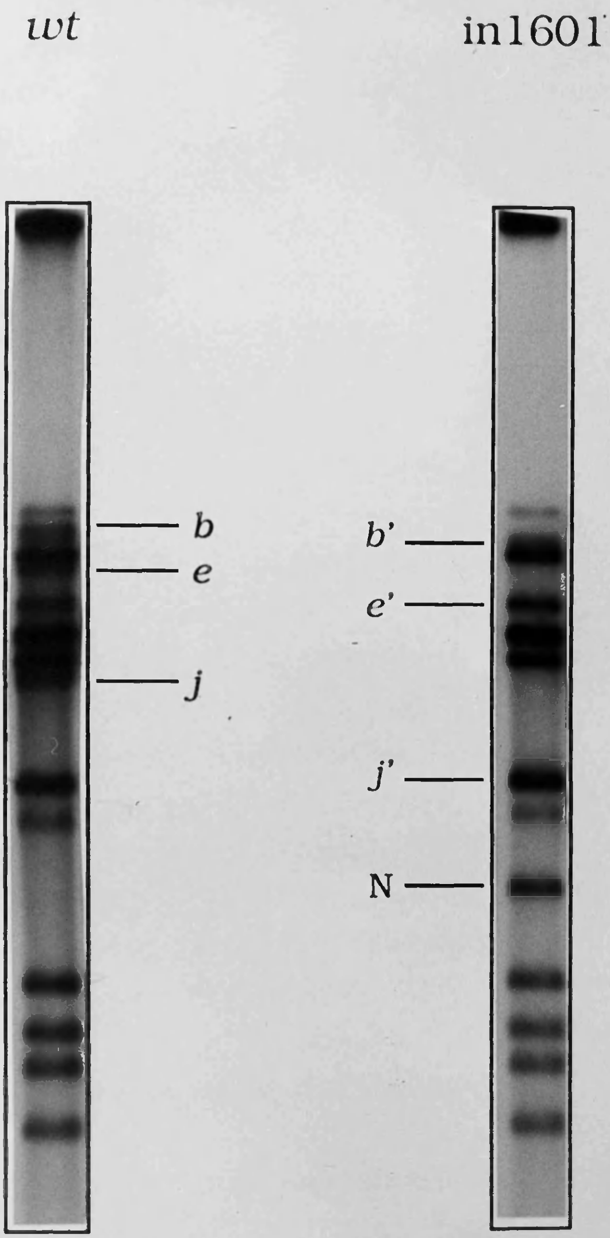

wt
j k o p n m i d g f h l
in1601
j' N k o p n m i d g f h l

Figure 3.10 : *Bgl*II restriction profiles of *wt* and in1601

In the case of digestion with *Bgl*II, some of the changes brought about the mutation are noticeable, but others are difficult to see. The novel band, N, is very obvious, as is the disappearence of the *j*, *b* and *e* fragments. However, it is difficult to discern the truncated *j*', *b*' and *e*' fragments. In the case of *j*', which being a terminal fragment, is half molar, the truncated fragment is the same size as another fragment, *k*, and therefore comigrates with it. *k* is a molar band and so it is difficult to determine any increase in intensity brought about by the half-molar *j*' fragment. The same is also true of *b*' and *e*', which are also half-molar and comigrate with the fragments *c* and *f*, respectively. The restriction fragments were separated on a 0.8% agarose gel.


wt
in1601
b
e
j
b'
e'
j'
N

are even more difficult to see than the increase in the *k* band. The novel band (N), composed of the insert plus part of the *j* fragment is seen migrating between the *l* and *m* bands, which is as expected. It is the case that all the differences observed in the mutant are as predicted.

d) *Hind*III

The insert, which has two *Hind*III sites, has been inserted into the *Hind*III *i* fragment. The predicted changes in the *Hind*III restriction profile of the prototypic orientation are :

The *i* fragment is 12.7 kbp in length and the insert increases this by 4.1 kbp. The insert contains two *Hind*III sites, separated by 3.7 kbp. Digestion of in1601 by *Hind*III should give rise to truncation of the *i* fragment by 2.1 kbp to *i'*, and to the appearence of two novel bands (N1 and N2), 3.7 kbp and 2.4 kbp in length respectively. Figure 3.11 is a partial *Hind*III restriction map of prototypic *wt* and in1601. *i* is a terminal fragment and so is only found in the prototype and I_S arrangements of the genome. In the other two orientations, *i* is incorporated into the *e* and *f* fragments, respectively. Thus, the mutation will also affect the *e* and *f* fragments, which will be truncated to *e'* and *f'*, respectively. These fragments will also be accompanied by the two novel bands N1 and N2.

The autoradiograph of the restriction profiles of both viruses digested with *Hind*III (figure 3.12) demonstrates the altered mobility of the *i*, *e* and *f* bands to *i'*, *e'* and *f'* respectively (although in this figure, the *i* fragment cannot be distinguished, due to its comigration with *h*, which is of a similar size and is present in molar amounts as opposed to *i*, which is only half molar). These bands are

Figure 3.11 : *Hind*III restriction maps of *wt* and in1601

This figure shows the location of the insert in the prototype arrangement of the genome, and the locations of the *Hind*III sites. The insert is located within the *i* fragment and introduces two novel *Hind*III sites into the genome. This results in the truncation of *i* to *i'*, and the appearence of two novel restriction fragments, N1 and N2. *i* is a terminal fragment and as such is only present in two arrangements of the genome (prototype and I_S). In the other two arrangements, *i* is incorporated into the *e* and the *f* fragments. Correspondingly, the mutation, which truncates *i*, will also truncate *e* and *f*, to *e'* and *f'*, repectively, and in both cases this will also be accompanied by the appearence of the two novel fragments, N1 and N2.

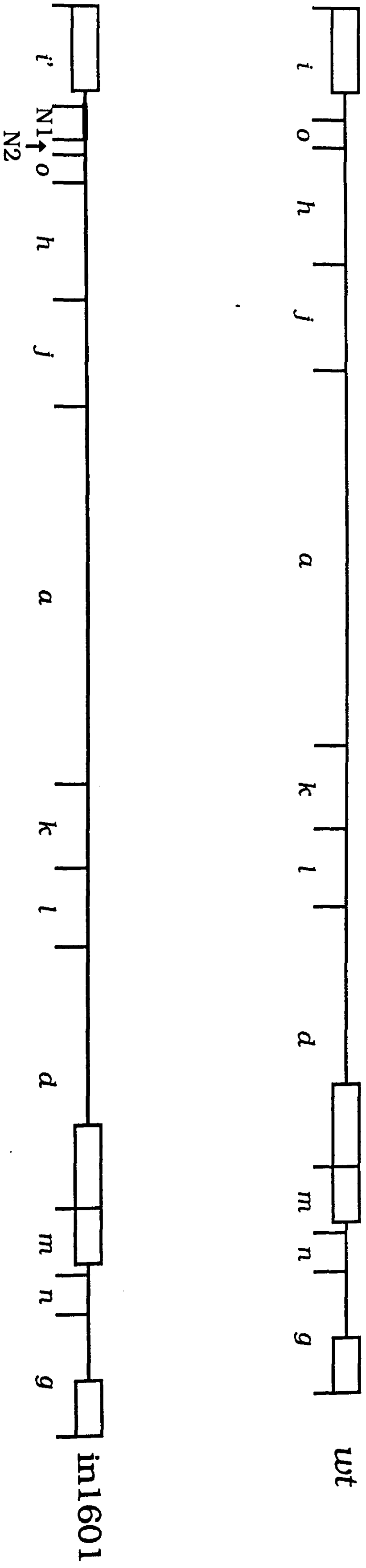
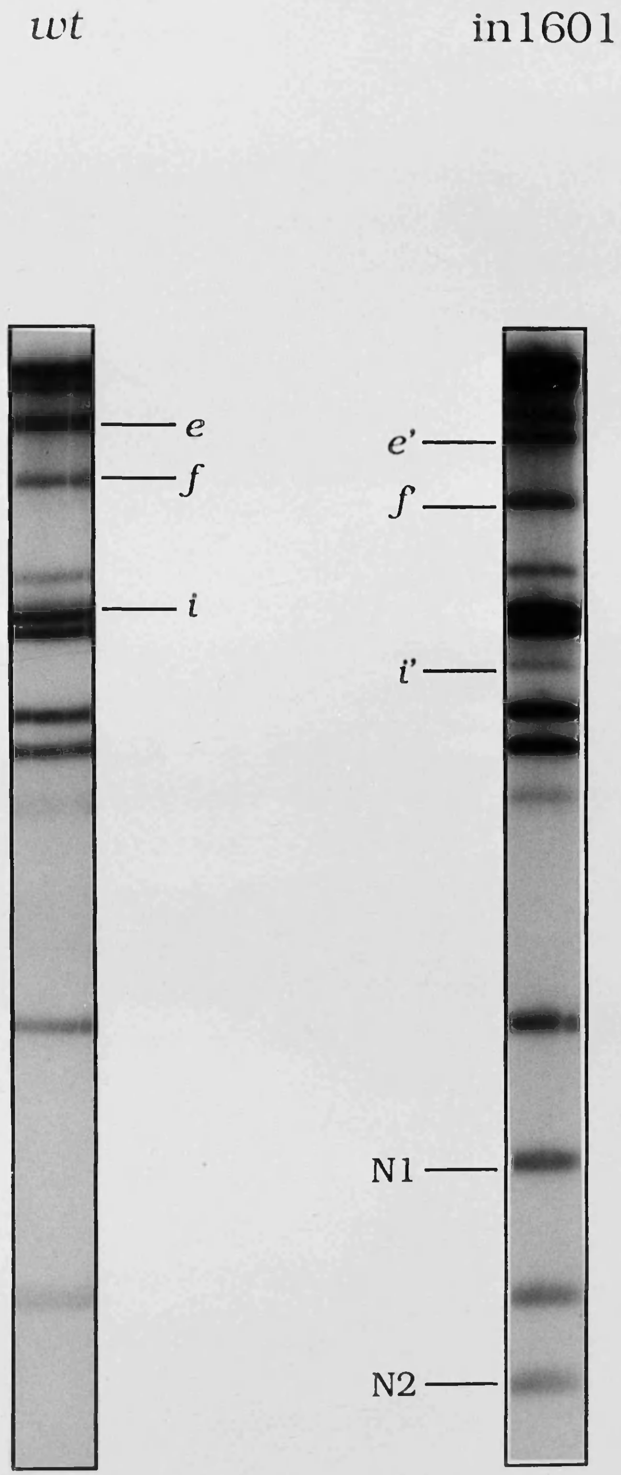

wt
i
o
h
j
a
k
l
d
m
n
g
in1601
i'
N1
N2
o
h
j
a
k
l
d
m
n
g

Figure 3.12 : *Hind*III restriction profiles of *wt* and in1601

Figure 3.12 is an autoradiograph of DNA prepared from *wt* and in1601 virus, and digested with *Hind*III. The *wt* profile is as expected; the *i* fragment co-migrates with *h* and so cannot be observed. However, *i'* can be seen in the in1601 profile, as can *e'*, *f'* and the two novel bands, N1 and N2, which are all of the predicted mobility. The restriction fragments were separated on a 1% agarose gel.


wt
in1601
e
f
i
e'
f
i'
N1
N2

of the predicted mobility, as are the two novel bands (N1 and N2) indicated.

All of these profiles together confirm that the insert has been introduced at the predicted location and that no other rearrangements are to be found in the genome of in1601. There have been reports of insertional mutagenesis by homologous recombination in HSV-1 giving rise to genome rearrangements other than those predicted. Longnecker and Roizman (1986) attempted to recombine a DNA fragment containing part of the HSV-1 IE175 gene and a copy of Ori_S into the TK gene. Analysis of the progeny revealed extensive, unpredicted rearrangements and deletions in U_S and in the L/S junction sequences. However, the authors have attributed this phenomenon to the inclusion of some *cis*-acting signal which was detrimental to the growth of the recombinant and bestowed a selective advantage to mutants arising spontaneously. Pogue-Geile *et al.* (1985) reported the isolation of mutants of HSV-1 exhibiting unpredicted rearrangements after cotransfecting DNA from the HSV-1 *Bam*HI *l* fragment into the TK gene. Presumably these rearrangements are a result of recombinational events between the sequences which are duplicated by the insertion of HSV-1 DNA. In the present study, the DNA used in the mutagenesis was not derived from the virus and so presumably there was not the same potential for recombinational rearrangements as there were no novel duplications in the genome of the progeny.

3.2.2 PROPERTIES OF THE RECOMBINANT in1601

i Growth

After the authenticity of the genome had been established, the mutant was subjected to a phenotypic investigation. The first part of this was an examination of the growth characteristics of this virus.

. The one-step growth curve shows the development of virus infection over a set period of time (24 hours) from a fixed inoculum (5 pfu/cell). The results obtained are displayed in figure 3.13. The graph shows that, under the conditions of the assay, growth of in1601 does not differ significantly from that of *wt*.

The experimental data presented so far confirm that an insertion mutation has been engineered into the UL2 gene and that the product of that gene is not absolutely required for normal growth, at least in the tissue culture system used in this study.

ii Enzyme assays

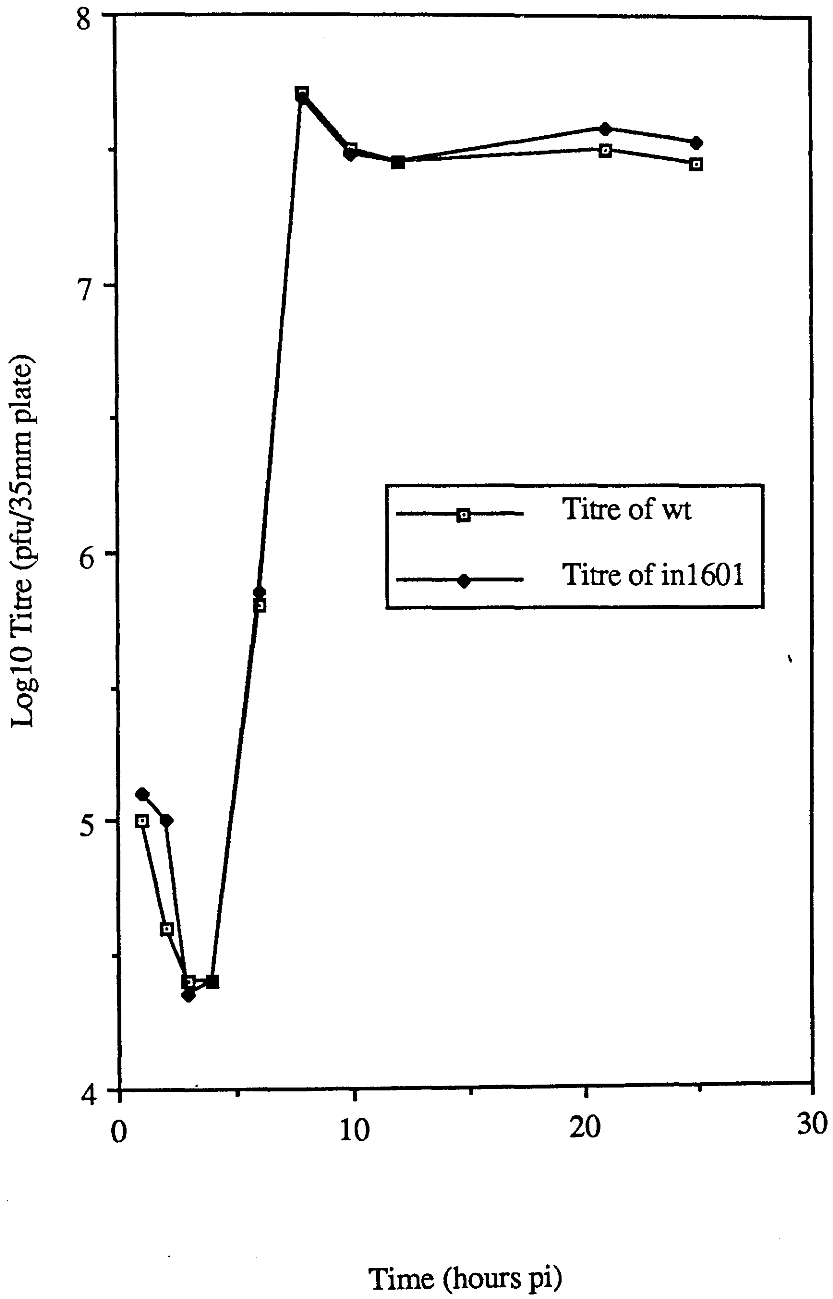
a) Uracil-DNA glycosylase

As was stated earlier, the primary candidate for the HSV induced uracil-DNA glycosylase activity, was the product of gene UL2. Once the insertion mutant had been constructed and its genome verified, the next step was determination of the uracil-DNA glycosylase activity of the mutant. Extracts were prepared from cells mock-infected or infected with *wt* or in1601 at 10 h pi as described in section 2.16. These extracts were assayed for uracil-DNA glycosylase activity as described in section 2.26. The principle of the assay is the release of [5-^{3}H]uracil from

Figure 3.13 : One step growth curves

Opposite is displayed the one step growth curves obtained for *wt* and in1601. The monolayers were infected at 5 pfu/cell and plates were harvested at 1, 2, 3, 4, 6, 8, 10, 12, 21 and 25 h pi, before being titrated on confluent monolayers of BHK C13 cells on 35 mm plates. All titrations were performed at 37°C.

One step growth curve



DNA which had been prepared containing [5-^{3}H]-dUMP (see section 2.25). The assays were performed in the presence of EDTA, which inhibits exonuclease activity. The levels of uracil-DNA glycosylase activity in these extracts are shown in figure 3.14. This figure shows a readily measurable level of uracil-DNA glycosylase activity in the extract prepared from cells infected with *wt*, with the level of activity in the mock-infected cell extract being much lower. There was no significant difference between the level of activity in the extract from cells infected with in1601 and the mock extract, indicating that this virus does not induce uracil-DNA glycosylase activity.

b) Other enzyme activities

In order to ensure that the low level of uracil-DNA glycosylase activity induced in cells infected with in1601 was not due to a reduced level of infection, two more enzyme activities were measured. The first of these was that of the HSV-induced alkaline exonuclease enzyme. The extracts used in the uracil-DNA glycosylase assays above were used in exonuclease assays which were performed in the absence of EDTA but at a pH of 9.0, which has been shown to inhibit any cellular exonuclease activity (Morrison and Keir, 1968). The level of alkaline exonuclease activity present in these extracts at 10 h pi is presented in figure 3.15. In this case, the levels of exonuclease activity in the extracts from *wt* and in1601 infected cells were very similar to each other, and the activity from the mock-infected extract was essentially zero. This indicates that the infection with in1601 was as productive as that with *wt*, and the low level of uracil-DNA glycosylase activity shown in the in1601 infected extract was not due to a reduced level of infection.

Figure 3.14 : Uracil-DNA glycosylase assays

Extracts prepared at 10 h pi were assayed for uracil-DNA glycosylase activity as described. The results of these assays are presented graphically. The enzyme activity present in each extract is displayed along the ordinate axis, as amount of radioactivity released/mg protein. The activities of extracts prepared from cells mock-infected, or infected with *wt* or in1601 virus are shown.

Uracil-DNA glycosylase assay

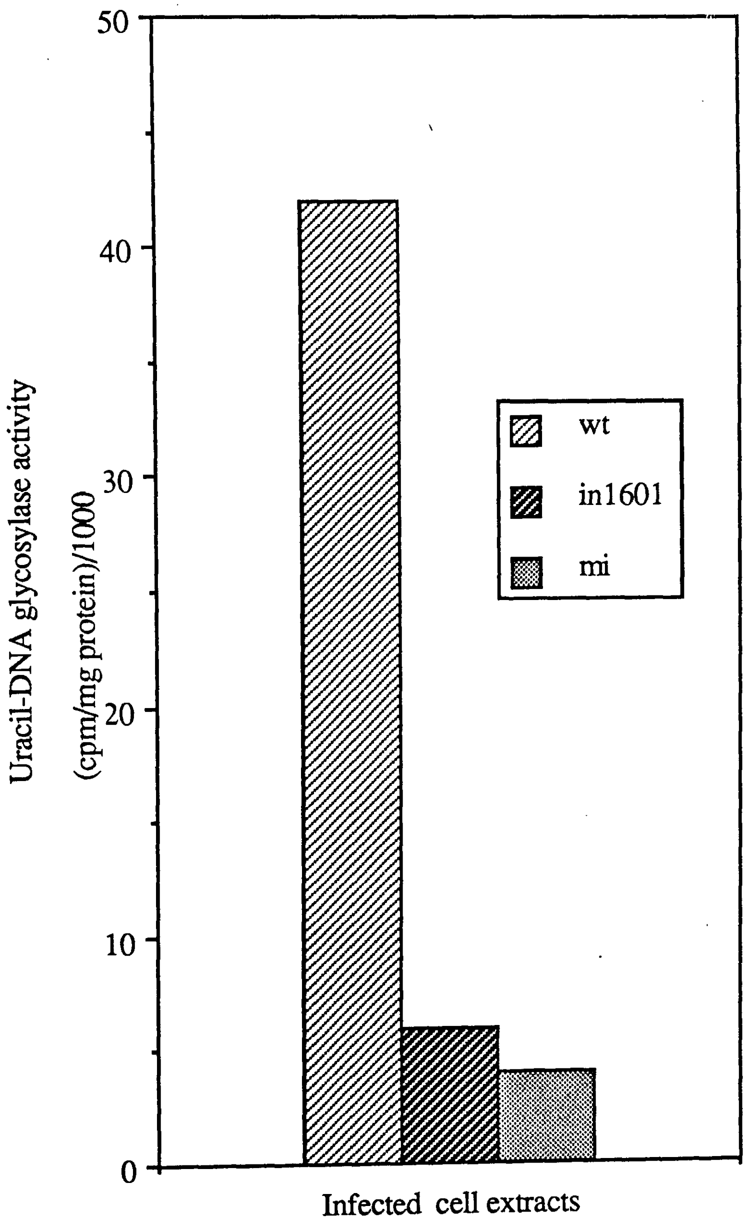
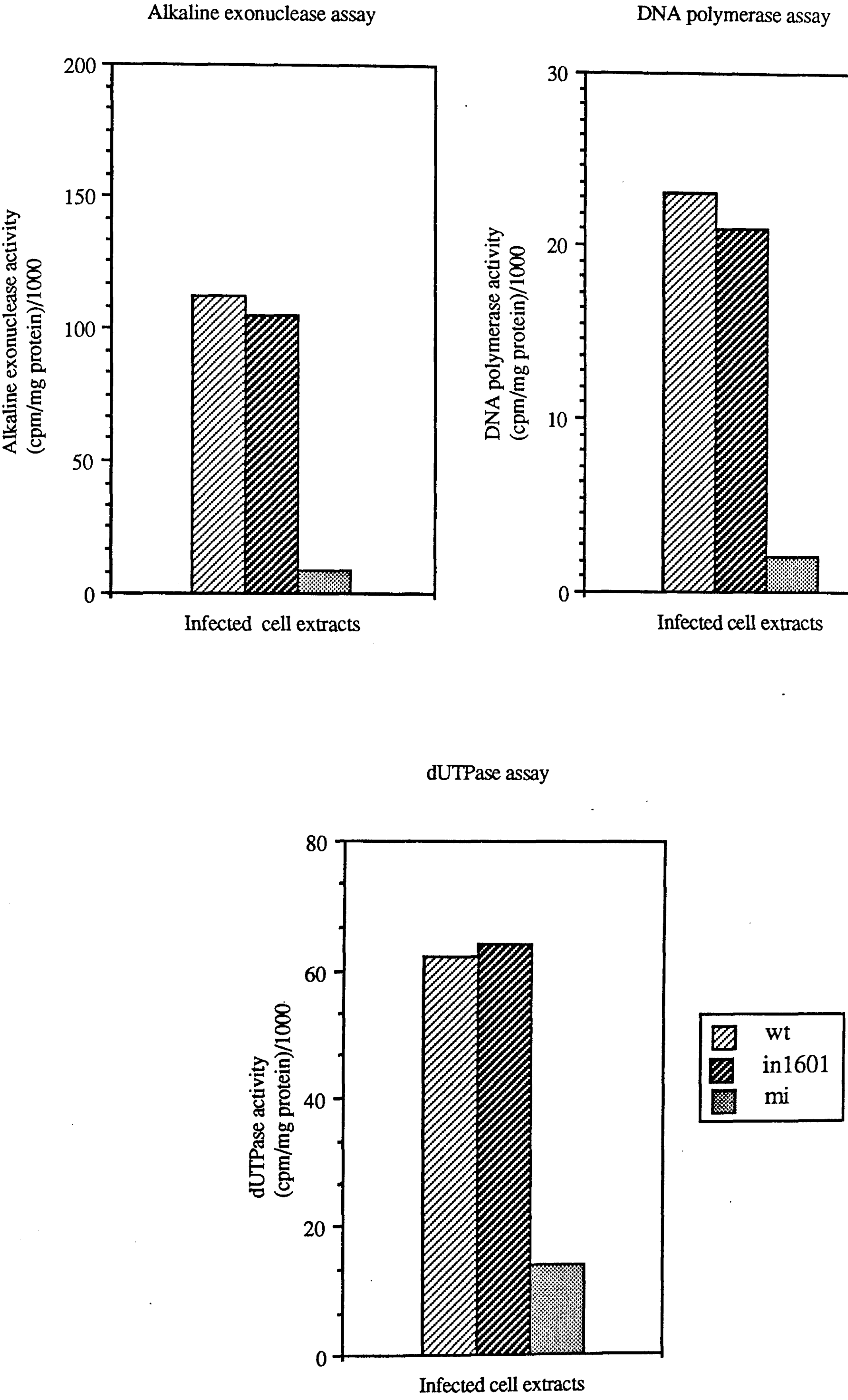


Figure 3.15 : Other enzyme assays

Extracts prepared at 10 h pi were assayed for alkaline exonuclease, DNA polymerase and dUTPase activity as described. The results of these assays are presented graphically, on graphs a, b and c, respectively. The enzyme activity present in each extract is displayed along the ordinate axis, as amount of radioactivity released (or incorporated, in the case of the DNA polymerase assay)/mg protein. The activities of extracts prepared from cells mock-infected, or infected with *wt* or in1601 virus are shown.



Alkaline exonuclease assay

DNA polymerase assay

dUTPase assay

The second enzyme activity to which the extracts were subjected was the virus induced DNA polymerase. The results of this assay are also presented in figure 3.15. Once again, there was no significant difference between the levels of polymerase activity in the extracts infected with *wt* and those infected with in1601, and the mock infected extract had an insignificant level of activity. These results corroborate those obtained for the exonuclease activities.

One other enzyme assay which was performed on the extracts was that for dUTPase. This assay was performed as part of the experiment to determine the phenotype of another recombinant virus, in1602 (to be discussed later), but the results are included here as further evidence of the level of infection of the in1601 extract. The results, which are also shown in figure 3.15, demonstrate a similar profile of activity between the respective extracts as the exonuclease and polymerase assays. This can be taken as further confirmation of the efficiency of infection with in1601.

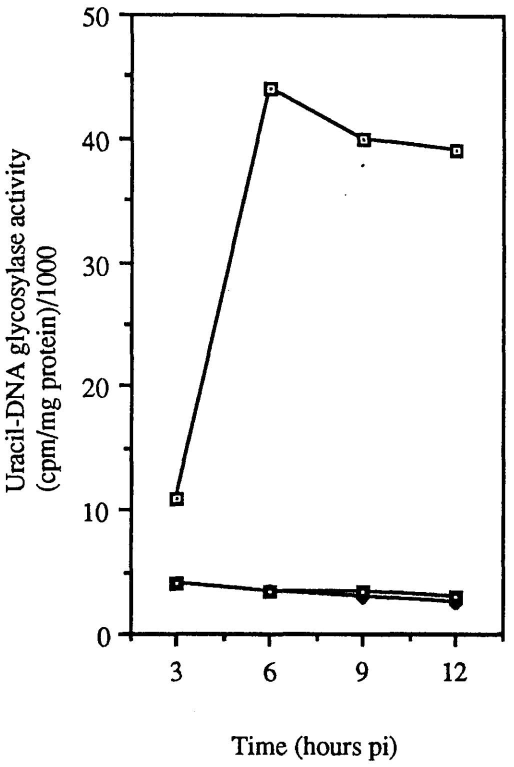
c) Time course of enzyme assays

In order to investigate the progression of infection in cells infected with these viruses, a series of extracts were prepared at 3, 6, 9 and 12 h pi. These extracts were assayed for the four enzyme activities described above. The results are displayed in figure 3.16. In general, the time courses show that, with the obvious exception of uracil-DNA glycosylase, there is no great difference in the levels of enzyme activities induced by *wt* and by in1601. There does appear to be a small lag in the level of

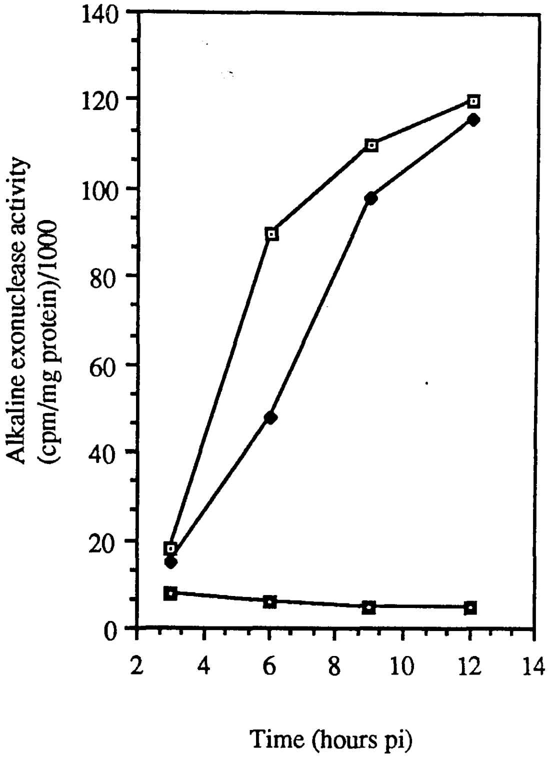
Figure 3.16 : Enzyme assay time courses

Extracts prepared at 3, 6, 9 and 12 h pi were assayed for uracil-DNA glycosylase, alkaline exonuclease, DNA polymerase and dUTPase activity as described. The results of these assays are presented graphically, in graphs a, b, c and d, respectively. The enzyme activity present in each extract is displayed along the ordinate axis, as amount of radioactivity released (or incorporated, in the case of the DNA polymerase assay)/mg protein. The activities of extracts prepared from cells mock-infected, or infected with *wt* or in1601 virus are shown.

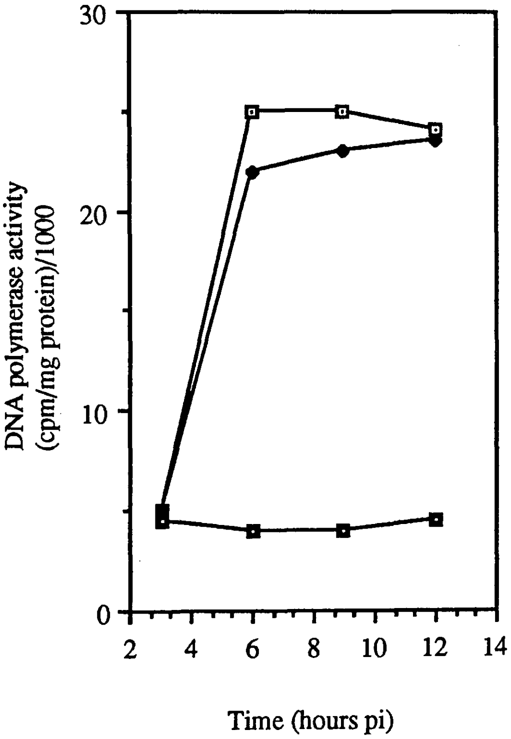
Uracil-DNA glycosylase time course



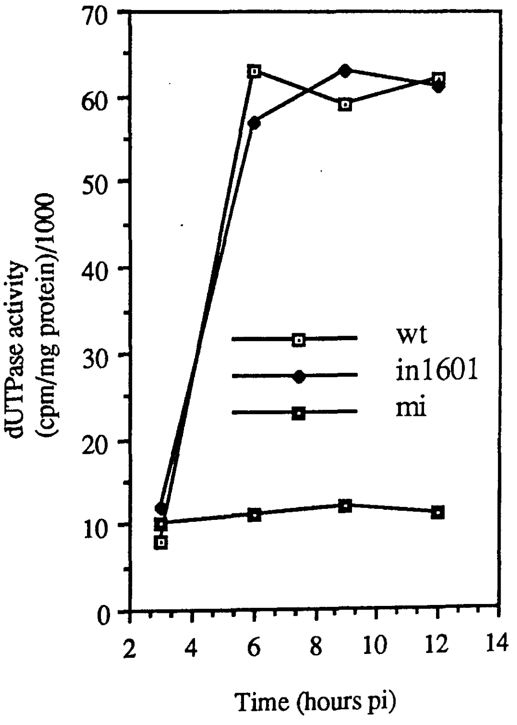
Alkaline exonuclease assay time course



DNA polymerase assay time course



dUTPase assay time course



exonuclease activity induced by in1601, but this is not observed in any of the other curves. Also, the lag is almost fully corrected by 9 h pi and so is not regarded as being significant.

d) Temporal regulation of the UL2 gene

As has already been discussed, HSV genes are regulated in three distinct temporal classes. In order to determine to which regulatory class UL2 belongs, uracil-DNA glycosylase assays were conducted on extracts of cells infected with *wt* HSV-1 in the continuous presence of phosphonoacetic acid (PAA), an inhibitor of virus DNA synthesis. The results of these assays are presented in figure 3.17. As can be seen from the figure, there is no significant difference between the levels of uracil-DNA glycosylase activity in the presence or absence of PAA. This indicates that the uracil-DNA glycosylase gene, UL2, is regulated as an early gene, i.e. it is transcriptionally active before the onset of virus DNA replication. This result is not unexpected as all enzyme activities concerning DNA replication are present before replication commences. For example, TK and RR are examples of early genes. Mutation and repair of DNA is a continuous process and as such it is advantageous if the level of mutation is minimised before replication commences, preventing any damage present in the parent molecule from being passed on to the progeny.

e) Radiolabelled HSV-1 induced polypeptides

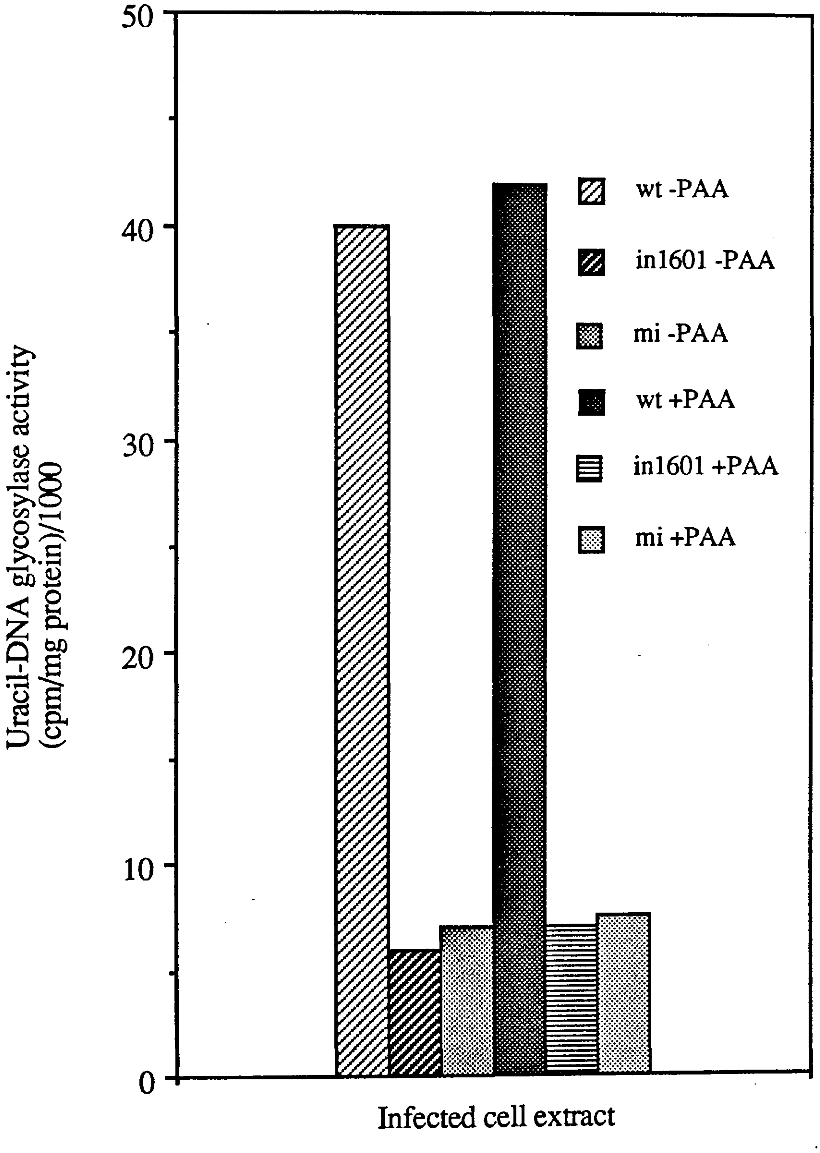
In order to discern what effect (if any), the mutation had on the proteins induced by in1601, radiolabelled cell extracts were prepared from cells infected with *wt* virus

Figure 3.17 : Temporal regulation of the UL2 gene

Cells which had been pre-treated with PAA were infected and incubated in the continuous presence of PAA. Extracts were prepared at 10 h pi and assayed for uracil-DNA glycosylase activity as described.
The results of these assays are presented graphically. The enzyme activity present in each extract is displayed along the ordinate axis, as amount of radioactivity released/mg protein. The activities of extracts prepared from cells mock-infected, or infected with *wt* or in1601 virus are shown, in the presence and absence of PAA.

Uracil-DNA glycosylase assays

in the presence and absence of PAA



and cells infected with in1601 in the presence of S^{35}-methionine. These extracts were examined by polyacrylamide gel electrophoresis, the results of which are shown in figure 3.18. Comparison of these profiles does not result in the identification of any specific polypeptide which can be assigned as the UL2 gene product, as there are no obvious differences between the two profiles, either in terms of a qualitative alteration or a modulation in the mobility of a specific band or bands. However, these profiles are complex and thus changes in minor bands may not be detectable by this method.

f) Reaction of virus-infected cell extracts with rabbit antisera raised against synthetic oligopeptides

Two synthetic oligopeptides were used to immunise a total of four rabbits. The peptide sequences used were derived from the amino acid sequence of the UL2 gene product and are shown in figure 3.19. Two rabbits (1 and 2) were immunised with peptide a and the other rabbits (3 and 4) were immunised with peptide b. The rabbits were boosted with more immunogen every four weeks and bled for testing two weeks after each boost. The bleeds were assayed for reactivity with virus-infected cell extracts by Western blotting. At seven months post-immunisation, the rabbits were sacrificed and terminal bleeds were taken. The sera from all four rabbits were assayed for reactivity to extracts obtained from cells either mock-infected, or infected with either *wt* HSV-1 or in1601. The figures 3.20 to 3.23 show the reactions of the sera obtained from rabbits 1 to 4, respectively, with these extracts. The sera from two rabbits (1 and 2) demonstrated reactivity with the extracts from cells infected with *wt* virus and this is shown in western blots, figures 3.20 and 3.21. These blots

Figure 3.18 : S^{35}-Methionine labelled HSV induced polypetides

Figure 3.18 is an autoradiograph of S^{35}-Methionine labelled virus induced polypeptides. There are no discernible differences between the two profiles. M_r are as indicated.

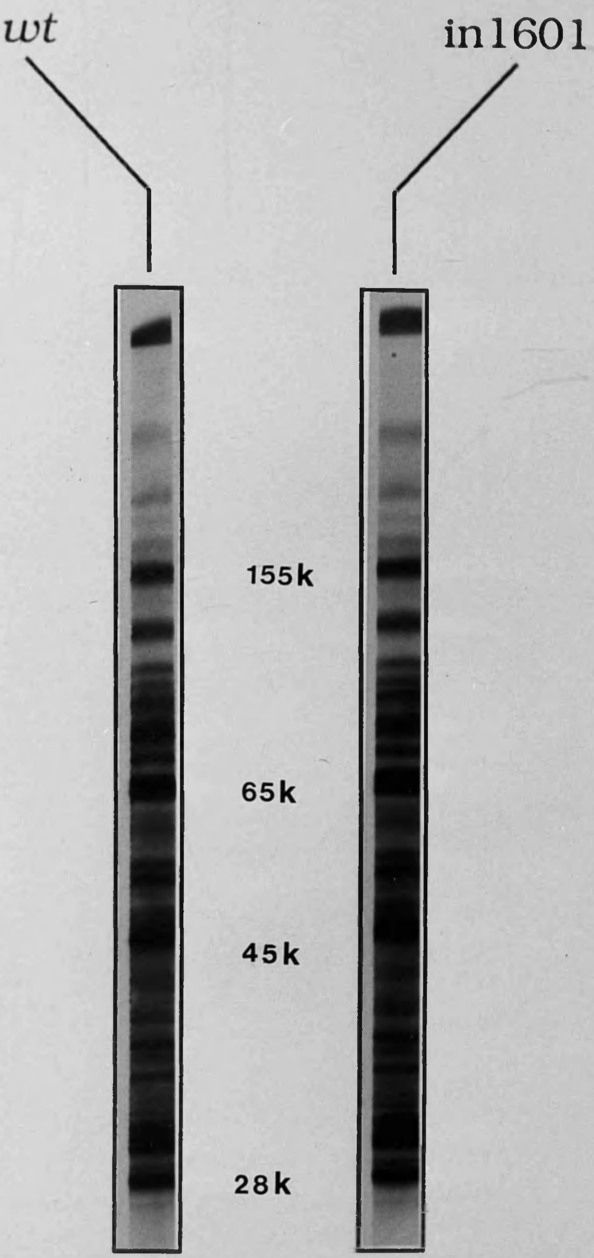
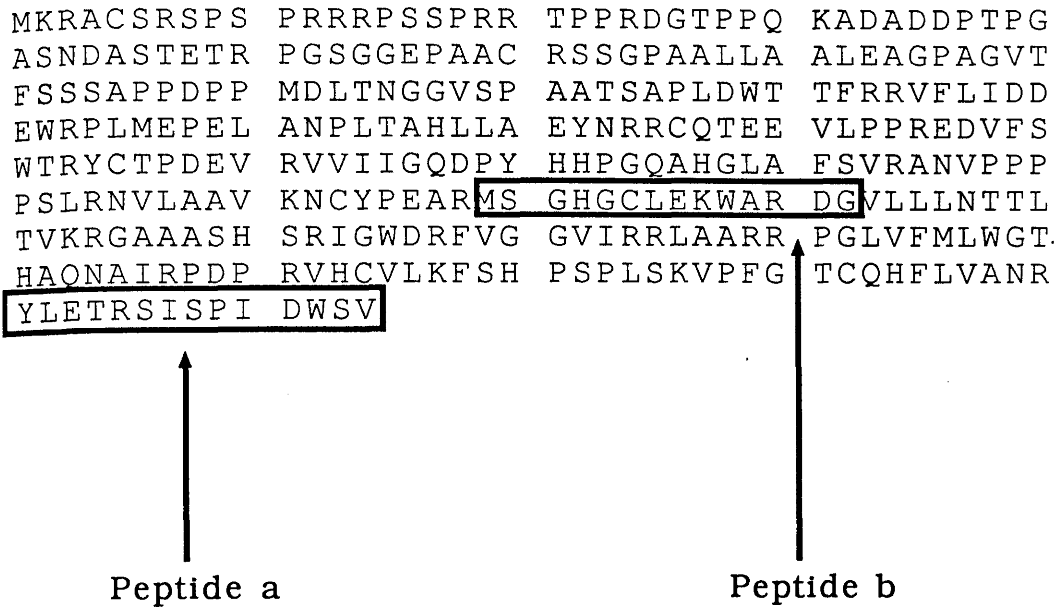

wt
in1601
155k
65k
45k
28k

Figure 3.19 : UL2 amino acid sequence

The amino acid sequence translated from the UL2 DNA sequence is depicted opposite. The sequences of the two synthetic oligopeptides (a and b) are shown.



```
MKRACSRSPS PRRRPSSPRR TPPRDGTPPQ KADADDPTPG
ASNDASTETR PGSGGEPAAC RSSGPAALLA ALEAGPAGVT
FSSSAPPDPP MDLTNGGVSP AATSAPLDWT TFRRVFLIDD
EWRPLMEPEL ANPLTAHLLA EYNRRCQTEE VLPPREDVFS
WTRYCTPDEV RVVIIGQDPY HHPGQAHGLA FSVRANVPPP
PSLRNVLAAV KNCYPEARMS GHGCLEKWAR DGVLLLNTTL
TVKRGAAASH SRIGWDRFVG GVIRRLAARR PGLVFMLWGT
HAQNAIRPDP RVHCVLKFSH PSPLSKVPFG TCQHFLVANR
YLETRSISPI DWSV
```

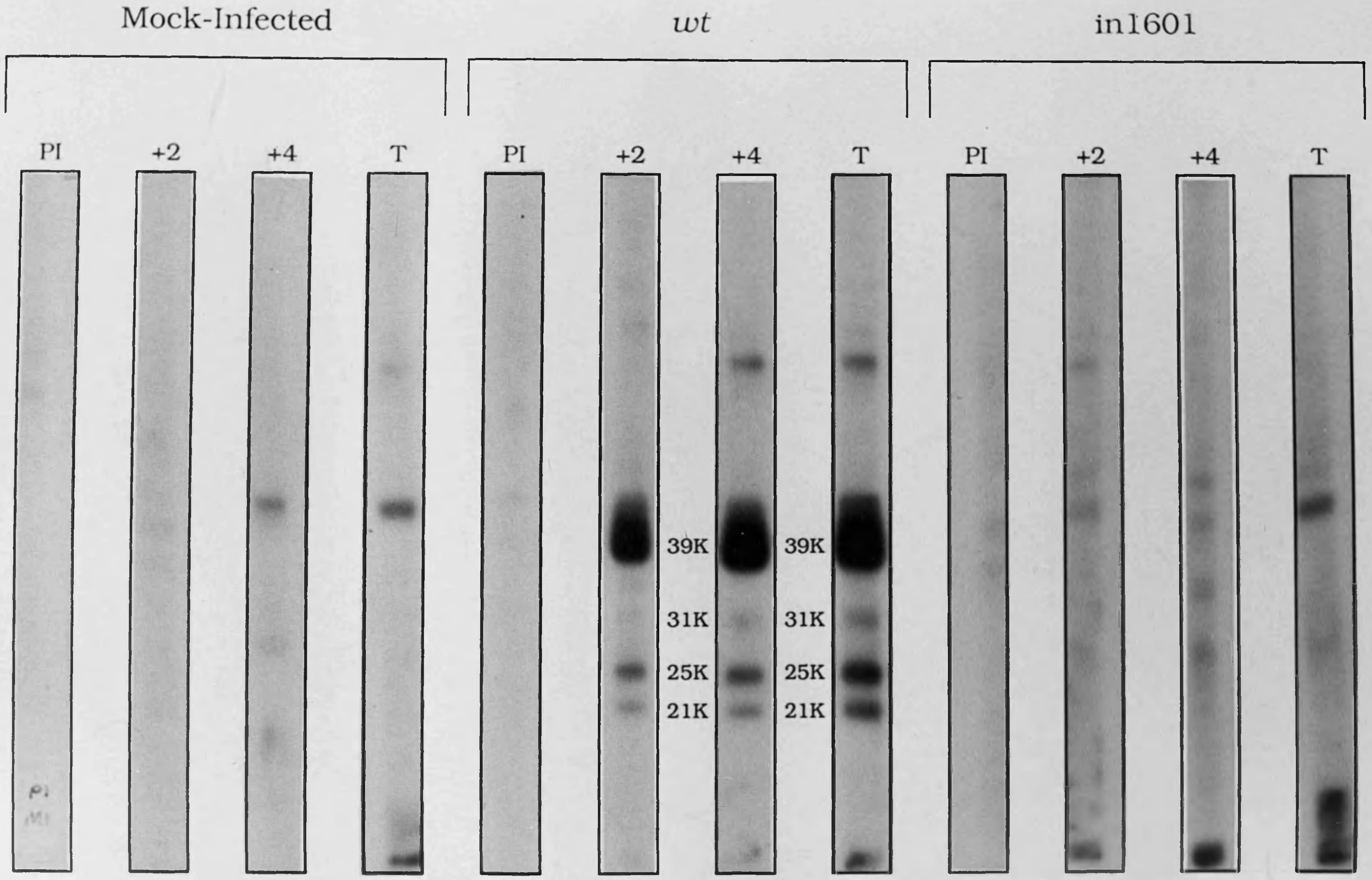
show that the sera from rabbits 1 and 2 react specifically with extracts from cells infected with *wt* HSV-1, but not in1601. The sera from these rabbits show an increasingly strong reaction over a period of months to a maximum at the terminal bleed, which was taken 7 months after the initial immunisation. None of the pre-immune bleeds from these rabbits shows any reaction with any of the extracts, and none of the sera incubated with mock-infected extracts show any reaction, apart from a few faint bands. There is no reaction between these sera and the extracts obtained from cells infected with in1601, indicating that the sera are reacting specifically with the uracil-DNA glycosylase protein.

There is one major band at M_r 39,000 with three minor bands at M_r 31,000, 25,000 and 21,000. The minor bands may be processed forms of the major band, but as the majority of the activity stays in the major band, it is more likely that the minor bands are degradation products of the more abundant species. Worrad and Caradonna (1988) estimated the M_r of the HSV-2 encoded uracil-DNA glycosylase enzyme to be 28-35,000 and predicted the M_r of the type 1 enzyme to be 42,300 although they did not state how the latter was calculated. Perry and McGeoch (1988) indicated the existence of two possible initiation codons for what was then regarded as the UL2 open reading frame (ORF). The first of these opens an ORF encoding a polypeptide of 334 amino acids with a predicted M_r of 36,300, and the second opens an ORF of 244 amino acids with a predicted M_r of 27,300. When the work described in this section was published (Mullaney *et al.*, 1989), the authors stated that the second methionine may have been the more likely candidate. This assumption was made on the basis of comparing the amino acid sequences of UL2 with its

3.20 : Immunoblot - Rabbit 1

Extracts from cells mock-infected, or infected with *wt* or in1601 were separated by SDS-PAGE and transferred to nitro-cellulose strips as described. Sera from rabbit 1 were reacted with the immobilised extracts as indicated. The abbreviations are as follows;

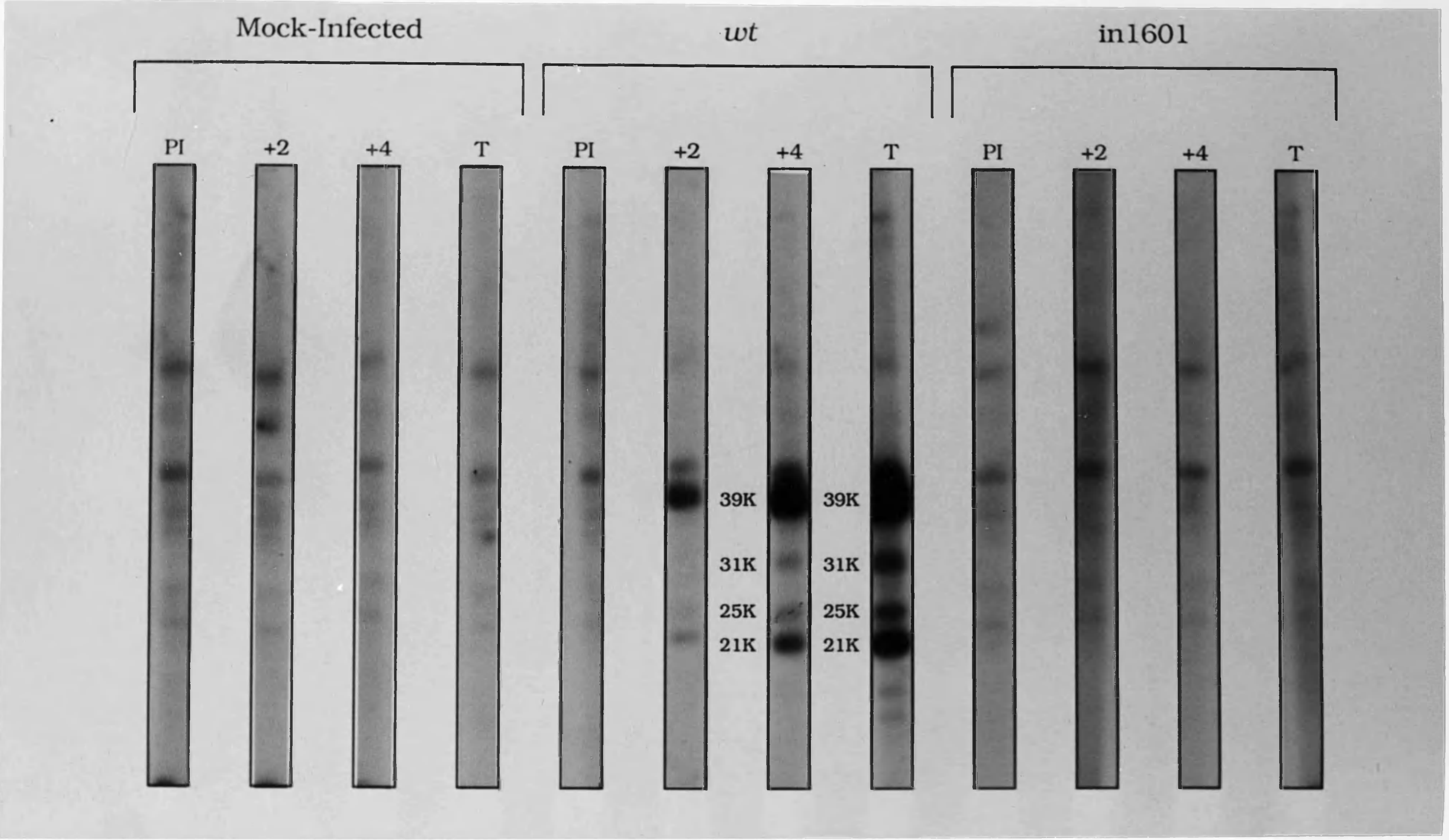
PI - Pre-immune serum
+2 - serum 2 months post initial immunisiation
+4 - serum 4 months post initial immunisation
T - terminal serum (7 months post initial immunisation)


Mock-Infected
wt
in1601
PI
+2
+4
T
PI
+2
+4
T
PI
+2
+4
T
39K
31K
25K
21K
39K
31K
25K
21K
PI
MI

3.21 : Immunoblot - Rabbit 2

Extracts from cells mock-infected, or infected with *wt* or in1601 were separated by SDS-PAGE and transferred to nitro-cellulose strips as described. Sera from rabbit 2 were reacted with the immobilised extracts as indicated. The abbreviations are as follows;

PI - Pre-immune serum
+2 - serum 2 months post initial immunisiation
+4 - serum 4 months post initial immunisation
T - terminal serum (7 months post initial immunisation)


Mock-Infected
wt
in1601
PI
+2
+4
T
PI
+2
+4
T
PI
+2
+4
T
39K
31K
25K
21K
39K
31K
25K
21K

homologues in VZV and EBV. This assignation was made without the benefit of the data obtained from the western blots (figures 3.20 and 3.21). The blots show that the protein has a M_r more consistent with transcription initiating at the first ATG codon, although it possible that the native protein could be smaller but is modified post-translationally. However, no conclusive assignment can be made with the data available at present.

The sera obtained from the other two rabbits (3 and 4) showed no reactivity with extracts prepared from cells infected either with *wt* or in1601 (figures 3.22 and 3.23). Once more there are a few faint bands, but these are not regarded as having any significance. These sera were obtained from rabbits which had been immunised with the other synthetic oligopeptide, b. This amino acid sequence was used as it is part of a motif which is conserved among the herpesviruses.

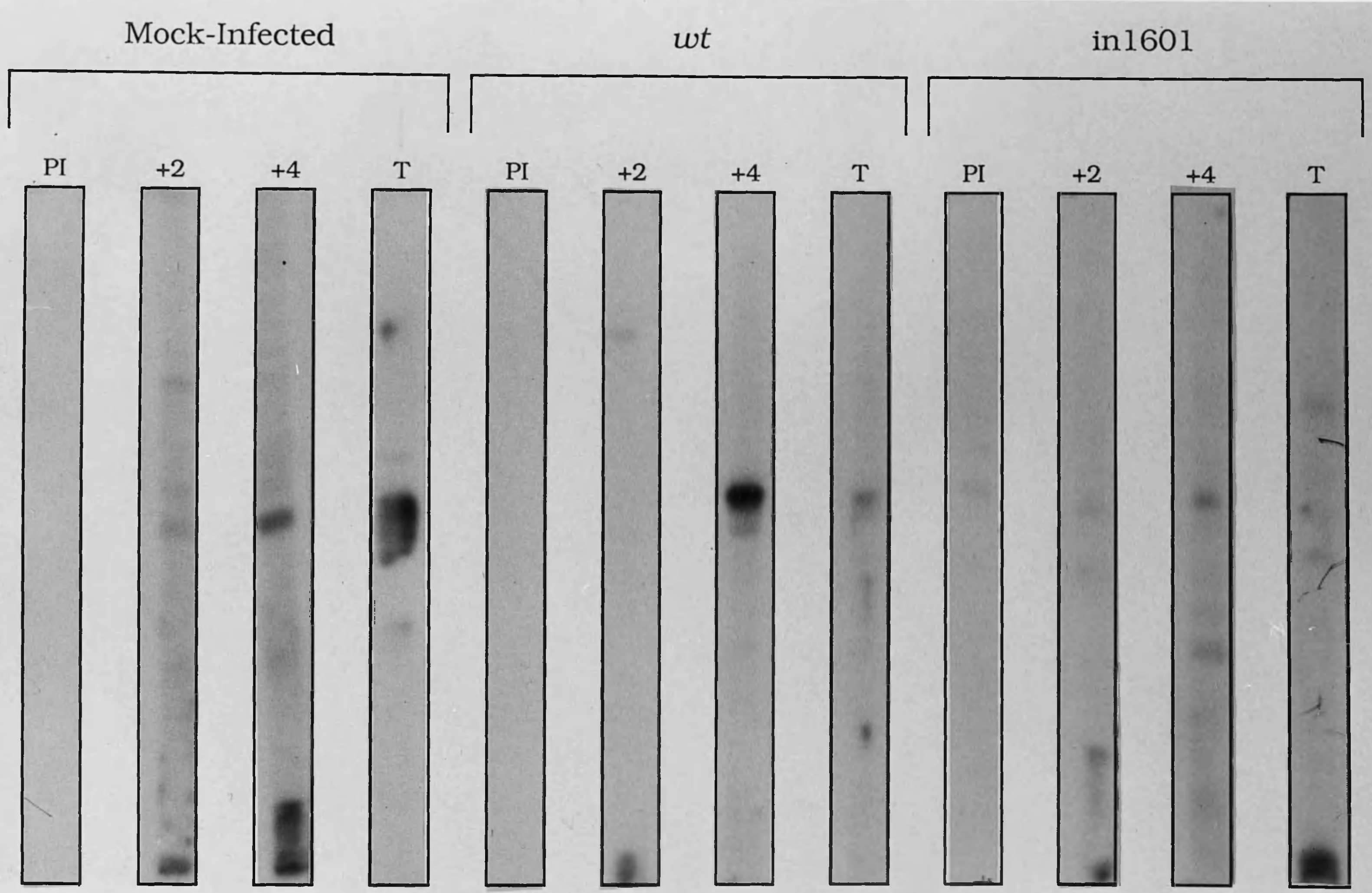
3.2.3 COMPARISON OF THE HSV-1 UL2 GENE PRODUCT AMINO ACID SEQUENCE WITH ITS HOMOLOGUES

Throughout the Herpesviridae there are many examples of genes being conserved between members of different subfamilies (for examples see McGeoch *et al.*, 1988). Comparison of the HSV-1 UL2 amino acid sequence with the proposed amino acid sequences in other human herpesviruses shows that there are UL2 homologues in all the other human herpesviruses whose genomes have been sequenced (ie HSV-2, Worrad and Caradonna, 1988; VZV, Davison and Scott, 1986; EBV, Baer *et al.*, 1984; and HCMV, Chee *et al.*, 1990). The homologues are as follows:-

3.22 : Immunoblot - Rabbit 3

Extracts from cells mock-infected, or infected with *wt* or in1601 were separated by SDS-PAGE and transferred to nitro-cellulose strips as described. Sera from rabbit 3 were reacted with the immobilised extracts as indicated. The abbreviations are as follows;

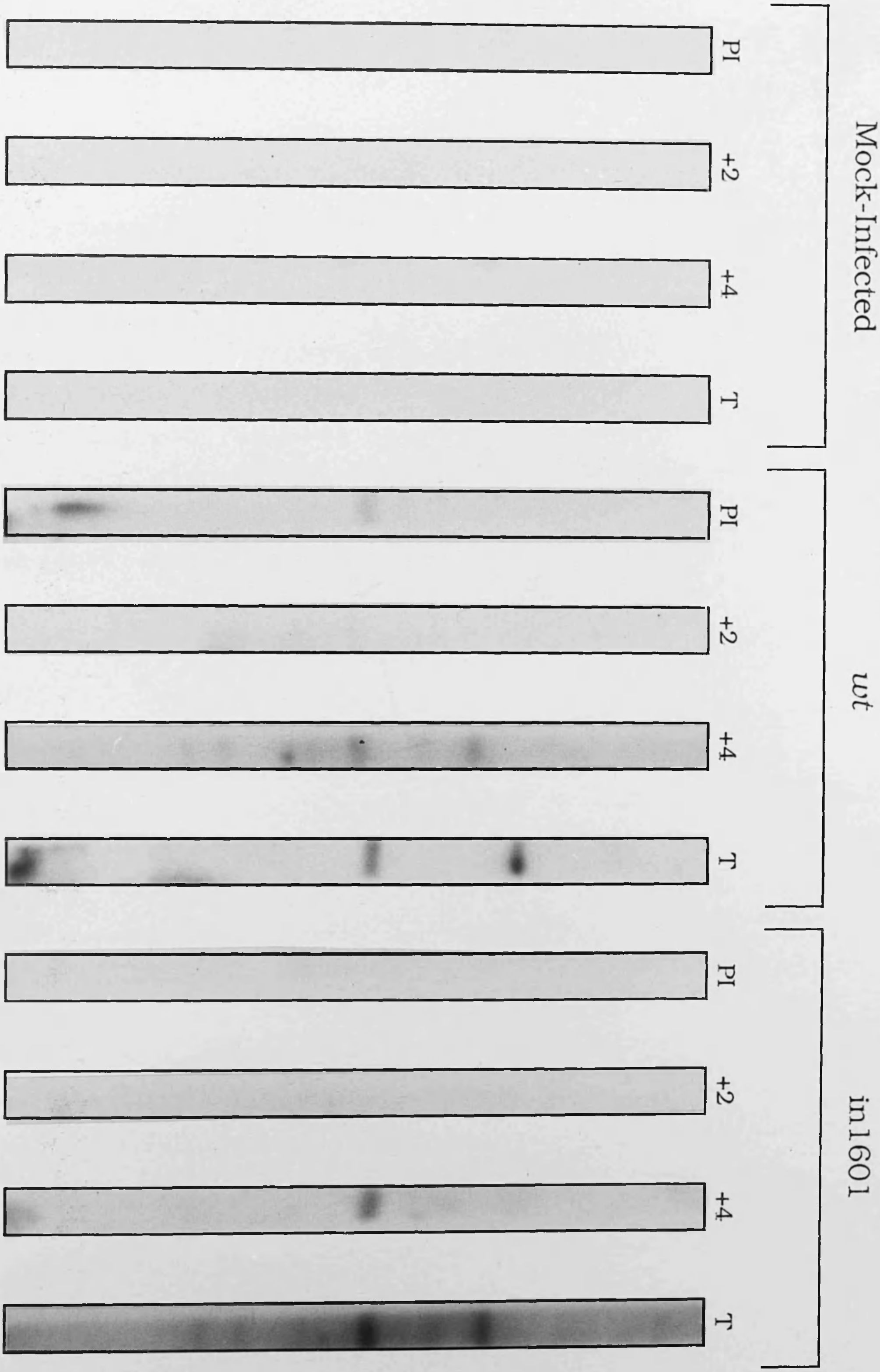
PI - Pre-immune serum
+2 - serum 2 months post initial immunisiation
+4 - serum 4 months post initial immunisation
T - terminal serum (7 months post initial immunisation)


Mock-Infected
wt
in1601
PI
+2
+4
T
PI
+2
+4
T
PI
+2
+4
T

3.23 : Immunoblot - Rabbit 4

Extracts from cells mock-infected, or infected with *wt* or in1601 were separated by SDS-PAGE and transferred to nitro-cellulose strips as described. Sera from rabbit 4 were reacted with the immobilised extracts as indicated. The abbreviations are as follows;

PI - Pre-immune serum
+2 - serum 2 months post initial immunisiation
+4 - serum 4 months post initial immunisation
T - terminal serum (7 months post initial immunisation)



Mock-Infected
PI
+2
+4
T
wt
PI
+2
+4
T
in1601
PI
+2
+4
T

HSV-2	UL2
VZV	Gene 59
EBV	BKRF3
HCMV	UL114

A comparison of these amino acid sequences with that of HSV-1 UL2 is depicted in figure 3.24. From this figure it can be seen that there are distinct motifs which are strictly conserved between all five sequences. This gene is one of the most highly conserved genes within the herpesvirus group, possessing percentage identities with the HSV-1 protein of 72% for the HSV-2 UL2 gene product, 42% for the VZV gene 59 product, 41% for the HCMV UL114 gene product and 40% for the EBV BKRF3 gene product. The amino acid comparisons show a striking degree of conservation, especially towards their carboxy termini.

All the genotypic and phenotypic evidence that gene UL2 of HSV-1 encodes a uracil-DNA glycosylase, presented so far has been of a circumstantial nature. Although all the data indicate that UL2 encodes the uracil-DNA glycosylase, none of it is direct. It is theoretically possible that UL2 may in fact encode an activator or enhancer function, although there is no evidence for this. However, work by Caradonna *et al.* (1987) indicated that the uracil-DNA glycosylase activity detected in these assays is virus encoded, and a cDNA clone was isolated, corresponding to 0.065-0.080 map units on the HSV genome, which expresses uracil-DNA glycosylase activity. UL2 lies within this region, but so do other genes whose role in the virus replicative cycle is still not clear. Final confirmation of UL2 as the uracil-DNA glycosylase gene came with the publication of the *E. coli* uracil-DNA glycosylase DNA sequence (Varshney *et al.*, 1988), which was subsequent to and independent of the

3.24 Amino acid sequence comparison UL2 and its homologues in other human herpesviruses

This figure is a comparison of the amino acid sequences translated from the HSV-1 gene UL2 and its homologues in HSV-2 (UL2), VZV (gene 59), EBV (BKRF3) and HCMV (UL114), using the computer program GAP (GCG software, University of Wisconsin) running on a VAX computer. The dots are pads inserted by the computer to achieve optimal alignment. The numbers refer to the relative positions from the start of the alignment. Position 1 is taken as the first potential methionine residue of the HSV-1 UL2 gene product and all other sequences are aligned relative to this. On the consensus line, dots are positions of poor conservation (residues common to less than two sequences or where there are two different pairs of identical residues), letters in lower case indicate where conservation is moderate (ie there is a single pair of identical amino acids or a group of three or four identical residues) and upper case letters indicate where the same amino acid is common to all five sequences.

```
                1                                                 50
    HSV1 UL2    MKRACSRSPSPRRRPSSPRRTPPRDGTPPQKADADDPTPGASNDASTETR
    HSV2 UL2    ..............................MAMKRNPSRVFCAYSKNGTH
      VZV 59    ...................MDVSGEPTVCSNAYANEMKLSDSKDIYVLAH
   EBV BKRF3    ..................................................
 HCMV UL114    ..................................................

        Cons    ...............................a..................

                51                                               100
    HSV1 UL2    PGSGGEPAACRSSGPAALLAALEAGPAGVTFSSSAPPDPPMDLTNGGVSP
    HSV2 UL2    RSAAPTTHRCIAGGGRGALDAGAENTQGHPESRCFPGGRPPQTGPSWCLG
      VZV 59    PVTKKTRKRPR.....GLPLGVKLDPPTFKLNNMSHHYDTETFT......
   EBV BKRF3    .........................MASRGLDLWLDEHVWKRKQEIGV..
 HCMV UL114    ...............MALKQWMLANIADNKGSLLTPDEQA..........

        Cons    .................ll.a..anpag.klsl..p...p...t......

                101                                              150
    HSV1 UL2    AATSAPLDWTTFRRVFLIDDAWRPLME.PELANPLTAHLLAEYNR.RCQT
    HSV2 UL2    AA.........FRRAFLIDDAWRPLLE.PELANPLTARLLAEYDR.RCQT
      VZV 59    .PDSSQLDSVEVFSKFNISPEWYDLLS.DELKEPYAKGIFLEYNRLLNSG
   EBV BKRF3    ..KGENLLLPDLWLDFL...QLSPIFQ.RKLAAVIA......CVR.RLRT
 HCMV UL114    .............RVFCLSADWIRFLSLPDHDTVLLRDTVAAVEG.ARQL

        Cons    ......l....frrvFli.dawrpll..pelanpl.a.llaeynr.rcqt

                151                                              200
    HSV1 UL2    EEVLPPREDV.FSWTRYCTPDEVRVVIIGQDPYHHPGQAHGLAFSVRANV
    HSV2 UL2    EEVLPPREDV.FSWTRYCTPDDVRVVIIGQDPYHHPGQAHGLAFSVRADV
      VZV 59    EEILPSTGDI.FAWTRFCGPQSIRVVIIGQDPYPTAGHAHGLAFSVKRGI
   EBV BKRF3    QATVYPEEDMCMAWARFCDPSDIKVVILGQDPY.HGGQANGLAFSVAYGF
 HCMV UL114    EMVYPAPEHV.HRWSYLCPPEQVRVVIVGQDPYCD.GSASGLAFGTLAGR

        Cons    eevlppredv.f.Wtr.CtPddvrVVIiGQDPYhhpGqAhGLAFsvragv

                201                                              250
    HSV1 UL2    PPPPSLRNVLAAVKNCYPEARMSGHGCLEKWARDGVLLLNTTLTVKRGAA
    HSV2 UL2    PVPPSLRNVLAAVKNCYPDARMSGRGCLEKWARDGVLLLNTTLTVKRGAA
      VZV 59    TPPSSLKNIFAALMESYPNMTPPTHGCLESWARQGVLLLNTTLTVRRGTP
   EBV BKRF3    PVPPSLRNIYAELHRSLPEFSPPDHGCLDAWASQGVLLLNTILTVQKGKP
 HCMV UL114    PPPPSLNNVFRELARTVDGFQRPASGCLDAWARRGVLLLNTVFTVVHGQP

        Cons    ppPpSLrNv.aalk..ype.r.pghGCLe.WAr.GVLLLNTtlTVkrGap

                251                                              300
    HSV1 UL2    ASHSRIGWDRFVGGVIRRLAARRPGLVFMLWGTHA.QN.AIRPDPRVHCV
    HSV2 UL2    ASTSKLGWDRFVGGVVRRLAARRPGLVFMLWGAHA.QN.AIRPDPRQHYV
      VZV 59    GSHVYLGWGRLVQRVLQRLCENRTGLVFMLWGAHA.QK.TTQPNSRCHLV
   EBV BKRF3    GSHADIGWAWFTDHVISLLSERLKACVFMLWGAKAGDK.ASLINSKKHLV
 HCMV UL114    GSHRHLGWQTLSNHVIRRLSERREHLVFMLWGADA.HTCEYLIDRRRHLV

        Cons    gShs.lGWdrfvg.VirrL.errpglVFMLWGahA.q..ai.pd.r.HlV

                301                                             349
    HSV1 UL2    LKFSHPSPLSKVP........FGTCQHFLVANRYLETRSISPIDWSV..
    HSV2 UL2    LKFSHPSPLSKVP........FGTCQHFLAANRYLETRDIMPITV.V..
      VZV 59    LTHAHPSPLSRVP........FRNCRHFVQANEYFTRKGEPEIDWSVI.
   EBV BKRF3    LTSQHPSPLAQNSTRKSAQQKFLGNNHVFLANNFLREKGLGEIDWRL..
 HCMV UL114    LKSCHPSPRNTTR.......AFVGNDHFILANAYLDTHYRETMDWRLCG

        Cons    Lk.sHPSPlskvp........Fg.cqHfllANrylet.gi..idw.v..
```

isolation and characterisation of in1601. When the two amino acid sequences (from *E. coli* and HSV-1) are compared, there are notable similarities, especially towards their carboxy termini. Such a comparison is presented in figure 3.25. This comparisons shows such a high level of conservation (45% of the amino acids in the *E. coli* sequence have identity with the UL2 sequence) that it confirms that UL2 does indeed encode the uracil-DNA glycosylase.

Since publication of the DNA sequence of the *E. coli* *ung* gene, the sequences of two more uracil-DNA glycosylase genes have been published. These are from yeast, *Saccharomyces cerevisiae* (Percival *et al.*, 1989) and from humans (Olsen *et al.*, 1989). When these are translated and the amino acid sequences are compared with HSV-1 (figure 3.26), the same patterns of similarity can be seen as were observed in the comparison with the *E. coli* sequence (figure 3.25). The degrees of identity in these cases are 37% for the yeast protein and 36% for the human. It is also the case that there are several motifs which are highly conserved between all the sequences compared. Whether or not these regions of strict conservation have any functional implications is discussed later.

The presence of this gene in all the herpesviruses studied so far would tend to suggest that the uracil-DNA glycosylase has an important function in the lytic cycle of these viruses. This is in contrast with the isolation of a virus, deficient in this function, but with growth characteristics unaffected by the mutation. However, it was the case that the isolation and characterisation of the mutant was performed under conditions which may not reflect those present in the replicative cycle of the virus *in*

Figure 3.25 : Amino acid sequence comparison UL2 and *E. coli ung* enzymes

This figure is a comparison of the amino acid sequences translated from the HSV-1 gene UL2 and the *E. coli ung* gene, using the computer program GAP (GCG software, University of Wisconsin) running on a VAX computer. The dots are pads inserted by the computer to achieve optimal alignment. The numbers refer to the relative positions from the start of the alignment. Position 1 is taken as the first potential methionine residue of the HSV-1 UL2 gene product and all other sequences are aligned relative to this. On the consensus line, dots are positions where the amino acids are different and upper case letters indicate where the same amino acid is common to both sequences.

```
          1                                                50
    UL2   MKRACSRSPSPRRRPSSPRRTPPRDGTPPQKADADDPTPGASNDASTETR
E. coli   ..................................................

   Cons   ..................................................

          51                                              100
    UL2   PGSGGEPAACRSSGPAALLAALEAGPAGVTFSSSAPPDPPMDLTNGGVSP
E. coli   ..................................................

   Cons   ..................................................

          101                                             150
    UL2   AATSAPLDWTTFRRVFLIDDAWRPLMEPELANPLTAHLLAEYNRRCQTE.
E. coli   ...............MANELTWHDVLAEEKQQPYFLNTLQTVASERQSGV

   Cons   .....................W......E...P.....L.......Q...

          151                                             200
    UL2   EVLPPREDVFSWTRYCTPDEVRVVIIGQDPYHHPGQAHGLAFSVRANVPP
E. coli   TIYPPQKDVFNAFRFTELGDVKVVILGQDPYHGPGQAHGLAFSVRPGIAI

   Cons   ...PP..DVF...R......V.VVI.GQDPYH.PGQAHGLAFSVR.....

          201                                             250
    UL2   PPSLRNVLAAVKNCYPEARMSGHGCLEKWARDGVLLLNTTLTVKRGAAAS
E. coli   PPSLLNMYKELENTIPGFTRPNHGYLESWARQGVLLLNTVLTVRAGQAHS

   Cons   PPSL.N......N..P......HG.LE.WAR.GVLLLNT.LTV..G.A.S

          251                                             300
    UL2   HSRIGWDRFVGGVIRRLAARRPGLVFMLWGTHAQ.NAIRPDPRVHCVLKF
E. coli   HASLGWETFTDKVISLINQHREGVVFLLWGSHAQKKGAIIDKQRHHVLKA

   Cons   H...GW..F...VI......R.G.VF.LWG.HAQ......D...H.VLK.

          301                                    344
    UL2   SHPSPLS.KVPFGTCQHFLVANRYLETRSISPIDWS.V......
E. coli   PHPSPLSAHRGFFGSNHFVLANQWLEQRGETPIDWMPVLPAESE

   Cons   .HPSPLS....F....HF..AN..LE.R...PIDW..V......
```

Figure 3.26 : Amino acid sequence comparison UL2 with yeast and human enzymes

The figure opposite is a comparison of the amino acid sequences translated from the HSV-1 gene UL2 with the yeast *Saccharomyces cerevesiae* gene and the human gene, using the computer program GAP (GCG software, University of Wisconsin) running on a VAX computer. The dots are pads inserted by the computer to achieve optimal alignment. The numbers refer to the relative positions from the start of the alignment. Position 1 is taken as the first methionine residue of the yeast amino acid sequence and all other sequences are aligned relative to this. On the consensus line, dots are positions of poor conservation (residues different in all three sequences), letters in lower case indicate where two of the residues are identical and upper case letters indicate where the same amino acid is common to all three sequences.

```
       1                                                50
  UL2  ...MKRACSRSPSPRRRPSSPRRTPPRDGTPPQKADADDPTPGASNDAST
Yeast  MWCMRRLPTNSVMTVAR..KRKQTTIEDFFGTKKSTNEAPNKKGKSGATF
Human  ....................................MGVFCLGPWGLGRK

 Cons  ...m.r....s....r.....t...d.....k.....p..g....a..

       51                                              100
  UL2  ETRPGSGGEPAACRSSGPAALLAALEAGPAGVTFSSSAPPDPPMDLTNGG
Yeast  MTITNG....AAIKTETKA...VAKEANTDKYPANSNAKDVYSKNLS...
Human  LRTPGKGPLQLLSRLCGDHLQAIPAKKAPAGQE.EPGTPPSSPLSAEQLD

 Cons  .t.pg.g...aa.r..g.a....a.ea.pag....s.app..p..l....

       101                                             150
  UL2  VSPAATSAPLDWTTFRRVFL.IDDAWRPLMEPELANPLTAHLLAEYNRRC
Yeast  .SNLRTLLSLELET.......IDDSWFPHLMDEFKKPYFVKLKQFVTKEQ
Human  RIQRNKAAALLRLAARNVPVGFGESWKKHLSGEFGKPYFIKLMGFVAEER

 Cons  .s...t.a.L...t.r.v...iddsW.phl..Ef.kPyf.kL..fv..e.

       151                                             200
  UL2  QTEEVLPPREDVFSWTRYCTPDEVRVVIIGQDPYHHPGQAHGLAFSVRAN
Yeast  ADHTVFPPAKDIYSWTRLTPFNKVKVVIIGQDPYHNFNQAHGLAFSVKPP
Human  KHYTVYPPPHQVFTWTQMCDIKDVKVVILGQDPYHGPNQAHGLCFSVQRP

 Cons  ...tV.PP..dvfsWTr.c....VkVVIiGQDPYH.pnQAHGLaFSV..p

       201                                             250
  UL2  VPPPPSLRNVLAAVKNCYPE.ARMSGHGCLEKWARDGVLLLNTTLTVKRG
Yeast  TPAPPSLKNIYKELKQEYPDFVEDNKVGDLTHWASQGVLLLNTSLTVRAH
Human  VPPPPSLENIYKELSTDIEDFVHP.GHGDLSGWAKQGVLLLNAVLTVRAH

 Cons  vPpPPSL.Niykelk..ypdfv...ghGdL..WA.qGVLLLNt.LTVrah

       251                                             300
  UL2  AAASHSRIGWDRFVGGVIRRLAARRPG....LVFMLWGTHAQNAIRP...
Yeast  NANSHSKHGWETFTKRVVQLLIQDREADGKSLVFLLWGNNAIKLVESLLG
Human  QANSHKERGWEQFTDAVVSWLNQNSNG....LVFLLWGSYAQKKGSA..I

 Cons  .AnSHs..GWe.Ft..Vv..L.q.r.g....LVFlLWG..Aqk.......

       301                                             350
  UL2  DPRVHC........VLKFSHPSPLS..KVPFGTCQHFLVANRYLETRSIS
Yeast  STSVGSGSKYPNIMVMKSVHPSPLSASRGFFGTNHFKMINDWLYNTRGEK
Human  DRKRHH........VLQTAHPSPLSVYRGFFGCRHFSKTNE.LLQKSGKK

 Cons  d..vh.........Vlk..HPSPLS..rgfFGt.hf...n..ll.trg.k

       351                        379
  UL2  PIDWSV.......................
Yeast  MIDWSVVPGTSLREVQEANARLESESKDP
Human  PIDWKEL......................

 Cons  pIDWsv.......................
```

vivo. There are instances of mutations in HSV-1 which are lethal under certain circumstances but are apparently tolerated under others. For example in UL39, which encodes the RR large subunit (Goldstein and Weller, 1988), a mutant was isolated in which the whole of the gene had been deleted. The authors demonstrated that the survival of viral RR mutants is dependent on the state of the infected cells ie cell type, presence of serum and temperature. The authors proposed that, in this case, the effect of the mutation is complemented by cellular factors. Host-cell complementation is also a possibility in the case of in1601. However, the *vhs* function of the virus would suppress transcription of any endogenous uracil-DNA glycosylase gene and levels of uracil-DNA glycosylase activity in extracts prepared from mock-infected cells are much lower than in those prepared from cells infected with *wt* HSV-1. Given the nature of the enzyme activity, it may be the case that uracil-DNA glycosylase is not required in the normal virus lytic cycle, but that the enzyme has an important role in the long term stability of the virus. It is proposed that the primary function of the uracil-DNA glycosylase enzyme is in the abatement of potentially deleterious mutations. If this activity is abolished, the potential for such mutations will increase. After replication, the number of progeny carrying mutations will be elevated. However, all progeny carrying a lethal mutation will fail to grow (although in the short term, the essential function may be complemented by virus present in the population not carrying this mutation, these mutants would not replicate upon subsequent rounds of growth). Other progeny may carry mutations in genes which are not required for lytic infection *per se*, but may have more subtle roles, for instance in the pathogenicity of the virus, or its ability to reactivate after latency. This would lead to a virus with reduced biological fitness. Any increase in the mutation rate over that observed naturally could have serious consequences, as the long term stability of the virus would be compromised. This may account for the apparent innocuous nature of a mutation which abolishes a gene with an important function.

3.3 RECOMBINANT VIRUS in1602

3.3.1 BACKGROUND

The two pathways which lead to the occurrence of uracil in DNA (ie misincorporation of dUTP during replication and *in situ* deamination of cytosine) have already been mentioned in the introduction. The effects of each of these pathways are minimised by distinct enzyme activities. Misincorporation of dUTP is suppressed by the dUTPase (Shlomai and Kornberg, 1978) and deamination of cytosine is repaired by uracil-DNA glycosylase (Lindahl, 1976). HSV-1 is known to encode both of these enzymes (Preston and Fisher, 1984; Caradonna *et al.*, 1987). Fisher and Preston (1986) reported construction of an HSV-1 mutant, dut-1218, in which the dUTPase was insertionally inactivated. The work reported here led to the construction of a mutant of HSV-1 with a mutation in the gene encoding the uracil-DNA glycosylase. It was decided to combine these two mutations, into a double mutant of HSV-1 which would carry both lesions, and so would not induce either enzyme activity.

3.3.2 COTRANSFECTION AND PURIFICATION

The DNA fragment from the plasmid pJM3 which was used to construct in1601 was cotransfected with DNA from the

HSV-1 mutant dut-1218, and gave rise to the mutant in1602.

As before all the blue plaques from the initial titration were picked and titrated. In subsequent rounds of plaque purification, ten blue plaques were picked and carried forward. The recombinant plaqued with an efficiency similar to *wt* and to in1601, which was included as a control. Plaque purification continued until all the progeny exhibited the recombinant phenotype. At this stage a single, isolated blue plaque was picked and used to generate a high-titre elite stock, designated in1602. This stock had a titre of 1 X 10^9 and was used in all subsequent experiments involving in1602. In tissue culture this virus grew as well as *wt* (and in1601), in that plaques picked from all three viruses yielded similar munbers of progeny (see also section 3.3.2). Plaques from in1602 were indistinguishable from those caused by infection with in1601 or *wt*, with the exception that the *wt* did not stain blue when grown under X-gal.

The genome of in1602 was subjected to a restriction endonuclease analysis similar to that performed on in1601, as described in section 2.13. The restriction enzyme used in this analysis was *Hind*III. The *Hind*III restriction profiles of *wt* and in1601 have already been discussed (see section 3.2.1 part d and figure 3.12).

dut-1218 was constructed by inserting an 8 bp oligonucleotide, containing a *Hind*III restriction site, into the *Kpn*I restriction site between the *Kpn*I fragments *u* and *o*. The relative positions of these sites are shown in figure 3.27, a *Hind*III restriction map of *wt*, and dut-1218 (as well as in1601 and in1602) viruses, all in the prototypic orientation.

The mutation is within the *Hind*III *c* fragment in the prototype arrangement (*c* is *d* + *m*), the *d* fragment in the I_L and I_{SL} arrangements (when it is terminal) and *b* fragment in the I_S arrangement (*b* is *d* + *g*). Correspondingly, changes in any of these fragments will also be observed in each of the others, due to orientation isomerisation.

Digestion of dut-1218 with *Hind*III will result in the truncation of the *b*, *c* and *d* fragments to *b*'', *c*'' and *d*'', respectively. In each case this will be accompanied by the appearence of a novel band, N3. This is seen in figure 3.28, an autoradiograph which has restriction profiles of *wt* and dut-1218 DNA (as well as in1601 and in1602 DNA) digested with *Hind*III.

The double mutant in1602 is predicted to carry the lesions from both single mutants (in1601 and dut-1218). The *Hind*III restriction profile of in1601 has already been discussed (see section 3.2.1 part d). Thus, the predicted genotypic changes present in the double mutant in1602 are the additive effects of those seen in both single mutants :

Figure 3.27 : *Hind*III restriction maps for *wt*, in1601, dut-1218 and in1602

This figure shows the location of the inserts in the prototype arrangement of the genome, and the locations of the *Hind*III sites. The *LacZ* insert is located within the *i* fragment and introduces two novel *Hind*III sites into the genome. This results in the truncation of *i* to *i'*, and the appearence of two novel restriction fragments, N1 and N2. *i* is a terminal fragment and as such is only present in two arrangements of the genome (prototype and I_S). In the other two arrangements, *i* is incorporated into the *e* and the *f* fragments. Correspondingly, the mutation, which truncates *i*, will also truncate *e* and *f*, to *e'* and *f'*, repectively, and in both cases this will also be accompanied by the appearence of the two novel fragments, N1 and N2.

The 8 bp oligonucleotide present in dut-1218 and in1602 also introduces a novel *Hind*III site into the *d* fragment, truncating it to *d''* and introducing a novel fragment N3. *d* is only present, as a terminal fragment, in two arrangements of the genome (I_L and I_{SL}). In the other genome arragements, *d* is incorporated into *b* and *c*. Consequently, the presence of the linker, which truncates *d* to *d''* and introduces the novel fragment N3, also results in the truncation of *b* and *c*, to *b''* and *c''*, repectively, accompanied in both cases by the appearence of the novel fragment, N3.

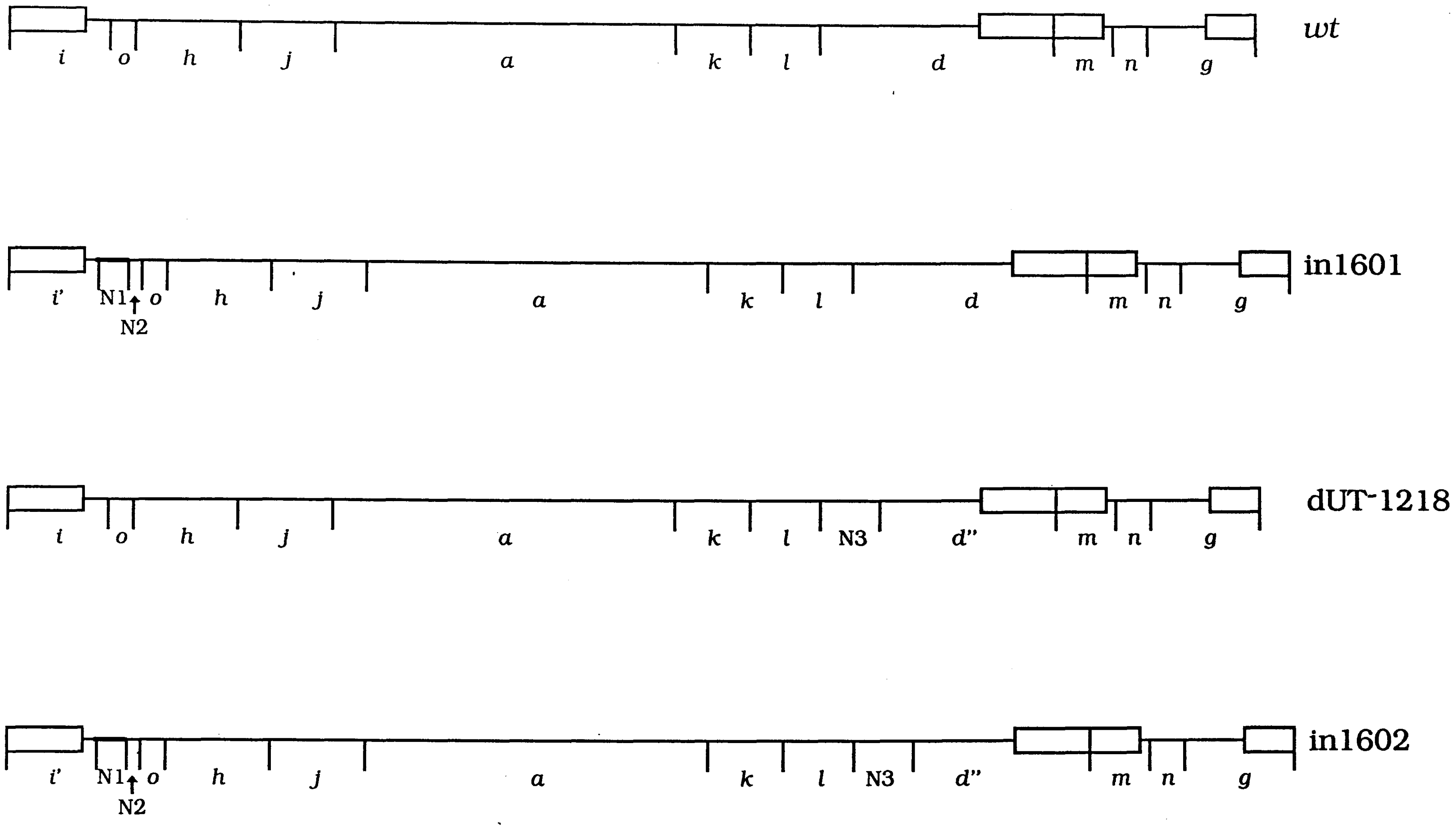
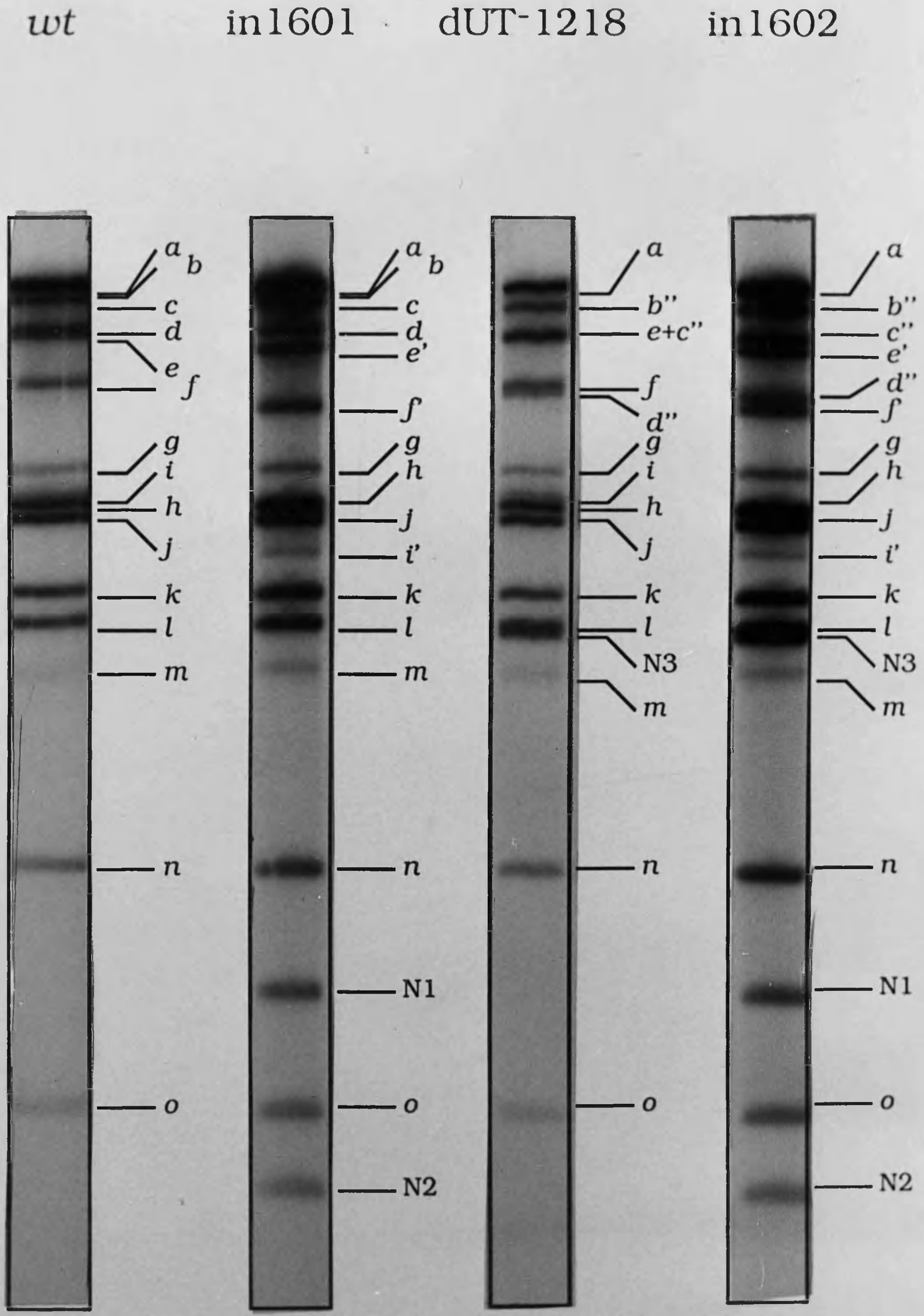

wt
i o h j a k l d m n g
in1601
i' N1 N2 o h j a k l d m n g
dUT-1218
i o h j a k l N3 d" m n g
in1602
i' N1 N2 o h j a k l N3 d" m n g

Figure 3.28 : *Hind*III restriction profiles of *wt*, in1601, dut-1218 and in1602

Figure 3.28 is an autoradiograph of DNA from *wt*, in1601, dut-1218 and in1602 digested with *Hind*III. The first two lanes are identical to figure 3.12. The changes in *wt* fragments brought about by this mutation are marked by ' (ie *i*, *e* and *f* are truncated to *i*', *e*' and *f*'), and the novel bands N1 and N2 are indicated. The third lane demonstrates the changes observed in the recombinant dut-1218. Changes in *wt* fragments are marked by '' (*b*, *c* and *d* are modulated to *b*'', *c*'' and *d*''), and the novel fragment is nominated as N3. It is predicted that in1602 will carry the mutations from both in1601 and dut-1218, and this is observed. The truncated fragments from in1601 (*i*', *e*' and *f*'), the truncated fragments from dut-1218 (*b*'', *c*'' and *d*'') and the novel bands N1, N2 and N3 are all indicated.


wt
in1601
dUT^{-}1218
in1602
a
b
c
d
e
f
g
i
h
j
k
l
m
n
o
a
b
c
d
e'
f'
g
h
j
i'
k
l
m
n
N1
o
N2
a
b''
e+c''
f
d''
g
i
h
j
k
l
N3
m
n
o
a
b''
c''
e'
d''
f'
g
h
j
i'
k
l
N3
m
n
N1
o
N2

The *LacZ* insert has two *Hind*III sites, separated by 3.7 kbp, and is situated in the *i* fragment in the prototypic arrangement of the genome. The changes in *i* also affect the *e* and *f* fragments, which are brought about by isomerisation. As was stated in section 3.2.1 d, insertion of the *LacZ* fragment truncates the *Hind*III *i*, *e* and *f* fragments by 2.1 kbp, to the fragments *i'*, *e'* and *f'*, respectively, and gives rise to two novel fragments, N1 and N2, 3.7 and 2.4 kbp in length, respectively. The presence of the linker truncates the *Hind*III *b* fragment to *b''*; truncates the *c* fragment with to *c''* and truncates the *d* fragment to *d''*. In each case, this is accompanied by the appearence of a novel band N3. As can be seen from figure 3.28, an autoradiograph of *Hind*III digests of *wt*, in1601, dut-1218 and in1602 DNAs, all the predicted alterations are present.

3.3.3 PROPERTIES OF THE RECOMBINANT in1602

i Growth

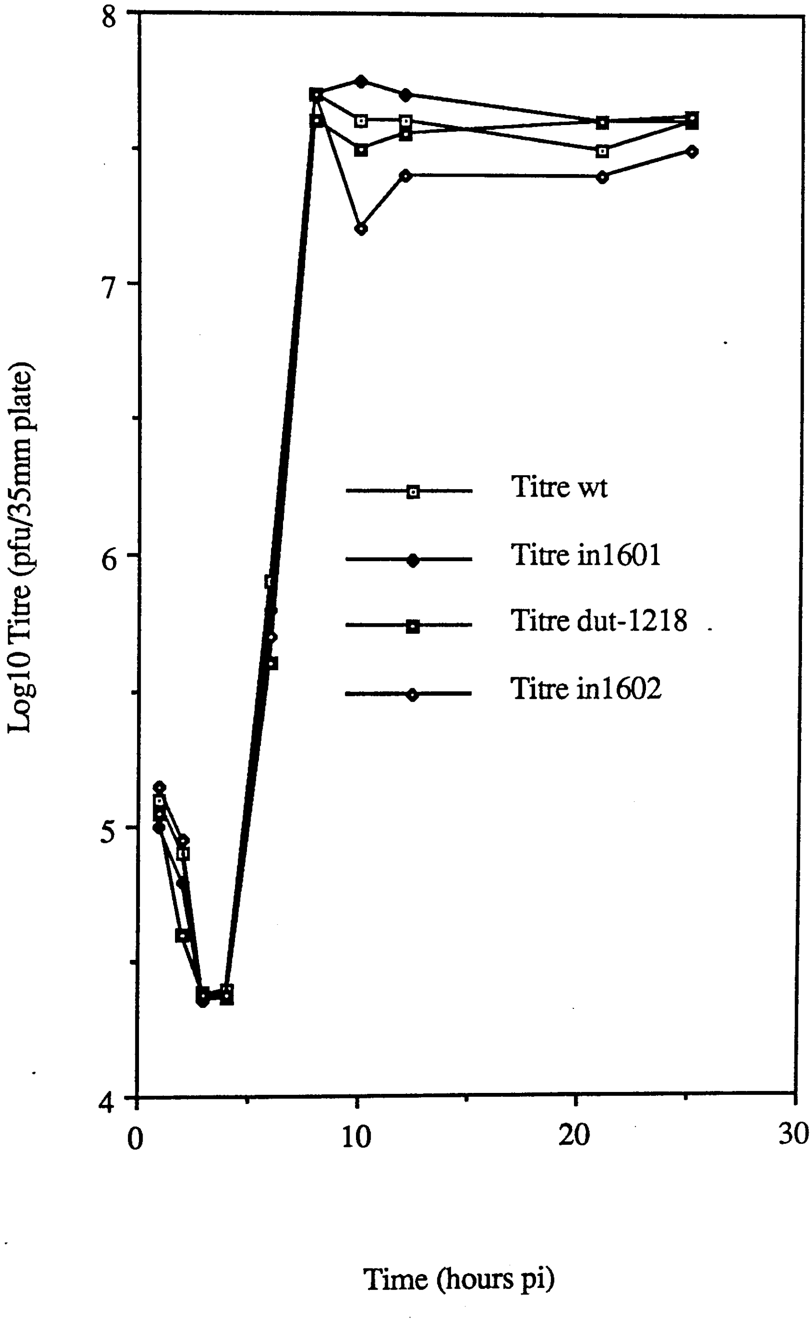
As in1602 had been constructed in a manner similar to in1601, it was subjected to the same phenotypic analyses as in1601. The first part of this was an examination of the growth properties of this recombinant.

The one-step growth curve shows the development of virus infection over a set period of time (24 hours) from a fixed inoculum (5

Figure 3.29 : One step growth curves

Opposite is displayed the onestep growth curves obtained for *wt*, in1601, dut-1218 and in1602. The monolayers were infected at 5 pfu/cell and plates were harvested at 1, 2, 3, 4, 6, 8, 10, 12, 21 and 25 h pi, before being titrated on confluent monolayers of BHK C13 cells on 35 mm plates. All titrations were performed at 37°C.

One step growth curve



pfu/cell). This is displayed in figure 3.29. The graph shows that, under the conditions of the assay, growth of in1602 is indistinguishable from that of *wt*, in1601 and dut-1218. This result is not unexpected as neither of the mutations engineered into in1602 has any effect on the growth properties of the single mutants. However, it is interesting to note that abolition of both functions together does not restrict the growth of the virus under the conditions of this experiment.

ii Enzyme assays

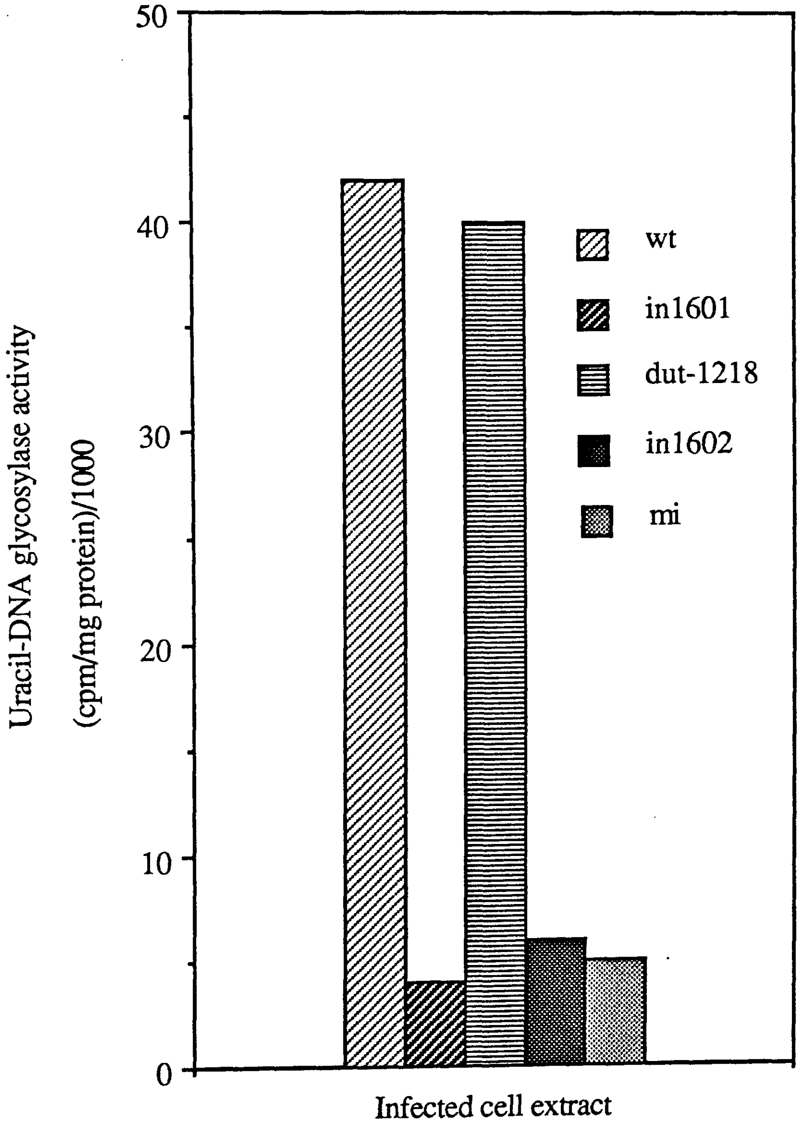
a) Uracil-DNA glycosylase

The predicted phenotype of in1602 is that both the uracil-DNA glycosylase and the dUTPase genes will be insertionally inactivated. In order to verify that in1602 carried the lesion in UL2, uracil-DNA glycosylase assays were performed. Extracts were prepared from cells mock-infected or infected with *wt*, in1601, dut-1218 or in1602 at 10 h pi and assayed for uracil-DNA glycosylase as described. The levels of uracil-DNA glycosylase activity in these extracts are shown in figure 3.30. This figure shows a high level of uracil-DNA glycosylase activity in the extract prepared from cells infected with *wt* and from those infected dut-1218, the level of activity in mock-infected cell extract being negligible. There was no significant difference between the level of activity in the extract from cells infected with in1602 and either in1601 or the mock-infected extract, indicating that this virus does not induce uracil-DNA glycosylase activity.

Figure 3.30 : Uracil-DNA glycosylase assays

Extracts prepared at 10 h pi were assayed for uracil-DNA glycosylase activity as described (Caradonna and Cheng, 1980). The results of these assays are presented graphically. The enzyme activity present in each extract is displayed along the ordinate axis, as amount of radioactivity released/mg protein. The activities of extracts prepared from cells mock-infected, or infected with *wt*, in1601, dut-1218 or in1602 virus are shown.

Uracil-DNA glycosylase assay



b) dUTPase activity

As was the case for in1601, in1602 infected cell extracts were also assayed for dUTPase activity. However, in this instance the reason for doing so was to confirm that in1602 also carried the lesion which incapacitated the dUTPase gene. The extracts used in the preceding assay were also used in the dUTPase assays, and the results are displayed in figure 3.31. This figure shows that while in1601 has *wt* levels of dUTPase activity, in in1602, this has been abated to the levels demonstrated by the extracts from mock-infected cells and cells infected with dut-1218. This, along with the genotypic evidence displayed by the restriction analysis, confirms that in1602 also carries the lesion from dut-1218, in the gene encoding the dUTPase.

c) Other enzyme activities

In this case it was also necessary to ensure that the low level of uracil-DNA glycosylase activity induced in cells infected with in1602 was not due to a reduced level of infection, so the two other enzyme activities were also quantified. The first of these was again the HSV-induced alkaline exonuclease enzyme. The extracts used in the uracil-DNA glycosylase assays above were used in exonuclease assays. The level of alkaline exonuclease activity present in these extracts at 10 h pi is evinced in figure 3.32. In this case, the levels of exonuclease activity in the extracts from *wt*, in1601, dut-1218 and in1602 infected cells were not significantly different from each other, and once again the activity from the mock-infected extract was essentially zero. This indicates that the infection with in1602 was as productive as that with *wt*, dut-1218 or in1601, and the low level of uracil-DNA

Figure 3.31 : Deoxyuridine triphosphatase assays

Extracts prepared at 10 h pi were assayed for deoxyuridine triphosphatase activity as described. The results of these assays are presented graphically. The enzyme activity present in each extract is displayed along the ordinate axis, as amount of radioactivity released/mg protein. The activities of extracts prepared from cells mock-infected, or infected with *wt*, in1601, dut-1218 or in1602 virus are shown.

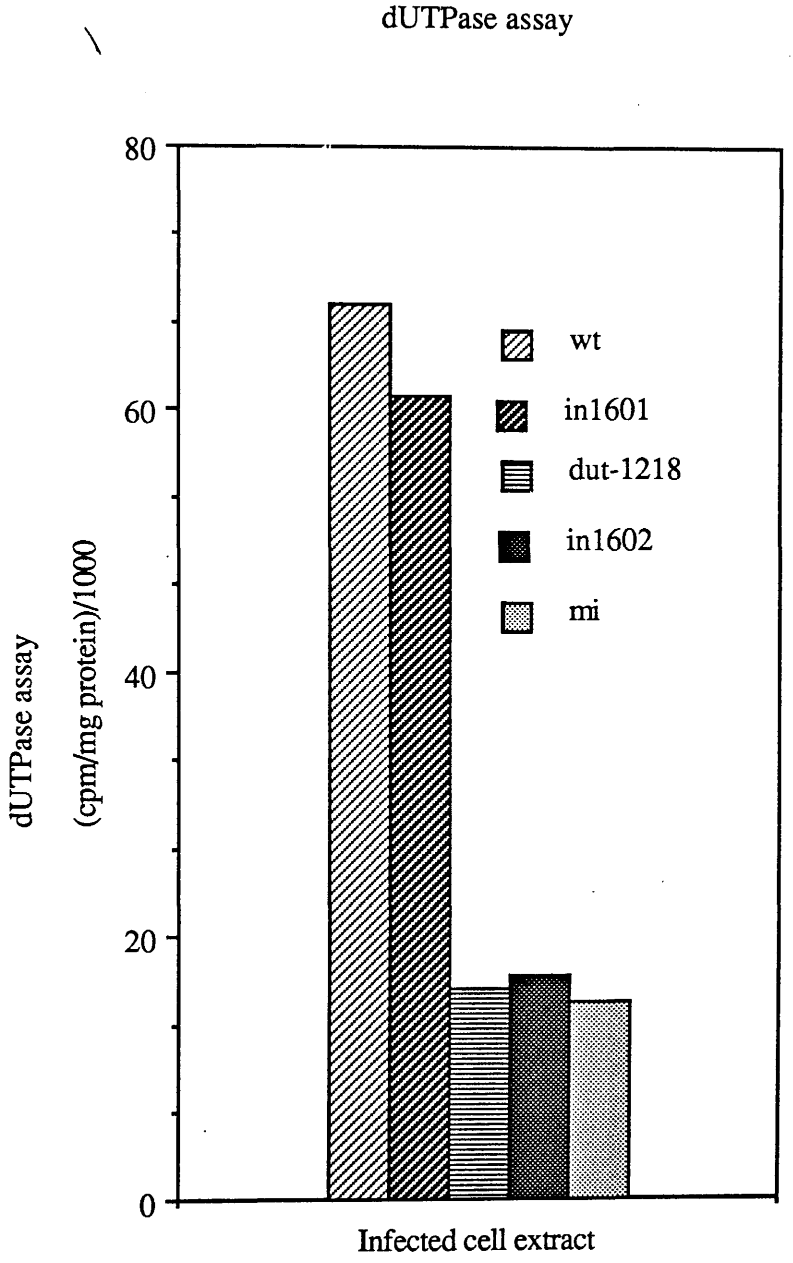
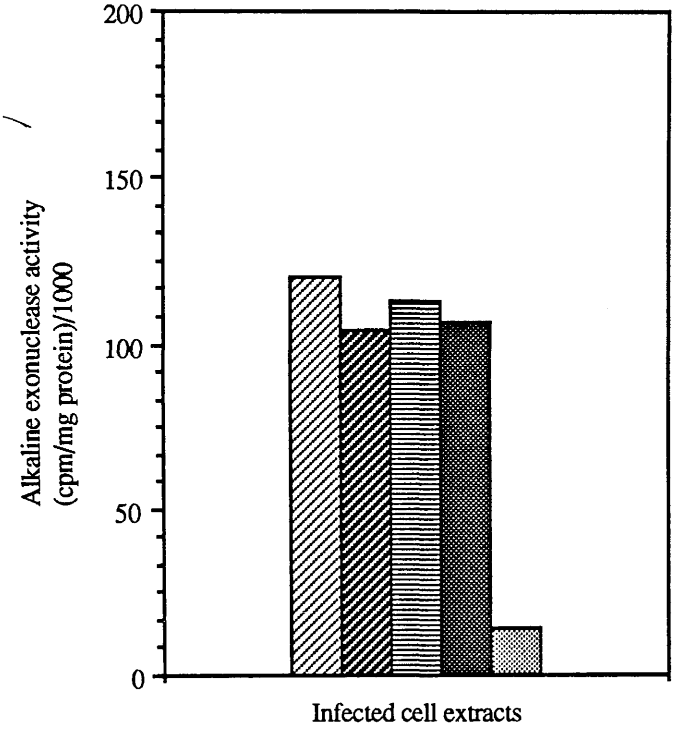

dUTPase assay
80
60
40
20
0
dUTPase assay
(cpm/mg protein)/1000
▨ wt
▨ in1601
▤ dut-1218
■ in1602
▩ mi
Infected cell extract

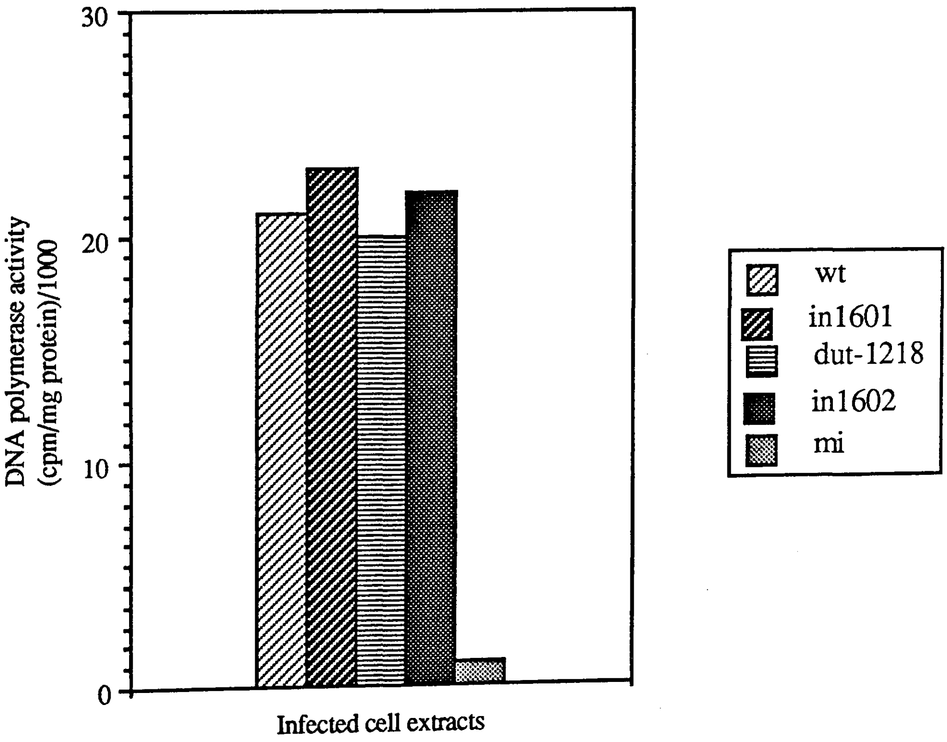
Figure 3.32 : Other enzyme assays

Extracts prepared at 10 h pi were assayed for alkaline exonuclease and DNA polymerase activity as described. The results of these assays are presented graphically, on graphs a and b respectively. The enzyme activity present in each extract is displayed along the ordinate axis, as amount of radioactivity released (or incorporated, in the case of the DNA polymerase assay)/mg protein. The activities of extracts prepared from cells mock-infected, or infected with *wt*, in1601, dut-1218 or in1602 virus are shown.

Alkaline exonuclease assay



DNA polymerase assay



glycosylase activity shown in the in1602 infected extract was not due to a reduced level of infection.

The second enzyme activity which the extracts were subjected to was the virus induced DNA polymerase. The results of this assay are also presented in figure 3.32. Again, there was no significant difference between the levels of polymerase activity in the extracts infected with *wt*, dut-1218 or in1601 and those infected with in1602, The mock infected extract had an insignificant level of activity. These results corroborate those obtained for the exonuclease activities.

d) Time course of enzyme assays

In order to investigate the progression of infection in cells either mock-infected or infected with *wt*, in1601, in1602, or dut-1218, a series of extracts were prepared at 3, 6, 9 and 12 h pi. These extracts were assayed for the four enzyme activities described above. The results are displayed in figure 3.33. In general, the time courses show that the four viruses assayed exhibit the spectrum of enzyme activities predicted from the 10 h pi assays. Again, there does appear to be a small lag in the levels of exonuclease activity induced by in1601, but this is almost fully corrected by 9 h pi and so is not regarded as being significant.

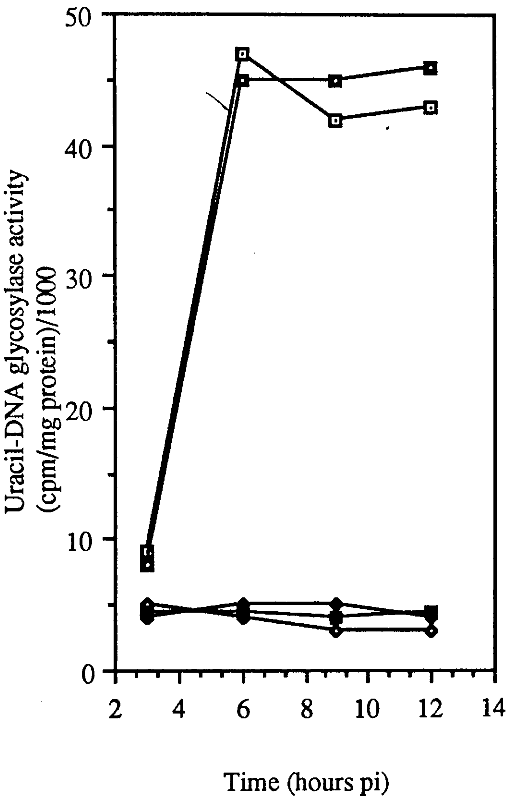
e) Radiolabelled HSV-1 induced polypeptides

In order to discern what effect (if any), the mutation had on the polypeptide profile of in1602, radiolabelled cell extracts were prepared from cells infected with *wt*, in1601, dut-1218 and in1602. As for in1601, these extracts

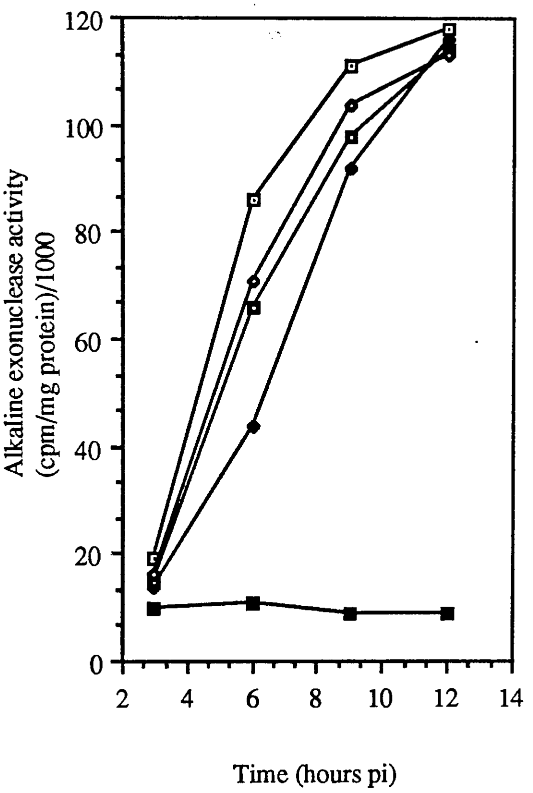
Figure 3.33 : Enzyme assay time courses

Extracts prepared at 3, 6, 9 and 12 h pi were assayed for uracil-DNA glycosylase, alkaline exonuclease, DNA polymerase and dUTPase activity as described. The results of these assays are presented graphically, in graphs a, b, c and d, respectively. The enzyme activity present in each extract is displayed along the ordinate axis, as amount of radioactivity released (or incorporated, in the case of the DNA polymerase assay)/mg protein. The activities of extracts prepared from cells mock-infected, or infected with *wt*, in1601, dut-1218 or in1602 virus are shown.

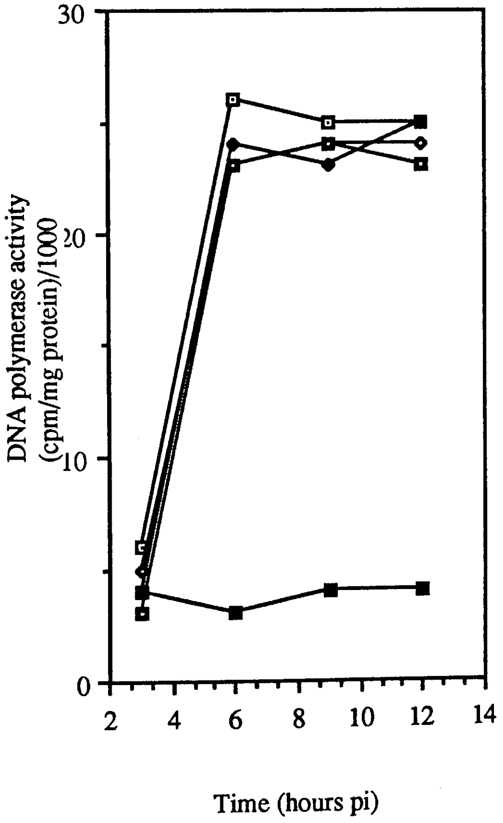
Uracil-DNA glycosylase assay time course



Alkaline exonuclease assay time course



DNA polymerase assay time course



dUTPase assay time course

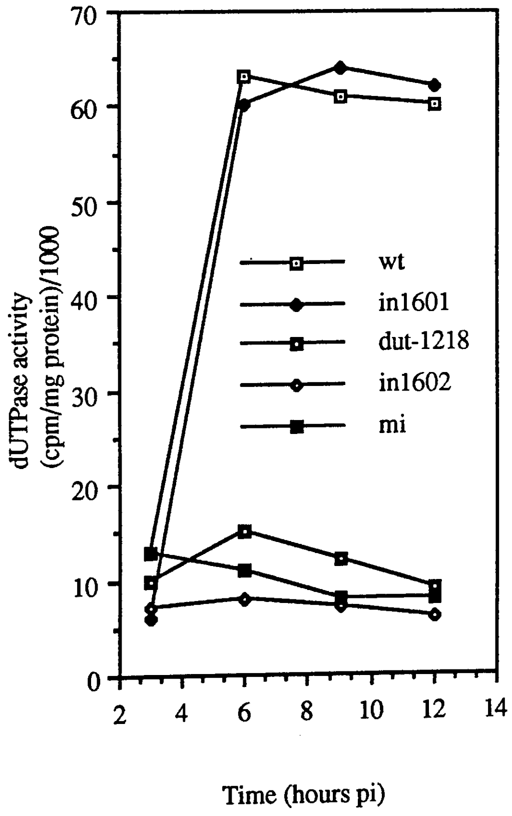
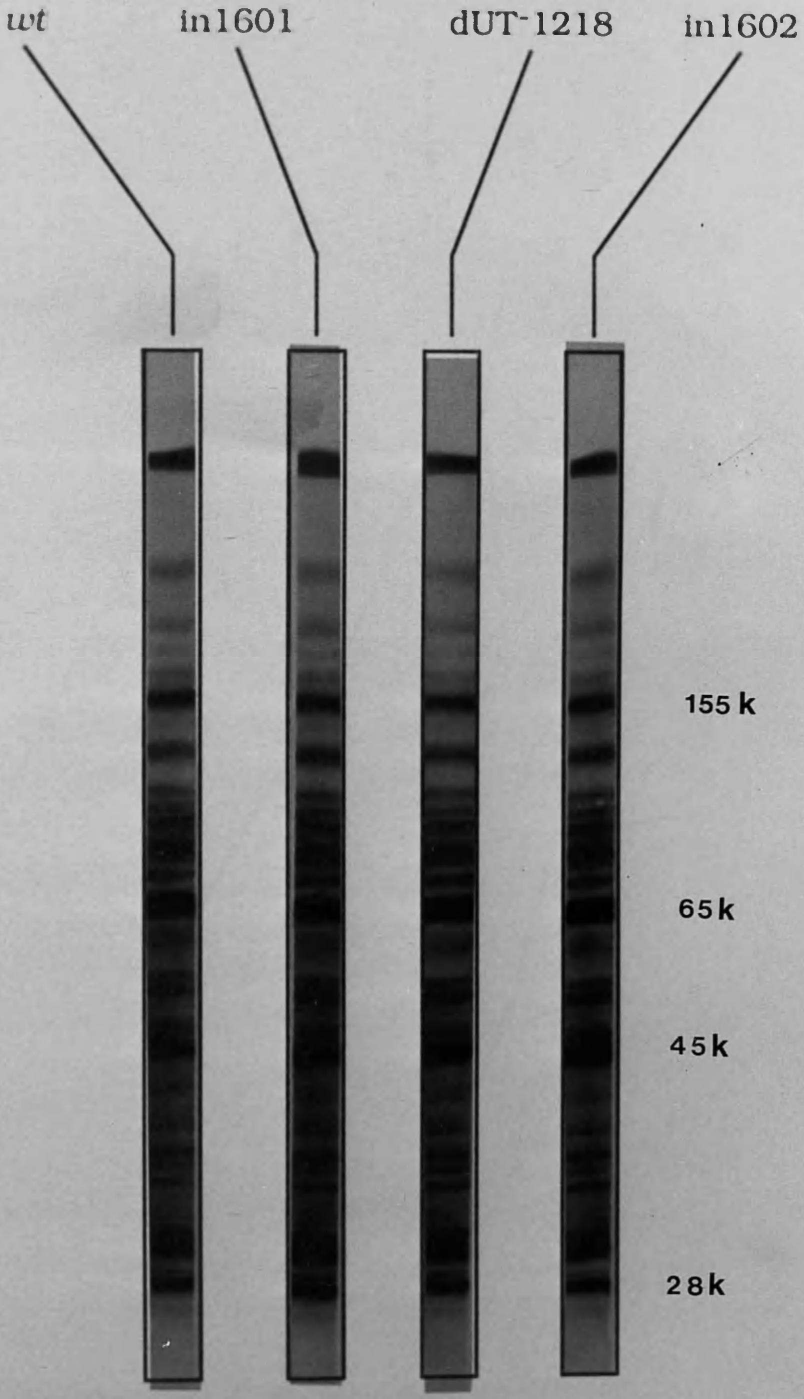


Figure 3.34 : ^{35}S-Methionine labelled HSV induced polypetides

Figure 3.45 is an autoradiograph of ^{35}S-Methionine labelled virus induced polypeptides. There are no discernible differences between the *wt* profile and any of the others, in1601, dut-1218 or in1602. M_r are as indicated.


wt
in1601
dUT-1218
in1602
155 k
65 k
45 k
28 k

The* fragment used was the segment between the *Not*I site at position 5092 and the *Eco*RI site at 3280.

were examined by polyacrylamide gel electrophoresis, the results of which are shown in figure 3.34. As before, there are no discernible differences between any of the viruses.

3.4 ATTEMPTS TO MUTATE GENE UL1

The technique of insertional mutagenesis was also used to investigate the function of the gene UL1 and its product, which has yet to be identified.

3.4.1 COTRANSFECTION AND ATTEMPTED PURIFICATION

DNA* from the plasmid pJM4 was used in a cotransfection experiment with *wt* virus DNA. This initial cotransfection yielded only 2 blue plaques. Attempts to purify these recombinants failed, as no blue plaques were seen in the next round. Two subsequent attempts to purify the initial 2 plaques were also unsuccessful. The cotransfection was repeated, but this gave only 3 blue plaques which again failed to purify. A third attempt, in which fresh BHK C13 cells of low passage were used yielded 20 blue plaques. All of these were picked and plaque purification was attempted.

However, it quickly became apparent that the recombinant was again failing to purify. By the third round of purification, no blue plaques were detected. This is in contrast to what was in observed in the purification of the recombinants in1601 and in1602. A further two attempts to isolate this recombinant also failed.

There are several possible explanations why isolation of this recombinant was not achieved. Perhaps the most likely of these are either that the mutation resulted in a genome which was unstable (for instance due to other, unpredicted alterations), or that there is a stringent requirement for the UL1 gene product in the lytic cycle of the virus. The unexpected appearence of unpredicted, and potentially unstable, rearrangements in recombinants constructed by homologous recombination has already been discussed. In the case of this construction, there were no duplications or rearrangements in the HSV-1 sequences within the plasmid. Although the plasmid contained a deletion in part of the HSV-1 sequence, the DNA used in the cotransfection was a single segment of DNA, contiguous apart from the *LacZ* insert. It seems unlikely that this construct would be unstable upon cotransfection.

The fact that recombinant blue plaques were observed in the initial transfection would tend to support the suggestion that there is an absolute requirement for the product of UL1 in the lytic cycle of the virus.

The function of the UL1 gene product is at present unknown. Extensive searches for homologues in the major sequence databases have failed to reveal any similar amino acid sequences of known function and little information can be deduced from the amino acid sequence on its own.

One way of potentially overcoming the problems associated with the mutagenesis of an essential gene is the construction of a cell line which expresses that gene and which would therefore be able to complement the function of its product, not expressed by the recombinant virus.

FINAL DISCUSSION

4.1 VALIDITY OF MUTAGENESIS AND COTRANSFECTION TECHNIQUES

Insertional mutagenesis has been used as a technique for investigating the functional potential of genes whose role in the lytic cycle of the virus has not been established. This has led to the positive identification of the gene encoding the HSV-1 specified uracil-DNA glycosylase enzyme as UL2. In this case the assignation was made after construction and analysis of a mutant which exhibited the predicted genotype and phenotype, and was subsequently confirmed by amino acid sequence homology with published sequences from other sources. That HSV encodes its own uracil-DNA glycosylase enzyme was first observed by Caradonna *et al.* (1987), who reported that the gene encoding this function was situated between 0.065 and 0.080 map units (mu) on the virus genome. However, in1601 is the first reported isolate of HSV-1 which is deficient in uracil-DNA glycosylase activity, due to a mutation in the gene UL2. When the amino acid sequence of the UL2 gene product is compared with amino acid sequences from uracil-DNA glycosylase enzymes isolated from other organisms, it can clearly be seen that gene UL2 encodes the HSV-induced uracil-DNA glycosylase enzyme.

The second recombinant virus constructed by this technique was a double mutant, carrying lesions in the genes encoding both the uracil-DNA glycosylase and the dUTPase. Both of these recombinant viruses (in1601 and in1602) were isolated and purified at the first attempt, and exhibited no rearrangements in their genomes, apart from those predicted. This technique has also been used in the construction of recombinant viruses with insertions in other genes. The availability of a facile screening procedure for recombinants means that they can be identified and selectively purified without the need to screen large numbers of plaque isolates, as is often the case with the isolation of other types of insertion mutants.

The effects of such mutations on the growth properties of HSV-1 are dependent on the target gene and range from lethality (for example in the DNA polymerase gene, Purifoy *et al.*, 1977) to having no apparent effect (the dUTPase, Fisher and Preston, 1986). Mutagenesis is a key step in the functional analysis of genes whose role in the replicative cycle of the virus is obscure. As the functions of more HSV-1 genes become known, the number whose products are not required for virus growth increases. At present, there are at least 19 genes in HSV-1 whose products appear to be dispensible for growth of the virus in tissue-culture (McGeoch, 1989). No doubt more will be discovered as functional assignments are made to the remaining genes whose in vivo roles are as yet unresolved.

The one case in which the technique failed was in the isolation of a recombinant virus carrying an insertion in the gene UL1 (in1603), which indicates that this gene may be essential for growth of the virus. However, throughout the attempts to plaque purify this recombinant it was apparent when the purification was failing.

The mutagenic technique employed to construct these recombinants could also be used to engineer deletions into target genes, in a manner analogous to that using the TK gene as a phenotypic marker. For instance, the entire UL2 gene could be deleted from the plasmid pGX23, and this plasmid could then be used in a cotransfection experiment with in1601 DNA. Homologous recombination would remove the

LacZ insert and the UL2 flanking sequences, and would yield clear plaques against a background of blue plaques. This has been performed in the construction of a recombinant with a deletion of the gene UL43, where the construction, identification and purification of the deletion mutant was no more difficult than that of the insertion mutant (Dr C A MacLean, personal communication).

4.2 GENOTYPIC CONFIRMATION OF RECOMBINANT VIRUSES

The ease with which recombinant progeny could be identified and purified was complemented by the utility of the screening procedure employed to confirm the genotypes of the recombinants. The restriction analysis used was based on the techique first described by Lonsdale (1979), who utilised it to discriminate between the different serotypes of HSV. This technique is useful not only when large alterations are made to the genome (ie the insertion or deletion of large DNA sequences) but also when smaller changes are made, which influence the characteristic restriction profiles of the virus. In the case of in1601 both of these occurred, with the insertion of a large DNA fragment containing restriction sites not found in the *wt* genome. The recombinant dut-1218, on the other hand, only contains a small 8 bp insertion, which would not normally affect the restriction profile. However, the introduction of a novel HindIII restriction site into the genome facilitated the identification of the recombinant by this method (Fisher and Preston, 1986).

4.3 ENZYME ASSAYS

After it had been confirmed that the genome of the recombinants were as expected and no other aberrations were present, the next step was to investigate the phenotypic alterations brought about by the mutations.

The results obtained from the uracil-DNA glycosylase assay show that mock-infected cells have a low level of uracil-DNA glycosylase activity. Uracil-DNA glycosylase appears to be ubiquitous in nature (Varshney *et al.*, 1988) and the activity observed in the mock-infected extract is presumably due to this enzyme being present in the tissue-culture cells.

Caradonna and Cheng (1981) reported a six-fold increase in uracil-DNA glycosylase activity in HeLa BU cells infected with HSV-1 and a forty-fold increase in the same cell line infected with HSV-2. The authors could not account for the disparity between the serotypes, but it has also been observed in BHK C13 cells infected with HSV-1 and with HSV-2 (Dr H Rixon, personal communication). The results presented in this thesis demonstrate an approximately equivalent increase in uracil-DNA glycosylase activity succeeding infecting with HSV-1, at almost ten-fold as opposed to six-fold. Both recombinants which carry the mutation in the UL2 gene (in1601 and in1602) have mock-infected levels of uracil-DNA glycosylase activity.

As stated previously, the infected cell extracts were assayed for three other virus-associated enzyme activities. The major reason for doing so was to confirm the results obtained from the purification of the recombinant; ie that infection with in1601 was as productive as with *wt* (although, the dUTPase assay was performed to confirm that in1602 carried the mutation in UL50). Therefore, the reduced levels of uracil-DNA glycosylase activity induced by in1601 were not a consequence of the recombinant being less virulent than *wt*.

The first of these assays was for alkaline exonuclease. This assay is performed at pH 9.0, which reduces any endogenous exonuclease activity to almost zero (Morrison and Keir, 1968). Under these conditions, the HSV-1 enzyme is still very active, inducing at least a ten-fold stimulation in exonuclease activity. This stimulation is apparent, to approximately equivalent levels, in extracts from cells infected with any of the four virus strains used in this study (ie *wt*, in1601, dut-1218 and in1602).

The second of these assays was for the HSV-1 DNA polymerase. As in the previous case, the conditions of the assay reduce any endogenous enzyme activity, almost to zero.

One other HSV-1-induced enzyme activity which was quantified in the course of this study was the dUTPase. The assay is performed at 4°C, which inhibits the host cell enzyme (Wohlrab *et al.*, 1982). Like that for the uracil-DNA glycosylase, the dUTPase assay was performed for confirmation of mutant phenotypes. The extracts from cells infected with viruses possessing intact dUTPase genes induced a four-fold increase increase in the enzyme activity over those extracts from cells infected with viruses carrying the mutation in the gene encoding the dUTPase, which were equivalent to extracts from mock-infected cells.

In all cases, the four virus isolates had levels of enzyme activity equivalent to those predicted by their phenotypes. All the assays yielded results which were quantifiable and which were unequivocal. The lowest level of virus-mediated stimulation was witnessed in the dUTPase assay, but in this case there was still approximately a four-fold increase in the appropriate extracts. This is not surprising as there are published examples of all the assays being previously performed in HSV-infected mammalian cells, with satisafactory results.

4.4 THE HSV-1 ENCODED URACIL-DNA GLYCOSYLASE

The experimental data presented in this thesis demonstrate that the gene UL2 of HSV-1 encodes the uracil-DNA glycosylase activity described by Caradonna and Cheng (1981). A recombinant virus constructed with an insertional mutation in the gene UL2 fails to induce uracil-DNA glycosylase activity and the amino acid sequence of the protein encoded by UL2 is endowed with highly conserved motifs which appear to be characteristic of the enzyme.

The in vivo role of uracil-DNA glycosylase appears to be relatively well characterised (Lindahl, 1974), as is the reaction which it catalyses (Lindahl, 1976; Lindahl *et al.*, 1977). No function has been attributed to the enzyme other than excision of uracil from DNA, leaving an AP site. It is proposed, therefore, that the enzyme must have a similar role in the lytic cycle of the virus. That this gene is common to all the herpesviruses (and is amongst the best conserved genes) is indicative of the protein having a fundamental role throughout the evolution of these viruses. It is now generally accepted that this role is in the prevention of mutations brought about by deamination of cytosine to uracil.

Duncan *et al.* (1978) reported the isolation and characterisation of a uracil-DNA glycosylase-deficient strain of E. coli (*ung*). In this strain, the rate of spontaneous mutations at cytosine residues was increased to the level observed at 5-methylcytosine residues in *wt* cells (Duncan and Miller, 1980). 5-methylcytosine residues are hotspots of spontaneous base substitutions (Coulondre *et al.*, 1978), but are deaminated to thymine which cannot be removed by uracil-DNA glycosylase. Thus, in an *ung* mutant, unmodified as well as modified cytosine residues become hotspots of mutation. It would be interesting to discover if in1601, which is deficient in uracil-DNA glycosylase activity, has an elevated amount of uracil in its DNA, due to spontaneous deamination of cytosine which will not be corrected. Presumably the DNA polymerase would pair the uracil with adenine upon the next round of replication, leading to a G.C to A.T transition mutation. It is not known to what extent this type of mutation occurs in *ung* mutants. Attempts were made to quantitate the levels of uracil present in the genomes of all four virus strains used in this study. Analysis using paper electrophoresis and chromatographic separation by HPLC after both formic acid digestion of virus DNA and enzymatic digestion with nuclease and phosphodiesterase were attempted. Unfortunately, none of these techniques gave satisfactory results in the time available and so cannot be included in this thesis.

It seems unlikely that the uracil-DNA glycosylase has potential as a possible target for anti-viral chemotherapy. Abolition of the function appears to have no detrimental effect on the infectivity or replication of the virus. Whether the mutation affects the long-term stability of the virus remains to be determined.

4.5 THE RECOMBINANT in1602

The recombinant in1602 carries insertional mutations in two genes involved in DNA metabolism, namely the uracil-DNA glycosylase and the dUTPase. The significance of the former has already been discussed (section 4.4). The HSV-encoded dUTPase is also dispensible for growth in tissue-culture (Fisher and Preston, 1986) but is responsible for preventing misincorporation of dUMP into DNA during replication (Shlomai and Kornberg, 1978).

Although it is known that the HSV-1 dUTPase is not essential for growth of the virus, it has been proposed that this enzyme might still be considered as a possible anti-viral target (Williams, 1988). Plaque reduction assays on HSV-1 dUTPase mutants, using synthetic nucleoside analogues, suggested that certain compounds, for example 5-fluoro-2'-deoxyuridine, can inhibit the HSV-1 dUTPase enzyme. This leads to an increase in the concentration of dUTP, which in turn may result in more dUTP being incorporated into the DNA. The action of uracil-DNA glycosylase on this DNA with an increased uracil content could result in fragmentation of the DNA, as the repair processes initiated by the enzyme are activated. In *E. coli*, *dut* mutants, which do not synthesise dUTPase, fail to grow exponentially, become filamentous at temperatures above 25oC and exhibit a hyper-rec phenotype (Warner *et al.*, 1981). These mutants were previously named *sof* mutants, because they accumulate short Okazaki fragments (Konrad and Lehman, 1975). This fragmentation is also recognised as being due to the action of repair processes initiated by uracil-DNA glycosylase. It is interesting to note that, by itself, incorporation of uracil instead of thymine in DNA does not appear to be markedly mutagenic. *E.*

coli mutants deficient in both dUTPase and uracil-DNA glycosylase (*dut ung)* grow exponentially, do not become filamentous and are not hyper-rec. Thus the phenotypic alterations apparently due to abolition of the dUTPase activity are in fact due to the processes of repair. The first step in this pathway is the excision of the uracil by uracil-DNA glycosylase, and abolition of this function prevents these processes from proceeding. These mutants retain their viability even when as much as 15-20% of their thymine residues are replaced by uracil (Warner *et al.*, 1981).

It seems likely, therefore, that the uracil-DNA glycosylase would not be a potential target for anti-viral chemotherapy, even when combined with the dUTPase. In fact, it would appear that any possible anti-viral effect brought about by mutation to the dUTPase, may in fact be reduced by a simultaneous mutation in the uracil-DNA glycosylase, as is the case in the *dut ung E. coli*.

Like in1601, nothing is known about the composition of the DNA of in1602. It is a possibility that in1602 will also contain uracil as a result of both lesions. This would lead to G.U base pairs due to the absence of uracil-DNA glycosylase activity and A.U pairs due to the absence of the dUTPase function. Attempts made to quantitate the amount of uracil in the genome of this virus were also unsuccessful.

4.6 ATTEMPTS TO MUTAGENISE THE GENE UL1

One limitation of the mutagenesis technique employed in this study is that it cannot be used to engineer mutations in genes whose products are essential in the normal lytic cycle of the virus. It is proposed that the product of the gene UL1 is essential for normal growth of the virus. Research by another group working on this gene has also indicated this (D. Johnson, personal communication). Perry (1986) suggested that the protein may be membrane translated, as the amino terminus of the protein is strongly hydrophobic. Apart from this analysis, little is known about the proposed product of UL1. Searches of the NBRF and Swissprot protein databases, using Wordsearch, a program which can search databases for conserved amino acid motifs, have produced no evidence of a homologue of known function for the product of this gene.

One piece of information which may concern the UL1 gene and its product is the report by Little and Schaffer (1981), that a *syn* locus maps to this region of the genome, map coordinates 0.040 to 0.064. However, the authors were not able to assign this lesion to a specific gene.

4.7 FUTURE PROSPECTS

Now that these recombinants have been constructed, there are a variety of studies which should be performed upon them. One of the most important of these is the determination of the amount of uracil present in the genomes of these viruses, and whether this changes after passage. Chromatographic separation of enzymatic digestion products, for example by HPLC, would provide a measure of the amount of uracil present in the genomes.

Because of the nature of the mutations they contain, it may be the case that these recombinants will exhibit an increased mutation frequency, relative to ***wt***. It would be interesting to see if this was the case, after serial passage of the virus in tissue culture. HSV-1 has a high G+C content (68.3%, McGeoch *et al.*, 1988), therefore there are many cytosine residues which could potentially be deaminated. The phenotype of these viruses dictates that the resulting defects would not be corrected. The DNA of these mutants, after serial passage, could be examined directly, for example by restriction enzyme analysis. Alternatively, a way to screen for such mutants on a phenotypic basis would be to look for mutations in the DNA polymerase gene. It has been reported that mutations in the *pol* gene can confer resistance to antiviral agents based on DNA polymerase substrate analogues (Hay and Subak-Sharpe, 1976; Honess *et al.*, 1984).

As the functions of more HSV-1 gene products become known, it is to be expected that some will be essential for growth in tissue culture and, by definition, will be difficult to study by mutagenesis. This has been the case with the UL1, the product of which may be essential for lytic infection and replication of HSV-1. Potentially, these genes are very important, as there is much active research into possible antiviral therapeutic agents. Any function which is indispensible for virus growth and proliferation is a valid target for such analysis. One way round the requirement for a particular function which has been incapacitated is to find a cell type which can provide the necessary function in trans. This can be an unmodified cell line which fortuitously compensates for the essential function, as is the case for mutants isolated with a deficiency in the RR large subunit which can grow in some cell types but not others (Goldstein and Weller, 1988). Alternatively, a cell line may be isolated which expresses the part of the HSV-1 genome in which the mutated gene lies. This is achieved by transfecting a specific restriction fragment of HSV-1 into the cells, which it is hoped will integrate into the cellular genome and be expressed. This can then be cotransfected with *wt* HSV-1 DNA and the mutating plasmid DNA and the intact cellular gene will complement the function which has been inactivated in the mutant. As the product of UL1 appears to be absolutely required for growth, it is a feasible target for such a study.

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