ANTISENSE RNA TRANSCRIPTION REGULATES IE2 EXPRESSION IN THE HSV-1 DELETION VARIANT 1703.

by

Mary Catherine Sinclair.

A thesis presented for the Degree of Doctor of Philosophy.

in

The Faculty of Science at the University of Glasgow.

Institute of Virology,
Church Street,
Glasgow G11 5JR.

March 1992.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ACKNOWLEDGEMENTS.

I would like to acknowledge the following people for their help and encouragement throughout the course of this study.

Professor J.H. Subak-Sharpe for the provision of facilities in the Institute of Virology and for his interest in this work.

Dr S.M. Brown for her excellent supervision and thorough criticism of this manuscript.

Drs J. McLauchlan and A.R. MacLean for their interest, helpful suggestions and for proof reading parts of this manuscript.

Dr L. Robertson for her help during the *in vivo* work, especially the LD50 experiments.

Dr H. Marsden for supplying antiserum, Dr V.G. Preston for plasmids pGX55 and pGX51, Dr J. McLauchlan for oligonucleotides and Mr J. Aitken for particle counts.

The inhabitants of labs 209 and 210.

Miss V. Evans, Miss G. McVey, Dr. L. Robertson and Miss E. McKie whose friendship was and is invaluable.

I would like to thank my parents and family whose patience, understanding and ever available transport made life easier.

I would like to recognise the assistance of the English department of Alness Academy, Alness.

I dedicate this manuscript to my husband, Brian.

The author was in receipt of a Medical Research Council training award. Unless otherwise stated, the results were obtained by the authors own efforts.

ABBREVIATIONS

A	deoxyadenylic acid in DNA.
AIDS	acquired immunodeficiency syndrome.
ALV	avian leucosis virus.
APS	ammonium persulphate.
ATP	adenosine triphosphate.
BCIG	5-chloro-4-bromo-3-indolyl-βD-galactoside.
BHI	brain heart infusion.
BHK21 C13	baby hamster kidney cells batch 21 clone 13.
bp	base pairs.
BSA	bovine serum albumin.
℃	degrees centigrade.
Ci	Curie.
cpe	cytopathic effect.
dATP	2'-deoxyadenosine-5'-triphosphate.
dCTP	2'-deoxycytosine-5'-triphosphate.
dGTP	2'-deoxyguanosine-5'-triphosphate.
dTTP	2'-deoxythymidine-5'-triphosphate.
ddATP	2'3'-dideoxyadenosine-triphosphate.
ddCTP	2'3'-dideoxycytosine-triphosphate.
ddGTP	2'3'-dideoxyguanosine-triphosphate.
ddTTP	2'3'-dideoxythymidine-triphosphate.
DMSO	dimethylsulphoxide.
DNA	deoxyribonucleic acid.
DTT	dithiothreitol.
EBV	Epstein-Barr virus.
E. coli	*Eschericia coli.*

EDTA	ethylenediaminetetra acetic acid.
G	deoxyguanylic acid.
HBLV	human B-cell lymphotropic virus.
HCMV	human cytomegalovirus.
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid.
HFL	human foetal lung.
HHV	human herpesvirus.
HSV-1	herpes simplex virus type 1.
HSV-2	herpes simplex virus type 2.
ICP	infected cell polypeptide.
IE	immediate-early.
IPTG	isopropyl-β-D-thiogalactoside.
IR_L	long internal repeat.
IR_S	short internal repeat.
k	kilo (ie $x10^3$).
kb	kilobase (s).
kbp	kilobase pairs.
L	long segment.
LAT	latency associated transcript.
mc	map coordinates.
mg	milligrams.
min	minutes.
ml	millilitre.
mM	millimolar.
moi	multiplicity of infection.
mol.wt.	molecular weight.
mRNA	messenger RNA.
NPT	non-permissive temperature.
OD	optical density.

ori$_L$	origin of replication in U_L.
ori$_S$	origin of replication in IR_S/TR_S.
PAGE	polyacrylamide gel electrophoresis.
PBS	phosphate buffered saline.
pfu	plaque forming units.
PIPES	piperazine-N,N'-bis(2-ethane sulphonic acid).
pi	post infection.
PRV	pseudorabies virus.
R	purine nucleotide.
RE	restriction enzyme.
RNA	ribonucleic acid.
rpm	revolutions per minute.
RR	ribonucleotide reductase.
RT	room temperature.
S	short segment.
SDS	sodium dodecyl sulphate.
TEMED	N, N, N', N',-tetramethylethylene diamine.
Tris	tris (hydroxymethyl) aminomethane.
TK	thymidine kinase.
TP	tryptose phosphate.
TR_L	long terminal repeat.
TR_S	short terminal repeat.
ts	temperature sensitive.
U_L	long unique.
U_S	short unique.
UV	ultraviolet radiation.
V	volts.
Vmw	molecular weight in kilodaltons of HSV induced polypeptides.

v / v	volume/volume (ratio).
VZV	varicella zoster virus.
w / v	weight/volume (ratio).
w / w	weight/weight (ratio).
µCi	microcurie.
µl	microlitre.

CONTENTS.

CHAPTER 1-INTRODUCTION.

CHAPTER 2-MATERIALS AND METHODS.

MATERIALS.

METHODS

CHAPTER 3-RESULTS.

SUMMARY.

The aim of this project was to further characterize the HSV-1 strain 17^+ deletion variant 1703. Initial characterization after isolation (MacLean & Brown, 1987a) demonstrated that 1703 had a deletion of approximately 7500 base pairs (bp); ($4.9x10^6$ mol. wt.) in the U_L/IR_L region of the genome. By restriction enzyme analysis, the deletion was shown to affect genes UL55, UL56 and one copy each of IE1 and LAT. Polypeptide analysis demonstrated that, although the deletion terminated an estimated 500 base pairs downstream of the 3' end of IE2, the IE2 gene product Vmw63 was apparently not produced during immediate-early times of infection.

Further characterization of 1703 was achieved by: **1**. The dideoxynucleotide sequence analysis of 1703 DNA fragments in which IE2 and the end points of the deletion were located, **2**. The analysis of 1703 IE2 gene products, **3**. The *in vivo* characterization of 1703 and **4**. The construction of a 1703 wild-type recombinant. Following the suggestion by Dr John McLauchlan that IE2 mRNA synthesis in 1703 infected cells may be controlled by the production of transcripts initiating from the promoter of the IR_L copy of IE1 which was antisense to IE2 RNA, the project was extended to determine if this could be substantiated.

Dideoxynucleotide sequence analysis of IE2 demonstrated that the promoter, promoter associated, terminator, terminator associated signals and most of the open reading frame were homologous to the published wild-type sequence (McGeoch *et al.*,1988a). Sequencing of the deletion end points has shown that it spans the region between np (nucleotide position) 123623 and np115839, removing UL56 and 343 base pairs of the

3' end of UL55 thus leaving 555 base pairs between the 3' end of IE2 and the deletion end point.

The methods of polypeptide analysis, Western blot analysis and S1 nuclease mapping were used to detect IE2 gene products at both protein and RNA levels. These techniques demonstrated that IE2 mRNA and Vmw63 synthesis were reduced, but not totally absent, at immediate-early times of infection. Western blot analysis of 17^+ immediate-early polypeptide extracts titrated in mock infected extracts compared to 1703 immediate-early polypeptide extracts demonstrated that, at most, Vmw63 production in 1703 infected cells was 1/8 that produced by 17^+. At early and late times of infection, Vmw63 synthesis by 1703 was equivalent to 17^+.

The *in vivo* effect of the loss of UL55, UL56, one copy of IE1 and LAT and the reduction in synthesis of Vmw63 during immediate-early times of infection was examined. Inoculation of 3 week old mice with 1703 via the intracranial route demonstrated that 1703 was as virulent as the wild-type virus. The latency characteristics of 1703 were also shown to be equivalent to those of 17^+ indicating that the products of the genes mentioned above are not required for intracranial virulence or for the establishment, maintenance or reactivation of latent genomes.

Construction of a wild-type recombinant of 1703 was achieved by recombination of the 1703 DNA fragment in which the end points of the deletion were located into 17^+ DNA. The characterization of the resultant recombinant's IE2 gene products indicated equivalence to those produced by 1703 and hence that the deletion was responsible for the underproduction of Vmw63 during immediate-early times of infection.

To examine the possibility of antisense transcripts controlling the production of IE2 mRNA, a polyadenylation signal was cloned between the 3' end of IE2 and the 5' end of the IR_L copy of IE1 in the correct

orientation to terminate the synthesis of a potential antisense transcript before IE2 coding sequences. A HSV-2 strain HG52 polyadenylation signal was chosen for this and, since the surrounding sequences were heterologous to 1703 DNA, two 1703 fragments were cloned around the polyadenylation signal and the construct recombined into 1703 DNA. The resultant recombinant was called 1703PA and analysis of 1703PA IE2 gene products demonstrated that IE2 mRNA and Vmw63 synthesis had returned to wild-type levels. The detection of the novel transcript generated as a result of the insertion of the polyadenylation signal substantiated the conclusion that antisense transcripts initiating from the IR_L copy of IE1 had the potential to control the production of IE2 mRNA in 1703 infected cells.

CHAPTER 1

INTRODUCTION

1.1 OBJECTIVES.

This project has involved the characterization of an HSV-1 17^+ deletion variant 1703 which underproduces the essential immediate-early polypeptide Vmw63 under immediate-early conditions despite the fact that the deletion does not extend into, nor is there an apparent deletion/insertion in IE2, the gene which codes for Vmw63. Characterization of 1703 involved the analysis of IE2 mRNA and Vmw63 protein synthesis, the dideoxynucleotide sequence analysis of IE2 and determination of the end points of the deletion and the construction of relevant recombinant viruses. The analyses have allowed the conclusion that the production of Vmw63 by 1703 is due to interference with IE2 transcription mediated by RNA initiating from the IR_L IE1 promoter under immediate-early conditions.

The aim of the introduction is to provide a general overview of herpes simplex virus (HSV) with emphasis on those topics relating to the project.

1.2 CLASSIFICATION OF HERPESVIRUSES.

Membership of the Herpesviridae family is based on the structure of the virion (Fenner,1976) which consists of **a**. a core made up of a fibrillar spool around which the double stranded DNA is wrapped, **b**. an icosahedral capsid consisting of 12 pentameric and 150 hexameric capsomeres, **c**. various amounts of an amorphous material asymmetrically arranged around the capsid and designated the

tegument and **d**. a membrane or envelope of 150-200nm surrounding the entire structure (Wildy *et al.*,1960; Roizman & Furlong, 1974).

Herpesviruses cannot be distinguished from each other on the basis of morphology, however they are readily separable using criteria such as biological properties, immunological cross-reactivity, and the size, base composition and structure of their genomes. More than eighty different members of the family have been identified by these criteria (Nahmias, 1972) and have been classified on the basis of their biological properties (Roizman *et al.*,1978; Matthews, 1982; Roizman, 1982) and genome structure (Roizman, 1982; Honess & Watson, 1977; Honess, 1984); these are outlined below.

1.2.a. Classification on the basis of biological properties.

Herpesviruses have biological as well as structural properties in common, for example, they specify a large array of enzymes involved in nucleotide metabolism, synthesis of viral DNA and encapsidation occurs in the nucleus, the host cell is destroyed after productive infection and herpesviruses are able to remain latent in their natural host where only limited transcription occurs. Biological properties provide an empirical means of grouping herpesviruses according to features such as host cell range, cytotoxicity and type of cell in which the viral genome establishes latency. These features have enabled the classification of herpesviruses into 3 groups, alpha, beta and gamma.

Alphaherpesviruses are classified on the basis of variable host range, relatively short replication cycle, rapid spread in culture, efficient destruction of infected cells and capacity to establish latency in infected cells primarily in the sensory ganglia. This subfamily includes 3 human

herpes viruses, herpes simplex viruses type 1 and 2 (HSV-1 & 2) and varicella-zoster virus (VZV).

Betaherpesviruses are mainly characterized by having a restricted host range, long replicative cycle and an infection that progresses slowly in tissue culture. The infected cells frequently become enlarged (cytomegalia) and carrier cultures are readily established. The virus can be maintained in the latent form in the secretory glands, lymphoreticular cells, kidney and other tissues. Human cytomegalovirus (HCMV) is a member of this subfamily.

The *in vivo* experimental host range of Gammaherpesviruses is limited to the family or order to which the natural host belongs. *In vitro*, all members of this family replicate in lymphoblastoid cells, and some also cause lytic infections in some types of epithelial and fibroblastic cells. Viruses in this group are specific for either T or B lymphocytes. In the lymphocyte, infection is at the pre-lytic or lytic stage, but without the production of infectious progeny. Latent virus is frequently demonstrated in the lymphoid tissues. Epstein-Barr virus (EBV) is a member of this subfamily.

1.2.b. Classification on the basis of genome structure.

It is relatively simple to assign herpesviruses to groups on the basis of visible criteria such as characteristics of infection, host/cell range and cell type in which the virus is capable of becoming latent. It is more difficult to do this on the basis of genome structure which requires detailed analysis (Honess & Watson, 1977; Roizman, 1982; Honess, 1984). Herpesviruses differ considerably in their overall base composition (32-75% G+C), the size of their genomes (80-150x10^6mol. wt.) and the arrangement of repeated elements. Classification on the

basis of genome structure is dependent on the latter property and is outlined below and in figure 1.1.

Group A

The DNA of members of this group is present as only one isomer and is characterized by a single direct repeat at both termini. Channel catfish virus (CCV) is a member of this group (Chousterman *et al.*, 1979)

Group B

The DNA of members of this group is present as only one isomer and is characterized by containing multiple copies of a sequence present as a direct repeat at both termini. The number of repeats in each genome is roughly constant, although the number at each end varies extensively (Stamminger *et al.*,1987). Herpes virus saimiri (HVS) is a member of this group.

Group C

Again the DNA of members of this group is present as only one isomer (Raab-Traub *et al.*,1980) whose genomes contain multiple copies of a sequence present as a direct repeat at both termini and internal tandem reiterations of a second set of sequences. A member of this group is Epstein-Barr virus (EBV).

Group D

The DNA of members of this group exists as two isomers since there are two unique regions (U_L and U_S) and the U_S region is flanked by inverted repeats (IR_L and TR_S) which allow inversion of Us. Pseudorabies virus (PRV) is a member of this group.

Group E

This group has been further divided into two subgroups, E1and E2.

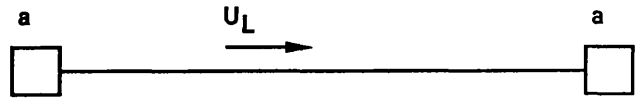
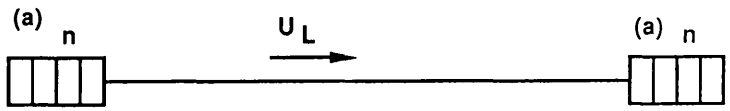
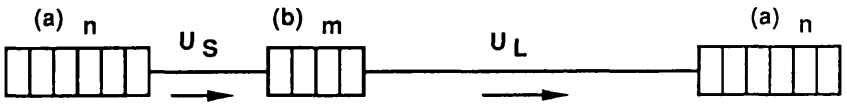
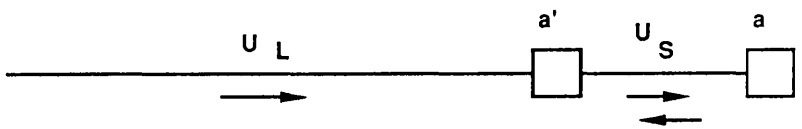
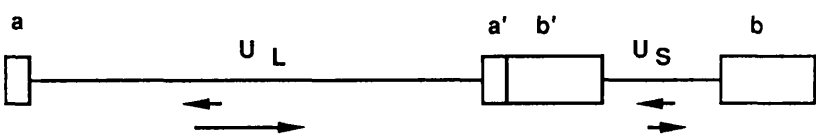
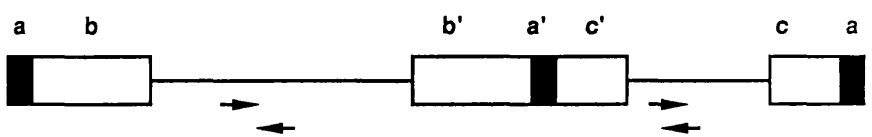
Subgroup E1 consists of those viruses eg. VZV whose unique regions (U_L and U_S) are flanked by inverted repeats (TR_L/IR_L, IR_S/TR_S). U_L is flanked by small inverted repeats (88bp in the case of VZV) and inverts

Figure 1.1 Genome structures of the herpesviruses.

The structures of herpesvirus genomes are demonstrated. Repeat sequences are shown as open boxes. U_S and U_L indicate the short and long unique sequences a, b and c indicate repeat sequences with a', b' and c' their complement. Arrows indicate the relative orientations of the unique segment. An example of each group A-E2, is illustrated and the numbers of isomers indicated. In VZV, U_S is in either orientation 50% of the time, while U_L is in one orientation 95% of the time.

CCV is channel catfish virus, HVS is herpesvirus saimiri, EBV is Epstein-Barr virus, PRV is pseudorabies virus, VZV is varicella-zoster virus and HSV is herpes simplex virus.

(Adapted from MacLean, 1988)

Group	No. of isomers.	Example	Genome structure
A.	1	CCV	
B	1	HVS	
C	1	EBV	
D	2	PRV	
E1	2 major 2 minor	VZV	
E2	4	HSV-1	

inefficiently. The short unique region is flanked by longer repeats and inverts to give both orientations of U_S in equimolar amounts. Hence U_L is found predominantly in one orientation and U_S is found equally in both orientations leading to the presence of 2 major and 2 minor isomers (Davison, 1984).

Viruses belonging to subgroup E2, eg HSV-1 and 2 have two unique segments (U_L and U_S) both of which are flanked by inverted repeats (TR_L/IR_L, IR_S/TR_S) which share a short region of DNA directly repeated at the termini and indirectly repeated at the junction between the internal inverted repeats. This allows equal isomerisation of both unique segments resulting in 4 isomers which are equally present in a population of DNA molecules.

1.3 HUMAN HERPESVIRUSES.

There are seven herpesviruses known to infect humans, herpes simplex virus types 1 and 2, (HSV-1&2) varicella-zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV) [these viruses are also known as human herpesviruses (HHV) 1-5 respectively], HHV-6 and HHV-7. Primary infection with HSV-1 occurs in early childhood and is often asymptomatic but may lead to a range of illnesses including fever, sore throat, ulcerative and vesicular lesions, oedema, localised general lymphadenopathy and general malaise (Whitely,1985). Following primary infection, a latent state is normally established (section1.9) usually in the trigeminal ganglia of the peripheral nervous system (Bastion *et al.*,1972). Reactivation of HSV-1 causes recurrent herpetic lesions (cold sores) localised to the dermatome supplied by the latently infected dorsal root ganglion (Wildy *et al.*,1982)

HSV-2 has similar biological properties to HSV-1 and shows 50% DNA homology. However HSV-2 is primarily a sexually transmitted disease and is associated with genital lesions (Dowdle *et al.*,1967; Kessler,1977). The virus normally establishes latency in the sacral dorsal root ganglia and periodically reactivates (Baringer & Swoveland, 1973). HSV-2 can also cause herpetic lesions on the mucocutaneous regions of the face, and HSV-1 can cause genital lesions. An association between HSV-2 and cervical carcinoma has been suggested (Naib *et al.*,1966; Eglin *et al.*, 1981; Park *et al.*,1983). A link between HSV-2, papilloma virus and cervical carcinoma has also been reported (zur Hausen, 1982).

Human cytomegalovirus infection in children and adults is mainly asymtomatic or associated with a mild fever. Infection of the foetus via their mothers is thought to be a major cause of intrauterine death and congenital defects (Weller, 1971; Rapp, 1980). Human CMV is also associated with the European form of Kaposi's sarcoma (Giraldo *et al.*,1975) and HCMV pneumonia is common among AIDS patients and those undergoing immunosuppressive therapy.

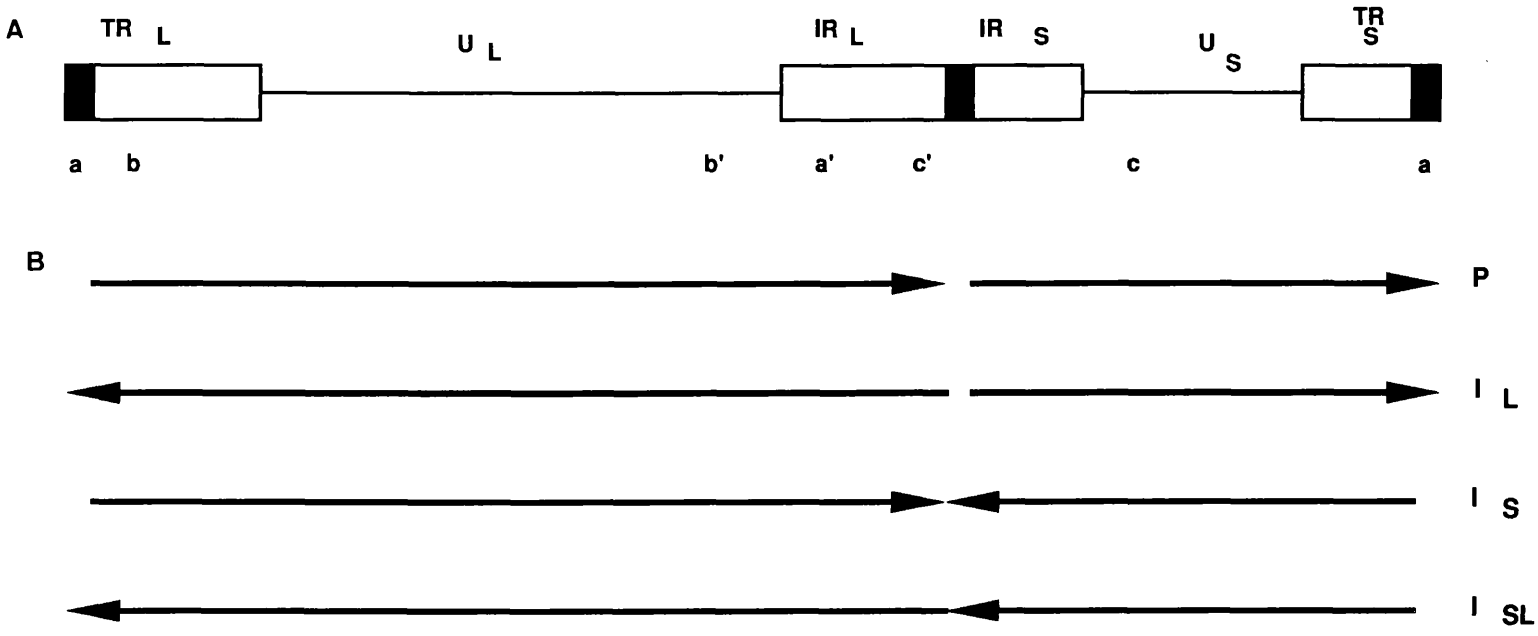
Varicella-zoster virus causes a primary childhood illness called chicken-pox (varicella). The virus becomes latent in the dorsal root ganglia (Gilden *et al.*,1978) and may reactivate mostly in older people to cause shingles (zoster). Reactivation may to some extent be dependent on the immunological status of the individual (Rifkind,1966; Gleb,1985).

Epstein-Barr virus (EBV) infection in childhood is often asymtomatic but can cause a debilitating infection in adolescents and adults, called glandular fever, when primary infection occurs at these times. EBV is also associated with Burkitts lymphoma and nasopharyngeal carcinoma in South East Asia.

Recently a novel herpes virus called HHV-6 (or human B-cell lymphotropic virus [HBLV]) was isolated from patients with AIDS and

Figure 1.2 Gross organisation of the HSV-1 genome.

A conventional representation of the HSV genome is shown, with unique sequences as solid lines (U_S and U_L) and the major repeat elements as open boxes (TR_L and IR_L, IR_S and TR_S). The a, b and c sequences and the same sequences in the opposite orientation a', b' and c' are indicated. Below the genome representation, the isomerisation of the HSV-1 genome is illustrated. The four isomers are: P (prototype), I_L (L inverted with respect to P), I_S (S inverted with respect to P) and I_{SL} (S and L inverted with respect to P).


A
TR L
U L
IR L
IR S
U S
TR S
a
b
b'
a'
c'
c
a
B
P
I L
I S
I SL

other lymphproliferative disorders (Salahuddin *et al.*,1986) and was later found to be the causative agent of exanthem subitum which is a childhood illness characterized by spiking fever and skin rash (Yamanishi *et al.*,1988).

A seventh herpesvirus called HHV-7 was isolated from activated $CD4^+$ lymphocytes of a healthy individual (Frenkel *et al.*,1990), and was shown to be unrelated to HHV-6 (Wyatt *et al*, 1991).

1.4 THE HERPES SIMPLEX VIRUS GENOME.

1.4.a. Structural features of the HSV genome.

The complete sequence of the HSV-1 genome has been established and shows that it has a total length of 152260 residues (McGeoch *et al.*,1988a) of base composition 68.3% G+C which is unevenly distributed throughout the genome. For example, the short repeat region has a base composition of 79.5% G+C (McGeoch *et al.*,1986) whereas the short unique segment has 64.3% G+C (McGeoch *et al.*,1985).

The genome is made up of a long (L) segment and a short (S) segment which are covalently linked and comprise 82% and 18% of the total DNA respectively (Roizman, 1979a,b). Each segment contains a unique sequence flanked by a pair of inverted repeat sequences (figure 1.2a), the long repeat (R_L) and short repeat (R_S) which are distinct from each other. The molecules also possess a terminal redundancy of between 250 and 500 base pairs (b.p.), termed the 'a' sequence; one or more additional copies of this sequence are located internally at the joint between the L and S segments in the opposite orientation to the 'a' sequence (Sheldrick & Berthelot, 1974). The HSV-1 genome isomerises to give equimolar amounts of each orientation (figure 1.2b).

Isomerization is thought to be mediated by the 'a' sequence (section 1.5). The four isomers are P (prototype), I_L (L inverted with respect to P), I_S (S inverted with respect to P), I_{SL} (S and L inverted with respect to P).

The number and size of 'a' sequences varies between and within strains of HSV-1 as does the number, size and location of tandem reiterations outwith the 'a' sequence (Rixon *et al.*, 1984). Both of these factors contribute to the variability in genome length found between strains. The length of the latter short reiterated sequences varies from 10-100 b.p. (McGeoch, 1989). The copy number of reiterations varies among virus isolates, serial passage of viral stocks, and upon recloning virus isolates (Watson *et al.*,1981a; Murchie & McGeoch,1982; Perry & McGeoch, 1988). These short tandem repeats may serve to promote exchange of genetic material between HSV DNA molecules thus maintaining homology (Umene, 1987) or they could promote a high degree of recombination, but the function of such reiterations is basically unknown.

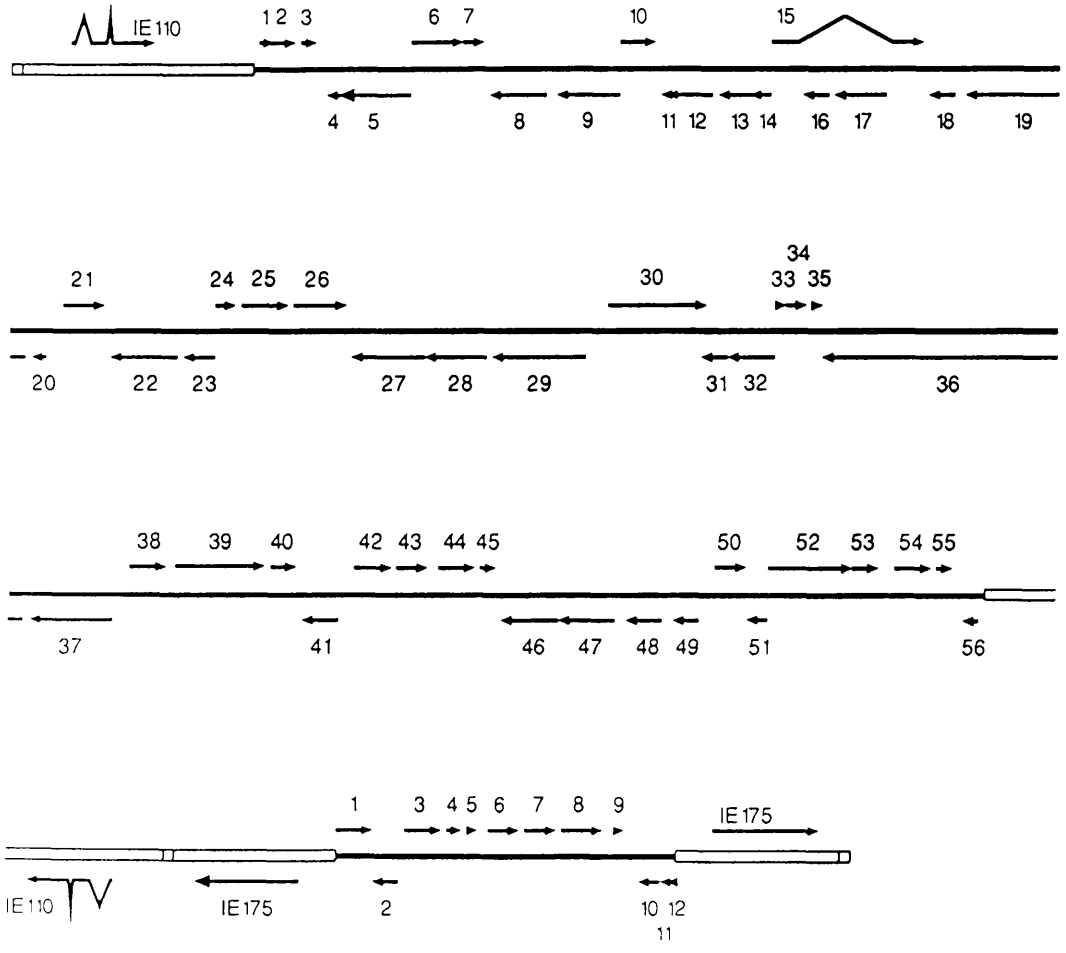
1.4.b. Organisation of HSV genes.

The genome of HSV-1 strain 17^+ has been sequenced and the genetic content analysed (McGeoch *et al.*, 1985, 1986, 1988a; Perry & McGeoch, 1988). The genome contains at least 75 genes which code for 72 distinct proteins, UL49.5 being the most recently identified gene (Barker & Roizman, 1992). Figure 1.3 shows the orientations and locations of these genes. Three open reading frames (IE1, IE3 and the RL1 gene encoding ICP 34.5) are within the repeats and are therefore diploid. The gene encoding ICP 34.5 was originally demonstrated in HSV strain F, as was the protein (Chou & Roizman, 1986; Ackerman *et al.*,1986; Chou & Roizman, 1990; Chou *et al.*,1990). The gene is positioned upstream of the

Figure 1.3 Layout of genes in the HSV-1 genome.

The HSV-1 genome is shown on four successive lines, with 40 kilo base pairs per line. Location of open reading frames are shown by arrows, with splicing within the coding regions indicated. In the top three lines, UL51 to UL56 are shown as 1-56, and in the bottom line, genes US1 to US12 as 1-12. Two recently characterized additional genes are UL49.5 (Barker & Roizman, 1992) and RL1 (Dolan *et al.*, 1992)

(Adapted from McGeoch, 1989)


IE110
1 2 3
6 7
10
15
4 5
8
9
11 12
13 14
16
17
18
19
21
24 25 26
30
34
33 35
20
22
23
27
28
29
31 32
36
38
39
40
42 43 44 45
50
52
53
54 55
37
41
46
47
48
49
51
56
1
3
4 5
6
7
8
9
IE175
IE110
IE175
2
10 12
11

5' end of IE1 in R_L in the same orientation as IE1. The assignment of a gene in this region has been confirmed in HSV-1 17^+ (Dolan *et al.*,1992) and in HSV-2 strain HG52 (McGeoch *et al.*,1991).

The detection of the latency associated transcripts (LATs) in latently infected animal and human ganglia (Stevens *et al.*,1987; Krause *et al.*, 1988) has demonstrated that, during latency, viral transcription is limited to the part of the genome that expresses LAT. However these transcripts are not thought to be essential for the establishment or maintenance of latency but may control the temporal regulation of reactivation from latency (Steiner *et al.*, 1989).

The sequence of U_L, and the functions of some of the genes contained therein, has been elucidated (McGeoch *et al.*,1988a) and is outlined below.

The only immediate-early gene contained within the long unique segment is UL54 (or IE2) which codes for Vmw 63 (Watson *et al.*,1979). Many genes in U_L are known to have a role in the replication of viral DNA. There is a set of seven genes whose products are required to promote the amplification of a test plasmid encoding an HSV origin of replication (Challberg, 1986; Wu *et al.*,1988; McGeoch *et al.*,1988b). These include the gene which encodes the viral DNA polymerase (UL30), the major DNA binding protein (UL29), the subunit of DNA polymerase (UL42) (Gottlieb *et al* .,1990), and an origin binding protein (UL9); (Weir *et al.*,1989; Weir & Stow,1990), but little is known about the other three (UL5, UL8 and UL52), although there is evidence indicating that these three gene products form a primase/helicase complex (Crute *et al.*,1989). The UL5 amino acid sequence exhibits an ATP binding site consensus and is proposed to represent an ATP using enzyme such as helicase (McGeoch *et al.*,1988a).

Other genes encoded in the U_L segment of the genome are known to specify proteins involved in DNA synthesis or nucleotide metabolism. These include UL1 (uracil DNA glycosylase) UL12 [alkaline deoxyribonuclease (Weller *et al.*,1990)], UL23 (thymidine kinase), UL39 and UL40 (the subunits of ribonucleotide reductase) and UL50 [deoxyuridine triphosphatase (dUTPase)]. Gene UL26 is known to encode a virion structural protein which is involved in processing and packaging of progeny DNA (Preston *et al.*,1983). Lui & Roizman, (1991) demonstrated that UL26 encodes a protease which cleaves the UL26 gene product to produce UL26a, which functions as a scaffolding protein.

Genes encoding virion structural proteins also lie within U_L. These include UL19 (the major capsid protein), UL48 (the major tegument protein); (Campbell *et al*, 1984), three surface glycoproteins (UL22 [gH], UL27 [gB] and UL44 [gC]) and three virion proteins (UL26, UL34 and UL36). The UL28 gene product is important for the formation of mature capsids (Addison *et al.*,1990) and UL41 codes for the virion host shut off protein (Fenwick & Everett, 1990).

Mutation in some U_L genes can result in a syncitial plaque phenotype which may arise from changes in structural proteins. One of these corresponds to UL27 which specifies the virion glycoprotein gB (Bzik *et al.*, 1984), another to UL53 which is multiply hydrophobic (Debroy *et al.*, 1985) and one corresponds to UL1 (Little & Schaffer, 1981).

Several U_L genes are dispensable in tissue culture. These include UL2 (Mullaney *et al.*,1989), UL10 (MacLean *et al.*,1991), UL41 (Fenwick & Everett,1990), UL43 (MacLean *et al.*,1991), UL13 (Miss L. Coulter, personal communication), UL23 [thymidine kinase (Jamieson *et al.*,1974; Sanders *et al.*,1982), UL24 (unknown function), UL39 [large subunit of ribonucleotide reductase (Goldstein & Weller,1988)], UL44 (gC) (Frink *et*

al.,1983), UL50 (dUTPase) (Preston & Fischer,1984), UL55 and 56 [functions unknown (MacLean & Brown, 1987a,b].

Analysis of the short unique segment of the HSV-1 genome (McGeoch *et al.*, 1985) has shown that it contains 12 genes most of which are arranged as 3' co-terminal families, that is, the genes have common 3' ends but distinct 5' termini and promoters. US1 and 12 both encode immediate-early polypeptides (McGeoch *et al.*,1985; Marsden *et al.*, 1982), US4,6,7 and 8 encode glycoproteins gG, gD, gI and gE respectively. US5 is thought to encode a small glycoprotein (McGeoch *et al.*, 1985). US11 encodes a sequence specific RNA binding protein (Roller & Roizman, 1990), US3 a protein kinase, US9 a tegument phosphoprotein (Frame *et al.*,1986) and US10 encodes a virion protein (Rixon & McGeoch, 1984).

In the synthesis and processing of virus specific RNAs and eukaryotic RNA, host encoded RNA polymerase II is utilised and transcription occurs in the nuclei of infected cells (Wagner & Roizman, 1969; Ben-Zeeve & Becker, 1977: Constanzo *et al.*, 1977). Most HSV genes possess upstream and downstream regulatory regions similar to those of host cell genes (McKnight, 1980). These include 5' promoter sequences such as a TATAA box, a CAAT box motif and a 3' pre-mRNA polyadenylation signal AATAAA (Zarkower *et al* 1986). There is another motif, YGTGTTYY, where Y represents C or T, and which is found downstream from the polyadenylation signal (McLauchlan et al 1985) and has been shown to be required for the efficient processing of the 3' end of mRNA (McLauchlan *et al.*,1985). One aspect in which HSV differs from host is the degree of gene splicing. There are relatively few spliced genes: (IE1, UL15, IE4, IE5 and UL44) (Rixon & Clements, 1982; Watson *et al.*,1981b Perry *et al.*,1986; McGeoch *et al.*,1988a Frink *et*

al.,1983). RL1 (ICP 34.5) is predicted to be spliced in HSV-2 but not in HSV-1 (McGeoch *et al.*, 1991; Dolan *et al.*, 1992).

1.5 THE HERPES SIMPLEX VIRUS 'A' SEQUENCE.

HSV-1 DNA possesses a direct terminal repeat of about 250-500 base pairs termed the 'a' sequence. The 'a' sequence is also located in inverted orientation at the joint between L and S segments. In some molecules of an HSV DNA preparation, the L terminus and the joint may possess multiple copies of the 'a' sequence.

The HSV genome can be represented as:

$$a_l\ a_n\ b\text{-}U_L\text{-}b'a'_m c'\text{-}U_S\text{-}c\ a_s$$

where a_l and a_s are terminal sequences with unique properties, and a_n and a_m are terminal 'a' sequences directly repeated 0-9 times (n) or present in 1-10 (m) copies (Davison & Wilkie,1981; Roizman, 1979 a, b; Wadsworth *et al.*,1975; Wagner & Summers, 1978).

1.5.a. Structure of the 'a' sequence.

The structure of the 'a' sequence is highly conserved and consists of a variable number of repeat elements. In HSV-1 strain F, the sequence is made up of a 20 base pair direct repeat (DR1), a 64 base pair unique sequence (Ub), a 12 base pair direct repeat (DR2) present in 19-22 copies per 'a' sequence, a 37 base pair direct repeat (DR4) present in 2-3 copies, a 58 base pair unique sequence (Uc), and a final copy of DR1 (Mocarski & Roizman, 1981, 1982). The size of the 'a' sequence varies between and within strains reflecting differing numbers of copies of DR2 and DR4, The structure of the 'a' sequence can be represented as follows and in figure 1.4:

$$DR1\text{-}U_b\text{-}DR2_n\text{-}DR4_m\text{-}U_c\text{-}DR1$$

Figure 1.4 Structure of the HSV-1 'a' sequence.

a. A HSV-1 genome in the prototype orientation. **b**. An expansion of the IR_L/IR_S junction showing the structure of the 'a' sequence. The 'a' sequence consists of unique and directly repeated elements (the information given concerns the HSV-1 strain F 'a' sequence [Mocarski & Roizman, 1981, 1982])

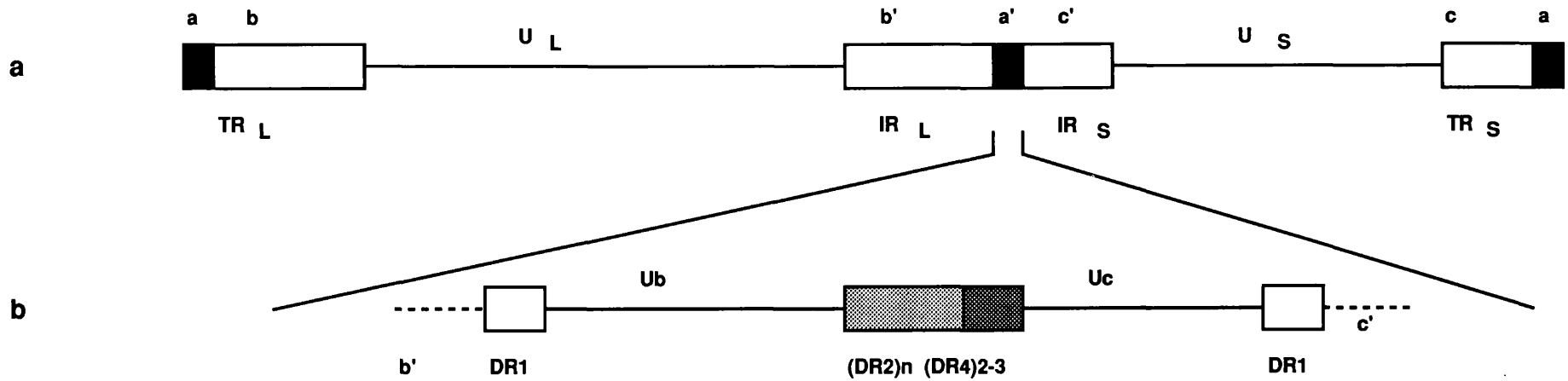
Ub- a unique region located towards the b' region of the genome.

Uc- a unique region located towards the c' region of the genome.

DR1- A 20 base pair element present as a direct repeat at the edge of the 'a' sequence.

DR2- a directly repeated 12 base pair element present in 19-22 copies.

DR4- a directly repeated 37 base pair sequence present in 2-3 copies.


a
a
b
U L
b'
a'
c'
U S
c
a
TR L
IR L
IR S
TR S
b
Ub
Uc
b'
DR1
(DR2)n (DR4)2-3
DR1
c'

with adjacent 'a' sequences sharing the intervening DR1. Linear virion DNA contains asymmetric ends with the terminal 'a' sequence of the L component (aL) ending with 18 base pairs and one 3' nucleotide extension, and the terminal 'a' sequence of the S component (aS) ending with a DR1 containing only 1 base pair and one 3' overhang (Mocarski & Roizman,1982). Thus genome inversion leads to formation of 1 DR1 between aL and aS. Variability of the copy number of DR elements accounts for most of the observed 'a' sequence polymorphism in different strains. For example, HSV-2 strain HG52 has only one copy of DR2 (Davison & Wilkie,1981) while HSV-1 strain F has 19-22 copies (Mocarski & Roizman,1982). Similarly, HSV-1 strain 17^+ and HSV-2 strain HG52 contain one copy of DR4 homology, which is thus a part of Uc (Davison & Wilkie,1981). Although there is variation in the copy number of the DR2 and DR4 elements, some regions of the 'a' sequence are highly conserved in different strains. These are within Ub and Uc and are represented by a short, well conserved sequence about 20bp in length located approximately 40bp and 35bp respectively from the end of the 'a' sequence (Davison & Wilkie,1981; Deiss & Frenkel,1986).

1.5.b. The 'a' sequence mediates circularization.

Following infection, the HSV genome rapidly circularizes: this is believed to be mediated by the 'a' sequence (Davison & Wilkie, 1983a; Poffenberger *et al.*, 1983; Poffenberger & Roizman, 1985). It is likely that circularization takes place by ligation of the two termini aided by the complementary single base at the 3' end overhang (Mocarski & Roizman, 1982)

1.5.c. 'a' sequence promoter activity.

Chou & Roizman (1986) have mapped the promoter of the gene coding for the neurovirulence factor ICP 34.5 (Chou *et al.*, 1990) to the Ub segment of the 'a' sequence. The 5' end of the transcript and the coding region is in the long repeat region of the genome in the same orientation as IE1 (Ackerman *et al.*,1986). Resequencing and confirmation of the presence of ICP 34.5 in HSV-1 strain F but not in HSV-1 strain 17^+ (Chou & Roizman, 1990) led to a more extensive analysis of this region of HSV-1 strain 17^+ and to the conclusion that HSV-1 17^+ does indeed encode a neurovirulence conferring gene in this region (Dolan *et al.*, 1992).

1.5.d. Isomerization of the HSV genome.

HSV-1 DNA contains equimolar amounts of four isomers (section 1.4a). The existence of four isomers was demonstrated by restriction enzyme analysis (Hayward *et al.*,1975; Clements*et al.*,1976). Studies of intertypic recombinants between HSV-1 and -2 demonstrated that inversion of the L and S segments was specifically dependent upon the 'a' sequence (Davison & Wilkie 1983b). Detailed analysis of the 'a' sequence (Chou & Roizman, 1985) has shown that deletion within the DR2 element resulted in a low frequency of inversion (Varamuza & Smiley, 1985), deletion of DR4 sequences resulted in completely abolishing inversion indicating that the presence of cis-acting signals for recombination and inversion in the DR2 and DR4 sequences. Harland & Brown (1989) reported an approximately 13.5kb deletion in HSV-2 strain HG52 across the L-S junction with loss of the 'a' sequence and complete loss of IR_L and half of the IR_S region, resulting in a fixed prototype orientation of the L segment. However, both IR_S and TR_S were present albeit in unequal proportions indicating that the 'a' sequence is not absolutely necessary

for the isomerization of HSV. Longnecker & Roizman (1986) reported similar findings.

(Section 1.6.d describes the cleavage packaging properties of the 'a' sequence and its protein binding properties).

1.6. VIRAL REPLICATION

Attachment of the virus to cell receptors is rapidly followed by fusion of the envelope with the plasma membrane. The de-enveloped capsid is then transported to the nuclear pores where the DNA is released into the nucleus. Transcription, replication of viral DNA and assembly of new capsids takes place in the nucleus. Viral DNA is synthesized by a rolling circle mechanism to yield concatemers that are cleaved into monomers and packaged into capsids. The virus then matures and acquires infectivity by budding through the inner lamellae of the nuclear membrane. In fully permissive tissue culture cells, the process takes approximately 18-20 hours.

1.6.a. Attachment and penetration of HSV to the cell.

Studies by WuDunn & Spear, (1989) indicate that the receptor molecules recognized in one of the initial binding events are heparan sulphate proteoglycans and that either gB or gC is required for this step. Neomycin and polylysine appear to block a similar step in attachment (Langeland *et al.*,1987, 1988) and mapping data suggests that this step appears to involve gC. It has also been demonstrated that gH may be required for an early step in viral replication (Buckmaster *et al.*,1984; Desai *et al.*, 1988)

Penetration is also mediated by the HSV surface glycoproteins. An HSV *ts* mutant expressing an altered gB attaches to but does not penetrate

into cells (Manservigi *et al.*,1977) and gB$^-$ viruses attach but do not penetrate (Cai *et al.*,1988). In the same manner, HSV-1 gD$^-$ viruses attach but do not penetrate (Johnson & Ligas, 1988). Cells expressing HSV gD allow attachment and penetration of both HSV-1 and HSV-2, however fusion of viral and cellular membranes and penetration do not ensue (Campadelli-Fiume *et al.*, 1988).

Hence, gB and gC recognize as well as attach to, cell receptors, gB and gD play an indispensable role in the fusion of the envelope with the plasma membrane, and gD sequesters the cell membrane proteins required for fusion of the viral and cellular membranes. Virions attaching to the plasma membrane which cannot fuse are internalized and degraded in endocytotic vesicles.

Upon entry into the cell, the capsids are transported to the nuclear pores (Batterson *et al.*,1983; Tognon *et al.*,1981) where viral DNA is released into the nucleoplasm, a step which requires a viral function (Batterson *et al.*,1983). Apart from DNA, other virion components are required for replication including a protein responsible for the shut off of host macromolecular synthesis (Fenwick & Everett,1990) and a tegument protein involved in the induction of immediate-early gene expression (Campbell *et al.*,1984).

1.6.b. Temporal control of gene expression.

Transcription of viral genes takes place in the nucleus and mRNA is translated in the cytoplasm. The expression of HSV genes is tightly controlled in that proteins form several groups whose expression is ordered in a sequential fashion (Honess & Roizman, 1973, 1974, 1975). (An extensive discussion of the regulation of HSV genes is given in section 1.7)

1.6.c. Synthesis of viral DNA.

The nature of the replicative forms of HSV DNA is obscure. It is known that newly replicated DNA does not posses detectable termini and is probably circular or in head to tail monomers (Jacob *et al.*,1979; Jongeneel & Bachenheimer, 1981). Late in infection replicated DNA is in a very rapidly sedimentable form, thought to be extensive concatemers (Jacob *et al* .,1979). It is therefore likely that replication is by a rolling circle mechanism. Concatemeric DNA is further processed in the cell nucleus by packaging into nascent nucleocapsids, and by cleavage into unit genome lengths (Vlazny *et al.*, 1982)

1.6.c.I. Origins of DNA replication.

HSV DNA has three origins of replication, one in U_L situated between divergently transcribed genes encoding the major DNA binding protein and the DNA polymerase. This origin is termed *ori*$_L$. The other origin is situated between the promoter of IE4/5 and that of IE3 in R_S and is therefore diploid. This origin is called *ori*$_S$.

***ori*$_S$.**

ori$_S$ activity has been localized to a region of 90 base pairs within the R_S sequence of HSV-1 (Stow & McMonagle,1983). The *ori*$_S$ sequence contains an imperfect palindrome, with each arm consisting of 21 residues. Although essential sequences are known to lie outside the palindrome (Stow & McMonagle,1983) there is $(A\text{-}T)_6$ at its centre essential for *ori*$_S$ function (Stow, 1985).

ori$_S$ has homologues in HSV-2 strain HG52 (Whitton & Clements,1984) and VZV (Stow & Davison,1986) both of whose sequences are known and are similar to that of HSV-1. There is a stretch of 11 base pairs identical

in HSV-1 and VZV which in HSV-1 lies across the boundary of the palindrome and in VZV lies just outside it. These 11 base pairs are included in an 18 base pair region which has been shown to bind a protein (Elias *et al.*,1986). This region has been further characterized by Weir & Stow, (1990) who demonstrated that the interaction of the UL9 gene product with *ori*$_S$ (Elias & Lehman,1988; Koff & Tegtmeyer,1988; Olivio *et al.*,1988; Weir *et al.*,1989) was at part of the 11 base pair conserved element and that the binding of the UL9 gene product to another related site was also required for efficient regulation.

***ori*$_L$**

DNA containing functional *ori*$_L$ could not be cloned intact into a standard bacterial vector system. Any recombinant clones obtained contained deletions of >100 base pairs (Spaete & Frenkel, 1982) which rendered the origin inactive.

Sequencing of *ori*$_L$ was eventually achieved by using non-cloned viral DNA fragments as sequencing substrates (Weller *et al.*,1985; Quinn & McGeoch,1985). Molecular cloning of the region in an undeleted form was reported using a yeast plasmid (Weller *et al.*,1985). It was found that the deleting region contained a long perfect palindrome with arms each of 72 residues which shows striking similarity to that of *ori*$_S$. As with *ori*$_S$, the *ori*$_L$ region contains an A-T rich region at the centre of the palindrome. Alignment of the two *ori* sequences demonstrates that the whole of *ori*$_S$ shows homology to *ori*$_L$ (McGeoch, 1987). The sequence similarity extends beyond the *ori*$_S$ palindrome on one side only which is within the mapped functional limits of *ori*$_S$ (Stow & McMonagle,1983) and contains part of the *ori*$_S$ binding site (Elias *et al.*,1986).

Lockshon & Galloway,(1986) found that the HSV-2 *ori*$_L$ equivalent was an almost perfect palindrome of total length 136 base pairs with high sequence homology to the HSV-1 palindrome. VZV does not appear to

contain any homology to ori_L in the equivalent genome location (Stow & Davison, 1986).

ori_S and ori_L structures and flanking sequences have several elements in common: **1.** a palindrome sequence. **2.** a repeating AT area **3.** a region conserved between HSV and VZV known to be protein binding* (in ori_S alone) and **4.** the origins are located close to transcriptional regulatory signals of divergently transcribed genes. In the case of ori_S these are IE4/5 and IE3, IE3 encodes an essential transcriptional regulator. ori_L is located between two genes whose products are components of the viral DNA replicating machinery.

A variant lacking ori_L has been isolated which grows normally *in vitro* and which establishes latent infection *in vivo* (Polvino-Bodnar *et al.*,1987). Viable variants of HSV types 1 and 2 lacking a single copy of ori_S have been isolated (Longnecker & Roizman,1986; Brown & Harland,1987). Failure to isolate viable HSV deletion variants lacking both copies of ori_S may not indicate that both are essential since there is evidence of a transcript spanning ori_S (Hubenthal-Voss *et al*, 1987). If both copies of ori_S were deleted then both copies of the proposed gene would also be disrupted making it difficult to dissociate the two effects. Recent evidence links these transcripts to replication of viral DNA (Wong & Schaffer,1991).

1.6.c.II. HSV-1 gene products required for DNA synthesis.

By transfecting cells with plasmids containing an HSV origin of DNA synthesis and various fragments of the HSV-1 genome, the identification of 7 genes encoding products required for viral origin dependent DNA synthesis has been achieved (Wu *et al.*,1988; McGeoch *et al.*,1988b). These are UL5, UL8, UL9, UL29, UL30, UL42 and UL52. UL30 encodes

the viral DNA polymerase (Chartrand et al., 1980; Quinn & McGeoch, 1985), a single stranded DNA binding protein is specified by UL29 (Conley *et al.*, 1981; Quinn & McGeoch, 1985) a protein binding to the origins of viral DNA synthesis is encoded by UL9 (Elias *et al.*,1986), a double stranded DNA binding protein encoded by UL42 and three additional proteins encoded by UL5, UL8 and UL52 are thought to be subunits of a primase-helicase complex (Crute *et al.*,1989).

Other HSV encoded enzymes also play a role in DNA synthesis. Two enzymes which catalyse reactions in the biosynthesis of DNA precursors are thymidine kinase and ribonucleotide reductase.

Thymidine kinase (TK) is not essential for virus growth in dividing tissue culture cells, but is required in resting cells (Jamieson *et al.*,1974). The pathogenicity of TK^- mutants in experimental animals is reduced (Field & Wildy, 1978). The enzyme functions to phosphorylate purine pentosides and a wide variety of nucleoside analogues that are not phosphorylated efficiently by cellular kinases.

Ribonucleotide reductase (RR) catalyses the reduction of nucleoside diphosphates to deoxynucleoside diphosphates (Cohen,1972; Dutia,1983), and is composed of two substrates which are encoded by separate but contiguous genes. (Preston *et al.*,1984; Bacchetti *et al.*,1986; Frame *et al.*,1985; McLauchlan & Clements, 1983à). The enzyme has been shown to be non-essential under *in vitro* conditions for viral DNA replication by the construction of a viable variant deleted within the ribonucleotide reductase coding region (Goldstein & Weller,1988). Preston *et al.*,(1988) have also described a mutant, *ts*1222, which has a single base pair deletion at the 3' end of the small subunit of RR. This mutant does not induce detectable amounts of RR activity at either 31°C or 39.5°C. In dividing cells at 31°C there is no growth defect, but 39.5°C was non permissive for growth of the mutant. This suggests that at 31°C growth

of the mutant is supported by cellular RR whereas virally encoded RR is necessary for viral replication at the higher temperature. RR has been demonstrated to be a determinant of pathogenicity in mice since mutants with lesions in either the large or small subunit were reduced in virulence by about 10^6-fold when compared to the parental virus (Cameron *et al.*, 1988).

1.6.d. Cleavage and packaging of HSV DNA.

Newly synthesized viral DNA is processed and packaged into empty capsids, a process involving amplification of the 'a' sequence and cleavage of 'endless' DNA (ie. in the circular or concatemeric form). Stow *et al.*,(1983) demonstrated that signals required for encapsidation of HSV-1 DNA are located within the 'a' sequence and it has since been proposed that cleavage of HSV-1 DNA after replication is coupled to encapsidation (Deiss & Frenkel, 1986). The net results of these processes are that the free S component terminus consists of one 'a' sequence with a terminal DR1 element containing only a single base pair and one 3' nucleotide extension (Mocarski & Roizman, 1982). The free L component terminus consists of one to several directly repeated 'a' sequences and ending in a DR1 element containing 18 base pairs and one 3' nucleotide extension. The 3' extensions are thought to mediate the circularization of the genome after infection (Mocarski & Roizman,1982). Two separate signals designated Pac1 and Pac2, located in the Ub and Uc regions respectively of the 'a' sequence, were essential for cleavage/packaging of viral genomes (Varmuza & Smiley,1985; Deiss *et al.*,1986). Nasseri & Mocarski, (1988) have shown that a 179 base pair fragment (containing Uc-DR1-Ub) from the junction of two tandem 'a' sequences carries all the elements necessary for cleavage recognition and encapsidation.

The analysis of the processes of cleavage and packaging was achieved by Deiss *et al.*, (1986) using a series of amplicons. A summary of the cleavage packaging model resulting from this analysis begins with a cleavage packaging protein attaching to the Uc element of the 'a' sequence (see section 1.5 for a description of the 'a' sequence structure). A potential structure on the surface of the capsid complexes with a Uc protein sequence, loops the viral DNA and scans from the bound 'a' sequence across the L component toward the end of the S component until it detects the first Uc-DR1-Ub domain of an 'a' sequence in an identical orientation. The DR1 sequence of one 'a' is then cleaved and the gap is repaired. Cleavage of the DR1 element shared by the two 'a' sequences then ensues.

Several proteins have been shown to bind the 'a' sequence. These include a small polypeptide attaching to the L-S junction of virus DNA (Wu *et al.*,1979). Late polypeptides encoded by the gene US11 (Rixon & McGeoch,1984; Johnston *et al.*,1986) have been shown to interact with the HSV-1 'a' sequence *in vitro* (Dalziel & Marsden, 1984) and are strong DNA binding proteins (MacLean *et al.*,1987). The function of the US11 gene products remain unclear since deletion mutants viable in tissue culture have been isolated (Umene,1986; Brown & Harland,1987). Two further proteins (>250KDa and 140KDa) which form complexes with *pac1* and the DR1 region of the 'a' sequence have been reported (Chou & Roizman,1989).

The model described by Deiss *et al.*,(1986) and above demonstrates that the 'a' sequences at the joint between the L and S component were not involved in cleavage and packaging is supported by the isolation of an HSV-2 variant lacking the internal 'a' sequence which was packaged normally (Brown & Harland,1987).

1.6.e. Envelopment and egress.

A notable feature of infected cells late in infection is the appearance of reduplicated membranes and thick concave or convex patches, particularly in nuclear membranes. Nuclear envelopment takes place at these patches. Because the enveloped virions do not contain detectable amounts of host membrane proteins, it is likely that these patches represent aggregations of viral membrane proteins presumably including viral glycoproteins on the outside surface and tegument proteins on the inside surface.

Nuclear DNA-containing capsids attach to these patches of the nuclear membrane and become enveloped in the process. It has been observed that only DNA containing capsids are enveloped (Roizman & Furlong,1974) and that the envelopment of empty capsids rarely occurs. Vlazny *et al.*,(1982) demonstrated that capsids containing fragments of HSV DNA less than standard genome length are retained in the nucleus leading to the suggestion that a capsid protein is modified after encapsidation of DNA and only modified capsids are able to bind to the patches of nuclear membrane containing viral proteins. The production of light particles indicates that capsids are not required for budding of envelopes (Szilagi & Cunninghan,1991; Rixon *et al.*,1992) and the possibility of cytoplasmic sites for acquisition of tegument and envelope has been indicated (Nii *et al.*,1968). Roffman *et al.*,(1990) detected structures called tegusomes in which HHV-6 virions acquire their tegument indicating a non-nuclear location for this process.

1.7. Regulation of viral gene expression.

The replication of HSV-1 is coordinated by temporal control of gene expression. The classification of three groups of genes, immediate-early

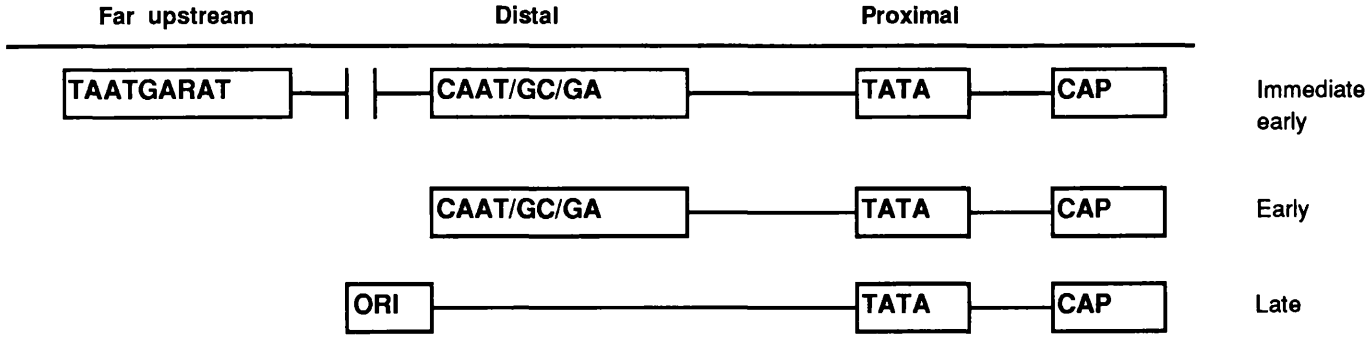
(IE), early (E) and late (L) (Clements *et al.*,1977) or alpha, beta or gamma (Honess & Roizman,1974) is based on their kinetics and expression in the presence and absence of metabolic inhibitors of translation or DNA replication (Kozak & Roizman,1974). The IE genes are the first to be transcribed and their expression does not require *de novo* protein synthesis, whereas early and late gene expression is dependent on prior synthesis of IE polypeptides (Honess & Roizman, 1974; Clements *et al,* 1977). IE genes are first expressed directly after release of DNA into the nucleus and their expression is stimulated by a component of the tegument (Post *et al.*,1981; Batterson & Roizman, 1983).

1.7.a. Regulatory elements of HSV-1 genes.

The transcription of HSV-1 genes begins with the five immediate-early genes which are expressed in the absence of prior viral protein synthesis. The immediate-early proteins in turn, activate early genes which encode mostly the viral DNA replicative machinery. The leaky late genes are induced soon after the early genes. They are first expressed prior to the onset of viral DNA replication, and expression increases at later times in a replication dependent fashion. Finally, the true late genes are activated only after DNA replication has begun. The replication step appears to induce a *cis*-acting modification of the template rendering it permissive for late gene expression (Mavromara-Nazos & Roizman, 1987). Each HSV gene bears its own promoter-regulatory region summarized by figure 1.5 and promoter transplant and nuclear run-on experiments indicate that temporal regulation occurs largely at the transcriptional level (Godowski & Knipe, 1986; Post *et al.*, 1981; Silver & Roizman, 1985).

Figure 1.5 Regulatory elements of HSV-1 genes.

A summary of the HSV-1 regulatory elements showing far-upstream, distal and proximal promoters of immediate-early, early and late genes. (Adapted from Everett,1986)


Far upstream
Distal
Proximal
TAATGARAT
CAAT/GC/GA
TATA
CAP
Immediate early
CAAT/GC/GA
TATA
CAP
Early
ORI
TATA
CAP
Late

Coordinate activation of HSV immediate-early gene expression is mediated through a class specific consensus sequence TAATGARAT that is found in one or more copies in each of the immediate-early 5' control regions (Cordingly *et al.*, 1983: Gaffney *et al.*, 1985; MacKem & Roizman, 1982a,b; Preston *et al.*, 1984; Triezenberg *et al.*, 1988). IE transcription is mediated by an abundant tegument component of the virion Vmw65 (also called VP16, aTIF and ICP25), that is delivered into cells upon infection (Batterson & Roizman, 1983; Campbell *et al.*, 1984; Post *et al.*, 1981). Vmw65 forms a complex with cellular factors, including OCT-1, and the resulting activator complex binds to the TAATGARAT sequence in part through the DNA binding domain of OCT-1 (McKnight *et al.*, 1987; Preston *et al.*, 1988). Four out of five immediate-early proteins (IE1, IE2, IE3, and IE4) contribute to the activation of the viral early and late genes. Extensive analysis of the IE3 promoter region (MacKem & Roizman, 1982b) has demonstrated that it possesses a TATA box at position -20 from the cap site, several SP1 binding sites scattered throughout the promoter region and two Vmw65 binding sites. The IE3 promoter also contains a site to which Vmw175, the IE3 gene product, binds to regulate its own expression. Each immediate-early promoter region in general has one or more Vmw65 binding sites, GC rich areas and a TATA box however the number of each varies according to the gene concerned. Ace *et al.*,(1989) demonstrated that each immediate-early gene has a different requirement for stimulation by Vmw65 since infection with the variant *in*1814 which contains a small insertion in the gene encoding Vmw65 rendering the tranactivating function of the protein inactive, resulted in the differential accumulation of immediate-early mRNAs. IE1 and 2 mRNAs accumulate to a lesser extent than that of IE4 and the accumulation of IE3 mRNA is unaffected. Transfection of a plasmid encoding Vmw110 before infection with *in*1814 results in the

accumulation of immediate-early mRNA to a similar quantity to wild-type indicating that the function of Vmw110 can substitute for that of Vmw65.

Although the classical definition of immediate-early genes is that they can be transactivated by Vmw65 in absence of prior protein synthesis, Elshiekh *et al.*,(1991) have demonstrated that only Vmw110 is maximally expressed in the absence of infected cell protein synthesis. Vmw68/12 and Vmw63 are maximally expressed after 1/2 hour of protein synthesis and Vmw175 after 1 hour.

The mechanisms of activation of early and late promoters remains unclear. Early and late promoters do not contain obviously conserved class-specific sequences analogous to TAATGARAT, and extensive mutational analyses have not uncovered *cis*-acting sequences solely and specifically required for activation by immediate-early polypeptides. The early and late promoter elements that are required for activation by immediate-early proteins are the same as those needed for basal level activity in uninfected cells (Everett,1987; McKnight & Tjian,1986). These data have been interpreted to suggest that immediate-early proteins induce early and late gene expression by altering the activity of one or more components of the transcription apparatus (Everett,1987; McKnight & Tjian,1986). Consistent with this hypothesis, the HSV immediate-early proteins display relatively relaxed target specificity and are able to activate a variety of heterologous genes that have been newly introduced into cells by transfection or as part of an infecting viral genome (Everett,1984 a,b,1985). Some evidence suggests that the TATA box factor TFIID serves as a critical target of the immediate-early polypeptides since changes in TATA box sequence can markedly affect the response of the simian virus 40 early promoter to immediate-early polypeptides (Everett,1988). Additional evidence for a functional

interaction between immediate-early proteins and TFIID comes from studies showing that the pseudorabies virus immediate-early protein (a homologue of HSV-1 Vmw175) activates transcription *in vitro* by increasing the rate of binding of TFIID to the TATA sequence (Abmayr *et al.*,1988).

HSV late promoters appear to have a simpler structure than early promoters, and some evidence suggests that this difference plays an important regulatory role during transfection (Homa *et al.*,1986,1988; Johnson & Everett, 1986). The early promoters so far examined consist of a near upstream region composed of binding sites for cellular transcription factors linked to a TATA box/cap site (Everett,1984a; McKnight & Tijan,1986). In contrast, the promoters of late genes US11, glycoprotein C and UL38 lack elements upstream of the TATA box (Flanagan *et al.*,1991; Homa *et al* .,1986,1988; Johnson & Everett,1986). Several observations suggest that true late promoters contain specialised *cis*-acting sequences that actively contribute to true late control. Imbalzano *et al.*,(1991) found that a mutant TK gene lacking all known promoter elements upstream of the TATA box remained under early control, implying that upstream elements are not always required for expression. Homa *et al.*,(1988) found that the TK TATA sequence was unable to function when translated to the gC locus, leading to the hypothesis that true late promoter activity requires a specific type of TATA box sequence. Mavromara-Nazos & Roizman, (1987) observed that sequences downstream of the TATA box of the true late g42 gene can confer some aspects of late regulation on an early promoter. Lastly, Kibler *et al.*,(1991) demonstrate that sequences downstream of the US11 TATA box play an active role in specifying true late temporal regulation.

1.7.b The immediate-early polypeptides.

There are five immediate-early genes in HSV-1 called IE1, IE2, IE3, IE4 and IE5, encoding Vmw110, Vmw63, Vmw175, Vmw68 and Vmw12 respectively. The location of each immediate-early gene in the HSV-1 genome is shown in figure 1.6.

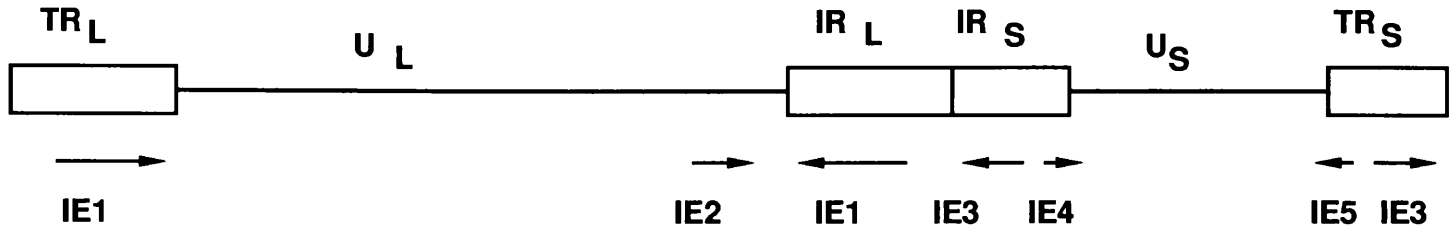
Vmw63

Central to this project is the immediate-early polypeptide Vmw63 since the subject of this thesis, the variant 1703, apparently fails to synthesize it under immediate-early conditions of infection (MacLean & Brown,1987a) despite it being classed as an essential regulatory protein (Sacks *et al.*,1985; McCarthy *et al.*,1989). Vmw63 is encoded by the gene IE2, which is also called UL54, reflecting its position near the right hand end of the prototype orientation of the HSV-1 genome. IE2 is the only immediate-early gene mapping entirely within a unique region of the genome (McGeoch *et al.*,1988a). It contains no splice sites and its product has a size of 63 KDa by SDS-PAGE. The IE2 promoter region has one TAATGARAT element situated 158 base pairs upstream of the IE2 cap site, several GC rich areas and a TATA box (Mackem & Roizman,1982a). The gene exhibits a strong requirement for stimulation by Vmw65 (Ace *et al.*,1989).

Phenotypic analysis of set of HSV-1 mutants with temperature sensitive (*ts*) lesions in IE2 (Sacks *et al.*,1985) demonstrated that, at the non-permissive temperature, the variants were unable to activate a subset of early and late genes and show enhanced expression of most immediate-early polypeptides. Transient expression assays have shown that Vmw63 expressed in transfected cells can alter the expression of

Figure 1.6 HSV-1 immediate-early genes.

A HSV-1 genome in the prototype orientation showing the locations of immediate-early genes. The genes are shown without introns, IE1 has two introns and IE4 and IE5 have one each.


TR L
U L
IR L
IR S
U S
TR S
IE1
IE2
IE1
IE3
IE4
IE5
IE3

many co-transfected HSV-1 target genes. Several studies have shown that Vmw63 can induce expression of chimeric target genes driven by specific early or late promoters (Everett,1986; Rice & Knipe,1988; Sekulovich *et al.*,1988; Su & Knipe.,1989). For some of these promoters, the effect requires the expression of the HSV-1 immediate-early proteins Vmw175, Vmw110 or both. However expression of Vmw63 alone is able to stimulate expression of the glycoprotein B promoter in transfected Vero cells (Rice & Knipe, 1988) indicating that at least part of the positive regulatory effect of Vmw63 is independent of other viral proteins. Vmw63 is also able to inhibit gene expression from another set of chimeric genes driven by early or late promoters (Sekulovich *et al.*,1988; Su & Knipe, 1989). The negative regulatory effect of Vmw63 is complex in that Vmw63 has little or no effect on the basal promoter activity of these genes but rather counteracts the stimulatory effects of the HSV-1 transactivating polypeptides Vmw110 and Vmw175.

Characterization of an HSV-1 IE2 null mutant (McCarthy *et al.*,1989) has shown that Vmw63 was required to repress early gene expression, to induce wild-type levels of delayed-early gene expression and to induce true late gene expression. The phenotypic characterization of IE2 deletion mutants lacking various parts of the IE2 coding region demonstrated that the deletion of different parts of the gene affect the regulatory functions of the product in various ways.

Hardwicke *et al.*, (1989) constructed a series of in frame insertion and deletion mutants which were used to map the repressor and activator functions of Vmw63. The repressor activity maps to the carboxy-terminal 78 amino acid residues, whereas the activator function maps to the carboxy-terminal half of the molecule encompassing a region of about 250 amino acids. Extensive analysis of these variants demonstrated that mutations in the activator domain can be *trans*

dominant, that is, when the mutant plasmids are co-transfected with a wild-type plasmid and one specifying Vmw175 or Vmw110, the activation of a reporter gene is affected (Smith *et al.*,1991). However mutants in the repressor domain can not interfere with either repressor or activator functions of the wild-type protein indicating that the repressor region is required for the binding of Vmw63 to its substrate. There is a putative zinc finger binding domain in this region (Hardwicke *et al.*, 1989) supporting the hypothesis that the region is DNA binding.

McMahon & Schaffer,(1990) pursued a similar strategy and demonstrated that there were two repressor domains within Vmw63, both lying in the activator domain, one of which was not absolutely required for the repressing activity of the protein. They also show that the enhancer function of Vmw63 involves a large portion of the carboxyterminal half of the molecule. The conclusion of McMahon's & Schaffer's work is that Vmw63 performs it regulatory activities over time and that these effects are mediated indirectly via interactions with and modifications of Vmw175 and perhaps other viral and cellular proteins.

It is interesting to note that there is extensive amino acid sequence conservation between this region of Vmw63 and the carboxyterminal halves of the related VZV gene 4 product, the EBV BMLF1 and the immediate-early 52K gene product of HVS (Davison & Taylor,1987; Gompels *et al.*,1988). Vmw63 has also been shown to be essential for the accumulation of the cellular protein p40 during lytic virus infection (Estridge *et al.*,1989). This finding is significant since p40 is overexpressed in cells transformed with HSV or other agents and in human cervical cancers. Other HSV regulatory gene products such as Vmw175 have also been shown to up-regulate the expression of particular cellular genes during infection.

Vmw110.

Vmw110 is an immediate-early polypeptide which has a size of 110KDa by SDS-PAGE and is encoded by two copies of the gene IE1 which is contained within R_L. IE1 possesses two spice sites which appear to be dispensable for virus growth in tissue culture (Everett,1991). Vmw110 is not an essential immediate-early polypeptide. A deletion variant which lacks 700bp of each IE1 gene is viable in tissue culture (Sacks & Schaffer, 1987). Stow & Stow, 1986 have shown that a HSV-1 variant which does not express Vmw110 because of a 2kb deletion in both copies of IE1 was able to replicate in BHK21 C13 cells and the results suggest that the effect of the deletion is manifest at low multiplicities of infection but can be overcome by increasing the infecting virus dose since at high multiplicities of infection the variant expressed similar amounts of viral proteins and replicated DNA as wild-type.

Vmw110 is able to stimulate expression from delayed early promoters and, in conjunction with Vmw175 this stimulation is increased (Everett, 1986). Mutations in different domains of Vmw110 can have different effects on function in the presence and absence of Vmw175 (Everett, 1988b), indeed the production of Vmw110 itself has been shown to be regulated by Vmw175 (Resnick *et al.*,1989).

Vmw175.

Vmw175 is an essential immediate-early polypeptide whose size on SDS-PAGE is 175KDa, is unspliced and encoded by IE3 which is completely contained within R_S and is therefore diploid (Rixon *et al.*,1982). Most *ts* mutants with lesions in IE3 only express immediate-

early polypeptides at the non-permissive temperature (Watson & Clements, 1978). Even when early and late transcription has commenced at the permissive temperature, shift up to the non permissive temperature results in the reversion to the immediate-early transcriptional phase (Preston,1979a,b).

The synergistic roles of Vmw175, Vmw110 and Vmw63.

Through using the bacterial chloramphenicol actyl transferase (CAT) assay, it has been shown that Vmw110 and Vmw175 together transactivate early genes to a much greater extent than either gene alone. However, this combination did not significantly transactivate a late promoter, but when a plasmid expressing Vmw63 was included in the transfection, transactivation of the late promoter was increased about 6-fold (Everett, 1986). This work indicates that together these polypeptides function to transactivate early and late genes. Although stimulation of immediate-early genes is achieved by the combination of Vmw65 and cellular transcription factors, HSV-1 DNA is itself infectious. Cai & Schaffer (1989) have shown that Vmw110 plays a critical role in the *de novo* synthesis of infectious virus following transfection of virus DNA.

There are two other immediate-early polypeptides encoded by HSV-1 whose function have not yet been fully elucidated.

Vmw68.

Vmw68 is coded by the gene IE4 and has a size of 68KDa on SDS-PAGE. The RNAs of IE4 and IE5 map across the junction between U_S and R_S and have common 5' portions located in IR_S and TR_S and the 3' portions extend into the opposite ends of U_S and are unique. The DNA sequence

encoding the common 5' end comprises a leader region and a single intron of variable size due to the variable copy number of a 22bp reiteration (Rixon & Clements, 1982).

The construction of a deletion mutant which removes the carboxy-terminus third of IE4 has shown that the gene appeared to be dispensable since the variant grew normally in tissue culture (Post & Roizman, 1981). Later analysis using several different cell lines indicated that in some circumstances the virus grew very poorly and the activity of at least one true late promoter was greatly reduced suggesting a host range function (Sears *et al.*,1985). The variant was not neurovirulent but could establish latency in mice. Since a truncated form of Vmw68 was still produced, these studies do not show a clear role for Vmw68.

Jaquemont *et al.* (1984) compared the production of HSV-1 immediate-early polypeptides in the presence of cycloheximide in HEp-2 cells which host a lytic HSV infection to that of XC cells which do not. There was an accumulation of all five immediate-early polypeptides in infected HEp-2 cells but infected XC cells Vmw68 could not be detected. Analysis of RNA from both cell types indicated that all five immediate-early RNAs were present. The conclusion from this work was that the XC cell environment was not conducive to the translation of IE4 mRNA. This suggests an essential role for Vmw68 in XC cells.

Vmw12.

Vmw12 is encoded by the gene IE5 and has a size of 12KDa on SDS-PAGE. The position of IE5 on the HSV-1 genome has already been described. Vmw12 is not essential and viable deletion mutants have

been isolated that lack the whole IE5 gene which indicates that it does not play an essential role in tissue culture (Brown & Harland, 1987).

1.7.c. Early gene expression.

After the immediate-early genes, the next set of genes to be expressed are the early genes. They appear after functional immediate-early gene products reach their peak, about 4-6 hours after infection, after which they decline (Honess & Roizman, 1974). Some early polypeptides, for example gD, require DNA synthesis for maximal expression (Johnson & Spear ,1984). Transfection assays have shown that individually Vmw110 and Vmw175 transactivate early gene expression and that a combination of these two immediate-early polypeptides resulted in very high levels of activation (Everett,1986). Early genes can be divided into two groups depending on the time they are first expressed: 1. eg. the major DNA binding protein or the large subunit of ribonucleotide reductase, and 2. eg. thymidine kinase and DNA polymerase.

1.7.d. Late gene expression.

Late gene products are detected 3 hours post infection and reach their peak 10-16 hour post infection (Roizman, 1979b). In contrast to early gene expression which is stimulated by Vmw110, Vmw175 or a combination of both, late gene expression is controlled by a combination of the above two immediate-early polypeptides and the IE2 gene product, Vmw63 (Everett, 1986b). There are two subclasses of late genes: **1.** The leaky-late genes, eg. the major capsid protein encoded by U_L19 and gB specified by U_L27. The leaky-late genes are induced soon after early genes: they are first expressed prior to the onset of viral replication, and expression increases at later times in a replication-

dependent fashion **2.** The true late genes, eg. gC encoded by U_L44. These genes are expressed only after DNA synthesis has begun (Holland *et al.*,1980) and are not expressed in the absence of Vmw63 (Rice & Knipe, 1990).

1.8. PATHOGENICITY OF HSV.

HSV is a neurotropic human herpesvirus responsible for a variety of conditions ranging from mild cutaneous lesions to a very rare fatal encephalitis. After acute infection at the surface of the body, the virus travels via axons at the sensory nerves to establish a latent infection in the dorsal root ganglia of the peripheral nervous system (Stevens & Cook, 1971). Following reactivation, virus mainly travels via the peripheral nervous system to peripheral tissue resulting in mucocutaneous lesions but transport to the central nervous system (CNS) can result in encephalitis. Acute necrotizing encephalitis is the most serious neurological disease caused by HSV (Finelli, 1975) and as a direct study in humans is impossible, animal model systems have been employed. Intracranial inoculation of mice with HSV produces lesions similar to those seen in humans.

The identification of HSV genes involved in viral pathogenicity and the elucidation of their precise functions is of fundamental importance to the understanding of the biology of herpes simplex virus. Many host factors have been shown to influence virulence including animal age (Kohl & Loo,1980), route of inoculation (Caspary *et al.*,1980) and strain of experimental animal (Lopez, 1975). Virus strain (Dix *et al.*,1983) and the consequences of serial passage of virus *in vivo* (Kaerner *et al.*,1983) and *in vitro*, (Goodman & Stevens, 1986) have also been implicated.

Mutation in, or deletion of, the viral thymidine kinase gene (TK) has been shown to impair virulence (Field & Wildy, 1978). Sequences between 0.25 and 0.53 m.u. have also been implicated (Thompson *et al.*, 1986) as has the viral DNA polymerase (Field & Coen, 1986). The virulence phenotype may be determined by sequences between 0.7 and 0.83mu (map units) (Thompson & Stevens, 1983; Thompson *et al.*,1985; Javier *et al.*,1986; Rosen *et al.*, 1986). Evidence indicating that the sequences in both copies of R_L between IE1 and the 'a' sequence are involved in neurovirulence has accumulated. Taha *et al.*, (1989 a,b) reported a 1488bp spontaneous deletion upstream of IE1 in a HSV-2 variant that abolished neurovirulence. Correction of the deletion restored the wild-type phenotype. Ackermann *et al.* (1986) demonstrated that an open reading frame reported by Chou & Roizman (1986) between the terminal 'a' sequence and IE1 in HSV-1 strain F specified a protein. Thompson *et al.*, (1989) mapped a neurovirulence locus to between 0.82 and 0.832mu. The protein designated ICP34.5 has been shown to be a neurovirulence factor and to be non-essential for growth in tissue culture (Chou *et al.*,1990). Recently MacLean *et al.* (1991b) reported the isolation of a spontaneous deletion HSV-1 17^+ variant with a non-neurovirulent phenotype. The deletion removed 759bp of sequences upstream of IE1 and correction of the deletion resulted in restoration of neurovirulence.

1.9. HSV LATENCY.

After infection with HSV, the viral genome is maintained in a latent state in neuronal cells. Periodically, in response to various stimuli, virus can be reactivated from latency, often resulting in the development of a lesion at the appropriate peripheral site (Roizman & Sears, 1987;

Stevens, 1989). During latency, viral transcription can only be detected from the long repeat region of the genome giving rise to products known as the latency associated transcripts (LATs) (Croen *et al.*,1987; Rock *et al.*,1987; Spivack & Fraser, 1987; Stevens *et al.*,1987). Latent HSV DNA is maintained in a non-linear configuration, probably as a circular episome, and is assembled into a chromatin like structure (Rock & Fraser, 1983,1985; Efstathiou *et al.*,1986; Melerick & Fraser, 1987; Deshmane & Fraser, 1989). Thus during latency, the transcription pattern and physical organisation of the HSV-1 genome is very different from that found during productive infection.

In vivo and *in vitro* models for HSV latency have been developed. The pioneering work in the mouse model latency system was described by Stevens & Cook (1971) who inoculated the mouse footpad with HSV. This was followed by centripetal movement of the virus through the peripheral and central nervous system. After 3 weeks the virus could be recovered from the dorsal root ganglia demonstrating that the virus was in a latent state in the nervous tissue.

This pattern of establishment of latency is similar in other animal models like the rabbit eye model (Stevens *et al.*,1972), the mouse ear model (Hill *et al.*,1972).

In vitro models have also been used to investigate HSV latency. The systems with most obvious relevance to latency in animals and humans utilize foetal neurons from rats or primates (Wigdahl *et al.*, 1983, 1984; Wilcox & Johnson,1988; Wilcox *et al.*,1990). However extensive use of this system is limited by the quality of such neurons available. An alternative method is to use non-primary tissue culture cells eg. human foetal lung (HFL) cells (Wigdahl *et al.*, 1981,1982). Russell & Preston (1986) reported the development of an *in vitro* latency system in which HFL cells were infected with low multiplicities of HSV-2 and incubated

at the supraoptimal temperature of 42°C for 6 days. Cultures were subsequently downshifted to 37°C and maintained without production of virus indicating that the HSV-2 virus was latent.

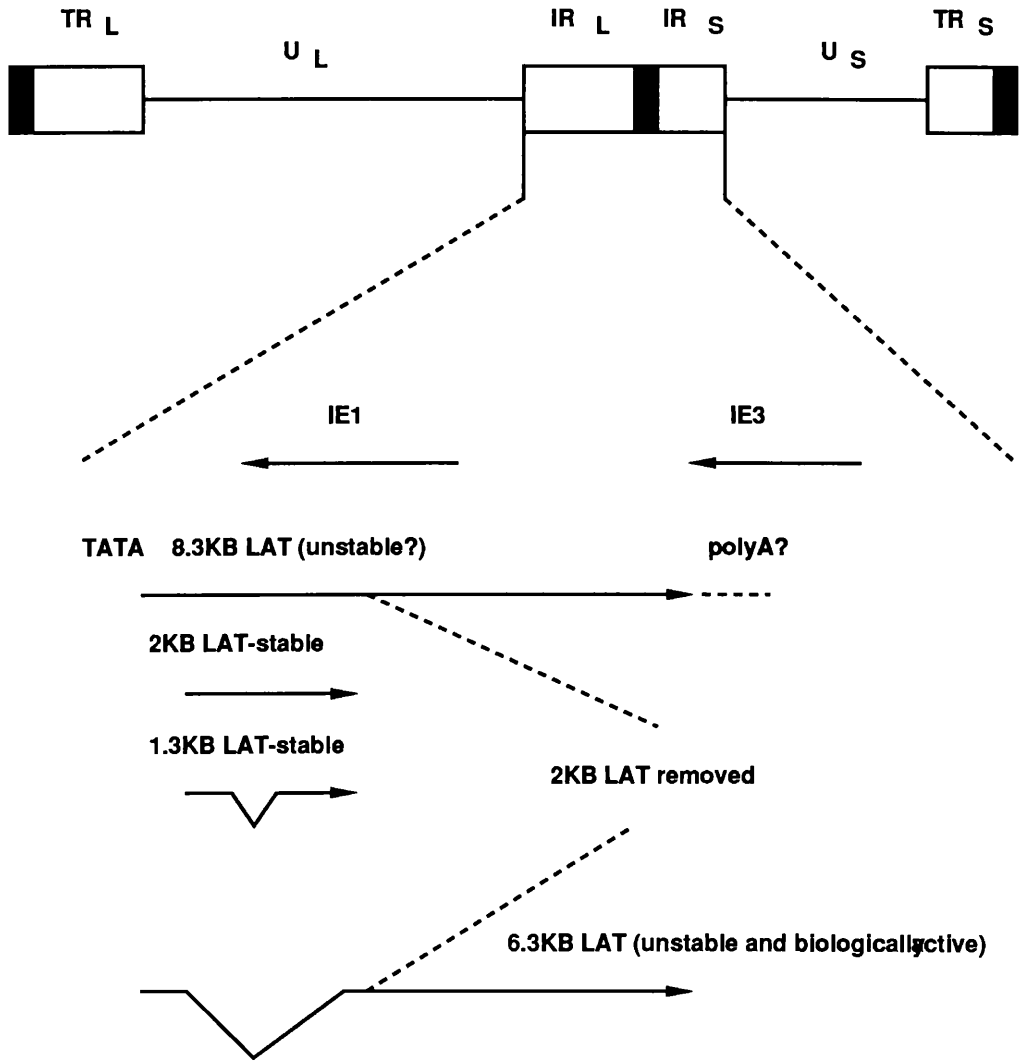
Using a mouse eye model (Tenser & Dunstan, 1979) and various deletion variants of HSV-1, Leib *et al.* (1989a) reported that the products of IE genes 2 and 3 were required for the establishment of latency *in vivo* and that the IE1 gene product was required to reactivate virus from the latent state. However, Clements & Stow, (1989) contradicted this result since the deletion variant *dl1403* reactivated normally. Harris & Preston (1991) used the *in vitro* latency system to demonstrate that a variant possessing an insertion mutation that abolished Vmw65 tranactivating ability could establish latency and that Vmw110 was required to reactivate virus.

The only transcription that occurs during latency is from the long repeat region of the genome and has been designated the latency associated transcripts (LATs). These consist of three herpes specific RNAs which map within IR_L and TR_L and are transcribed in the opposite direction to IE1 (Spivack & Fraser, 1987). These transcripts are present in reduced amounts in acutely infected mice (Spivack & Fraser, 1988b) and infected tissue culture cells (Spivack & Fraser,1987).

The 2 and 1.3 Kb LATs share their 3' and 5' ends and are derived by alternative splicing (Wechsler *et al.*, 1988). These LATs partially overlap the 3' end of IE1 and are antisense (complementary) to IE1 mRNA (Stevens *et al* .,1987). Zwaagstra *et al.* (1989) have demonstrated that the LAT promoter is 660 nucleotides upstream of the mapped 5' end of the stable LAT and have proposed that the LATs are in fact splice products of an 8.3 Kb primary transcript (Zwaagstra *et al.*,1990); (figure1.7) and suggest that the 6.3Kb exon may be the biologically active LAT. That the 8.3Kb primary transcript exists is supported by

Figure 1.7 A diagram of the LAT.

Diagram of the LAT as proposed by Zwaagstra et al.,(1990).


TR L
U L
IR L
IR S
U S
TR S
IE1
IE3
TATA
8.3KB LAT (unstable?)
polyA?
2KB LAT-stable
1.3KB LAT-stable
2KB LAT removed
6.3KB LAT (unstable and biologically active)

reports of viral mutants which lack this promoter failing to produce LAT RNA during latent infection (Dobson *et al.*,1989; Leib *et al.*,1989b; Mitchell *et al.*,1990; Steiner *et al.*,1989). However this would imply that the 2.0 and 1.3 Kb LATs are stable introns. Farrel *et al.* (1991) have proposed that LAT is a stable intron which controls latency by an antisense mechanism.

The function of LAT is unclear but recent studies of LAT deletion variants suggest that LAT may play a role in reactivation of the virus from the latent state (Dobson *et al.*, 1989; Leib *et al.*,1989b; Steiner *et al.*, 1989; Trousdale *et al.*, 1991). However this is not supported by all LAT mutants (Block *et al.*, 1990, Ho & Mocarski, 1989).

1.10. ANTISENSE NUCLEIC ACIDS IN BIOLOGICAL SYSTEMS.

Gene expression in prokaryotes and eukaryotes is normally controlled by regulatory polypeptides, for example, the immediate early gene products of HSV-1 (section 1.7). The products of these genes are called activators or repressors. The isolation of naturally occuring regulatory genes which direct the synthesis of RNA antisense to target RNA has led to the discovery of a new category of repressors which are highly specific inhibitors of gene expression. Binding of the antisense RNA to the target RNA renders the target RNA unavailable for translation. The fate of the RNA:RNA complex is unclear.

1.10.a. Systems in which antisense RNA is naturally involved.

Natural, prokaryotic antisense systems have been studied extensively. Inouye, (1988) separated the mechanisms by which antisense RNAs affect gene expression into three groups. This section discusses these

groupings and gives an example of each. This is followed by examples of eukaryotic systems in which antisense RNAs are thought to function.

Class 1 antisense RNAs inhibit translation of mRNA by binding to the transcriptional initiation region of target RNA and it is likely that control takes place by the inhibition of ribosome binding. Some class 1 antisense RNAs require RNAseIII for inhibition (Krinke & Wulff, 1987). This form of control is involved in the lysis/lysogeny pathways of two temperate bacteriophage, P22 and λ. Phage P22 encodes an antisense repressor, Ant, which inhibits DNA binding by the principal repressors of many lamboid phages including P22 (Susskind & Youderian, 1983). It is advantageous for P22 to express Ant early in infection as resident lamboid prophages will be induced, increasing the opportunity for recombination. However, Ant repressor must be carefully controlled, too much Ant will prevent the establishment of P22 lysogeny. Ant expression is controlled in part by a P22 specified antisense RNA, *sar*, which binds to the translational activation region of the Ant message and prevents its activation (Wu *et al.*, 1987).

Class 2 antisense RNAs also inhibit at the level of translation but at a region distant from translational start signals of the target transcript. An extensively studied example of this is the plasmid ColE1. ColE1 and its relatives are small multicopy plasmids whose replication is initiated by the transcription of a primer RNA termed RNAII (Tomizawa, 1987). To prime DNA synthesis, RNAII must hybridize to its DNA template strand and then be cleaved by RNAse H at a specific site approximately 500 nucleotides from its 5' end. The ColE1 antisense RNA, RNAI, is an approximately 110 nucleotide RNA species expressed from the primer region and complementary to the 5' end of RNAII. When RNAI binds RNAII, it triggers a conformational change in RNAII preventing

productive hybridization to the template DNA (Matsukata & Tomizawa, 1986).

Class 3 antisense RNAs control target RNA at the level of transcription. This is not as well characterized a form of antisense RNA as class 1 and class 2, however, the *Eschericia coli* CRP protein is thought to be regulated in this way. CRP mediates catabolic repression by modulating the activity of many bacterial promoters (Gottesman, 1984). For some time it has been known that the *crp* gene is negatively autoregulated (Aiba, 1985). On the basis of *in vitro* experiments, Okamoto & Freundlich (1986) proposed that *crp* autoregulation results from antisense control by an RNA called *tic*. The *tic* promoter, which is activated by CRP in the presence of cAMP, initiates transcription a few nucleotides upstream and divergently from the *crp* message. The *tic* RNA is partially antisense to the 5' end of the *crp* message and it is thought that this binding induces a conformational change in the message resulting in the termination of transcription. This is called transcriptional attenuation and has been invoked as a possible control mechanism of the human and murine *myc* gene families (Nepveu & Marcu, 1986; Krystal *et al.*, 1988).

Some examples of eukaryotic antisense RNA control mechanisms are the following. Katsuki *et al.*, (1988) constructed an antisense myelin basic protein (MBP) gene and used it to generate a transgenic mouse strain in which the antisense MBP was expressed. The transgenic mouse strain was converted to the shiverer phenotype characteristic of myelin deficient mice demonstrating that is was possible to control the MBP gene with antisense RNA.

A role for 'natural' antisense RNA in a eukaryotic system has been identified (Tosic *et al.*,1990). This group investigated the reasons why, although myelin deficient mice produced only about 2% of the wild-type concentration of myelin basic protein mRNA, the overall transcription

rate of the myelin basic protein RNA is normal indicating a block during the processing or transport of the RNA. The group also found that, of the two genes which code for myelin basic protein in myelin deficient mice, one gene contains a large inversion of the 3' end resulting in the inversion of the polyadenylation signal and associated termination signals preventing processing of the RNA. The inverted region is also antisense (complementary) to the intact gene remaining. Tosic *et al.* (1990) conclude that since the concentration of sense myelin basic protein RNA in the nucleus is relatively high as is the concentration of nuclear antisense RNA and the concentration of myelin basic protein in the cytoplasm is relatively low, post transcriptional regulation occurs through the formation of double stranded RNA molecules. The fate of the duplex RNA is uncertain and they do not assign the antisense RNA to any of the classes described above, but it is thought that it may eventually interfere with the processing or transport of mature RNA to the cytoplasm, that is, class 1 or class 2.

The antisense theory has also been implicated as a possible control mechanism for the maintenance of the HSV latent state (Stevens *et al.*,1987; Deatly *et al.*,1987). This group demonstrated by *in situ* hybridization that during latency the only region of the genome that could be visualised corresponded to IE1. Later work has shown that there are at least two stable latency associated transcripts (LATs) and that part of these transcripts is complementary to IE1 (Wechsler *et al.*,1988). Zwaagstra *et al.* (1989, 1990) suggested that the primary LAT transcript is 8.3 Kb and is spliced into products including the stable LATS, which are possibly introns, and an unstable 6.3 Kb LAT which is possibly an exon involved in the maintenance of latency.

It has also been proposed that the introns themselves may control latency by an antisense mechanism (Farrell *et al.*,1991). It has been

demonstrated that the stable LATs can inhibit transactivation by Vmw110 in transient transfection assays. The proposed mechanism by which this occurs is by the hybridization of the stable LAT to the 3' end of IE1 RNA leading to the production of hybrid molecules (class 1 or class 2 antisense RNA). It has previously been shown that Vmw110 which is encoded by IE1 is implicated in the *de novo* synthesis of HSV proteins (Cai & Schaffer,1989). In co-transfection experiments with infectious DNA of an IE1 null mutant and a plasmid expressing wild-type Vmw110, titres of infectious virus were significantly enhanced relative to transfection with mutant DNA alone, demonstrating a role for Vmw110 in *de novo* protein synthesis. These findings were consistent with the proposed role for Vmw110 in the reactivation from latency (Leib *et al.*,1989a), a process which is also thought to require *de novo* protein synthesis, and it is thought that by the hybrid arrest of IE1 RNA no Vmw110 is synthesized and HSV transcription is prevented.

The above theory is not supported by the isolation of an HSV-1 deletion variant (MacLean & Brown,1987) which does not express detectable LATs during latency (Steiner *et al.*,1989). The variant is able to establish latency but is delayed with respect to reactivation compared to wild-type virus.

1.10.b. Antisense nucleic acids as control mechanisms.

With the development of oligonucleotide technology, it has become possible to use antisense oligonucleotides to regulate gene expression. Smith *et al.* (1986) synthesized an oligo which was complementary to the splice acceptor junction of HSV immediate-early polypeptides -4 and -5 and demonstrated that this could negatively control viral growth. Kawamura *et al.* (1991) used an oligo which was complementary to the

•The locations of transcriptional termination signals in HSV-1 are not known, however termination of transcription by RNA polymerase II is achieved by a combination of a polyadenylation signal and transcriptional termination regions in the mouse Bmaj globin gene (Logan et al., 1987). The termination signals for that gene are located 600-1500 bp downstream of the polyadenylation signal. This is in agreement with results previously obtained by Whitelaw and Proudfoot, (1986) who demonstrated that transcriptional termination and 3' end processing of mRNA are coupled events for the mouse α2 globin gene.

splice donor site of a Mareks disease virus (MDV) transformed cell line to control its growth.

Sandri-Goldin *et al.*,1987 constructed several cell lines which expressed different amounts of antisense IE1 mRNA due to the cells harbouring variable copy numbers of a recombinant plasmid expressing a portion of the IE1 gene in an antisense manner. During *in vitro* transfection assays and lytic HSV-1 infections, the quantity of Vmw110 produced from these cell lines was lower than from wild-type, indicating that the antisense transcript was controlling Vmw110 production. The study also demonstrated that the antisense transcript had to be present in a ten fold excess to obtain this effect.

1.11. AIMS OF PROJECT.

Initial characterization of the HSV-1 17^+ deletion variant 1703 demonstrated a $7.5x10^6$mol. wt. deletion in U_L/IR_L which removed the 3' end of UL55, all of UL56 and the 3' end of the IR_L copy of IE1 (MacLean & Brown, 1987a).Polypeptide analysis of 1703 had demonstrated that Vmw63 was apparently not produced by 1703 under immediate-early conditions despite the fact that the deletion terminated about 500bp downstream of the 3' end of IE2.

Vmw63 is an essential immediate-early polypeptide (Sacks *et al.*, 1985; McCarthy *et al.*,1989) and the isolation of a variant which apparently did not express the protein under immediate-early conditions presented an anomaly for which MacLean & Brown gave three possible explanations: 1. There was a small deletion/point mutation in the promoter, open reading frame or termination signals of IE2, 2. Lack of the products of

the deleted genes UL55 or UL56 led to incomplete transactivation of IE2 under immediate-early conditions, or alternatively the deletion removed a downstream transactivator element of IE2, **3.** The reagents available for polypeptide analysis were not sensitive enough to detect low levels of Vmw63 produced by 1703.

The aim of this project was to find out why 1703 apparently failed to produce Vmw63 under immediate-early conditions. This was done by: **1.** The dideoxynucleotide sequence analysis of IE2 .i.;and the end points of the deletion in 1703, **2.** The detection of IE2 mRNA and protein, **3.** The construction of a recombinant virus to demonstrate that there was no deletion or mutation in any other region of the 1703 genome which would result in the underproduction of Vmw63 under immediate-early conditions, **4.** The *in vivo* characterization of 1703. The aim of this project was extended by a suggestion that Vmw63 production by 1703 could be controlled by the production of an antisense transcript initiating from the IE1 promoter (Dr J. McLauchlan personal communication). The termination signals of the IR_L copy of IE1 in 1703 have been deleted thereby allowing the possibility that transcripts are not terminated until reaching the termination signals of the next gene in the correct orientation, that is, UL51. There were two ways of establishing that antisense transcripts were controlling Vmw63 production: **1.** The detection of the long antisense transcript, **2.** The insertion of a polyadenylation signal in the correct orientation to terminate the long antisense transcript before reaching IE2 coding sequences. This study reports the characterization of an HSV-1 variant which controls Vmw63 production in infected cells under immediate-early conditions by the production of an antisense transcript.

*genotype: *supE*44 *hsdR*17 *recA1 endA1 gyrA*46 *thi relA*1 *lac*⁻. F
[*proAB*⁺ *lacI lacZ* M13 Tn10 (*tet*ʳ)].

CHAPTER 2

MATERIALS

Cells

Unless otherwise stated, baby hamster kidney 21 clone 13 (BHK21 C13) cells were used for this work (Macpherson and Stoker, 1962). Human foetal lung cells (HFL) were used when stated.

Virus

Herpes simplex virus type 1 Glasgow strain 17 syn$^+$ (HSV-1 17$^+$) was the wild-type used throughout this study (Brown *et al.*, 1973). The deletion variant 1703 which is the subject of this project has been partially characterized (MacLean and Brown, 1987a).

Bacteria

The bacteria used for the growth of plasmids was *E.coli* strain XL-1$^•$ which were grown in L-broth or 2YT broth, ampicillin being added (100μg/ml) where appropriate.

HSV-1 recombinant plasmids

The following recombinant plasmids carry restriction enzyme fragments of HSV-1 cloned into pAT153 (Twigg and Sherratt, 1980). They were supplied by Dr V.G. Preston.

Plasmid	Restriction fragment	Map Location
pGX51	*Sal*1 digested *Bam*H1b	114517-120902
pGX55	*Sal*1 digested *Bam*H1z	143481-144677

Plasmids used for cloning 1703 DNA fragments

Commercially available pUC19 mp18 and 19 and M13 mp8 (cut *Sma1*); (Messing, 1983) were used throughout this study.

Tissue culture media

BHK21 C13 cells were routinely cultured in Glasgow modified Eagles medium (Busby et al, 1964) supplied by Gibco-BRL. The medium was supplemented with 100 units/ml penicillin, 100µg/ml streptomycin, and 0.002% (w/v) phenol-red. To this was added 10% (v/v) tryptose phosphate broth and 10% newborn calf serum (ETC 10%). HFL cells were cultured in the same medium with the exception that the 10% newborn calf serum was replaced with 10% foetal calf serum (ETF 10%). Variants of the media were:

E met/5C2 : Eagles medium containing one-fifth the normal concentration of methionine and 2% newborn calf serum.

E met/5F2 : Eagles medium containing one fifth the normal concentration of methionine and 2% foetal calf serum.

PIC : Phosophate free Eagles medium containing 1% newborn calf serum.

EMC 10% : Eagles medium containing 1.5% methyl cellulose and 10% newborn calf serum.

Stock solutions.

Phosphate buffered saline-A (PBS-A): 170mM NaCl, 3.4mM KCl, 1mM Na_2HPO_4, 2mM KH_2PO_4 ph7.2 (Dulbecco & Vogt, 1954)

Phosphate buffered saline (PBS): PBS-A supplemented with 6.8mM CaCl and 4.9mM $MgCl_2$.

PBS/calf serum: PBS containing 5% newborn calf serum.

TBE : 90mM Tris base, 90mM Boric acid, 2.5 M EDTA pH8.3.

30% acrylamide: 29.25% (w/v) acrylamide, 0.75% (w/v) N-N'-methylene bisacrylamide.

Trypsin: 0.25% (w/v) Difco trypsin dissolved in Tris-saline (140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1mg/ml glucose, 0.0015% (w/v) phenol-red, 25mM Tris. HCl (pH7.4), 100 unit/ml penicillin, 100µg/ml streptomycin.

Versene: 0.6 mM EDTA dissolved in PBS containing 0.002% (w/v) phenol-red.

Trypsin-versene: One volume of trypsin plus four volumes of versene.

Giemsa stain: 1.5% suspension of Giemsa in glycerol heated at 56°C for 1.5 - 2 hours and diluted with an equal volume of methanol.

L-broth: 170mM NaCl, 0.5% (w/v) yeast extract (Difco) 1% bactotryptone (Difco).

2 YT broth: 85mM NaCl, 1% (v/v) bactotryptone (Difco), 1% yeast extract.

L-broth agar: L-broth containing 1.5 (w/v) agar (Difco).

Top agar: 1% (w/v) agar in water.

Chemicals

Most chemicals were supplied by BDH Chemicals UK or by the Sigma Chemical Company and were of analytical grade. Ammonium persulphate (APS) N,N,N',N'-tetramethylethylenediamine (TEMED) and colour development reagent were supplied by Bio-rad Laboratories, Boric acid was supplied by May and Baker, Nytran paper by Amersham, nitrocellulose by Schliecher and Schuell and unlabelled nucleotides by Pharmacia Ltd.

Radiochemicals

Radiochemicals were obtained from Amersham International plc at the following specific activities:

5' [α-^{32}P] dNTPs~3000 Ci/mmol

[γ-^{32}P] dATPq~5000 Ci/mmol

[^{35}S] methionine > 1000 Ci/mmol

[^{35}S] dATP > 1000 Ci/mmol

[^{32}P] orthophosphate 3000 Ci/mmol

[Me-3H] thymidine 1mCi/ml

Enzymes

Restriction enzymes were obtained from Bethseda Research Laboratories (BRL), New England Biolabs, or NBL enzymes Ltd. DNA polymerase 1, Klenow polymerase, T4 DNA ligase and T4 polynucleotide kinase were supplied by the Boehringer Mannheim Corporation. Lysozme and BSA were supplied by the Sigma Chemical Company.

Restriction enzyme buffers

In general, the buffers used were as recommended by BRL or the Boehringer Mannheim Corporation. These were supplied with enzymes as 10x stock solutions which were stored at -20°C. A buffer was made to digest HSV DNA with *Hpa*1 and is also suitable for *Xba*1, *Bgl*II, *Hind*III and *Eco*R1 digestions. The buffer consists of: 60mM Tris pH 7.4, 1M NaCl, 60mM $MgCl_2$, 0.01% (w/v) BSA.

Chemicals used for the purification of DNA or RNA

Phenol : Phenol (BDH) was saturated before use by mixing 2:1 with phenol saturation buffer (10mM Tris, HCl pH 7.5, 10mM EDTA, 100mM NaCl) and stored at either -20°C or 4°C for up to one month.

Chloroform : Chloroform was mixed 24:1 with isoamylalcohol to reduce foaming during extraction and to facilitate the separation of the organic and aqueous phases.

Phenol : Chloroform (1:1) : This was a 1:1 mixture of saturated phenol and chloroform isoamylalcohol (24:1).

Animals.

3 and 4 week old Balb/c mice were obtained from Bantin and Kingman.

Anti-peptide serum.

Anti-peptide serum was supplied by Dr H Marsden and raised against the carboxy-terminus of Vmw63 (NATDIDMLI DLGLDLS).

METHODS

2.1 Growth of cells.

BHK21 C13 cells were grown in 80oz roller bottles containing 100ml ETC 10% at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Confluent cells (approximately $3x10^8$/roller bottle) were harvested by washing the monolayers twice with trypsin/versene, and the detached cells were resuspended in 20ml of ETC 10%. Cells from one roller bottle were sufficient to seed a further 10. For experiments cells were plated on 50mm or 30mm petri dishes or limbro trays at a density of $4x10^6$, $2x10^6$ or $2x10^5$ cells per plate respectively. HFL cells were grown in a similar manner, except that ETF 10% was used and for routine passage 800ml flasks were split 1 in 3.

2.2 Growth of virus stocks.

Almost confluent BHK21 C13 cells in 80oz roller bottles were infected at a multiplicity of infection (moi) of 0.001 plaque forming units (pfu) per cell in ETC 10% at 31°C. The infection was allowed to proceed until the cytopathic effect (cpe) was complete (usually 3-4 days). The virus was then harvested by shaking the cells into the medium and pelleting by centrifugation at 2000 rpm for 10 minutes at 4°C in a Beckman benchtop centrifuge. The cell pellet was resuspended in 5ml of supernatant, sonicated in a bath sonicator (50W at 4°C) until homogeneous and centrifuged as before. The supernatant was removed and stored on ice (supernatant 1). The pellet was resuspended in a further 5ml of the original supernatant, sonicated and centrifuged as

above to give supernatant 2. The cell pellet was discarded and supernatants 1 and 2 combined to give cell associated virus stock.

The supernatant from the original 2000 rpm spin was centrifuged at 12000 rpm for 2 hours at 4°C in a Sorval GSA rotor, and the virus containing pellet resuspended in 5-10ml of the supernatant and sonicated until homogeneous. This was termed the supernatant virus stock.

Virus stocks were checked for sterility, titrated at 37°C and stored in 1ml aliquots at -70°C.

2.3 Titration of virus stocks.

Stocks to be titrated were serially diluted 10 fold in PBS/calf serum. Aliquots of 0.1ml were added to BHK21 C13 cell monolayers which were 70% confluent from which the medium had been removed. Following incubation at 37°C for one hour, the plates were overlaid with 4ml EMC 10% and incubated for 2 days at 37°C or 3 days at 31°C. Monolayers were fixed and stained with Giemsa at room temperature for not less than one hour. After washing, plaques were counted using a dissection microscope.

2.4 Sterility checks on virus and cell stocks

Brain heart infusion agar (BHI) plates, and BHI plates containing 10% horse blood (BHI blood agar), were obtained from the Cytology Department. Cell or virus stocks were checked for fungal contamination by streaking on BHI plates in duplicate. The plates were sealed with parafilm and incubated at room temperature for seven days. Similarly yeast or bacterial contamination was detected by streaking on BHI blood

agar plates and incubating at 37°C for 7 days. If no contamination became apparent within that time the stocks were considered sterile.

2.5 Preparation of virion DNA.

This method is based on that described by Wilkie (1973) and Stow and Wilkie (1976). BHK21 C13 cells in 80 oz roller bottles were infected and harvested as described previously (method 2.2). The cell supernatant was stored at 4°C. To extract cytoplasmic virus the cell pellet was resuspended in RSB (10mM KCl, 1.5mM $MgCl_2$, 10mM Tris. HCl pH 7.5 containing 0.5 % [v/v] Nonidet P40) incubated on ice for ten minutes and centrifuged at 2000 rpm in a Beckman benchtop centrifuge for 5 minutes. The supernatant from this spin was added to the previous cell supernatant. The pellet was suspended in RSB/Nonidet P40, extracted as before, and the final supernatant added to the initial cell supernatant. The supernatant pool was centrifuged at 12000 rpm in a Sorval GSA rotor for 2 hours. The virus pellet was resuspended in 8ml NTE (10mM Tris.HCL pH 7.5, 10mM NaCl, 1mM EDTA) by sonication and lysed by the addition of SDS and EDTA to a final concentration of 2% (v/v) and 0.8mM respectively. The released virus DNA was carefully extracted three times with an equal volume of phenol and once with chloroform and precipitated by the addition of 2 volumes of ethanol. The DNA was pelleted by centrifugation at 2000 rpm in a Beckman benchtop centrifuge for 10 minutes, dried at 37°C for 1 hour, redissolved in water containing 50µg/ml Rnase A and quantified by spectrophotometry.

1 0D unit$_{260nm}$ = 50µg/ml DNA.

2.6 Transfection of virus DNA by $CaPO_4$ precipitation/DMSO boost

To 50mm Petri dishes containing semi-confluent BHK21 C13 was added 400ml HEBS buffer pH 7.05 (130 mM NaCl, 1.6 mM Na_2HPO_4, 5.5 mM D-glucose, 21 mM HEPES) containing 10μg/ml calf thymus DNA at a 5:10 molar ratio of plasmid:virus DNA and 110 mM $CaCl_2$. Following incubation at 37°C for 40 minutes, the plates were overlaid with 4ml ETC 10%, 4 hours later 1ml of HEBS containing 25% (v/v) dimethylsulphoxide (DMSO) was added, and the plates incubated at room temperature for 4 minutes. The DMSO was removed and the plates were washed twice with ETC 10% and overlaid with EMC 10%.

2.7 Growth of plaques isolated from a transfection

EMC 10% was removed from the transfection plate and the monolayer was washed twice with PBS/calf serum. Separated plaques were picked using a 100ml Eppendorf pipette into linbro trays containing confluent BHK21 C13 cells. The trays were then incubated at 37°C until cpe was complete and the viral DNA was analysed by the method of Lonsdale (1979).

2.8 Preparation and isolation of ^{32}P labelled virion DNA

This method is a modification of that described by Lonsdale (1979). Monolayers of BHK21 C13 cells were maintained in ETC 10% in linbro trays until confluent, and then infected with 50μl of virus from a plaque which had been isolated and grown from a transfection. After one hour at 37°C the medium was removed and the monolayer washed twice with PIC and overlaid with 450μl PIC. After a further incubation at 37°C for 2 hours, 1μCi of ^{32}P-orthophosphate was added and the incubation

continued at 37°C for 48 hours. The cells were lysed by adding sodium dodecyl sulphate (SDS) to a final concentration of 2.5% (v/v) and incubating at 37°C for 5 minutes. The DNA was extracted once with an equal volume of phenol and precipitated by the addition of 2 volumes of ethanol. The DNA was dried at 37°C for 15-20 minutes (with the tubes in an inverted position) and redissolved in 150µl of H_2O containing 50µg/ml Rnase A. Usually 10% of each sample (15µl) was digested with the appropriate restriction enzyme and electrophoresed in an agarose gel (method 2.9) for 16-24 hours at 70-85 V. Gels were air dried and set up for autoradiography against Kodak X-Omat XS-1 film and developed after 48 hours at room temperature.

2.9 Agarose gel electrophoresis

Agarose gels [0.5-1.5%(w/v)] were prepared by boiling the appropriate quantity of agarose in 250ml of 1xTBE until dissolved. Once cool, the solution was poured onto a glass plate (152cmx85cm) whose edges had been sealed with masking tape onto which had been placed a teflon coated well forming comb (26, 15 or 12 teeth). Once set, the gel was placed in a horizontal gel tank containing 1xTBE. For non-radioactive samples, the gel contained 0.5µg/ml ethidium bromide. For plasmid DNA, gels were electrophoresed at either 20-50V overnight or 80-120V for 3-6 hours. For HSV DNA, gels were electrophoresed at 60-80V overnight. As a rule, to resolve DNA fragments of high molecular weight, low percentage agarose gels were used, while for low molecular weight fragments, higher percentage agarose gels were used.

2.10 Thymidine kinase assay.

The method used is a modification of that of Jamieson and Subak-Sharpe (1974). BHK21 C13 cells in 35mm petri dishes were infected at a moi of 20 pfu/cell. After adsorption at 37°C, cells were overlaid with 2ml of ETC 10% and incubated for 6 hours at 37°C. Cells were washed twice with cold PBS, scraped into 1ml cold PBS and pelleted in a microfuge for 2 minutes. The pellet was resuspended in 150µl ice cold lysis buffer (20mM Tris.HCl pH7.5,2mM $MgCl_2$, 10mM NaCl, 0.5% (v/v) NP40, 6.5mM 2-mercaptoethanol), maintained on ice for 5 minutes, mixed briefly and placed on ice for a further 5 minutes. The samples were then centrifuged and the supernatant retained and stored at 70°C. 5µl of extract was mixed with the reaction buffer in a total volume of 50µl (Reaction buffer: 0.5M Na_2PO_4 pH6.0, 100 mM $MgCl_2$, 2m M dTTP, 100 mM ATP, 5µl aqueous [Me-^{3}H] thymidine 1mCi/ml). After incubation for 1 hour at 30°C, the reaction was stopped by the addition of EDTA and thymidine to a final concentration of 20mM and 1mM, respectively. The samples were heated for 3 minutes at 100°C and placed on ice for 3 minutes. After centrifugation, 40µl of the supernatant was spotted onto DE81 discs and washed three times (10 minutes each at 37°C) with 4mM ammonium formate pH6.0 containing 10µM thymidine. After a further two washes with ethanol, the discs were dried and radioactivity due to ^{3}H thymidine was determined using a scintillation counter.

2.11 Glycerol stocks of bacteria

Bacterial stocks were prepared from 5ml shaking cultures grown overnight in L-broth in the presence of the appropriate antibiotic at

37°C. These stocks were stored at both -20°C and -70°C in 50% (v/v) glycerol.

2.12 Restriction enzyme digestion

Viral and plasmid DNA was digested with the appropriate restriction enzymes (2.5 units/μg DNA), in the presence of 1x recommended buffer, in a final volume of 20-200μl and incubated at 37°C for 2-16 hours. The reaction was stopped by the addition of 20% restriction enzyme stop solution (RE stop- 5xTBE, 100 mM EDTA, 10% v/v Ficoll, 0.1% (w/v) bromophenol blue) prior to electrphoresis (Maniatis *et al*, 1982), or by purifying the digested DNA once with chloroform, and precipitating in ethanol, washing with 70% ethanol, and vacuum drying prior to ligation (method 2.14), transfection (method 2.6) or end labelling (method 2.28).

2.13 Elution of DNA fragments from agarose gels

The DNA was digested with appropriate restriction enzymes and electrophoresed on agarose gels in the presence of 5μg/ml ethidium bromide. The gel was visualised under long wave U.V. and the appropriate fragment cut out using a sharp scalpel. The isolated gel slice was placed in an electrophoresis chamber containing 1xTBE. The DNA was electroeluted from the agarose gel onto the dialysis membrane of the electrophoresis chamber (preboiled in 1xTBE for 10 minutes) at 200V for 1 hour. The DNA was collected, extracted with phenol:chloroform (1:1) and precipitated at -20°C overnight with three volumes ethanol and 0.1 volume 3M sodium acetate. DNA was washed with 70% ethanol, vacuum dried and redissolved in water.

2.14 Construction of recombinant pUC19 plasmids.

Plasmid vectors pUC19 and pUC18 were linearised with *Sma1* and treated with calf intestinal phosphatase (both at concentrations of 5 units/μg of plasmid DNA). After incubation at 31°C for 1-3 hours, the DNA was extracted twice with phenol:chloroform (1:1), once with chloroform and precipitated with two volumes of ethanol in the presence of 0.1 volume 3M sodium acetate. The DNA pellet was washed with 70% ethanol, dried and resuspended in the appropriate amount of water to give 40ng/μl. A 3-4 molar excess of the purified HSV DNA fragment relative to the phosphatase treated vector (40ng) was ligated overnight at room temperature in a 20μl reaction containing 2 units of T4 ligase and 1x ligase buffer (10 mM Tris.HCl pH7.5, 10m M $MgCl_2$, 10 mM DTT, 1 mM ATP).

2.15 Transformation of bacterial cells with plasmid DNA.

E.coli strain XL-1 cells were made competent for transformation with plasmid DNA by the method of Chung and Miller, (1988). 5μl of a glycerol stock of XL-1 cells was added to 10ml of L-broth and incubated with shaking at 37°C overnight. The cells were then diluted 1/100 in L-broth and incubated at 37°C with shaking until an OD_{600} of 0.3-0.6 was reached. The suspension was then pelleted at 2000 rpm for 10 minutes in a benchtop centrifuge and resuspended in a 1/10 of the original volume of ice cold transformation and storage buffer (TSB: L-broth pH 6.1, 10% PEG Mw 3350, 5% DMSO, 20 mMMg^+[10 mM $MgCl_2$, 10 mM $MgSO_4$]).The cells were then incubated on ice for 10 minutes. 5μl of each ligation mixture (method 2.14) was added to 100μl of competent bacterial cells and incubated on ice for 10-30 minutes. 900μl of TSB containing 20mM glucose was then added and the cells were incubated

at 37°C with shaking for 1 hour. 100µl of each sample was spread on L-broth agar plates containing 100µg/ml ampicillan and incubated overnight at 37°C. Single bacterial colonies were picked from the plates and analysed. (method 2.16).

2.16 Small scale preparation of plasmid DNA by alkaline lysis

This method is essentially as described by Birnboim and Doly (1979). Colonies from an agar plate were inoculated into 5ml L-broth containing the appropriate antibiotic and shaken overnight at 37°C. 1.5ml of cells were added to an Eppendorf tube and pelleted by centrifugation in a Beckman microfuge for 30 seconds. The pellet was resuspended in 100µl of solution 1: (50mM glucose, 10mM EDTA, 25mM Tris. HCl, pH8.0) containing 4mg/ml lysozyme, and incubated at room temperature for 5 minutes, 200µl of solution 2: (0.2M NaOH, 1% SDS [w/v]) was added and incubation continued on ice for 5 minutes. 150µl of ice cold solution 3 (3M KAc, pH4.8) was added, and following incubation on ice for 5 minutes, the cell debris was pelleted by centrifugation in a Beckman microfuge for 5 minutes. DNA was extracted by mixing with an equal volume of phenol:chloroform (1:1), centrifuging for 2 minutes in a Beckman microfuge and removing the upper aqueous layer. This was added to 2 volumes of ethanol and the DNA precipitated at room temperature for 2 minutes, pelleted by centrifugation for 5 minutes in a Beckman microfuge, washed in 70% ethanol, dried in a vacuum desiccator and redissolved in 50µl water containing 50µg/ml RNase A. Usually 10µl of this was used per restriction digest.

2.17 Large scale preparation of plasmid DNA by alkaline lysis

This method is essentially that described by Birnboim and Doly (1979), and modified by Maniatis *et al.*, (1982). Single colonies from an agar/L-broth or 25µl from a bacterial glycerol stock were inoculated into 5ml of L-broth containing the appropriate antibiotic and shaken at 37°C overnight. The culture was transferred into 500ml L-broth containing the appropriate antibiotic in a 2 litre flask and shaken at 37°C for 12-24 hours. The bacteria were pelleted by centrifugation at 8000 rpm for 5 minutes in a Sorval GSA rotor, the pellet resuspended in 7ml solution 1 (method 2.16) containing 4mg/ml lysozyme and incubated at room temperature for 10 minutes. Freshly made solution 2 (14ml) was added and incubation continued for a further 10 minutes on ice. Ice cold solution 3 (10.5ml) was added, incubation continued for a further 10 minutes on ice and the bacterial cell debris pelleted by centrifugation at 12000 rpm for 30 minutes in a Sorval SS34 rotor. DNA was extracted twice with an equal volume of phenol:chloroform (1:1) and once with an equal volume of chloroform. The DNA was precipitated at room temperature for 15 minutes by the addition of 2 volumes of ethanol, centrifuged at 12000 rpm for 30 minutes in a Sorval SS34 rotor at RT, washed in 70% ethanol, pelleted as before, and dried in a vacuum desiccator. The DNA pellet was dissolved in water containing 50µg/ml RNase A. The DNA was quantitfied by running a small sample on an agarose gel containing 0.5µg/ml ethidium bromide and comparing its intensity against that of a known standard under UV light.

2.18 Synthesis and purification of a synthetic oligonucleotide.

A synthetic oligonucleotide was synthesized on a Biosearch 8600 DNA synthesizer. The DNA was eluted from the column by resuspending the beads in 1ml of ammonia and incubating at 55°C for 5 hours. The ammonia was removed by lyophilisation in the 'speedivac' vacuum desiccator, and the dried sample purified by denaturing polyacrylamide gel electrophoresis. 75ml of 16% sequencing gel mix (16% acrylamide, containing 1 part in 30 n-n'-methylene bisacrylamide, and 8.3M urea in 1xTBE), polymerised with 400µl of 10% ammonium persulphate and 40µl TEMED, was poured between two 20x22cm glass plates separated by 1.5mm spacers. Wells of approximately 1cm in width were formed using an 8-tooth teflon comb. The DNA samples were resuspended in 50µl of water by vortexing, then microfuged for 3 minutes. The supernatant was transferred to 50µl of sample buffer (28µl 10xTBE, 117µl H_2O, 800µl deionised formamide), boiled for 10 minutes then quenched on ice and loaded immediately. 2µl of formamide dye mix was loaded in a separate well to act as a molecular weight marker. The gels were electrophoresed slowly, at 3.5-4mA overnight in TBE.

To visualise the DNA the gel was removed, wrapped in cling film and viewed against a white chromatographic plate by angled long wave light. If the synthesis had been successful then a predominant, strong band with possibly a few minor lower molecular weight bands was seen. The top band was cut out with a scalpel, mashed with a glass rod, and incubated at 42°C for 16 hours in 1ml elution buffer (0.5m ammonium acetate, 1mM EDTA, 0.5% SDS). This was filtered through glass wool to remove the acrylamide, phenol:chloroform (1:1) extracted, ethanol precipitated, washed in 70% ethanol, dried and dissolved in water. To

quantify the DNA, the OD_{260} was read. The conversion factor for synthetic oligonucleotides is taken as 1 OD unit = 20μg/ml.

2.19 ^{32}P-labelling of a synthetic oligonucleotide.

100-200ng of oligonucleotide was labelled in a buffer containing 60mM Tris pH7.5,10mM $MgCl_2$, 10mM DTT, 50μCi γ^{32}PdATP, and 1 unit T4 polynucleotide kinase made up to a final volume of 9.5μl. The mixture was then incubated at 37°C for 30 minutes after which an additional unit of T4 polynucleotide kinase was added and the mixture incubated at 37°C for a further 30 minutes. The labelled probe was then denatured by boiling for 10 minutes and used for Southern blotting (method 2.21).

2.20 ^{32}P-labelling of non-synthetic DNA.

Purified virion, plasmid or infected cell DNA was ^{32}P-labelled using the method of random priming. 10-100ng of DNA was boiled for 10 minutes before labelling in a buffer which was made from 3 components:

5x reaction mix for random priming.

Reaction mix was made up from solutions A, B and C at a ratio of 10: 25: 15 respectively:

Solution A: 1ml solution q: 1.25M Tris.HCl pH7.8, 0.125M $MgCl_2$.

18μl 2-mercaptoethanol

5μl 100mM dGTP

5μl 100mM dATP

5μl 100mM dTTP.

Solution B: 2M HEPES pH6.6.

Solution C: Hexadeoxyribonucleotide (Pharmacia) evenly resuspended in TE at 90OD units/µl.

5µl of this buffer is added to the boiled DNA together with 1µl of 1% BSA, 20µCi α^{32}P dCTP, 1unit of Klenow polymerase and made up to 25µl with water. The mixture was incubated at room temperature overnight, or at 37°C for 30 minutes, boiled and used for Southern blotting (method 2.21).

2.21 Southern blotting and hybridization to ^{32}P-labelled DNA

This method is based on one described by Sambrook *et al.*, (1989). Purified virus, plasmid or infected cell DNA was digested with the appropriate restriction enzyme and run on an agarose gel for 16-24 hours at 60-80 V (method 2.9). The gel was visualised under short wave UV to confirm digestion of the samples and to partially fragment large DNA molecules to aid their transfer. The gel was placed in 1 litre of gel soak 1: (200mM NaOH, 600mM NaCl) for 1 hour and transfered to 1 litre of gel soak 2: (1H Tris. HCl pH8.0, 0.59M NaCl) for 1 hour. The gel was then transferred to 1 litre of 20xSSC (3MNaCl, 0.3M trisodium citrate) for 1 hour and blotted onto a nytran membrane (Amersham) in the following manner: one or two pieces of membrane, cut to the size of the gel, was placed together with 3-6 pieces of 3mm Whatman chromatography paper, also cut down to gel size, in 20xSSC and allowed to soak. If a single blot only was required two pieces of dry chromatography paper were placed upon a stack of correctly sized "Hi-Dri" towels followed by 3 pieces of soaked chromatography paper, the soaked nytran membrane, and the gel; ensuring that no bubbles were left between the gel and the membrane. A glass plate was then placed on top of the gel followed by a weight. This set-up acted as a wick to

draw the SSC through the gel and onto the nytran membrane. If desired, the gel can be double blotted by placing another piece of soaked membrane onto the gel instead of the glass plate and building up another wick on this side of the gel. The glass plate then goes on top of the final stack of "Hi-Dri" towels followed by the weight. After 12-24 hours, the DNA is fixed to the membrane using a UV crosslinker (Stratagene) set at 12000μjoules/cm^2.

The nytran was hybridized to the ^{32}P-labelled probe in hybridization buffer (7% SDS, 0,5M Na_2HPO_4 pH7.4) at 42-65°C, 65°C for non-synthetic DNA, and 42-65°C for synthetic DNA depending on the size and G+C content of the oligonucleotide. Hybridization was allowed to proceed overnight after which the filter was washed twice with 2xSSC containing 0.1% SDS for 1/2 hour each time. The filter was then sealed into another hybridization bag and set up for autoradiography against Kodak XS-1 film either without a Dupont image intensifying screen at room temperature or with one at -70°C.

2.22 Construction of M13 recombinant plasmids

The double stranded replicative form of M13 mp8 was commercially obtained from Amersham cut with the restriction enzyme *Sma*1. The fragments were purified once with phenol:chloroform (1:1), ethanol precipitated, washed in 70% ethanol, dried and redissolved in water. 40ng of M13 vector DNA, 120-160ng of the DNA insert, 2 units of T4 DNA ligase in ligase buffer (method 2.14) were mixed and incubated for 24 hours at room temperature. (Sanger *et al.*, 1977).

*10 mins, 13000rpm, room temperature on a Beckman benchtop centrifuge.

2.23 Transfection of bacterial cells with M13

E.coli strain XL-1 was grown in 2xYT broth to an OD of 0.3 to 0.6 and made competent as described (method 2.15). 5-20µl of ligation mix was added to 100µl of competent cells and incubated on ice for 10-30 minutes. 3ml of melted top agar at 42°C containing 25 µl of 2.5% isopropyl-βD-thiogalactoside (IPTG) in water and 25µl of 2% 5-chloro-4-bromo-3-indolyl-ß-D-galactoside (BCIG or x-gal) in dimethylformamide was added to the sample and the mixture poured onto 90mm L-broth agar plates and incubated at 37°C overnight.

2.24 Preparation of template DNA.

A culture of XL-1 cells was grown to saturation in 2YT broth. Colourless plaques from the M13 transformation were picked into 1.5ml of saturated XL-1 cells diluted 1/100 in 2YT broth and grown with vigorous shaking at 37°C for 4.5-6 hours. Samples were then transferred to 1.5ml Sarsedt tubes and pelleted for 5 minutes (MSE microfuge). The supernatant was then transferred to another tube and the procedure repeated ensuring that the supernatant was free from bacterial contamination. To precipitate the M13, 150µl of a solution of 20% PEG, 2.5M NaCl was added, the sample vortexed and left at room temperature for 10 minutes. The M13 was pelleted by centrifugation, and repelleted to get rid of residual PEG. The M13 was then resuspended in water by vigorous vortexing, lysed and purified by the addition of an equal volume of saturated phenol, and precipitated in the presence of 100% ethanol. The purified M13 DNA was then washed with 70% ethanol, dried in a vacuum desiccator and dissolved in 20µl of water. 5µl of this was used for each sequencing reaction.

2.25 Sequence analysis of recombinant M13 clones.

5µl of single stranded DNA template (method 2.24) was annealed to 3ng of commercial oligonucleotide primer (Bio-rad) in 40mM Tris.HCl pH7.5, 25mM $MgCl_2$, 50mM NaCl in a total volume of 10µl at 55-60°C for 10 minutes in a 1.5ml sarsted tube and left at room temperature for 15-30 minutes. The templates were then labelled and extended in a buffer containing 0.75µM dCTP, 0.75µM dGTP, 0.75µM dTTP, 10mM DTT, 10µCi ^{35}S dATP, and 2 units of klenow polymerase in a total volume of 60µl. This was added to each primer annealed template (6µl in each sample) and labelling was carried out at room temperature for 5 minutes. The extended templates were then terminated by the addition of ddNTP termination mix (figure 2.1). The plate contents were mixed by centrifugation in a Beckman benchtop centrifuge and the reactions carried out at 37°C for 10-30 minutes. The reaction was stopped by the addition of 2µl of formamide dye mix to each well (95% formamide, 20mM EDTA, 0.5% (w/v) bromophenol blue, 0.5% (w/v) xylene cyanol). The samples were then boiled for 1 minute and electrophoresed through a polyacrylamide gel (method 2.26).

2.26 Electrophoresis and autoradiography of sequencing gels.

Electrophoresis was carried out through vertical gels 42x34x0.04 cm in size. Spacers and gel combs were obtained from Gibco-BRL. Both plates were treated with repelcote enabling the gel to be transferred to Whatman 3mm chromatography paper to be dried down after electrophoresis under vacuum. Dried gels were then exposed to XS-1 film (35x43 cm) and developed using an X-omat processor.

Figure 2.1

a. The concentration of nucleotides used to make the termination mixes. b. 2.5µl of each termination mix is added to the correct well, that is; 2.5µl of termination mix A is added to A1-A10, 2.5µl of G to G1-G10, and so on. To this is added 3µl of each extended template, that is; 3µl of sample 1 to each of wells 1A-1T, 3µl of sample 2 to 2A-2T etc.

a.

Component	Termination mix (ul)			
dNTPs (10mM)	**A**	**C**	**G**	**T**
dTTp	12.5	12.5	12.5 1.25	
dCTP	12.5	1.25	12.5	12.5
dGTP	12.5	12.5	1.25	12.5
dATP	1.25	12.5	12.5	12.5
ddNTPs (5mM)	**A**	**C**	**G**	**T**
ddTTP	-	-	-	50
ddCTP	-	10	-	-
ddGTP	-	-	15	-
ddATP	30	-	-	-
H_2O	431.5	451.25	446.25	411.25

b.

1 **2** **3** **etc. 10**

A

C

G

T

2.27 The formation of gradient sequencing gels.

Electrophoresis was carried out through TBE gradient gels (Biggin *et al*, 1983). In this system a buffer gradient is produced in the gel with 0.5xTBE in the top and 5xTBE in the bottom of the gel. This was achieved using two acrylamide gel mixes. The top mix was of 0.5xTBE, 6% acrylamide and 9M urea. The bottom mix was composed of 5xTBE, 6% acrylamide, 9M urea, 5% sucrose (w/v) and 0.1% bromophenol blue (w/v) to visualise the gradient. When preparing a 42x33x0.04 gel, ammonium persulphate and TEMED were added to both the top (0.016% and 0.16% respectively) and bottom (0.02% and 0.2% respectively) gel mixes. 45ml of top mix was drawn into a 50ml syringe followed by 15ml of bottom mix. A few bubbles were passed through to allow some mixing. The syringe contents were slowly expelled between plates to the bottom. The gel comb was inserted into the gel plain side down and the gel rested in a near horizontal position until polymerisation was complete. The tape was removed from the bottom of the gel, and the plates set up with 1xTBE in the gel kit. After removing the comb, the space was filled with 1xTBE, the comb was reversed to form wells and the boiled DNA samples loaded. The gel was run at a constant speed of 70W for 2 hours.

2.28 Filling in 5' overhangs with klenow polymerase.

Restriction enzyme digested DNA containing 5' single strand extensions was extracted once with phenol: choroform (1:1), ethanol precipitated, washed in 70% ethanol, dried and redissolved in water at 1µg/10µl. 1µg of DNA was incubated in the presence of 1xNT buffer (50mM Tris. HCl pH7.8, 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 10µg/ml BSA) containing 0.2mM of the cold nucleotide triphosphates required to fill the overhang,

and 2 units of klenow polymerase at 37°C for 1 hour. The reaction was stopped by extraction with phenol:chloroform (1:1). If labelled DNA was required, one of the cold nucleotide triphosphates was replaced by 20μCi of the corresponding α-^{32}P labelled nucleotide. The labelled DNA was purified by agarose gel electrophoresis (methods 2.9 and 2.13).

2.29 Preparation of cytoplasmic and nuclear RNA.

Immediate early RNA was prepared as follows: BHK21 C13 monolayers were maintained in ETC10% until confluent and treated with 100μg/ml cycloheximide in ETC10% both 15 minutes prior to and continually throughout infection. Roller bottles were infected with 20 pfu/cell of virus, and allowed to adsorb at 37°C for 1 hour, The cells were then washed twice with ETC10%/ cycloheximide and maintained in that media for a further 7 hours.

Early and late RNA was made in BHK21 C13 cells again in roller bottles. In both cases, 10 pfu/cell of virus was adsorbed at 37°C for 1 hour. Early RNA was harvested 7 hours post-infection and late RNA was harvested 16 hours post-infection.

2.30 Purification and harvesting cytoplasmic and nuclear RNA.

Immediate-early, early and late cytoplasmic and nuclear RNA were prepared from roller bottles by a modification of a method by Kumar and Lindberg, (1972). Following removal of the medium, cells were washed with 30ml of cold PBS and harvested into 10ml of cold PBS. Cells were recovered by centrifugation in the cold at 2000 rpm for 2 minutes. The pellet was then washed with a further 10ml cold PBS and centrifugation was repeated. Cells were carefully resuspended in 1.5ml of cold isotonic lysis buffer (ILB-150mM NaCl, 1.5mM $MgCl_2$, 10mM

Tris.HCl pH 7.8, 0.65% (v/v) NP40), left on ice for a further 5 minutes and centrifuged at 3000 rpm for 10 minutes to pellet the nuclei. Following the addition of an equal volume of phenol extraction buffer (PEB-7.0M urea, 350mM NaCl, 10mM EDTA, 10mM Tris.HCl pH 7.9, 1% (w/v) SDS) to the cytoplasmic fraction and the addition of 3ml PEB to the nuclear fraction, both fractions were extracted three times with phenol:chloroform (1:1) and once with chloroform. Both RNA fractions were precipitated by the addition of 3 volumes of ethanol and left overnight at -20°C. RNA was recovered by centrifugation at 3000rpm for 15 minutes, dissolved in 200µl of water and re-ethanol precipitated by the addition of 20µl 5MNH_4Ac, 700µl ethanol. The RNA was then pelleted, dried and dissolved in 30µl of water. The concentration was then determined by spectrophotometry.

1 OD unit$_{260}$= 40µg RNA/ml.

2.31 S1 nuclease mapping.

This method is an adaptation of that used by Whitton *et al*, (1983). >300 counts per second of an end labelled probe (method 2.28) was added to 15µg of RNA (method 2.30) and vacuum dried. The samples were then resuspended in 20µl of hybridization buffer [90% (v/v) deionised formamide, 0.4M NaCl, 40mM PIPES, 1mM EDTA] and denatured by boiling for 3 minutes, quickly transferring to a water bath at 57°C and incubating for 16 hours to allow the formation of DNA/RNA hybrids. Prior to nuclease S1 digestion, the samples were quenched on ice. 200µl of a solution containing 0.25mM NaCl, 30mM NaAc pH4.5, 1mM $ZnSO_4$ and 150 units of the enzyme S1 nuclease was added, and S1 nuclease digestion of regions of single stranded DNA and RNA was carried out at 30°C for 2 hours. The RNA/DNA duplexes were then

purified once with phenol: chloroform (1:1) and once with chloroform, precipitated in the presence of carrier tRNA and ethanol, washed with 70% ethanol, vacuum dried and resuspended in 5µl of sequencing dye mix (method 2.25), denatured and electrophoresed through a top sequencing gel (method 2.26) until the xylene cyanol dye mix was about half way down the gel. The gel was dried in the same maner as a sequencing gel and exposed to XS-1 Kodak film.

2.32 Purification of immediate-early, early and late polypeptides.

Immediate-early polypeptides were prepared in the following manner: Confluent HFL or BHK21 C13 were incubated in Emet/$5F_2$ or Emet/$5C_2$ (depending on which cell line was used) containing 100µg/ml cycloheximide for 15 minutes at 38.5°C. 50 pfu/cell of virus was adsorbed onto the cells for 1 hour at 38.5°C, then the monolayer was washed twice with the appropriate medium containing cycloheximide and maintained in the same medium for a further 5 hours. 15 minutes prior to the end of the 5 hours, the media was replaced with the appropriate media containing 2.5 µg/ml actinomycin D and maintained at 38.5°C until the 5 hour time point was reached. The media was then removed and the monolayer washed at 37°C 4 times, 1 minute each wash with PBS containing 2.5µg/ml actinomycin D which had been prewarmed to 37°C. The monolayer was then incubated at 38.5°C in the presence of PBS containing 50µCi/ml ^{35}S methionine for 2 hours. Then the monolayer was washed twice with PBS and harvested into 200-300µl of boiling mix [1ml stacking gel buffer (method 2.33),1ml glycerol, 0.5ml ß-mercaptoethanol, 20µl bromophenol blue, 0.8ml 25% SDS- the working solution is a 1/3 dilution of this].

Early polypeptides were made in BHK21 C13 cells. Confluent cells which have been grown in ETC10% were adsorbed with 20pfu/cell virus and incubated at 37°C for 1 hour. The monolayer was washed twice with Emet/5C_2 and incubated in this medium for 1 hour at 37°C. The medium was then replaced with the same medium containing 10μCi/ml ^{35}S methionine. After a further 5 hours at 37°C, the monolayer was washed twice with PBS and harvested into 200-300μl of boiling mix.

Late polypeptides were made in BHK21 C13 cells. 20pfu/cell of virus was adsorbed onto the monolayer for 1 hour at 37°C in Emet/5C_2. Next, the medium was replaced with the same medium containing 10μCi/ml ^{35}S methionine and incubated for a further 12 hours at 37°C. The monolayer was then washed twice with PBS and harvested into 200-300μl of boiling mix.

2.33 Analysis of polypeptides by SDS-PAGE.

This is the method of Marsden *et al*, (1978). Slab gels were cast vertically in a sandwich consisting of two glass plates separated by 1mm thick perspex spacers and sealed with teflon tape. Single concentration gels containing 7.5% acrylamide cross-linked with 2.5% N,N'-methylene bisacrylamide in resolving gel buffer (375mM Tris.HCl pH8.9, 0.1% [w/v] SDS) and polymerised with ammonium persulphate (0.006% [w/v]) and 0.004% TEMED were poured between the two glass plates and overlaid with butan-2-ol in order to ensure a smooth surface after polymerisation. Prior to addition of stacking gel, the butan-2-ol was washed off with deionised water. The stacking gel contained 5% acrylamide (cross-linked with the same ratio of N,N'-methylene bisacrylamide used in the resolving gel) in stacking gel buffer (0.11mM Tris.HCl pH 6.7, 0.1% [w/v] SDS), and was polymerised with ammonium

persulphate and TEMED as above. After the stacking gel was poured, a teflon coated comb was inserted to form the wells. Samples were prepared for analysis by boiling for 5 minutes and loading directly onto the gels which were electrophoresed in running gel buffer (52mM Tris base, 53mM glycine, 0.1% [w/v] SDS) at either 60mA for 3-4 hours or 10-15mA for 16 hours (Marsden *et al*, 1976, 1978). Following electrophoresis, the gels were fixed or stained for 1 hour in a solution of methanol: acetic acid: water 53:7:50, in the presence or absence of Coomassie brilliant blue, and destained for 3x30 minutes in methanol: acetic acid: water 5:7:88. The gels were then dried under vacuum and exposed for autoradiography at room temperature.

2.34 Analysis of proteins by Western blotting.

The Western blotting technique is that used by Towbin *et al.*, (1979) with several modifications. Cells were harvested into boiling mix at a concentration of 10^7cells/ml (method 2.32). Samples were boiled for 5 minutes and loaded onto a 7.5% SDS- polyacrylamide gel at approximately 10^6 cell equivalents per well. Following electrophoretic separation at 10mA overnight, proteins were then transferred to nitrocellulose using a Bio-rad transblot apparatus. Three foam pads and two sheets of Watman No 182 filter paper were presoaked in transfer buffer (192mM glycine, 25mM Tris.HCl pH 8.3, 20% methanol) along with the nitrocellulose sheets to be used for the transfer. The gel was layered down on one sheet of the filter paper on top of two of the presoaked foam pads and then covered with nitrocellulose. This assemblage was rolled with a glass rod to exclude air bubbles. The nitrocellulose was then covered with the other sheet of filter paper and a further foam pad and the plastic folder was closed tightly over the

sandwich. The sandwich was placed in the transfer tank with the gel towards the cathode and the nitrocellulose towards the anode. Proteins were transferred onto the nitrocellulose electrophoretically in transfer buffer at 250mA for a minimum of 3 hours.

Following electrophoretic transfer, nitrocellulose was removed from the sandwich and incubated for 2x30 minutes with shaking at 30°C in blocking buffer (20mM Tris.HCl, 500mM NaCl pH 7.5, 3% gelatin). Sheets were then washed 2x5 minutes in wash buffer (TBS: 20mM Tris, 500mM NaCl ph 7.5, 0.5% Tween 20). Antibody were made up to the desired concentrations in wash buffer containing 1% BSA and 0.01% sodium azide and added to the nitrocellulose in plastic dishes and incubated overnight at room temperature with shaking. Antisera was removed with two washes in wash buffer. Bound antibody was visualised using the enzyme horseradish peroxidase. Goat anti-rabbit conjugated horse radish peroxidase was diluted 1/1000 in wash buffer containing 1% gelatin and incubated with the nitrocellulose filter for 1 hour at room temperature with shaking. The membrane was washed twice with wash buffer and twice with wash buffer without Tween, prior to colour reaction. The colour development solution was prepared from solution A and solution B. Solution A consists of 60mg HRP colour development reagent in 20ml ice cold high grade methanol. This should be protected from light and made fresh daily. Solution B was made immediately prior to use by adding 60µl of ice cold hydrogen peroxide (H_2O_2) to 100ml TBS at room temperature. Solution A and B are then mixed and added to the nitrocellulose membrane. The membrane should not be in colour development solution for longer than 40 minutes. After developing, the colour development solution was removed by washing the membrane with water.

2.35 Particle counts.

5µl sodium silicon tungstate, 5µl of a known concentration of laytex beads and 5µl of virus sample were mixed. 2-3 drops of the mixture were added to a support grid, left for 5 minutes and the excess liquid drained with blotting paper. Particles and laytex bead were visualised by electron microscopy. Corresponding fields of vision were then counted. By comparing the number of particles to the number of laytex beads, which are of a known concentration, the number of virus particles/ml can be estimated.

2.36 Virus growth properties.

One step growth experiments were carried out essentially as described by Dargan & Subak-Sharpe, (1985). Confluent BHK21 C13 cells monolayers in 35mm petri dishes were infected at a moi of 5 pfu/cell and virus adsorbed at 37°C for 1 hour. Following two washes with PBS/calf serum, the cells were overlaid with 2mls ETC10% and incubated at 37°C. Samples were harvested at 0, 2, 4, 6, 8, 10, 12 and 24 hours post infection. Virus was released by ultrasonic disruption and titrated (method 2.3) and the titre expressed as pfu/10^6 cells. (This is equivalent to pfu/ml since $2x10^6$ cells were harvested into 2mls of medium). Long term virus growth experiments involved infecting cells at a multiplicity of 0.001pfu/cell. Virus was absorbed for 1 hour at 37°C. Following two washes with PBS/calf serum, the cells were overlaid with 2ml ETC10% and incubated at 37°C. Samples were harvested at 0, 6, 12, 24, 36, 48 and 72 hours post infection and treated as for one step growth experiment.

2.37 Procedure for LD50 *in vivo* virulence experiments.

Three week old male Balb/C mice (Bantin and Kingman) were anaesthetized with ether and inoculated with 25µl of the appropriate virus, which had been diluted in PBS/calf serum, into the central region of the left cerebral hemisphere. Groups of 4 mice were inoculated with a single dilution of each virus stock (between 10^2 and 10^7 pfu/animal). The virus stocks were retitrated on BHK21 C13 cells on the day of inoculation to determine the precise quantity of virus inoculated. Mice were observed daily for 14 days post inoculation and their clinical states recorded. The 50% lethal dose LD50 was then calculated.

2.38 Procedure for *in vivo* latency studies.

Four week old male Balb/C mice (Bantin and Kingman) were inoculated into the right rear footpad as described previously (Clements and Subak-Sharpe,1983,1988). At the time of inoculation the virus was titrated on BHK21 C13 cells to quantify the precise dose administered. A series of 10-fold dilutions of the virus were made up and inoculated into the right rear footpad and mice were examined daily and scored for clinical symptoms. Mice surviving 6 weeks were examined for the presence of latent virus. The mice were killed, dissected, and the two lower thoracic, six lumbar and the upper two sacral ganglia were removed from the inoculated side, placed in culture medium and screened for the release of infectious virus every two days by transferring the culture medium to control BHK21 C13 cells. The inoculated BHK21 C13 cells were incubated at 37°C for 2 days before examining for the presence of virus plaques or cytopathic effect and were then stained.

CHAPTER 3

RESULTS.

3.1. INITIAL CHARACTERIZATION OF 1703.

1703 was partially characterized after isolation (MacLean & Brown, 1987a). A brief summary of this analysis is presented below (sections 3.1.1 and 3.1.2).

3.1.1. The DNA profile of 1703.

1703 DNA was labelled *in vivo* and prepared and analysed by the method of Lonsdale, (1979). Figure 3.1 shows *Hpa*1 and *Bam*H 1 restriction enzyme maps of the prototype orientation of HSV-1 17^+ DNA. A *Hpa*1 restriction enzyme analysis of 1703 DNA compared to 17^+ DNA gave the following information (figure 3.2). Two new bands are present in the 1703 track, one (0.25M) of $11x10^6$ mol. wt. running with *b* and one (0.5M) of approximately $1.7x10^6$ mol. wt. running below *s* . *Hpa*1v is absent. This indicates that *Hpa*1m has a $1.8x10^6$ mol. wt. deletion located within IR_L sequences which generates a novel 0.5M band of $1.7x10^6$ mol. wt. (designated *m'*), whereas *Hpa*1m generated from TR_L is unaltered and migrates normally. Thus joint fragments containing *m*, usually 0.5M, will now consist of undeleted 0.25M copies, *a* and *d*. *a'* would run as the novel 0.25M $11x10^6$ mol. wt. band comigrating with *b*, while the *d'* band would run at about $7.2x10^6$ mol. wt. comigrating with *g*/*h* and is therefore not detected. As *Hpa*1v is absent and *Hpa*1m is deleted, the *Hpa*1r fragment is also expected to be absent. The 2M *q*/*r* band is reduced in comparison to the 1703 2M *o*/*p* band and the

Figure 3.1 *Hpa*1 and *Bam*H1 maps of HSV-1.

*Hpa*1 and *Bam*H1 restriction enzyme maps of HSV-1 17^+ DNA (Wilkie, 1973; Davison,1981).

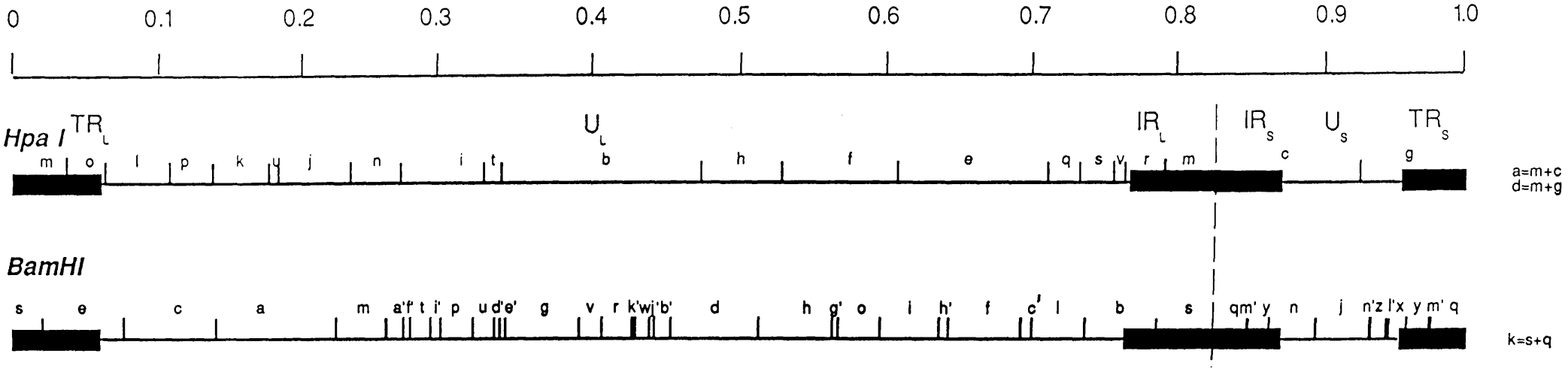
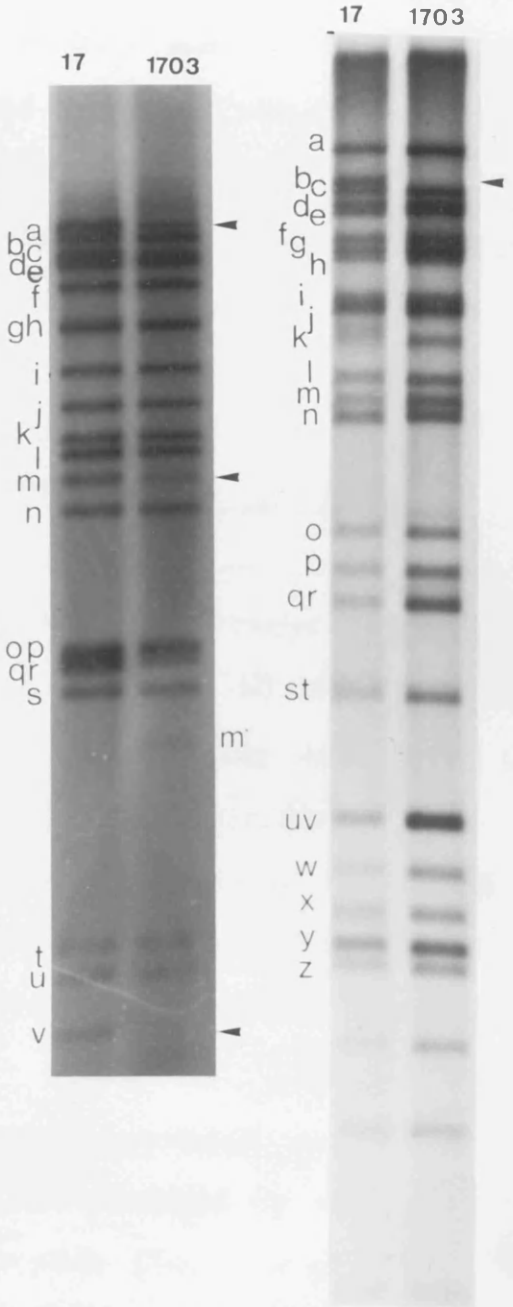

0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Hpa I
TR L
U L
IR L
IR S
U S
TR S
m o l p k u j n i t b h f e q s v r m c g
a=m+c
d=m+g
BamHI
s e c a m a' f' t i' p u d' e' g v r k' w j' b' d h g' o i h' f c' l b s qm' y n j n' z l' x y m' q
k=s+q

Figure 3.2 Analysis of 17^+ and 1703 DNA.

17^+ and 1703 DNA was *in vivo* ^{32}P labelled and digested with *Hpa*1 or *Bam*H1 (0.8% gels). Missing or reduced bands are marked and novel bands are indicated with the letter of the band from which they are derived and a prime symbol (').


17
1703
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
m'
17
1703
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
qr
st
uv
w
x
y
z

*although a band runs in the Vmw63 position in the 1703 track. This appears to be a cellular band.

corresponding band in 17^+. On a *Bam* H1 digest (figure 3.2), the profile of 1703 again differed from that of 17^+. The *Bam*H1b band ($6.7x10^6$ mol. wt.) is absent and a *b'* band now comigrates with *s/t* ($1.9x10^6$ mol. wt.) indicating a deletion of about $4.8x10^6$ mol.wt. at the U_L/IR_L junction. This data and the data from other restriction enzyme digests and Southern blots (MacLean & Brown, 1987a; MacLean,1988) are consistent with a $4.9x10^6$ mol. wt. deletion in U_L/IR_L.

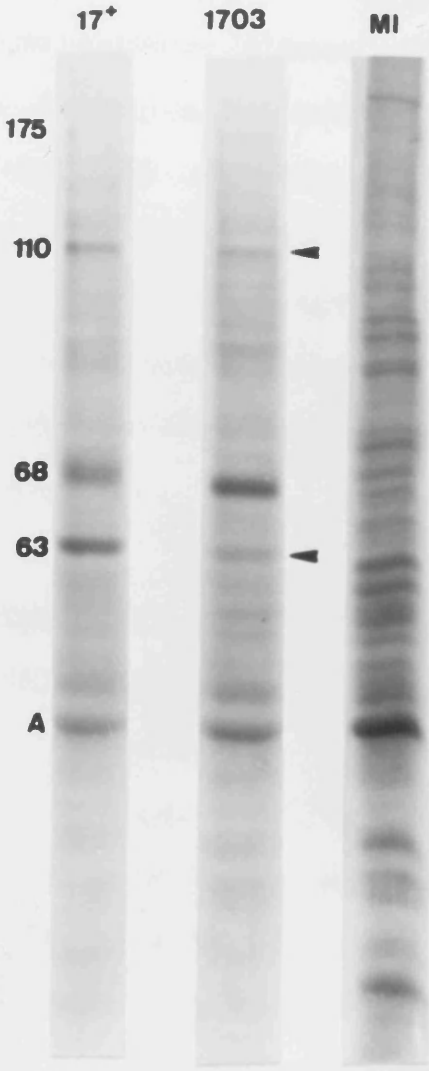
3.1.2. The polypeptide profile of 1703.

The deletion described above results in the removal of the 3' end of the IR_L copies of IE1 and LAT, all of UL56, and the 3' end of UL55 terminating about 500bp (base pairs) downstream of the 3' end of IE2. The immediate-early polypeptide profile of 1703 (figure 3.3) shows a reduction in the amount of Vmw110 synthesized by 1703 due to the deletion of one copy of IE1. Vmw63 was apparently not produced by 1703. This was confirmed by Southern blot analysis of 17^+ and 1703 DNA using *in vivo* ^{32}P labelled 17^+ RNA as a probe (MacLean & Brown, 1987a). Dot blot assays demonstrated that IE2 mRNA produced by 1703 was at most 1/32 of that produced by wild-type. Vmw63 is an essential immediate-early polypeptide (Sacks *et al.*, 1985; McCarthy *et al.*, 1989) and the isolation of a HSV-1 variant that apparently did not produce this protein presented an anomaly.

Thus the initial characterization of 1703 had shown that although the $4.9x10^6$ mol. wt. deletion in U_L/IR_L did not affect IE2, the IE2 gene product Vmw63 was underproduced during immediate-early conditions of infection. A number of possible explanations have been suggested by MacLean & Brown, (1987a) to rationalise this anomalous data: **1.** A secondary mutation in the promoter, open reading frame or termination

Figure 3.3 Polypeptide profiles of 17^+ and 1703.

Immediate-early polypeptide extracts were labelled with ^{35}S methionine and run on 7.5% polyacrylamide gels. Molecular weights ($x10^3$) are indicated on the left and reduced bands are marked. A is actin.


17+
1703
MI
175
110
68
63
A

signals of IE2, **2.** The deletion removed a downstream transactivator of IE2. UL55 and UL56 gene products are both absent as a result of the deletion and neither of these genes has been assigned a function (McGeoch *et al.*, 1988a). There is also the possibility that the deletion removed a downstream enhancer element of IE2, although none has been demonstrated in the region deleted in 1703 and **3.** The levels of Vmw63 synthesized by 1703 under immediate-early conditions were undetectable with the reagents available.

The aims of this project are outlined in section 1.11 of the introduction. The results section reports the experimental work which led to the conclusion that antisense transcripts control the production of Vmw63 in 1703 infected cells.

3.2. CLONING AND SEQUENCING 1703 DNA FRAGMENTS; 1. IN WHICH THE DELETION END POINTS WERE LOCATED AND 2. THE FRAGMENT CONTAINING IE2.

Analysis of 1703 began with the cloning and sequencing of *Hpa*1s in which IE2 is located, in order to detect any deletion/insertion in the promoter, open reading frame or termination signals of the gene. Such a mutation was one of the possible reasons given for the defect in Vmw63 synthesis at immediate-early times. Although restriction enzyme analysis of 1703 DNA had de-limited the end points of the deletion to about 500bp downstream of the 3' end of IE2, it was necessary to find the exact end points in order to determine the genes affected. The deletion end points were located within *Hpa*1m' as shown by restriction enzyme analysis of 1703 ^{32}P *in vivo* labelled DNA (MacLean & Brown, 1987a; MacLean 1988). The cloning and didoxynucleotide sequence analysis of both fragments are detailed in the next two sections.

3.2.1 Sequencing the Vmw63 encoding gene IE2.

1703 DNA was digested with the restriction enzyme *Hpa*1, electrophoresed through a 0.8% TBE agarose gel and the DNA fragment in which IE2 was located, *Hpa*1s excised and purified. *Hpa*1s was then cloned into pUC19 (*Sma*1 digested) and transformed into competent bacterial cells giving rise to recombinant colonies whose DNA was analysed using restriction enzymes. One recombinant plasmid, pUC19/*Hpa*1s, thought to contain *Hpa*1s as an insert, was further analysed by Southern blotting. Figure 3.4 shows these results. The probe used for this experiment was a 70 base oligonucleotide corresponding to a region of 1703 DNA at the 5' end of IE2 (np113714-np113784). In lane 1, the probe detects the *Hpa*1s fragment which had been separated from its plasmid backbone using the restriction enzymes *Eco*R1 and *Hind*III (3251 base pairs). The fragment detected by the oligo in lane 2 corresponds to linearised pUC19/*Hpa*1s and is therefore larger than *Hpa*1s alone (5941 base pairs). These results demonstrate that *Hpa*1s has been cloned into pUC19.

In order to sequence IE2, *Hpa*1s was subcloned into M13 mp8 which was supplied cleaved with *Sma*1. pUC19/*Hpa*1s was also digested with *Sma*1 and the resultant subfragments purified and randomly cloned into M13 mp8. The DNA from recombinant plaques was prepared and sequenced by the Sanger dideoxynucleotide sequencing method. The results from this experiment are outlined by figure 3.5.

The top of the figure 3.5 is a representation of the HSV-1 genome in the prototype orientation and the next line is an expanded representation of *Hpa*1s showing the positions of *Sma*1 restriction enzyme sites within that fragment. Each subfragment was then given a

Figure 3.4 Southern blot analysis of pUC19/*Hpa*1s.

Autoradiograph of a Southern blot in which a ^{32}P *in vitro* labelled 70 base oligonucleotide, corresponding to a region of the 5' end of IE2 (np113714-np113782), was hybridized to *Eco*R1/*Hind*III digested pUC19/*Hpa*1s (lane 1) and *Eco*R1 digested pUC19/*Hpa*1s (lane 2). Numbers indicate the size of specific fragments in base pairs.

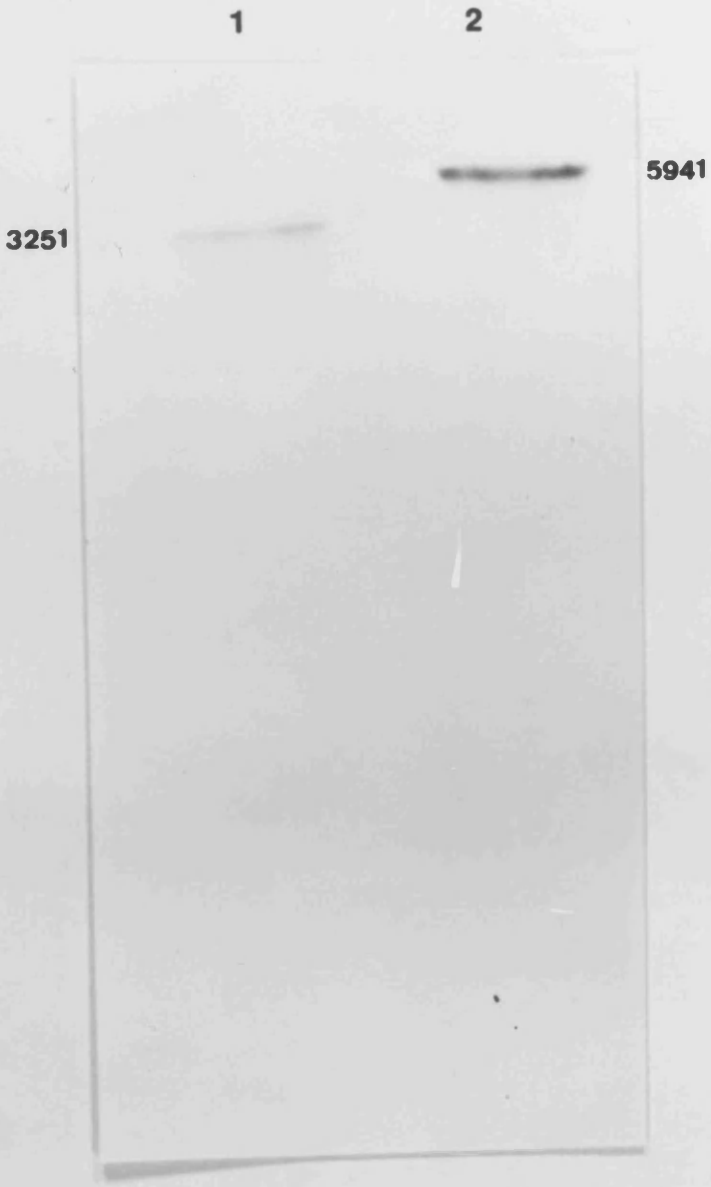
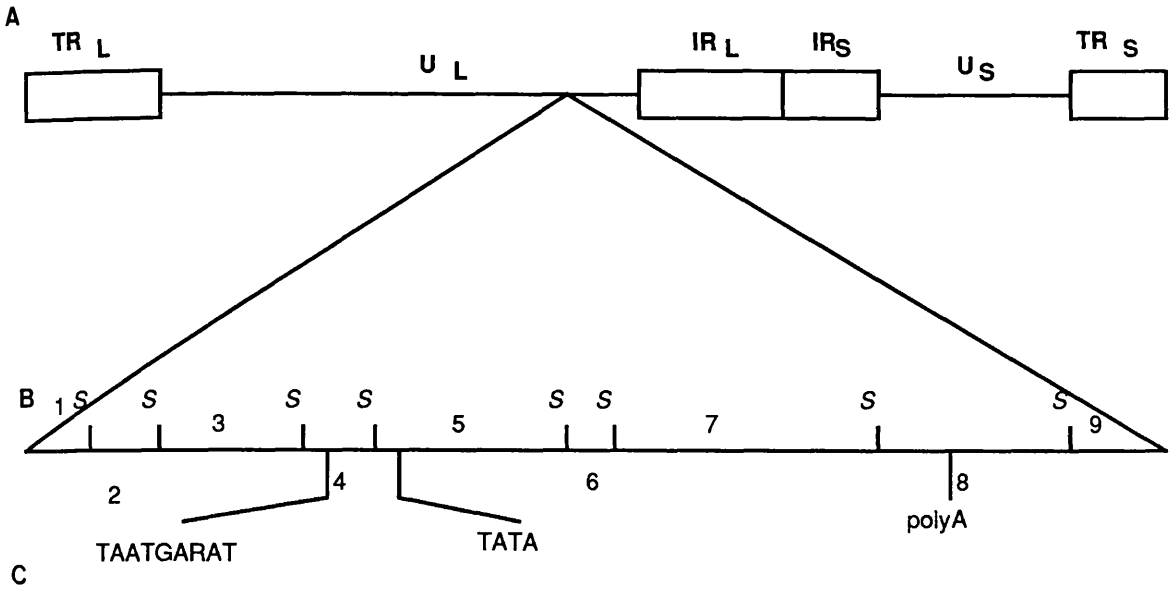

1
2
5941
3251

Figure 3.5 IE2 sequencing results.

Structure of the HSV-1 genome (a) showing U_L and U_S flanked by TR_L/IR_L and IR_S/TR_S respectively. *Hpa*1s is expanded (b) and the *Sma*1 sites contained within (*S*) are indicated as are the approximate locations of the promoter/regulatory regions of IE2. The *Sma*1 fragments are designated 1-9 from left to right. These fragments were subjected to dideoxynucleotide sequence analysis and the results of this analysis are given (c). 'No coding sequences' means that there are no IE2 coding sequences in the subfragment, 'intact' means that the sequence is homologous to the published wild-type sequence (McGeoch *et al.*, 1988a).



C

Fragment no.	Size	Comment
1	12511 → 12896 385bp	no coding sequences.
2	12896 → 13035 139bp	no coding sequences.
3	13035 → 13403 368bp	no coding sequences. intact.
4	13403 → 13517 114bp	promoter sequences. intact.
5	13517 → 14192 675bp	promoter associated sequences. 13517-13690 intact.
6	14192 → 14267 75bp	intragenic region. intact.
7	14267 → 15128 861bp	intragenic region. 14267-14497 intact.
8	15128 → 15472 344bp	poly A signal. intact.
9	15472 → 15703 231bp	no coding sequences. intact.

number 1-9 in a 5' to 3' direction and the sequencing status of each is detailed. Fragment 4, 5 and 8 contain the promoter, promoter associated and termination signals of IE2 respectively and sequencing has shown that each is homologous to the published wild-type sequence (McGeoch *et al.*,1988a). The IE2 intragenic region has been partially sequenced and was shown to be free from alteration compared to the wild-type sequence (fragments 5, 6, 7 and 8). Fragments 2 and 9 are upstream and downstream of IE2 coding sequences respectively and since both are intact, demonstrate that any IE2 promoter or termination signals 139bp upstream or 231bp downstream respectively of the gene are homologous to the wild-type sequence.

Thus the sequencing of IE2 and the surrounding DNA has conclusively demonstrated that the promoter, promoter associated, termination, termination associated sequences and most of the open reading frame are homologous to the wild-type sequence, showing that the apparent lack of Vmw63 under immediate-early conditions is not due to a secondary mutation within the IE2 gene.

3.2.2. Sequencing the end points of the deletion in 1703.

The restriction enzyme fragment in which the end points of the deletion in 1703 were located is *Hpa*1m'. 1703 DNA was cleaved with the restriction enzyme *Hpa*1, the resultant fragments separated by electrophoresis through a 0.8% agarose/TBE gel, the *m'* subfragment excised, purified and ligated to pUC19 which had been cleaved at the *Sma*1 site. The ligation mixture was then transformed into competent XL-1 cells, the plasmid DNA of recombinant colonies purified and the presence of *m'* within pUC19 detected by restriction enzyme analysis and Southern blotting.

Restriction enzyme analysis of a potential pUC19/*Hpa*1m' recombinant with *Eco*R1 and *Hind*III gave two distinct bands, one corresponding to the plasmid backbone (2686 base pairs), and one which was slightly bigger, the *Hpa*1m' insert (approximately 3000 base pairs). Figure 3.6 is an autoradiograph of the Southern blot analysis of 1703 DNA *Hpa*1 digested and electrophoresed through a 0.8% agarose/TBE gel. The probe, pUC19/*Hpa*1m' had been ^{32}P labelled *in vitro* and hybridized to *Hpa*1m', *Hpa*1m, the associated joint fragment *a, d, a'* and *d'* and all of these fragments plus or minus extra 'a' sequences. The lanes on either side of the 1703 track are kilobase markers to aid the sizing of the fragments on the 1703/*Hpa*1 track.

*Hpa*1m' appeared to be a fragment of about 3000bp and, although the end points of the deletion were thought to be near one end of the fragment, it was decided that the fragment was too big to be directly cloned into M13 mp8. Thus a large scale stock of pUC19/*Hpa*1m' was grown, a portion of this was digested with *Sma*1 and the resultant fragments randomly cloned into M13 mp8. The M13 mp8 DNA from recombinant plaques was prepared and sequenced by the Sanger dideoxynucleotide sequencing method.

The sequencing profile of a clone in which the end points of the deletion are located is shown in figure 3.7. The sequence 'CGG' appears to be common to both deletion end points. Figure 3.8 demonstrates the end points of the deletion diagrammatically. The total length of the deletion is 7784bp, and the region deleted is between np (nucleotide position) 123623 and np 115839 removing UL56, 343bp of the 3' end of UL55, leaving 555bp between the deletion end point and the 3' end of UL54 (IE2). This demonstrates that the genes affected by the deletion are UL55, UL56 and one copy of IE1 and correspondingly, one copy of LAT. Neither of the two UL genes has been assigned a function (McGeoch *et*

Figure 3.6 Southern blot analysis of pUC19/*Hpa*1m'.

Autoradiograph of a Southern blot in which random primed ^{32}P *in vitro* labelled pUC19/*Hpa*1m' was hybridized to *Hpa*1 digested 1703 DNA (0.8% gel). The fragments detected are indicated as are the sizes of marker DNA (M) ($x10^3$ bp).

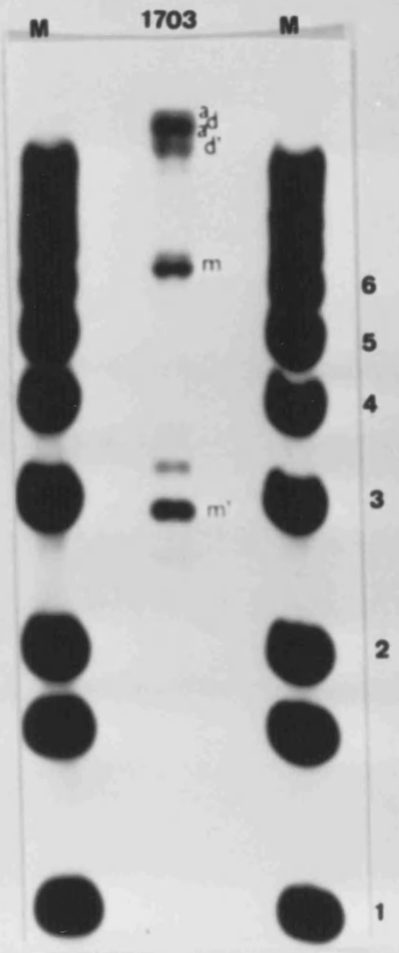

M
1703
M
d
d'
m
m'
6
5
4
3
2
1

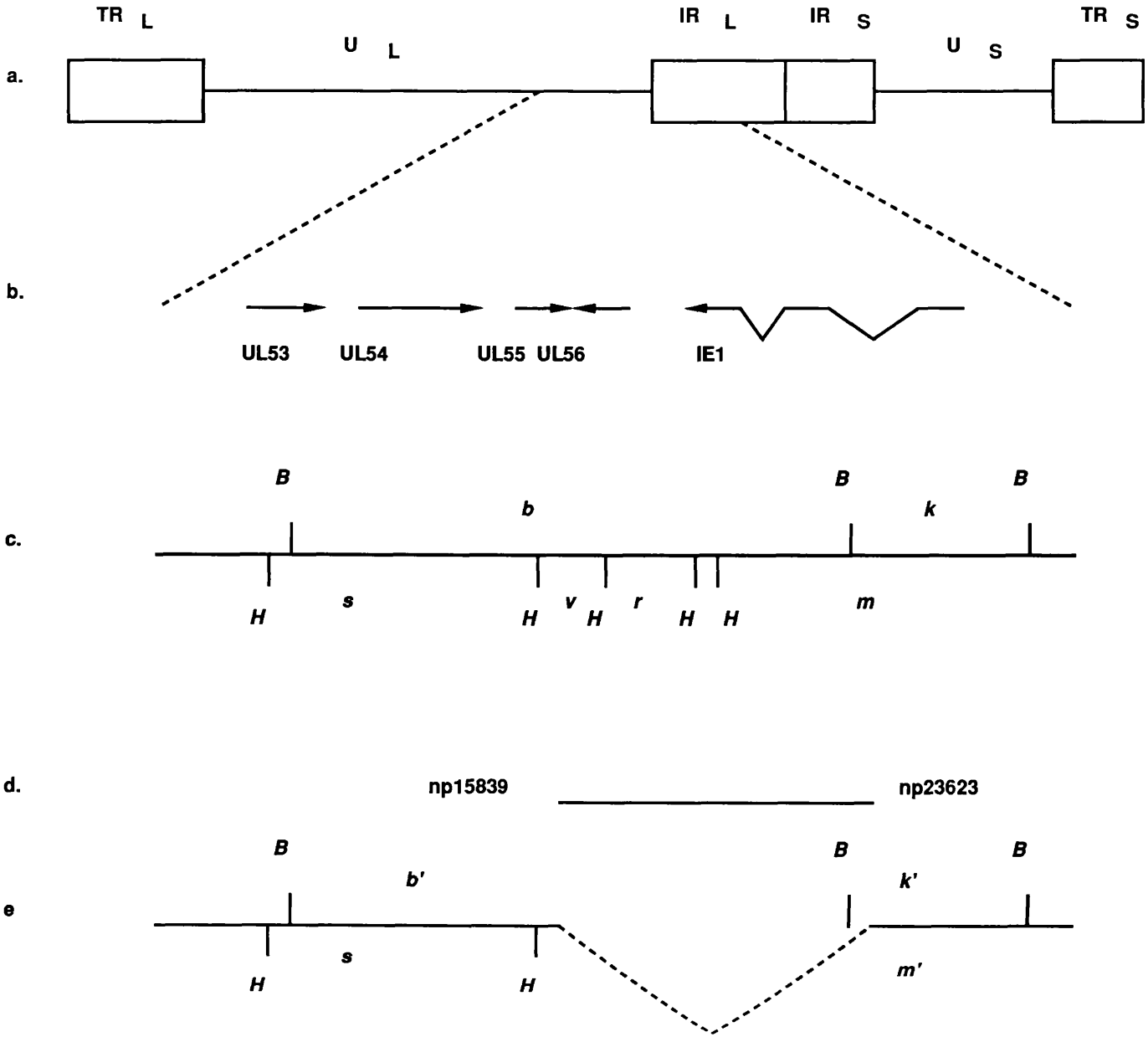
Figure 3.7 The DNA sequence of the 1703 deletion end points.

A portion of an autoradiograph of a sequencing gel showing the deletion in 1703. The deletion is between np23623 and np15839. The sequence 'cgg' is common to both deletion end points.


A C G T
ccccagcggcctgcgat

Figure 3.8 Exact map of the deletion in 1703.

Structure of the HSV-1 genome (a) showing U_L and U_S flanked by TR_L/IR_L and IR_S/TR_S respectively. A portion of U_L/IR_L is expanded and the genes contained within are shown (b). *Bam*H1 (***B***) and *Hpa*1 (***H***) restriction enzyme sites are indicated (c) as is the deletion (d). The resultant 1703 *Hpa*1 (***H***) and *Bam*H1 (***B***) DNA fragments are shown (e).

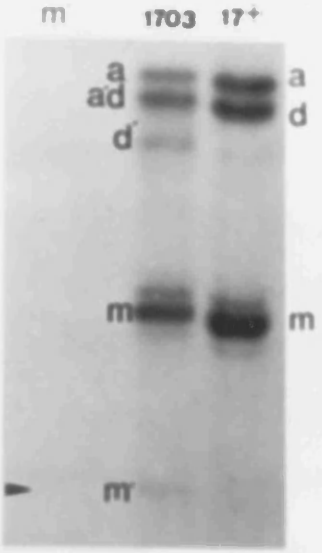

a.
TR L
U L
IR L
IR S
U S
TR S
b.
UL53
UL54
UL55 UL56
IE1
c.
B
b
B
k
B
H
s
H
v
H
r
H
H
m
d.
np15839
np23623
e
B
b'
B
k'
B
H
s
H
m'

al., 1988a) and only one copy of IE1 is required for the virus to grow as wild-type (Harland & Brown, 1985; MacLean & Brown 1987a,b). Since the deletion terminates 555bp downstream of the 3' end of IE2 leaving the 3' termination signals intact, an explanation for the apparent lack of Vmw63 under immediate-early conditions had not yet been established. The data gained from sequencing the end points of the deletion is in agreement with the restriction enzyme analysis (MacLean & Brown, 1987a) in that the *Hpa*1 restriction enzyme fragments affected are *v,r*, and *m* and the partially deleted *v* and *m* fragments together become *m'*.

The sequence analysis indicated that the deletion also removed a *Bam*H1 site which had been previously shown to be present (MacLean & Brown, 1987a). This was confirmed by Southern blot analysis of pUC19/ *Hpa*1m' which had been removed from its plasmid backbone (figure 3.9), 1703 DNA *Hpa*1 cleaved and 17^+ DNA also *Hpa*1 cleaved. The probe was an 30-base oligonucleotide which corresponded to a region between the sequenced end point of the deletion and the apparently deleted *Bam*H1 site (np123495-np123524). The ^{32}P *in vitro* labelled oligonucleotide did not hybridize to the pUC19/ *Hpa*1m' fragment, but did hybridize to the following 1703 DNA fragments: *m'*, *m*, *a*, *b*, *a'* and *b'* and the *m*, *a*, and *b* fragments of 17^+ indicating that the deletion in the pUC19/*Hpa*1m' plasmid was not identical to that in 1703. Since the small scale preparation of pUC19/*Hpa*1m' was cleaved with *Bam*H1 in order to find its orientation in pUC19 (section 3.7.3) it is probable that the deletion was extended during the large scale preparation of plasmid DNA due to the instability of this region in *E.coli* strain XL-1, the host bacteria for the plasmid.

Figure 3.9 Southern blot analysis of pUC19/*Hpa*1m'.

Autoradiograph of a Southern blot in which a ^{32}P *in vitro* labelled 30 base oligonucleotide (np123695-np123524), corresponding to a region of 1703 DNA between the *Bam*H1 site deleted in pUC19/*Hpa*1m' and the sequenced deletion end point of 1703 and hybridized to pUC19/*Hpa*1m' *Eco*R1/*Hind*III digested, 1703 DNA *Hpa*1 digested and 17^+ DNA *Hpa*1 digested. 17^+ and 1703 *Hpa*1 fragments detected by the oligonucleotide are indicated, reduced bands indicated by the letter of the band from which they are derived plus a prime symbol, and missing bands marked.


m
1703
17+
a
ad
d
m
m'
a
d
m

3.3. WESTERN BLOT ANALYSIS OF 17±, 1703 AND MOCK INFECTED POLYPEPTIDES.

HSV protein synthesis in infected cells falls into three categories: **1.** Immediate-early polypeptides which are produced in the absence of prior protein synthesis and are stimulated by the virion tegument protein Vmw65 in the presence of cellular transcription factors including oct-1. There are five HSV-1 immediate-early proteins, IE1, IE2, IE3, IE4 and IE5, the first three of which function to transactivate both themselves and later classes of genes and the last two which are dispensable in tissue culture (for a detailed description of the function of immediate-early polypeptides see section 1.7). **2.** Early genes eg. thymidine kinase and **3.** The late genes. This section of the results describes the detection of the immediate-early polypeptide Vmw63 whose production is affected by the deletion in 1703. By comparing the amount of Vmw63 produced by 1703 to that produced by 17^+ under immediate-early, early and late conditions it was possible to asses the temporal regulation of the protein in 1703 infected cells.

To synthesize immediate-early polypeptides, BHK21 C13 or HFL monolayers were infected with 50pfu/cell of 17^+ or 1703 in the presence of cycloheximide and with 20pfu/ cell in the absence of any inhibitor for early or late polypeptide synthesis. After 7 hours, immediate-early and early polypeptides were harvested into denaturing buffer. Before harvesting, immediate-early polypeptides were translated from the accumulated immediate-early mRNA by washing the infected cell monolayer with PBS containing actinomycin D to remove the cycloheximide and incubating in the presence of actinomycin D to prevent further mRNA synthesis. Late polypeptides were harvested after 16 hours. A mock infected protein preparation was used as a

control. ^{35}S-methionine was incorporated into the polypeptides to facilitate analysis by SDS-PAGE. The antiserum used throughout these experiments was directed against the carboxyterminus of Vmw63 (peptide NATDIDMLIDLGLDLS) and could also detect the HSV-2 Vmw63 equivalent, Vmw65. This was supplied by Dr Howard Marsden.

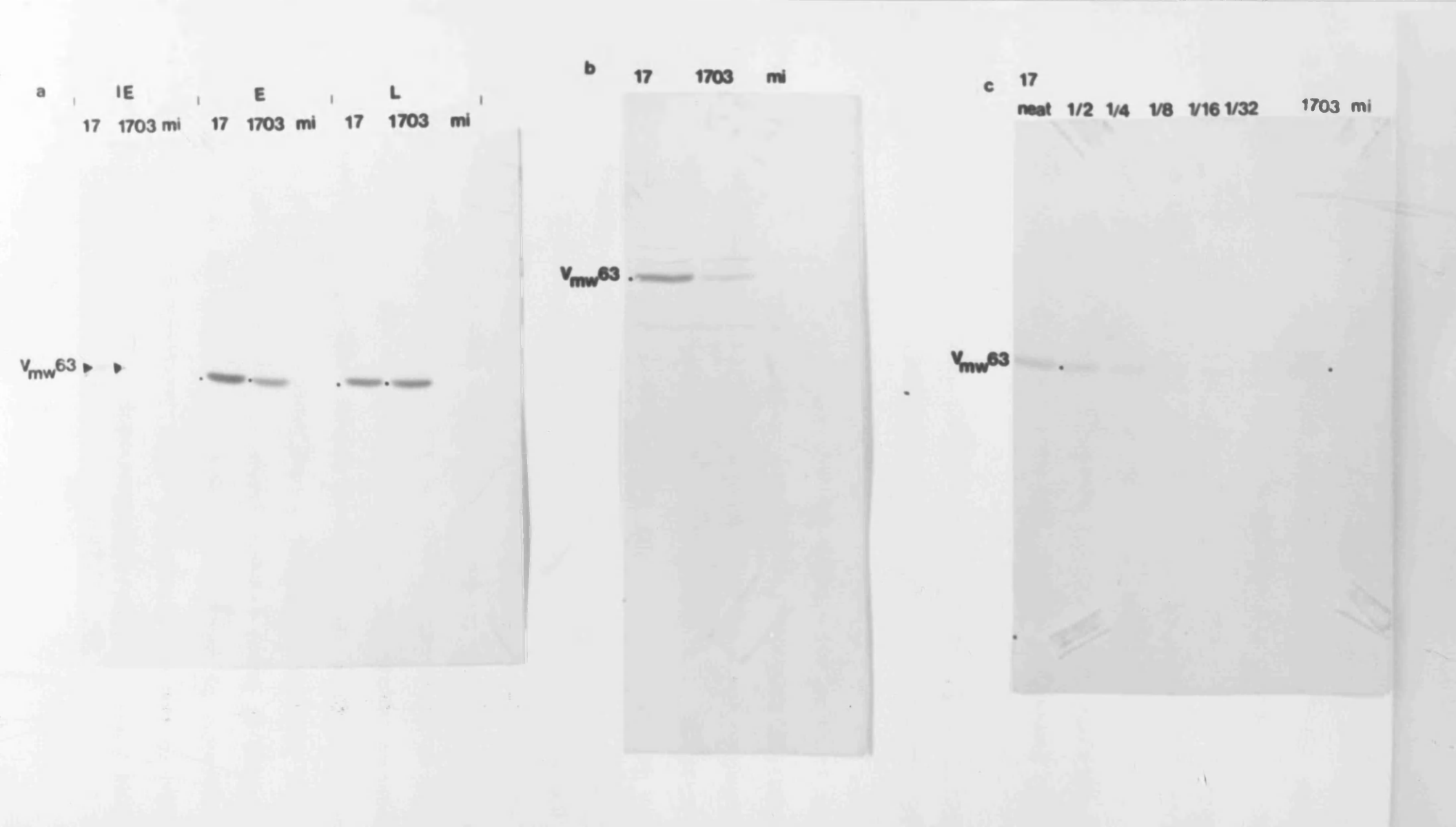
Polypeptides were separated by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with antiserum. Figure 3.10a shows the results of this experiment. Western blot analysis of immediate-early preparations of 17^{+}, 1703 and mock infected polypeptides demonstrate that Vmw63 is produced by wild-type but that the amounts produced are relatively small. Neither mock infected or 1703 lanes show a band that corresponds to Vmw63. Analysis of the early extract demonstrates that Vmw63 was produced by 1703 but that the amounts were about half that of wild-type. Later results (figure 3.18) suggest that Vmw63 production under early conditions is equivalent to wild-type. Late extracts show that Vmw63 production by 1703 is equivalent to wild-type. In both early and late mock infected tracks no band appears in the Vmw63 position indicating that the strong band appearing in the 17^{+} and 1703 lanes is indeed Vmw63.

The low amount of Vmw63 synthesized by 17^{+} at immediate-early times was unacceptable and it was therefore decided to use HFL cells to synthesize immediate-early polypeptides and to carry out Western blots. This was because HFL cells have previously been demonstrated to synthesize immediate-early polypeptides more efficiently than BHK21 C13 cells (MacDonald, 1980). Figure 3.10b shows the result of this experiment. Substantial amounts of Vmw63 are present in the wild-type lane and reduced amounts are demonstrable in the 1703 track. In the mock infected lane there is no band at the Vmw63 position proving that the band in the 17^{+} and 1703 tracks are virus specific. Thus 1703

Figure 3.10 Western blot analysis of 17^+, 1703 and mock infected polypeptide extracts.

a. Immediate-early, early and late extracts of 17^+, 1703 and mock infected BHK21 C13 cells. **b**. Immediate-early extracts of 17^+, 1703 and mock infected HFL cells. **c**. Immediate-early extracts of 17^+, serially diluted in mock infected extract as indicated, 1703 and mock infected HFL cells.

All polypeptides are run on a 7.5% polyacrylamide gel, blotted and hybridized to an antiserum which recognises the carboxyterminus of Vmw63 (peptide sequence NATDIDMLIDLGLDLS) and was supplied by Dr Howard Marsden. The position of Vmw63 is indicated and faint bands are marked.


a
IE
E
L
17 1703 mi 17 1703 mi 17 1703 mi
$V_{mw}63$
b
17 1703 mi
$V_{mw}63$
c
17
neat 1/2 1/4 1/8 1/16 1/32 1703 mi
$V_{mw}63$

produces Vmw63 under immediate-early conditions, albeit in reduced amounts compared to wild-type virus infections. This result supports the suggestion by MacLean & Brown,(1987a) that the reagents available at the time of initial characterization of 1703 were not sensitive enough to detect Vmw63 synthesized during immediate-early times of infection. Vmw63 production by 1703 was further analysed by titrating the 17^+ polypeptide extract in mock infected extract and comparing the intensity of the bands obtained by Western blotting to that produced by 1703. Figure 3.10c is the result of this experiment and demonstrates that the amount of Vmw63 produced by 1703 is approximately 1/8 of that produced by 17^+.

Hence Western blot analysis has conclusively demonstrated that Vmw63 is produced by 1703 under immediate-early conditions at 1/8 the level of that produced by wild-type virus. Exposing the nitrocellulose filter to an autoradiograph and ensuring that each lane demonstrated equivalent loading and band intensity acted as a control for this series of experiments (results not shown).

3.4. S1 NUCLEASE MAPPING OF MRNA PRODUCED BY 1703 UNDER IMMEDIATE-EARLY, EARLY AND LATE CONDITIONS.

To determine whether the impairment of Vmw63 synthesis at immediate-early times was the result of interference with transcription, post-transcription or translation, the synthesis of mRNA by 1703 was analysed. Although originally used to map the 3' and 5' ends of genes, S1 nuclease mapping was the method of choice since it was sensitive enough to detect low abundance transcripts of which IE2 mRNA produced by 1703 under immediate-early conditions was typical. Synthesis and purification of immediate-early, early and late RNA was

carried out as described in the materials and methods section. Two probes were used: pGX51 and pGX55, which detected IE2 (and IE1 mRNA) and IE5 mRNA respectively. The latter was a control for the experiment.

pGX51: is the *Sal*1 fragment of *Bam*H1b cloned into the plasmid pAT153. *Sal*1 digestion gives two fragments, one of about 6000bp corresponding to the HSV-1 fragment, and one of about 3000 bp which is the plasmid backbone. The fragments are end labelled by incorporating a ^{32}P labelled nucleotide. The *Sal*1 fragment is purified by agarose gel electrophoresis, excised, and electroeluted from the gel. At this stage the probe can be used for S1 nuclease mapping. pGX51 has been used previously to map the 3' end of IE2 mRNA (Whitton *et al.*,1983).

pGX55: is the *Sal*1/*Bam*H1 fragment of HSV-1 *Bam*H1z cloned into pAT153. *Bam*H1 digestion of pGX51 gives one fragment of about 6000bp. The fragment is ^{32}P end labelled as described. pGX55 has been previously used to map the 3' end of IE5 (Rixon & Clements, 1982).

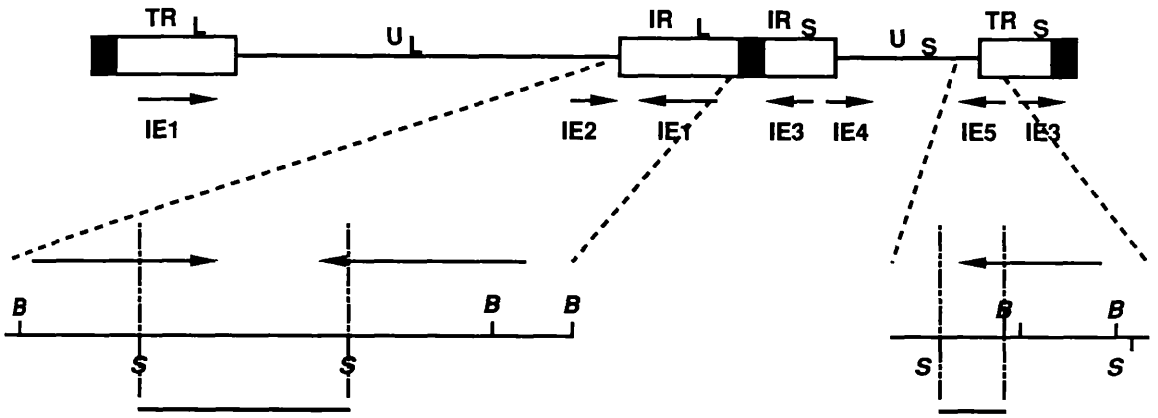
The derivation of both plasmids is outlined by figure 3.11. Figure 3.12 demonstrates the method of S1 nuclease mapping which relies on the fact that the enzyme S1 nuclease digests areas of single stranded RNA and DNA leaving double stranded molecules intact.

3.4.1. S1 nuclease mapping of mRNA synthesized by 1703 under immediate-early conditions.

The result of this experiment is given in figure 3.13. The RNA has been separated into cytoplasmic and nuclear fractions demonstrating that the amount of IE5 mRNA produced by 17^+ and 1703 in both fractions is approximately equivalent, with a slight reduction of intensity in the

Figure 3.11 Map of pGX51 and pGX55.

Structure of the HSV-1 genome showing U_L and U_S flanked by TR_L/IR_L and IR_S/TR_S respectively. Portions of U_L/IR_L and U_S/TR_S are expanded to show the locations of the HSV-1 DNA fragments contained within pGX51 and pGX55.



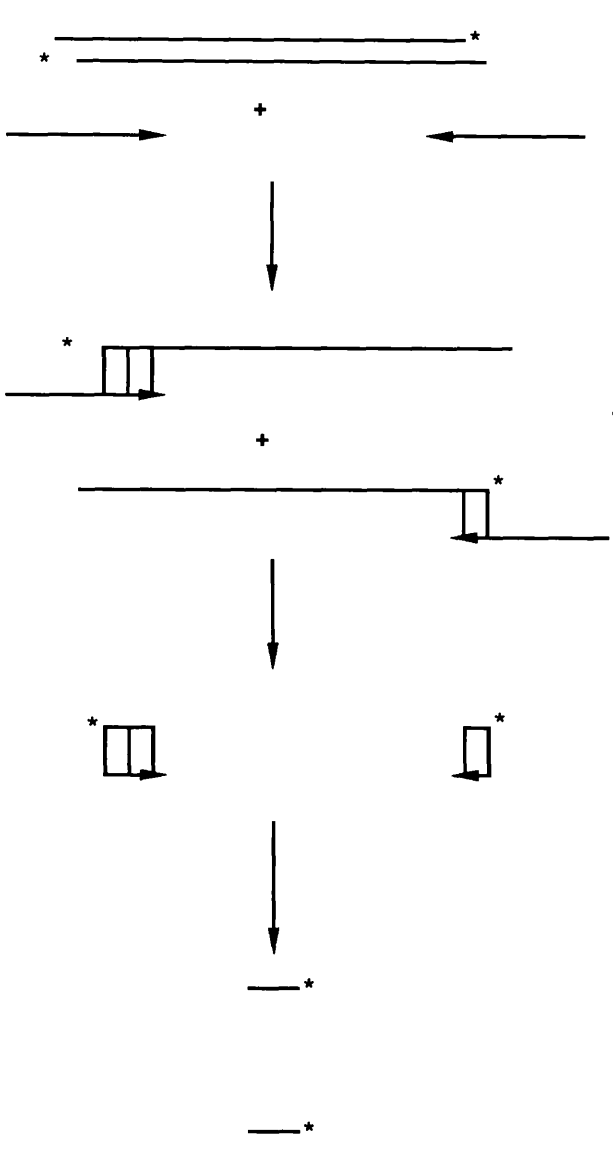
pgx51: a Sal1 digest of BamH1b cloned into pAT153.

pgx55: a Sal1 digest of BamH1z cloned into pAT153.

Figure 3.12 The method of S1 nuclease mapping.

pGX51, ^{32}P *in vitro* end labelled is used to illustrate the S1 nuclease mapping procedure.

* represents the site of end labelling



pGX51 Sal1 digested, 32P-labelled.

HSV-1 mRNA.

Production of RNA/DNA hybrids. (pGX51 hybridises to IE2 and IE1 mRNA).

Protected RNA/DNA hybrids.
(Single-stranded areas of DNA and RNA digested with S1 nuclease).

Hybrids denatured and separated. (790bp for IE2, 240bp for IE1).

Figure 3.13 S1 nuclease map of 17^+ and 1703 immediate-early RNA.

Immediate-early preparations of 17^+, 1703 and mock infected RNA (BHK21 C13 cells) subjected to S1 nuclease mapping procedures. Lanes are labelled along the top of the gel and the size of the resultant fragments (base pairs) indicated. Bands reduced in intensity are marked.

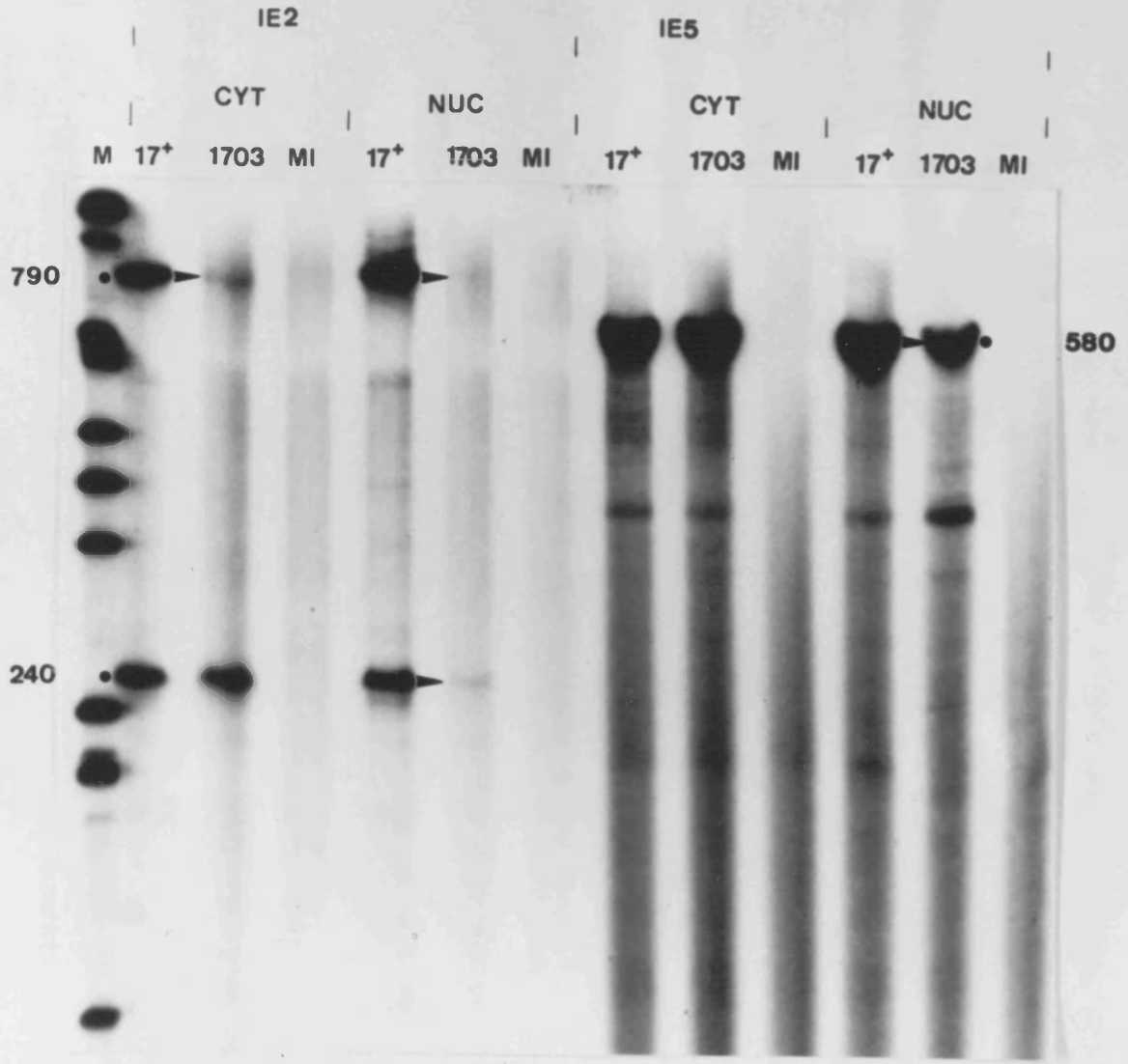
nuc-nuclear

cyt-cytoplasmic

IE2-fragments detected by pGX51

IE5-fragments detected by pGX55

MI-mock infected


IE2
IE5
CYT
NUC
CYT
NUC
M
17+
1703
MI
17+
1703
MI
17+
1703
MI
17+
1703
MI
790
240
580

1703 nuclear IE5 band. Similarly, the amount of IE1 mRNA produced by 17^+ and 1703 in the cytoplasmic fraction is equivalent whereas IE2 production by 1703 is much less than that produced by wild-type virus. This result is consistent with the amount of Vmw63 synthesized by 1703 as shown in Western blotting experiments (section 3.3). However IE1 mRNA synthesis by 17^+ and 1703 is not equivalent in the nuclear fraction. The IE2 mRNA band in the 1703 nuclear track is seen as a slight darkening of the area in which the band would be expected to run, and the IE1 mRNA band in the same track is correspondingly less intense than the 1703 IE1 mRNA band in the cytoplasmic track. The band in the 1703 IE5 nuclear track is also less intense, hence an explanation for the reduced amounts of IE1 mRNA in the 1703 nuclear track could be that the concentration of total RNA in the 1703 nuclear fraction is less than in the other RNA preparations. Thus it would appear that production of IE2 mRNA by 1703 under immediate-early conditions corresponds to the amount of Vmw63 produced by the virus implying that the block in Vmw 63 synthesis occurs at the transcriptional level. This is supported by the low amounts of IE2 mRNA in the 1703 nuclear fraction which implies that only a small amount of IE2 RNA is polyadenylated. The 1703 IE2 mRNA and protein bands run at the same position as the equivalent wild-type bands indicating that an uninterrupted mRNA molecule is synthesized excluding the possibility of a mutation within the IE2 coding region.

3.4.2. S1 nuclease mapping of mRNA synthesized by 1703 under early and late conditions.

At early and late times, the amount of IE2 mRNA produced by 1703 returns to normal, in agreement with the Western blot analysis data.

Figure 3.14 is an S1 map of early RNA. Although not as clear as the immediate-early map, the synthesis of IE1, IE2 and IE5 mRNA in the cytoplasmic and nuclear tracks of 17^+ and 1703 appears to be equivalent. At late times it was obvious that the amount of IE2 mRNA synthesized by 1703 was directly comparable to wild-type (figure 3.15). In all S1 nuclease mapping experiments a mock infected control was incorporated. This consisted of uninfected cells which had gone through an identical process to that of infected cells.

3.5. THE *IN VIVO* CHARACTERIZATION OF 1703.

Vmw63 is an essential immediate-early polypeptide both for *in vitro* virus growth (Sacks *et al.*,1985; McCarthy *et al.*,1989) and the establishment of latency (Leib *et al.*,1989a). It was therefore necessary to examine the neurovirulence and latency characteristics of 1703 to determine if the underproduction of Vmw63 under immediate-early conditions affected these characteristics. As reported by MacLean & Brown, (1987a), 1703, then called X2D, did not express thymidine kinase. For this reason the fragment of X2D in which the deletion end points were located was co-transfected with 17^+ DNA to give a wild type recombinant, 1703 (MacLean, 1988). In order that 1703 could be used in virulence experiments, and as thymidine kinase is a virulence determinant (Field & Wildy, 1978), thymidine kinase production by 1703 was determined.

3.5.1. Thymidine kinase production by 1703.

Table 2.1 gives the results of this experiment. ^3H thymidine is incorporated into thymidine kinase and the results are expressed in

Figure 3.14 S1 nuclease mapping of 17^+ and 1703 early RNA.

Early preparations of 17^+, 1703 and mock infected RNA (BHK21 C13 cells) subjected to S1 nuclease mapping procedures. Lanes are labelled along the top and the resultant protected fragments (base pairs) indicated.

nuc-nuclear

cyt-cytoplasmic

IE2-fragments protected by pGX51

IE5-fragments protected by pGX55

MI-mock infected

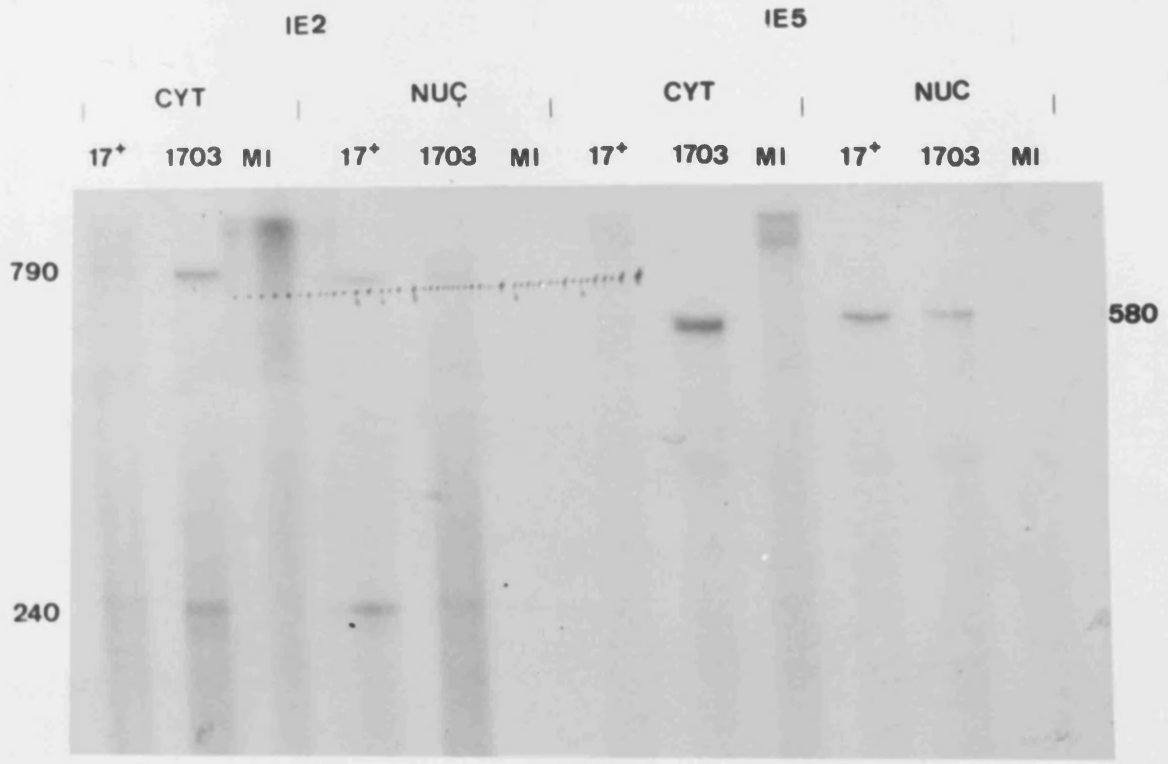

IE2
IE5
CYT
NUÇ
CYT
NUC
17+
1703
MI
17+
1703
MI
17+
1703
MI
17+
1703
MI
790
240
580

Figure 3.15 S1 nuclease map of 17^+ and 1703 late mRNA.

Late preparations of 17^+, 1703 and mock infected cell RNA (BHK21 C13) subjected to S1 nuclease mapping procedures. Lanes are labelled along the top of the gel and fragment sizes (base pairs) are given.

nuc-nuclear

cyt-cytoplasmic

IE2-fragments detected by pGX51

IE5 fragments detected by pGX55

MI-mock infected

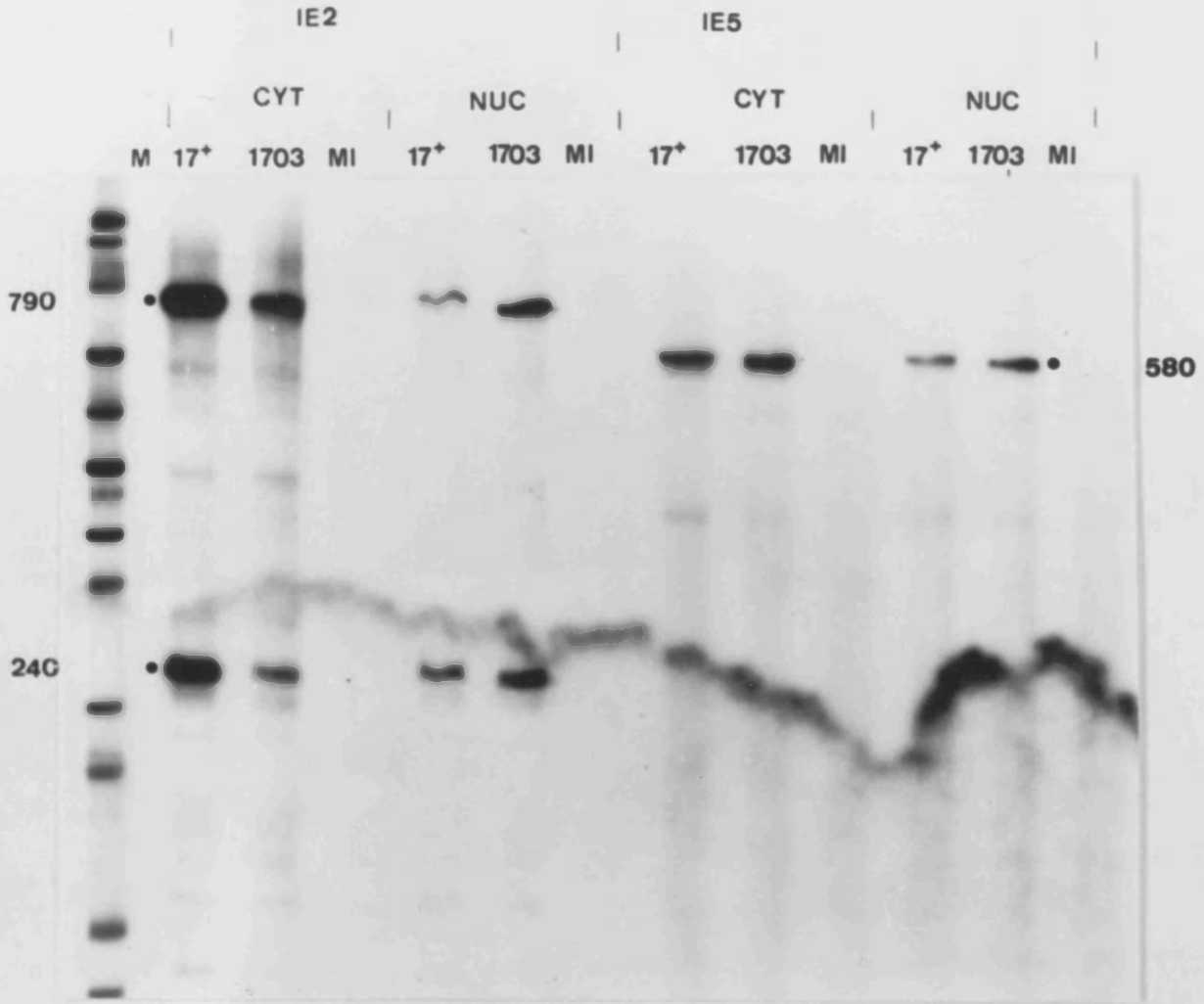

IE2
IE5
CYT
NUC
CYT
NUC
M
17+
1703
MI
17+
1703
MI
17+
1703
MI
17+
1703
MI
790
240
580

Table 3.1. Thymidine kinase characteristics of 17^{+}, 1703, X2D and mock infected cells

	Radioactivity (cpm)/5ul sample	
Virus	Experiment 2	Experiment 2
Mock infected.	7350	5183
17 $^{+}$	19511	24225
1703	29063	23091
X2D	4796	5030

counts per minute due to ^{3}H thymidine. The thymidine kinase positive viruses, 17^{+} and 1703, had 2-3 times more counts per minute than the 1703 parental virus X2D and mock infected cells indicating that 1703 expressed thymidine kinase.

3.5.2. 1703 neurovirulence.

Serial ten-fold dilutions of 1703 and 17^{+} were made in PBS/calf serum and inoculated into 3 week old male Balb/c mice via the intracranial route. Deaths were recorded and the LD50 found to be $<10^{2}$pfu/mouse (Table 2.2). The LD50 of 17^{+} in 3 week old Balb/c mice has recently been found to be <10pfu/mouse (MacLean *et al.*, 1991b), but this was not known at the time of this experiment, hence, it is probable that 1703 is as virulent as wild-type although its virulence has not been determined at <10pfu/mouse.

3.5.3. 1703 latency.

Serial ten-fold dilutions of 1703 and 17^{+} were made in PBS/calf serum and inoculated into the right rear footpad of 4 week old male Balb/c mice. 17^{+} was the control for this experiment but the inoculum did not contain $>10^{4}$ pfu/ mouse due to the ability of 17^{+} to kill at higher dilutions via this route. 1703 was able to establish and reactivate from latency in the same manner as wild-type.

3.6. Transfer of the 1703 deletion to $17^{\pm}$ DNA.

The results of the sequence analysis of the 1703 IE2 gene demonstrated that the alteration of the temporal regulation of Vmw63

Table 3.2. LD50 of 17 and 1703 following intracranial inoculation of 3 week old Balb/c mice.

	Dose (pfu / animal)				LD50
Virus	10^2	10^3	10^4	10^5	**pfu/animal**
1703	4/4*	4/4	4/4	4/4	$<10^2$
17 +	4/4	4/4	ND	ND	$<10^2$

* number of animals dead/number of animals inoculated.

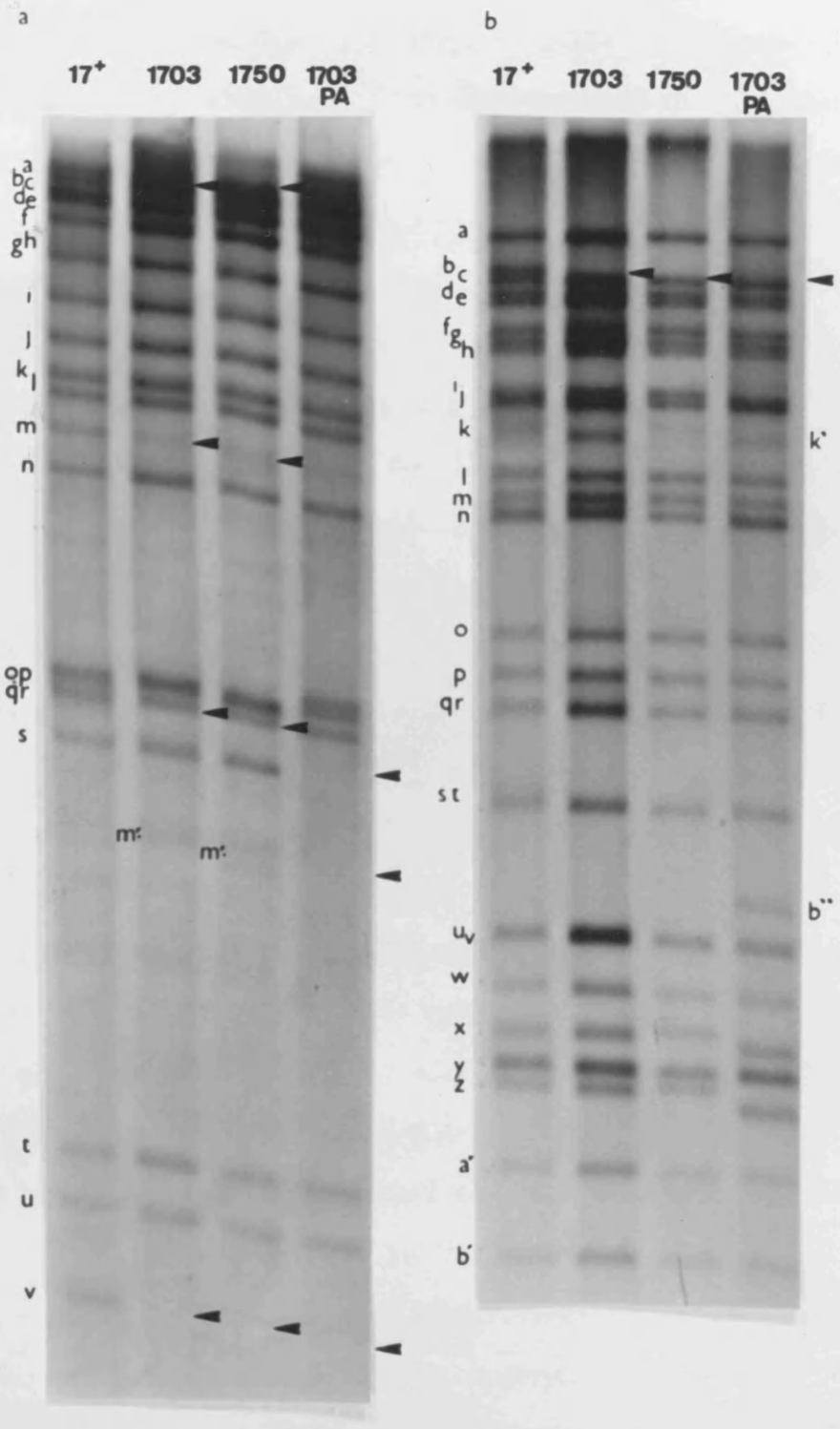
probably
in 1703 infections was not due to a secondary mutation in IE2. Confirmation that it was a direct result of the downstream deletion has been achieved by transfer of a fragment spanning the deletion into a 17^+ genome. The 1703 *Bgl*11f' DNA fragment was used since *Hpa*1m', the fragment used to sequence the deletion end points was not large enough for efficient recombination. The *Bgl*IIf' fragment from 1703 DNA was obtained by separation of *Bgl*II cleaved 1703 DNA through an agarose/TBE gel and electroelution and co-transfected with 17^+ DNA into BHK21 C13 cells. Resultant plaques were analysed by the method of Lonsdale, (1979). One recombinant plaque with the required DNA profile (outlined below) designated 1750 was isolated and plaque purified three times.

3.6.1. Analysis of 1750 DNA.

^{32}P *in vivo* labelled 17^+, 1703 and 1750 DNA was digested with the enzymes *Bam*H1 and *Hpa*1. DNA profiles are shown in figure 3.16. Evidence that *Bgl*IIf' had been recombined into 17^+ DNA was provided by a reduction in intensity of bands *Hpa*1m and *Hpa*1a. The presence of *Hpa*1m' in the *Hpa*1 DNA profiles of both 1703 and 1750 is not clear as the band comigrates with a cellular band present in all four tracks, however the reduction in bands *Hpa*1m and *Hpa*1a and the absence of *Hpa*1v and *Hpa*1r, characteristic of the 1703 *Hpa*1 DNA profile, demonstrates that the 1703 and 1750 DNA profiles are identical. The loss of *Bam*H1b in the 1703 and 1750 DNA profiles compared to wild-type conclusively demonstrated that 1750 was a *Bgl*IIf'/17^+ recombinant. 1750 was further characterized by the methods of Western blot analysis and S1 nuclease mapping.

Figure 3.16 Analysis of 17^+,1703, 1750 and1703PA DNA.

17^+, 1703, 1750 and 1703PA DNA was *in vivo* ^{32}P labelled and digested with *Hpa*1 (a) or *Bam*H1 (b). Missing or reduced bands are marked and novel bands are indicated with the letter of the band from which they are derived and a prime symbol (').


a
17+
1703
1750
1703 PA
b
17+
1703
1750
1703 PA
k'
b''

3.6.2. Analysis of 1750 IE2 transcripts and protein products.

S1 nuclease mapping of 17^+, 1703 and 1750 transcripts using the probes discussed in section 3.4 (figure 3.17) demonstrated that IE5 mRNA production by 1703 and 1750 was reduced in comparison to 17^+ and indicated that the total RNA in the former samples was less than the latter. IE1 mRNA production by all four viruses demonstrated the same pattern, 1703 and 1750 reduced in comparison to 17^+, IE2 mRNA production by both 1703 and 1750 under immediate-early conditions was not apparent, that is, below the level of detection. Western blot analysis of Vmw63 production by 17^+, 1703 and 1750, synthesized under immediate-early conditions (figure 3.18) demonstrated that, compared to wild-type, the production of Vmw63 by 1703 and 1750 was reduced. The reduction in Vmw63 seen in the 1750 track appears to be less marked than the same band in the 1703 track indicating that there may have been a loading discrepancy between the tracks. However the mock bands in all five lanes are identical in concentration implying that the difference in the intensity of the 1703 and 1750 Vmw63 bands reflected a genuine difference in the synthesis of the polypeptide by the two viruses. At early and late times of infection, the synthesis of Vmw63 by 1750 is directly comparable to that of wild-type. At all times of infection, a mock infected cell extract (uninfected cells) was analysed with the infected extracts to allow the elimination of cellular bands in the analysis of the blot.

This information demonstrates conclusively that the underproduction of Vmw63 under immediate-early conditions was not due to an mutation in a part of the 1703 genome distant from the deletion.

Figure 3.17 S1 nuclease map of 17^+, 1703, 1750 and 1703PA immediate-early mRNAs.

Immediate-early preparations of 17^+, 1703, 1750, 1703PA and mock infected cell (BHK21 C13) cytoplasmic RNA was subjected to the S1 nuclease mapping procedure. Bands reduced in intensity are marked and the sizes of resultant fragments in base pairs indicated.

IE2-fragments detected by pGX51

IE5-fragments detected by pGX55

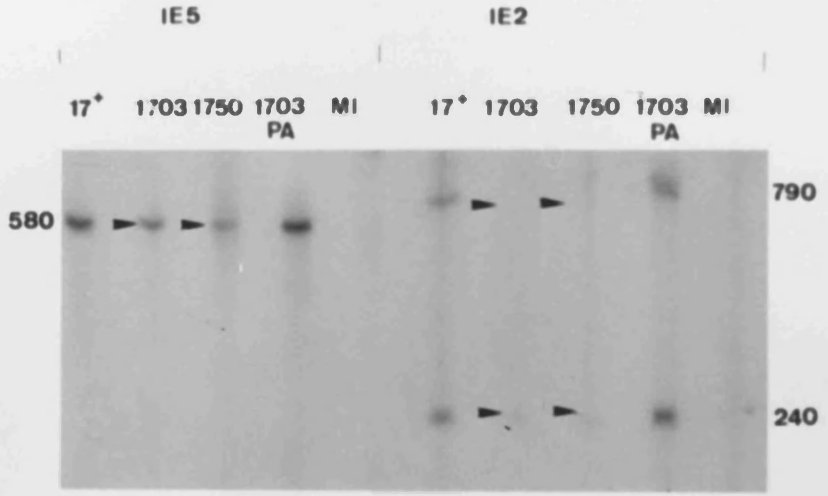
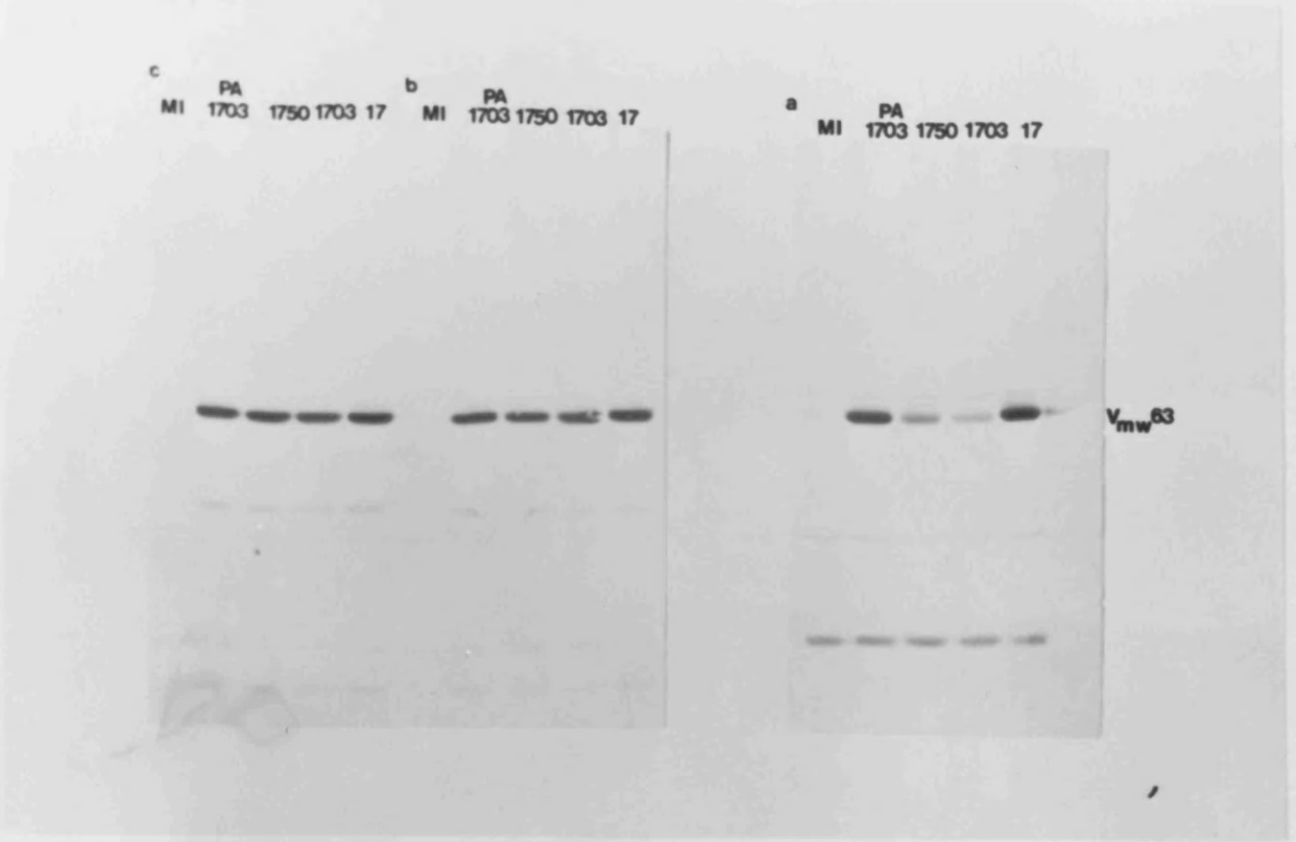

IE5
IE2
17+
1703
1750
1703 PA
MI
17+
1703
1750
1703 PA
MI
580
790
240

Figure 3.18 Western blot analysis of 17^+, 1703, 1750, and 1703PA immediate-early, early and late polypeptide extracts.

Immediate-early (a), early (b) and late (c) extracts of 17^+, 1703, 1750, 1703PA and mock infected polypeptides run on a 7.5% polacrylamide gel, blotted and hybridized to an antiserum which recognised the carboxyterminus of Vmw63 and was supplied by Dr Howard Marsden. The position of Vmw63 is indicated.


c
MI
PA
1703
1750
1703
17
b
MI
PA
1703
1750
1703
17
a
MI
PA
1703
1750
1703
17
V_{mw}63

3.7. THE INTRODUCTION OF A POLYADENYLATION SIGNAL BETWEEN THE 5' END OF IE1 AND THE 3' END OF IE2.

It became increasingly apparent that the downregulation of transcription of IE2 was due to the production of a transcript initiating from the IE1 promoter and interfering with the synthesis of IE2 mRNA. Deletion of the normal IE1 polyadenylation signal and of UL56 (the only adjacent gene whose polyadenylation signal is in the same orientation as that of IE1) could result in the production of a long transcript (about 15kb), part of which would be antisense to transcripts initiating from the IE2 promoter. Previous data (sections 3.3 and 3.4) has demonstrated that small amounts of IE2 mRNA and protein are produced by 1703 under immediate-early conditions implying that a small amount of IE2 mRNA is processed. To confirm that IE2 mRNA synthesis was being controlled by an antisense transcript, a polyadenylation signal was introduced between the 5' end of IE1 and the 3' end of IE2 in 1703 in the correct orientation to polyadenylate mRNA initiating from the IR_L copy of the IE1 promoter before reaching IE2 coding sequences. This required the manipulation of plasmids containing relevant 1703 DNA fragments.

3.7.1. The polyadenylation signal.

A plasmid pSAU3 containing a polyadenylation signal which had been cloned into the *Bam*H1 site of pGEM, was supplied by Dr John McLauchlan. As the polyadenylation signal was HSV-2 in origin it was anticipated that the construct could be used as a probe when detecting 1703/polyadenylation signal recombinant viruses since its surrounding DNA should be heterologous to HSV-1 1703. However this proved not to

be the case as the plasmid hybridized to other 1703 DNA fragments (figure 3.23).

There was about 150bp 5' to the polyadenylation signal which was used to transcript map the novel RNA synthesized as a result of the insertion of the signal into 1703 DNA. The structure of pSAU3 is shown in figure 3.19a.

The construct will be referred to as the polyadenylation signal (PA). It could not be directly cloned into 1703 DNA as its surrounding sequences were not present in sufficient quantity, nor were they compatible with 1703 DNA. Thus, the 1703 DNA fragments *Hpa*1s and *Hpa*1m' were cloned around the signal in the orientation described below:

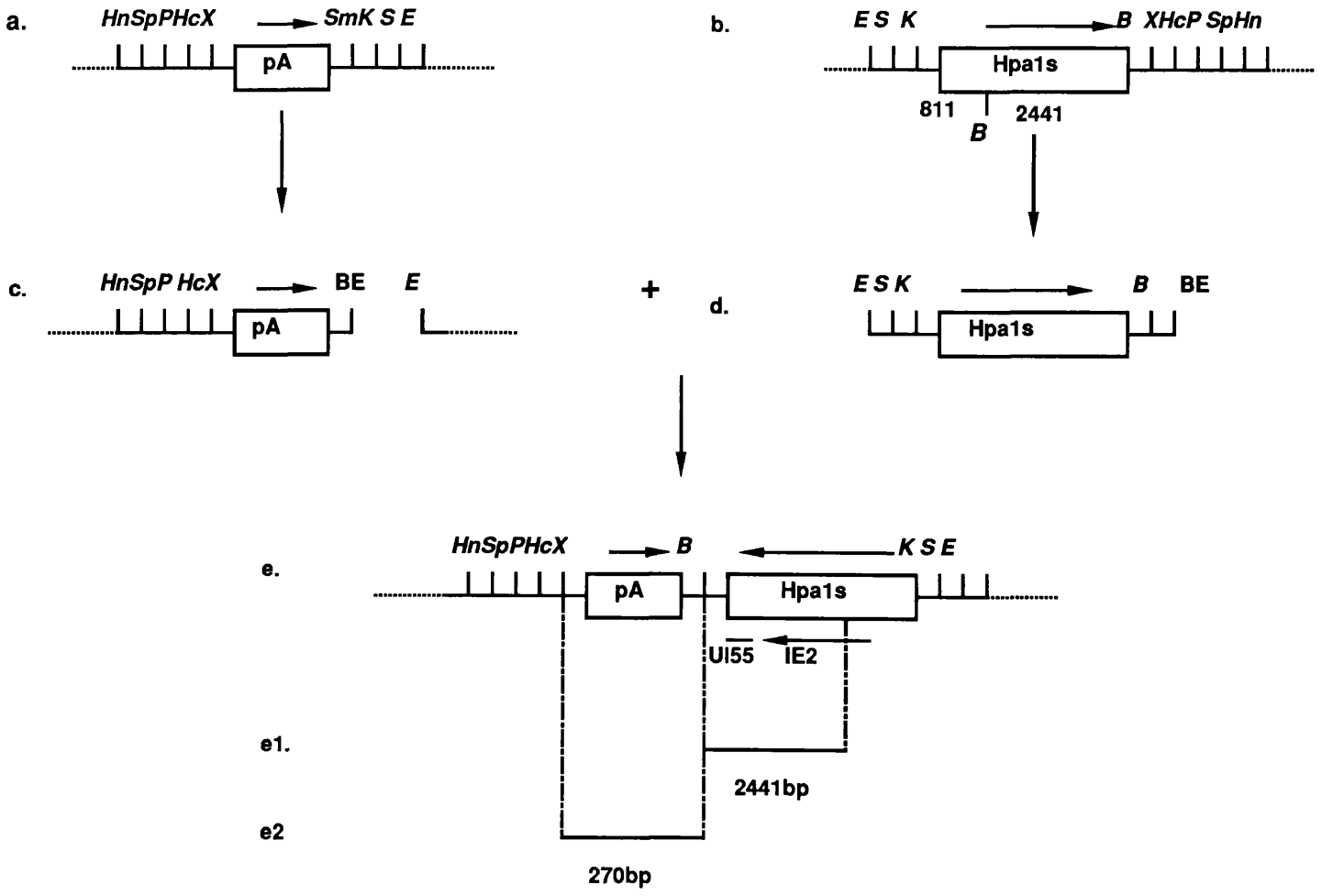
3.7.2. Cloning *Hpa*1s into pSAU3.

*Hpa*1s was previously cloned into pUC19 in order to sequence the end points of the deletion in 1703 (section 3.2.1) since IE2 was located within this fragment. To find the orientation of *Hpa*1s within pUC19, the construct was cleaved with the restriction enzyme *Bam*H1 (figure 3.19b). A *Bam*H1 site is located on the HSV-1 genome at np113322 cleaving *Hpa*1s into one 2441bp fragment and one 811bp fragment. Upon digestion of *Hpa*1s with *Bam*H1, one fragment of 2441bp (a portion of *Hpa*1s), and one of about 3500bp (the remaining 811bp of *Hpa*1s plus the plasmid) were detected demonstrating that *Hpa*1s had the orientation within pUC19 shown by figure 3.19b. To clone *Hpa*1s into pSAU3, pUC19/*Hpa*1s was cleaved with the restriction enzyme *Xba*1, blunt ended at that site and digested with *Eco*R1 resulting in a fragment of about 3251bp (figure 3.19d). pSAU3 was linearised by cleaving at the *Sma*1 and *Eco*R1 sites (figure 3.19c) resulting in both pSAU3 and

Figure3.19 Cloning *Hpa*1s into pSAU3.

a and **b** are pSAU3 and pUC19/*Hpa*1s respectively. The dotted lines indicate the plasmid backbone and the orientation of each fragment within the plasmid is marked by an arrow. Restriction enzyme fragments are represented by the first one or two letters of the enzyme name (a key for this is given below). **d** and **c** demonstrate the ends obtained by digestion and blunt ending of pUC19/*Hpa*1s and pSAU3. The ligation of both gives a fragment whose structure is shown in **e**. The genes contained within the HSV-1 fragments are shown and the fragments resulting from a *Bam*H1 (**e1**) and *Bam*H1/*Xba*1 (**e2**) digest are indicated.

BE-Blunt ended	***S***-*Sst*1
B-*Bam*H1	***Sp***-*Sph*1
E-*Eco*R1	***Sa***-*Sal*1
Hn-*Hind*III	***P***-*Pst*1
Hc-*Hinc*II	***X***-*Xba*1
K-*Kpn*1	--- plasmid backbone
	⟶ orientation of fragment


a.
HnSpPHcX
SmK S E
pA
b.
E S K
B XHcP SpHn
Hpa1s
811
2441
B
c.
HnSpP HcX
BE
E
pA
+
d.
E S K
B
BE
Hpa1s
e.
HnSpPHcX
B
K S E
pA
Hpa1s
UI55
IE2
e1.
2441bp
e2
270bp

pUC19/*Hpa*1s having one blunt and one sticky end each and ensuring that the orientation of *Hpa*1s within pSAU3 was correct. Both fragments were purified by agarose gel electrophoresis and ligated together. As there was no colour selection available to identify recombinants, colonies arising from the transformation were all picked and the resultant plasmid DNA analysed using restriction enzymes (figure 3.19e). Digestion of plasmid DNA with *Bam*H1 (figure 3.19e1) resulted in two fragments, one of 2441bp (a portion of *Hpa*1s) and one of 3831bp (270bp of the polyadenylation signal, 2700bp of the plasmid backbone and 811bp of the rest of *Hpa*1s). Cleavage of pSAU3/*Hpa*1s recombinant plasmids with the enzymes *Bam*H1 and *Xba*1 (figure 3.19e2) gave three fragments: one of 2441bp (a portion of *Hpa*1s), one of 270bp (the polyadenylation signal) and one of about 3500bp (the rest of *Hpa*1s together with the pGEM backbone). Thus it was demonstrated that *Hpa*1s was cloned into pSAU3 in the correct orientation. The recombinant plasmid was called pSAU3/*Hpa*1s.

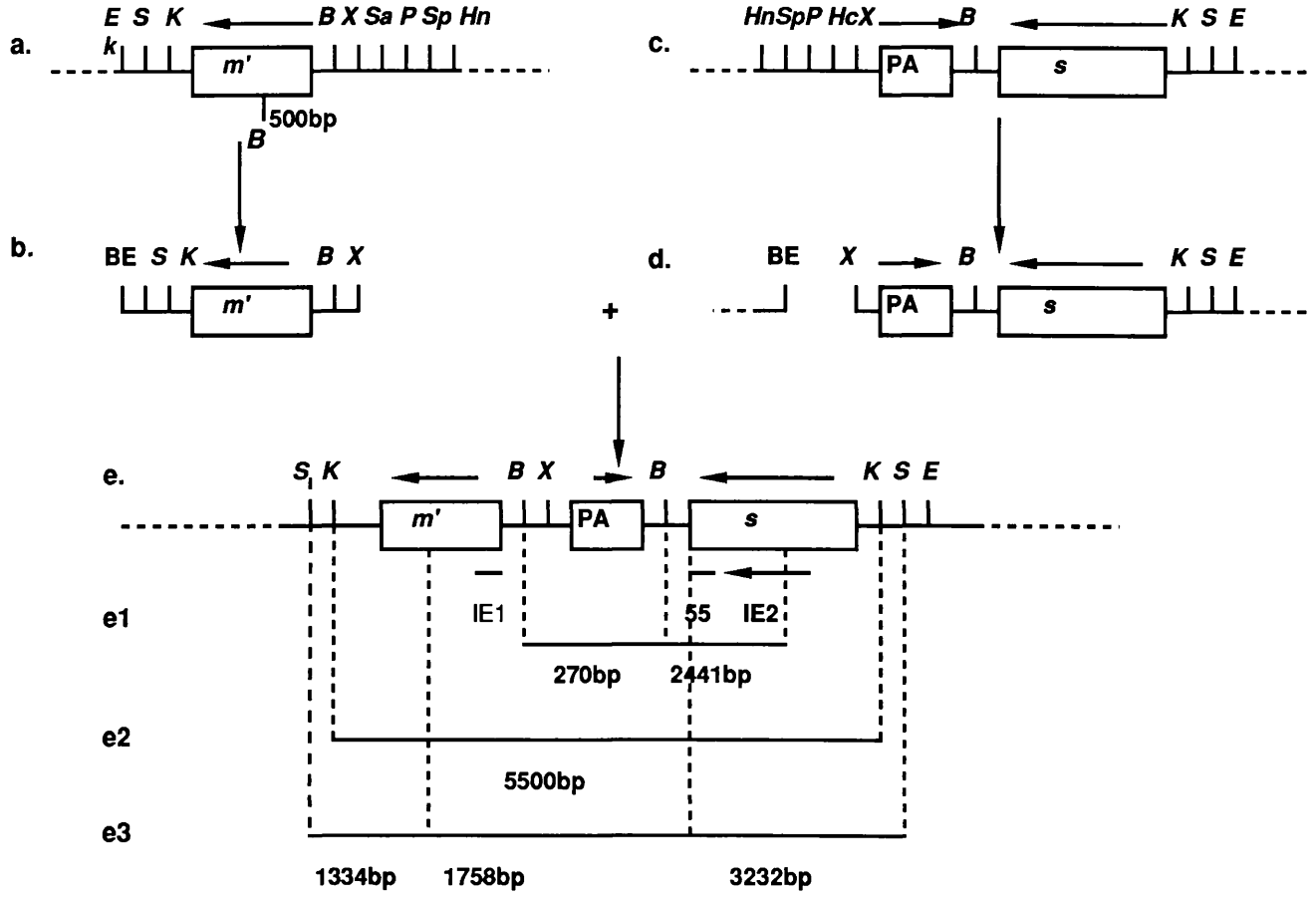
3.7.3. Cloning *Hpa*1m' into pSAU3/*Hpa*1s.

The end points of the deletion within 1703 were located within *Hpa*1m' and this fragment was already cloned into pUC19 (section 3.2.2). The orientation of *Hpa*1m' in pUC19 was demonstrated by *Bam*H 1 restriction enzyme analysis of the construct (figure 3.20a). A BamH1 restriction enzyme site is located within the HSV-1 genome at np123459 and cleaves Hpa1m' into two distinct fragments, one of approximately 500bp (a portion of *Hpa*1m') and one of about 5000bp (the rest of *Hpa*1m' plus the plasmid backbone). At this point, a large scale plasmid preparation of pUC19/*Hpa*1m' was grown and the *Bam*H1 site within *Hpa*1m' was lost (section 3.2.2). The structure of pUC19/*Hpa*1m' is

Figure3.20 Cloning *Hpa*1m' into pSAU3/*Hpa*1s.

a and **c** are pUC19/*Hpa*1m' and pSAU3/*Hpa*1s respectively. Dotted lines indicate the plasmid backbone and the orientation of each fragment within the plasmid is marked by arrows. Restriction enzyme sites are represented by the first one or two letters of the enzyme name (a key for this is given below). **b** and **d** demonstrate the fragments obtained by digestion and blunt ending to give *Hpa*1m' and pSAU3/*Hpa*1s with suitable ends for ligation. **e** is the result of ligation of both fragments and the genes within are indicated. **e1** shows the fragments obtained by a *Bam*H1 digest, **e2** is a *Kpn*1 digest and **e3** is a *Sst*1 digest.

BE-blunt ended
B-*Bam*H1
E-*Eco*R1
Hn-*Hind*III
Hc-*Hinc*II
K-*Kpn*1
S-*Sst*1
Sp-*Sph*1
Sa-*Sal*1
P-*Pst*1
X-*Xba*1
--- plasmid backbone
⟶ orientation of fragment.


a.
E S K
B X Sa P Sp Hn
k
m'
500bp
B
b.
BE S K
B X
m'
+
c.
HnSpP HcX
B
K S E
PA
s
d.
BE
X
B
K S E
PA
s
e.
S K
B X
B
K S E
m'
PA
s
e1
IE1
55
IE2
270bp
2441bp
e2
5500bp
e3
1334bp
1758bp
3232bp

shown in figure 3.20 as are the manipulations of pSAU3/*Hpa*1s and pUC19/*Hpa*1m' which resulted in the isolation of pSAU3/*Hpa*1s/*Hpa*1m'. These are summarized as follows:

pUC19/*Hpa*1m' was cleaved with the enzyme *Eco*R1, blunt ended at that site, purified and further digested with *Xba*1 (figure 3.20b). This separated *Hpa*1m' from its plasmid backbone and resulted in it having one blunt end and one sticky end. The same ends were given to pSAU3/*Hpa*1s by first digesting with *Hind*III, blunt ending at that site, and cleaving with *Xba*1 (figure 3.20d). The fragments were ligated in the correct orientation. All colonies from the transformation were picked, and the resultant DNA analysed using restriction enzymes (figure 3.20e). Cleavage of the construct with *Bam*H1 (figure 3.20e1) gave one 2441bp fragment (a portion of *Hpa*1s), one of 270bp (the polyadenylation signal), and one of 5500bp (the plasmid backbone, *Hpa*1m', and the rest of *Hpa*1s). Digestion with *Kpn*1 (figure 3.20e2) gave two fragments one of which is the *Hpa*1s/polyadenylation signal/*Hpa*1m' whose size was about 6000bp, and one of 2770bp corresponding to the plasmid backbone. Confirmation of the orientation of the 1703 DNA fragments within pSAU3 was obtained by digestion with *Sst*1 (figure 3.20e3) which gave the following restriction enzyme cleavage pattern: a 3232bp fragment (*Hpa*1s), one of 1752bp (part of *Hpa*1m' and the polyadenylation signal), one of 1334bp (the rest of *Hpa*1m') and one of 2770bp (made up of the plasmid backbone). Further confirmation of the orientation of the fragments within pSAU3 was achieved by Southern blot analysis.

3.7.4. Confirmation of the structure of pSAU3/*Hpa*1s/*Hpa*1m' by Southern blot analysis.

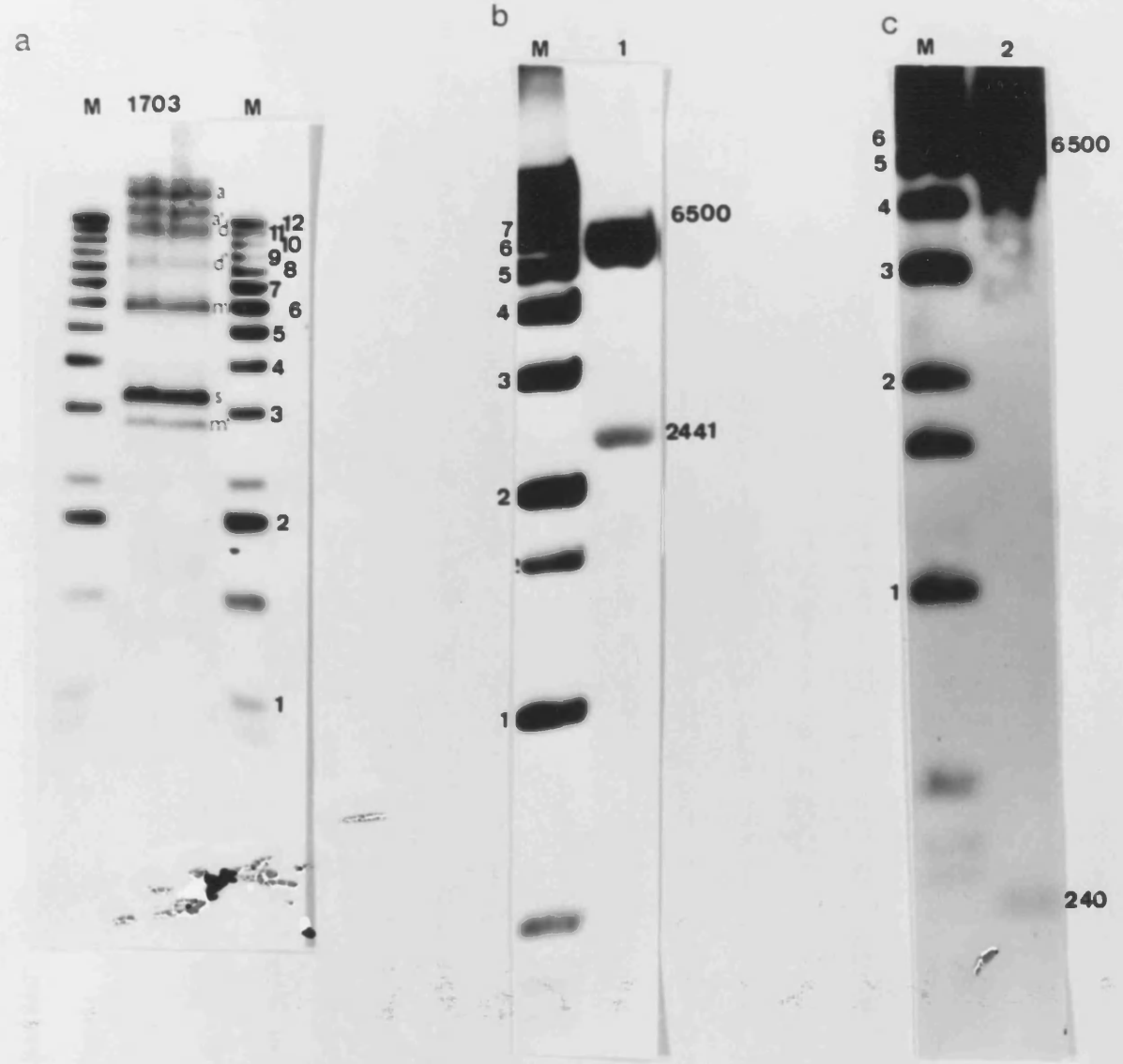
Figure 3.21 is an autoradiograph of the Southern blot analysis of pSAU3/*Hpa*1s/*Hpa*1m' and 1703 DNA hybridized to various probes. 3.21a shows 1703 DNA cleaved with *Hpa*1 and electrophoresed through a 0.8% TBE/acrylamide gel. The probe is random primed pSAU3/*Hpa*1s/*Hpa*1m', which hybridizes to the following *Hpa*1 fragments: *m'*, *s*, *m*, and the *m'* and *m* joint fragments *a*, *d*, *a'*, and *d'*. The probe detects several bands other than those described above indicating that the polyadenylation signal hybridizes non-specifically to 1703 DNA. 3.21b demonstrates the hybridization of random primed ^{32}P *in vitro* labelled 1703 DNA to *Bam*H1 digested pSAU3/*Hpa*1s/*Hpa*1m' which gives two fragments, one of 2441bp (a portion of *Hpa*1s) and a large fragment of about 7000bp (the rest of *Hpa*1s, the plasmid backbone, and *Hpa*1m'). The probe hybridizes to all fragments except that containing the polyadenylation signal. Hybridization of random primed pSAU3 to the *Bam*H1 digested construct (3.21c) gave a signal corresponding to the polyadenylation signal and the fragment containing pGEM, the plasmid backbone. This data is consistent with the plasmid construct first discussed in section 3.7 which was subsequently used to construct the 1703 recombinant virus containing a polyadenylation signal in the correct orientation to polyadenylate the antisense transcript.

3.7.5. Construction and analysis of the 1703PA recombinant genome.

The plasmid construct described in section 3.7.4 was cleaved with the restriction enzyme *Kpn*1 and the *Hpa*1s/polyadenylation signal/*Hpa*1m'

Figure 3.21 Southern blot analysis of pSAU3/*Hpa*1s/*Hpa*1m'.

Autoradiograph of Southern blots in which: **a**. random primed ^{32}P *in vitro* labelled pSAU3/*Hpa*1s/*Hpa*1m' is hybridized to *Hpa*1 digested 1703 DNA (0.8% agarose), **b**. random primed ^{32}P *in vitro* labelled 1703 DNA is hybridized to *Bam*H1 digested pSAU3/*Hpa*1s/*Hpa*1m' (1% agarose: lane 1) **c**. random primed ^{32}P *in vitro* labelled pSAU3 is hybridized to *Bam*H1 digested pSAU3/*Hpa*1s/*Hpa*1m' (1% agarose; lane 2). Marker tracks are indicated (M) and the size of bands labelled (kbp). The size of fragments detected by the probes are indicated.


a
M
1703
M
a
a'
d
d'
m'
s
m'
12
11
10
9
8
7
6
5
4
3
2
1
b
M
1
7
6
5
4
3
2
1
6500
2441
c
M
2
6
5
4
3
2
1
6500
240

fragment was purified by agarose gel electrophoresis. Co-transfection of the fragment with 1703 DNA and analysis of resultant progeny by the Lonsdale (1979) method identified a 1703/polyadenylation signal recombinant called 1703PA whose restriction enzyme profile is now described:

Figure 3.16 shows *Hpa*1 and *Bam*H1 profiles of 17^{+}, 1703, 1750 and 1703PA ^{32}P *in vivo* labelled DNA. Sections 3.1.1 and 3.6.1 summarize the restriction enzyme profiles of 1703 and 1750 respectively, thus only the profile of 1703PA will be discussed here. The *Hpa*1 site between *Hpa*1s and *Hpa*1m' has been lost due to insertion of the polyadenylation signal at that site resulting in the loss of *Hpa*1s and *Hpa*1m'. A novel 0.5M band consisting of *Hpa*1s, the polyadenylation signal, and *Hpa*1m' which has a molecular weight of $4.1x10^6$, runs with *Hpa*1m and is therefore not detected. Insertion of the construct also results in the *Hpa*1m' containing joint fragments *Hpa*1a' and *Hpa*1d' returning to almost the equivalent size as wild-type, that is $14.5x10^6$ mol. wt. (*Hpa*1a") and $11.1x10^6$ mol. wt. (*Hpa*1d") respectively. Due to the intact copy of *Hpa*1m in TR_L, the joint fragments *a* and *d* are also present. The *Bam*H1 profile of 1703PA is slightly more complicated (figure 3.16b) since insertion of the polyadenylation signal also results in the introduction of two novel *Bam*H1 sites, one on either side of the signal. This results in a *Bam*H1 site at almost exactly the position of the *Hpa*1 site deleted during the construction of pSAU3/*Hpa*1s/*Hpa*1m'. Thus *Bam*H1b' becomes *Bam*H1b", a 1M fragment of $1.63x10^6$ mol. wt. which migrates between *u*/*v* and *s*/*t*. *Bam*H1k is also slightly altered due to the extension of the deletion in the *Hpa1*m' fragment used to construct pSAU3/*Hpa*1s/*Hpa*1m'. *Bam*H1k becomes *Bam*H1k', a 1M fragment of $3.90x10^6$ mol. wt., only slightly smaller than *Bam*H1k. In *Bam*H 1

digested 1703PA DNA, the polyadenylation signal is cleaved but runs off the gel because it is only 270bp in length.

The above analyses are shown diagrammatically by figure 3.22. The data is consistent with the insertion of the polyadenylation signal in the desired region of 1703 DNA. 1703PA DNA was further analysed by Southern blot analysis.

3.7.6. Confirmation of the presence of PA within 1703.

Southern blot analysis of 1703PA DNA which had been digested with *Hpa*1 and hybridized with ^{32}P *in vitro* labelled pSAU3 (figure 3.23) shows that the polyadenylation signal is capable of hybridizing to a variety of fragments demonstrating that it has sequences in common with HSV-1 17^{+} polyadenylation signals. However, the probe hybridizes to a band near the 6000bp marker band which is diffuse in appearance indicating that it is probably an end. The *Hpa*1s fragment in which the polyadenylation signal was recombined is an end and is thought to have a size of approximately 6500bp (3251bp [*Hpa*1s], 3000bp [*Hpa*1m'] and 270bp [polyadenylation signal]). Restriction enzyme analysis of 1703PA DNA indicated that the polyadenylation signal lies within this fragment. Restriction enzyme analysis and Southern blotting has demonstrated that the polyadenylation signal has been recombined into the correct fragment of 1703 DNA and careful cloning of *Hpa*1s and *Hpa*1m' into pSAU3 has ensured that the polyadenylation signal is in the correct orientation within 1703PA to terminate the antisense transcript before reaching IE2 coding sequences. Knowing these facts, it was now possible to proceed with the analysis of 1703PA IE2 products.

Figure 3.22 Map of 1703 showing inserted polyadenylation signal.

Structure of the HSV-1 genome (a) showing U_L and U_S flanked by TR_L/IR_L and IR_S/TR_S respectively. A portion of the U_L/IR_L junction is expanded and the genes within are shown (b). *Bam*H1 (***B***) and *Hpa*1 (***H***) restriction enzyme sites are indicated (c) as is the deletion (d), the resultant 1703 DNA fragments (e) and those obtained after insertion of the polyadenylation signal (e1).

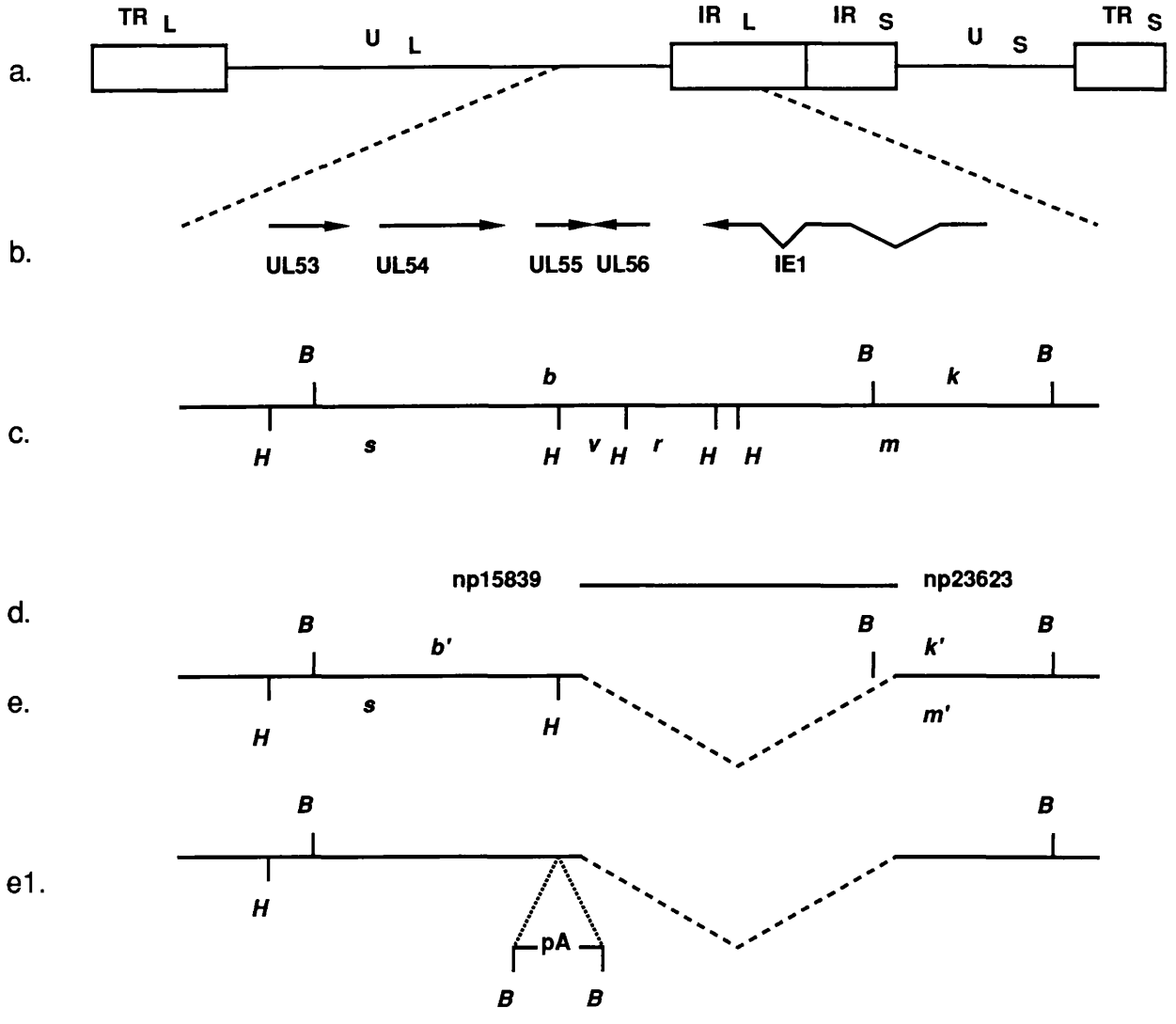
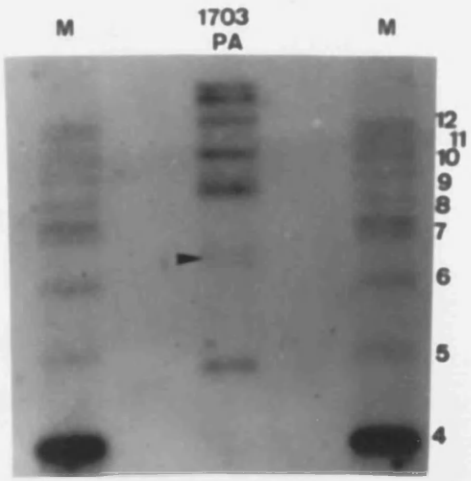

a.
TR L
U L
IR L
IR S
U S
TR S
b.
UL53
UL54
UL55
UL56
IE1
c.
B
b
B
k
B
H
s
H
v
H
r
H
H
m
d.
np15839
np23623
e.
B
b'
B
k'
B
H
s
H
m'
e1.
B
B
H
pA
B
B

Figure 3.23 Southern blot analysis of1703PA.

Autoradiograph of Southern blot in which random primed ^{32}P *in vitro* labelled pSAU3 (the plasmid containing the polyadenylation signal) is hybridized to *Hpa*1 digested 1703PA DNA (0.8% gel). Marker fragments are labelled (Mx10^3 base pairs) and the 1703PA DNA fragments detected are indicated.


M
1703
PA
M
12
11
10
9
8
7
6
5
4

3.8. ANALYSIS OF 1703PA IE2 GENE PRODUCTS.

The methods of S1 nuclease mapping and Western blot analysis have been described elsewhere (sections 3.4 and 3.3 respectively), however an additional probe was used in the S1 nuclease mapping experiments and is described in detail in section 3.8.2.

3.8.1. Western blot analysis of 1703PA polypeptides.

Figure 3.18 is a Western blot analysis of 17^+, 1703, 1750 and 1703PA polypeptides which demonstrates that under immediate-early conditions, the production of Vmw63 by 1703PA has returned to wild-type levels. In the lane before 17^+, a low intensity band at the same molecular weight as Vmw63 in the other four track is present. This band is also present in the mock infected tract implying that it is either a cellular band or is a result of overflow from the 17^+ and 1703PA tracks. Repeating this experiment demonstrated that the band was a result of overflow (results not shown). Synthesis of Vmw63 by 1703PA is also comparable to wild-type at times other than immediate-early. In section 3.3, it was indicated that production of Vmw63 by 1703 under early conditions was about half that produced by wild-type implying that the block in the synthesis of Vmw63 may not be fully overcome at early times. This blot demonstrates that Vmw63 production by 1703 was equivalent to wild-type during early conditions of infection and that the slight underproduction demonstrated in the other blot (figure 3.10a) may have been due to a loading artifact, or to variation from one experiment to another.

Thus, Western blot analysis of 1703PA has demonstrated that the production of Vmw63 in 1703 infected cells is at the transcriptional

The 1703PA IE2 mRNA fragment appears to be increased is size relative to the 17^+ IE2 mRNA fragment. This may reflect a genuine increase in molecular weight of the fragment or may be due to unequal migration of the bands (ie. a gel artifact).

level and is controlled by the production of transcripts that are antisense to IE2 mRNA initiating from the IR_L copy of the IE1 promoter. This was shown conclusively by S1 nuclease mapping IE2 mRNA and by detection of the novel transcript resulting from the integration of the polyadenylation signal.

3.8.2. S1 nuclease mapping of 1703PA transcripts.

There were three different probes used during this experiment, pGX55, pGX51 (described in section 3.4) and pSAU3/*Hpa*1s/*Hpa*1m'. The last probe was prepared by cleaving the construct with *Bam*H1, filling in the 5' overhang incorporating a ^{32}P labelled nucleotide and isolating the 270bp fragment in which the polyadenylation signal was situated. This ensured that the novel transcript resulting from the insertion of the polyadenylation signal would be detected. Figure 3.24 demonstrates the manner in which pSAU3/*Hpa*1m'/*Hpa*1s was cleaved with restriction enzymes to give the DNA fragment used as a probe and the protected fragment obtained after S1 nuclease mapping. Figure 3.17 shows the results of an S1 nuclease map with the probes which detected IE2, and IE5 mRNA. The results agree with the Western blot analysis since synthesis of IE2 mRNA by 1703PA has returned to wild-type levels. Because the protein data had demonstrated that the production of Vmw63 was equivalent to wild-type during early and late times, it was obvious that S1 nuclease mapping of these mRNAs would give identical results. For this reason, these mRNAs were not mapped. Detection of the novel transcript using the polyadenylation signal as a probe (figure 3.25) conclusively demonstrated that a transcript antisense to the IE2 mRNA initiated from the IE1 promoter

This is the first example of a variant which

Figure 3.24 The probe used to detect the novel transcript.

Diagram of the probe used to detect the novel transcript created by insertion of the polyadenylation signal.

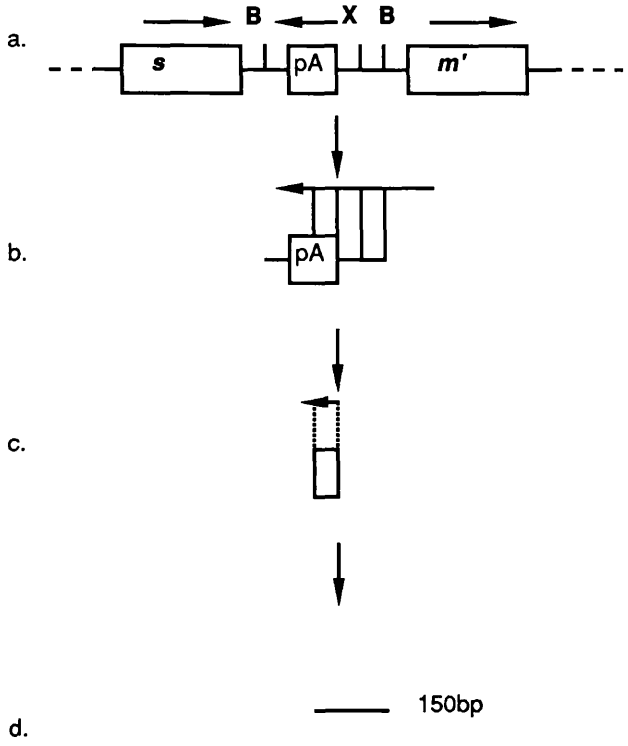
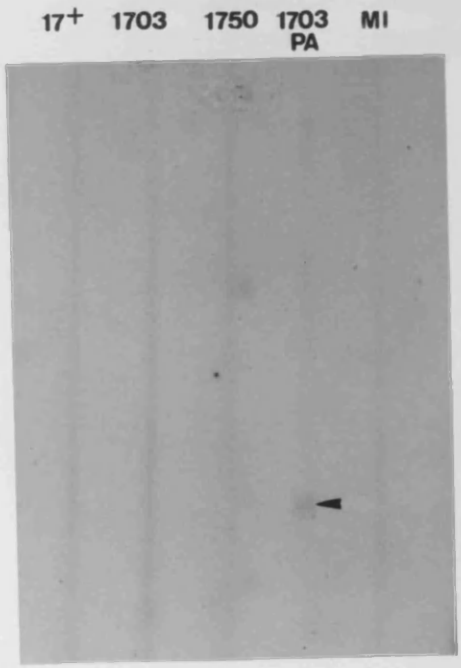

a.
B
X
B
s
pA
m'
b.
pA
c.
d.
150bp

Figure 3.25 S1 nuclease map of 17^+, 1703, 1750 and 1703PA immediate-early RNA.

S1 nuclease map of 17^+, 1703, 1750 and 1703PA RNA subjected to S1 nuclease mapping. The probe used is described in figure 3.24. Lanes are labelled along the top of the gel and fragments detected are indicated.


17+
1703
1750
1703
PA
MI

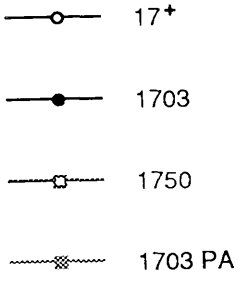
has been shown to control the product of one of its genes by the synthesis of an antisense transcript.

3.9 Growth properties of the three variants.

Growth characteristics of 1703, 1750 and 1703PA are similar to strain 17^+. A one-step growth experiment over 24 hours showed no marked difference in the growth characteristics of the three variants compared to 17^+ (figure 3.26). The long term growth properties of 1703, 1750 and 1703PA indicated that the three variants were slightly growth restricted compared to 17^+ since 1703, 1750 and 1703PA all clustered around a final titre of about one log lower than 17^+, although the slope of all four curves was similar indicating that their growth rates were the same. 1703PA, the virus in which Vmw63 production has returned to wild-type virus levels, showed a similar curve to that of the other two variants indicating that the defect in growth exhibited by the three variants was not due to reduced production of Vmw63 during immediate-early conditions of infection. The particle/pfu ratios of 17^+, 1703, 1750 and 1703PA are 12:1, 57:1, 9:1 and 94:1 respectively and typical stock titres are $1x10^{10}$, $8x10^9$, $6x10^9$ and $2x10^9$ pfu/ml respectively. These results show that long term growth of the three variants is one log lower than 17^+ but the stock titres and particle:pfu ratios are acceptable for wild-type virus. Long term growth experiments mimic the growth of virus stocks in that the infecting dose (0.001pfu/cell) and the time allowed for growth (typically 72 hours) are the same. This implies that the defect in virus growth seen in this experiment is the result of variation among BHK21 C13 cells.

Figure 3.26 One-step growth characteristics of 17^+, 1703, 1750 and 1703PA.

One step growth curves of 17^+ , 1703 , 1750 and 1703PA in BHK21 C13 cells. Cells were infected at a multiplicity of infection of 5pfu/cell, the monolayers washed twice in PBS/calf serum, overlaid with ETC10% and incubated at 37°C. Plates were harvested at 0, 2, 4, 6, 8, 10, 12 and 24 hours post infection and titrated as normal.



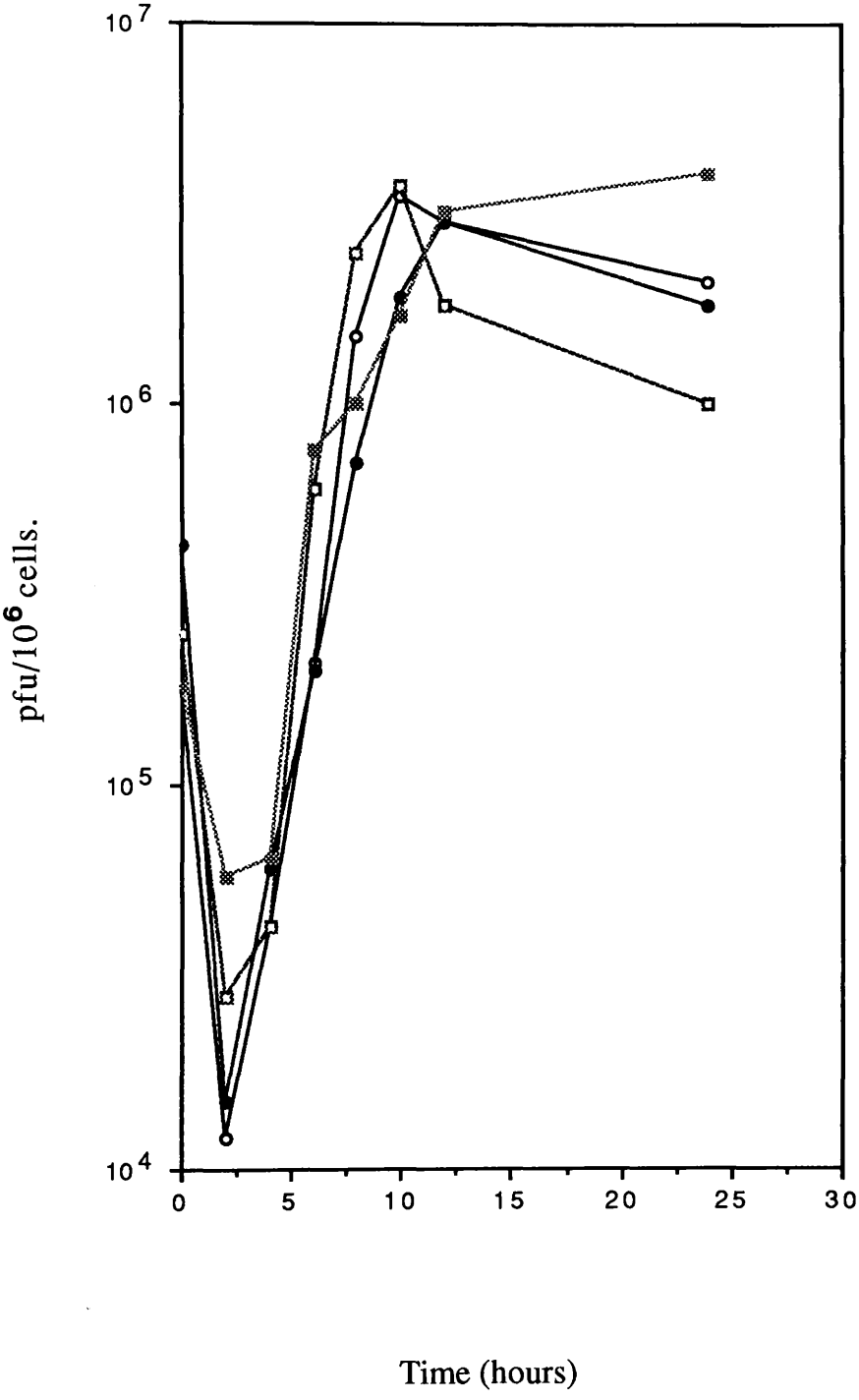
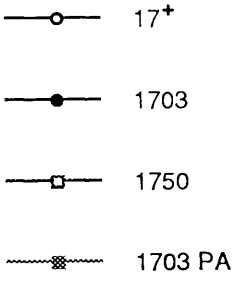
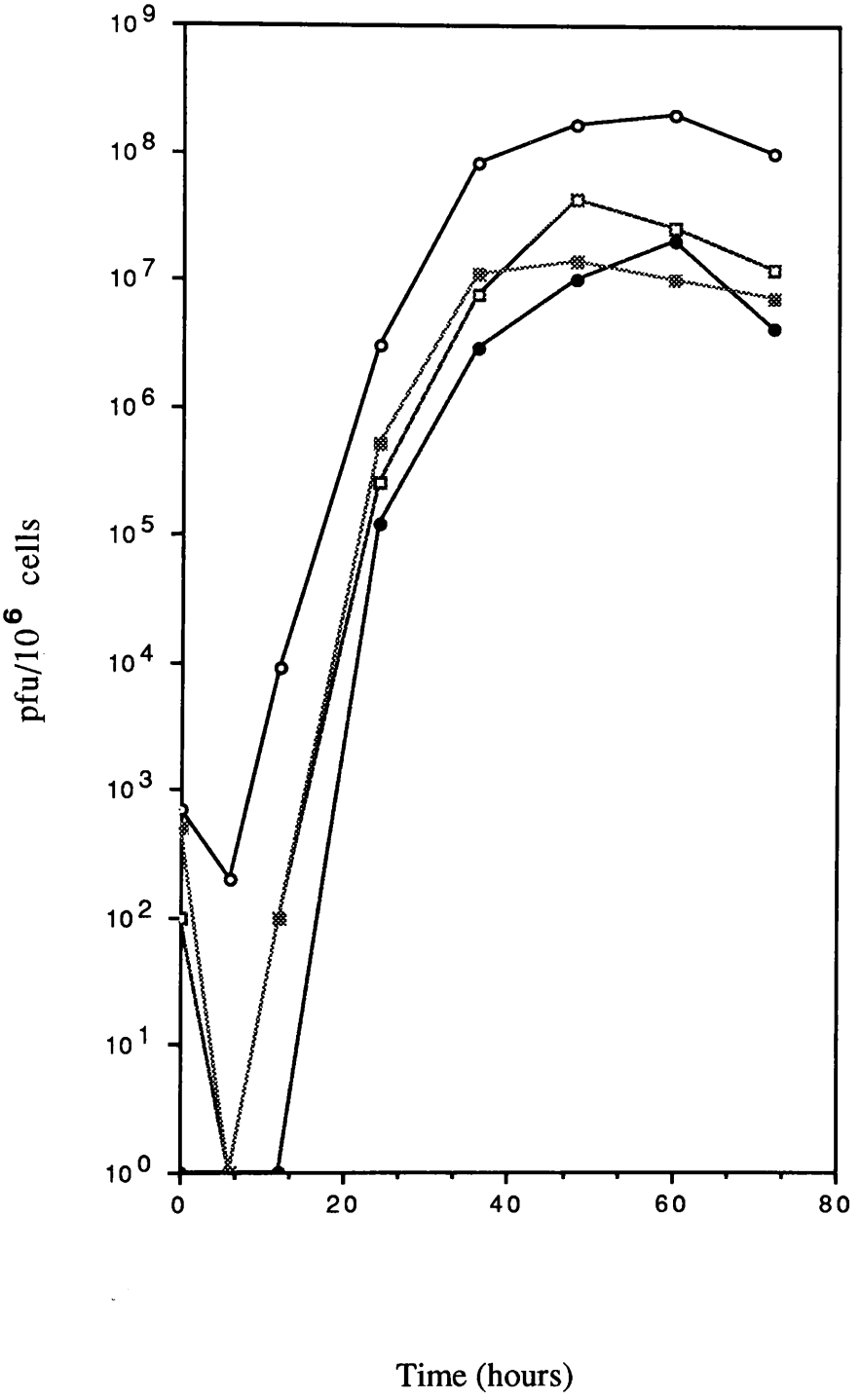

10^7
10^6
10^5
10^4
pfu/10^6 cells.
0
5
10
15
20
25
30
Time (hours)

Figure 3.27 Long term growth characteristics of 17^+, 1703, 1750 and 1703PA.

Long term growth curves of HSV-1 strain 17^+ , 1703 , 1750 and 1703PA in BHK21 C13 cells. Cells were infected at a multiplicity of 0.001pfu/cell, the monolayers washed twice in PBS/calf serum, overlaid with ETC10% and incubated at 37°C. Plates were harvested at 0, 6, 12, 24, 36, 48, 60 and 72 hour post infection and titrated as normal.




10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
pfu/10^6 cells
0
20
40
60
80
Time (hours)

Chapter 4

Discussion.

The original aim of the project described in this thesis was to further characterize the HSV-1 deletion variant 1703 which, on initial analysis (MacLean & Brown,1987a; MacLean,1988), apparently failed to synthesize the essential immediate-early polypeptide Vmw63 at immediate-early times of infection. In depth characterization as detailed in the results section was achieved by: **1**. The dideoxynucleotide sequence analysis of the 1703 DNA fragments in which IE2 and the deletion end points were located. **2**. The *in vivo* characterization of 1703. **3**. The analysis of 1703 IE2 gene products and **4**. The production of a 1703 wild-type recombinant. The purpose of the investigation was to find a reason for the apparent lack of production of Vmw63 under immediate-early conditions and to examine its *in vivo* effect. The project was extended to investigate an idea proposed by Dr John McLauchlan, that synthesis of Vmw63 in 1703 infected cells could be controlled by a transcript antisense to IE2 mRNA.

Vmw63 is an essential immediate-early polypeptide (Sacks *et al.*,1985; McCarthy *et al.*,1989) which functions to transactivate later classes of genes, especially those which are not synthesized in the absence of viral DNA replication, that is, the true late genes. IE2 deletion variants are not viable in tissue culture (except in cell lines expressing Vmw63), they induce synthesis of greatly reduced levels of viral DNA and early-late polypeptides and overexpress early proteins. IE2 deletion variants are also unable to establish latency (Leib *et al.*,1989a). 1703 exhibited no aberrant characteristics, growing almost normally in tissue culture (MacLean & Brown,1987a; this thesis). The growth characteristics of

1703 as established by MacLean & Brown, (1987a) were similar to 17^{+}. However, results obtained during this project indicate that 1703 and the two recombinants 1750 and 1703PA exhibited a reduction in growth of one log during a long term growth experiment. The shape of the curves obtained indicated that the rates of growth were equivalent but eventual titres different. The stock titres of the three variants were within the acceptable range for HSV-1 indicating that perhaps the batch of BHK21 C13 cells used for the latter experiment did not support the growth of the variants as well as those used in the former, demonstrating a possible requirement for the products of the genes deleted in the variants in some batches of BHK21 C13 cells. Alternatively, the difference in results obtained in this thesis and those obtained by MacLean & Brown, (1987a) may be due to variation between experiments and would have to be repeated several times to establish the involvement of the products of genes deleted in 1703, 1750 and 1703PA, in long term growth.

Western blot analysis and S1 nuclease mapping had established that Vmw63 production in 1703 infected cells was only affected during immediate-early conditions of infection and the block in synthesis was at the level of transcription. The synthesis of IE2 mRNA and Vmw63 returned to levels equivalent to wild-type during early and late times of infection, and the small amount of polyadenylated IE2 mRNA present during a 1703 immediate-early infection was reflected in the small amount of Vmw63 synthesized, demonstrating that the block was not at the translational level. During immediate-early conditions of infection, IE2 mRNA was present in both the nuclear and cytoplasmic RNA fractions indicating that a limited quantity of IE2 mRNA was polyadenylated and therefore transported to the cytoplasm. To explain this, several ideas were proposed. These ranged from a secondary

mutation in the IE2 immediate-early specific promoter, TAATGARAT, to the possibility that the deleted genes in 1703 functioned to transactivate IE2, or that antisense transcripts initiating from the IR_L copy of the IE1 promoter controlled IE2 mRNA production. Systematically, each of these possibilities was examined by: **1**. DNA sequencing the IE2 promoter region, **2**. An extensive review of the literature examining the role of the 1703 deleted genes UL55 and UL56, and **3**. The construction of a recombinant 1703 virus in which a polyadenylation signal had been inserted to terminate synthesis of the antisense transcript before reaching IE2 coding sequences.

A requirement for transactivation of IE2 by the Vmw65/cellular factor complex was demonstrated by Ace *et al.*, (1989). This group mutated the Vmw65 encoding gene in such a way as to abolish the transactivating function of the protein. Cells infected with the resultant recombinant virus expressed reduced amounts of IE2 mRNA demonstrating that the gene had a requirement, though not absolute, for functional Vmw65. 1703 expressed 1/8 of the wild-type amount of Vmw63 during immediate-early times of infection indicating that perhaps the IE2 immediate-early promoter was not recognised by the Vmw65/cellular factor complex, that is, a mutation within the TAATGARAT immediate-early consensus sequence. The products of immediate-early genes 3 and 4 were synthesized in amounts equivalent to wild type indicating that Vmw65 was able to transactivate normally. Dideoxynucleotide sequence analysis of IE2 demonstrated that its immediate-early promoter was homologous to the published wild-type sequence (McGeoch *et al.*, 1988a). This demonstrated conclusively that a mutation in the promoter region of 1703, IE2 was not responsible for the pattern of Vmw63 production during immediate-early times.

Sequencing of the 1703 DNA fragment containing the end points of the deletion has shown that there are 555 base pairs remaining between the 3' end of IE2 and the deletion end point. The deletion removes 343 base pairs of the 3' end of UL55, leaving its promoter elements and part of the open reading frame intact. All of UL56 and the 3' end of the IR_L copy of IE1 are deleted as far as the first intron of IE1. It has already been shown that UL55 and UL56 are dispensable in tissue culture and that HSV-1 deletion variants lacking one complete copy of IE1 are viable in tissue culture (Brown *et al.*, 1984; Harland & Brown, 1985; MacLean & Brown,1987a,b). UL55 and UL56 have, however, been implicated as possible HSV-1 gene regulators. Block *et al.*,(1991) used several plasmid constructs in a transient expression assay to demonstrate that UL55 and UL56, when co-transfected with plasmids encoding IE1, IE2 or IE3 and one encoding a region upstream of the 5' end of IE2 can together repress the activity of a Rous sarcoma virus-CAT hybrid and that when UL55 contained a point mutation, or when UL55 and UL56 were not included in the assay, this effect was not seen. This indicates that either UL55 or UL56 gene products may act alone or together to repress HSV-1 promoter activity. 1703 infected cells exhibit reduced synthesis of Vmw63 during immediate-early conditions of infection, the opposite of what would happen if UL55 and/or UL56 did indeed have the function described above.

Several variants have been isolated with deletions affecting UL55 and/or UL56 (table 4.1; information for this table was taken from the following sources; MacLean, 1988; MacLean & Brown, 1987 a, b; Junejo, 1991: Junejo *et al.*, 1991 and Rosen & Darai, 1985). Three variants, 1704, 1705 and 1706 were isolated by MacLean & Brown, (1987b) whose deletions were within the U_L/IR_L region of the HSV-1 genome and terminated 1232, 183, and 80 base pairs respectively downstream of

	Deletion state of the following genes:				
Virus	**UL55**	**UL56**	**IE1**	**LAT**	**Vmw63 production**
HFEM	Present	Absent	Present (both copies)	Absent (one copy)	affected (quantity not known)
1703	Absent	Absent	Absent (one copy)	Absent (one copy)	affected (1/8)
1704	Present	Absent	Present (both copies)	Absent (both copies)	normal
1705	Absent	Absent	Present (both copies)	Absent (one copy)	affected (1/2)
1706	Absent	Absent	Present (both copies)	Absent (one copy)	affected (1/2)

Table 4.1. HSV-1 IR_L/U_L deletion variants.

the 3' end of IE2 (Junejo, 1991; Junejo *et al.*, 1991). 1705 and 1706 demonstrate a two-fold reduction in Vmw63 synthesis (MacLean,1988) probably reflecting the proximity of the deletion end point to the 3' termination associated signals of IE2. 1704 does not demonstrate any change in Vmw63 synthesis again reflecting the distance between the deletion endpoint and the 3' end of IE2. A YGTGTTYY (Y= pyrimidine, either C or G) motif located downstream from the polyadenylation signal is required for efficient formation of 3' end termini (McLauchlan *et al.*,1985). This motif is present 40 base pairs downstream of the 3' end of the IE2 gene in HSV-1. According to the sequence analysis of 1705 and 1706, their deletion end points do not encompass this motif but the proximity of the deletion to the YGTGTTYY sequence may result in the slight inhibition of termination and reduced levels of product. There may be other, as yet undiscovered, termination associated signals in the deleted region, the loss of which results in reduced levels of Vmw63, and the possibility of a secondary mutation in IE2 cannot be ruled out as the IE2 promoter region of neither variant has been sequenced. It is interesting to note that in 1704, UL55 is intact and UL56 is deleted whereas 1705 and 1706 have both genes deleted possibly indicating a link between UL55 and the transactivation of IE2.

A deletion variant has been isolated in which UL55 remains, UL56 is deleted and Vmw63 is underproduced. This variant, HFEM, isolated by Rosen & Darai, (1985), has a 4.1kbp deletion in U_L/IR_L which affects UL56, removing its promoters. The data from MacLean & Brown, (1987b), MacLean, (1988) and Junejo, (1991) indicate that UL55 may encode an IE2 transcriptional enhancer and that the deletion of this element could result in the reduced synthesis of Vmw63. This is not supported by the HFEM data which indicates that UL56 may encode a function which transactivates IE2, and that presence of UL55 has no

bearing on Vmw63 synthesis. In addition, the data from Block *et al.*, (1991) implicating UL55 and UL56 in the repression of HSV-1 genes complicates matters since it attributes no enhancing activity to either gene product. The deletion of an as yet undiscovered enhancer element which is not encoded by either gene may be responsible for the pattern of Vmw63 production demonstrated by the variants described above and cannot be discounted as a possible mechanism by which Vmw63 is underproduced by these variants.

The experimental work has shown that the relevant regions of the 1703 gene IE2 are homologous to the wild-type sequence and the putative functions of UL55 and UL56 makes it impossible to conclude that either gene is a positive regulator of IE2.

In vivo characterization of 1703 demonstrated that the deletion of parts of UL55, one copy of LAT and IE1, the total deletion of UL56 and the reduced production of Vmw63 had no effect on the ability of 1703 to grow in mouse brains, or to establish, maintain and reactivate from latency.

These results correlate with those obtained for the variants HFEM, 1704, 1705 and 1706. The deleted regions of these variants differ in genes removed (table 4.1). Virulence of the four viruses via the intracranial route was, in all cases, comparable to that of wild-type, (Becker *et al.*, 1986; Junejo, 1991) indicating that wild-type amounts of Vmw63, LAT, Vmw110, and the products of genes UL55 and UL56 are not required for virulence via this route. HFEM was characterized in terms of pathogenicity by inoculating via the intraperitoneal, subcutaneous and intravenous routes where it was found to be avirulent, thereby implicating the product of either UL56 or LAT in the ability of this HSV-1 strain to replicate in the organs encountered during inoculation by these routes. Correction of the HFEM deletion by replacing

it with the wild-type fragment from the identical region restored pathogenicity in tree shrews but in mice only 19% of inoculated animals died, showing that the above two genes products may be necessary for the pattern of pathogenicity demonstrated by HFEM in tree shrews but not absolutely required for virulence in mice (Rosen *et al.*,1986).

Wollert *et al.*,(1991) implicate the region of the HSV-1 genome deleted in HFEM with its ability to grow in macrophages of mice and indicate that HFEM is unable to do so. This may be the reason that HFEM is unable to kill via routes other that intracranial, although intraperitoneal virulence appears to be dependent on the animal used. The use of tree shrews during the pathogenicity experiments by Rosen *et al.*,(1986) is of interest since these animals occupy a phylogenetic niche between rodents and primates and therefore may mimic the responses of man to virus infection in a manner which is more genuine than the mouse model.

1703 has not been characterised for virulence by any route other that intracranial and such an experiment, incorporating perhaps, 1704, 1705 and 1706 which differ with respect to the presence or absence of UL55, with UL56 being absent in all cases, may give information concerning the roles that these genes play during pathogenicity. It is possible that, from the evidence presented by Rosen *et al.*,(1986) and since none of the variants encode the UL56 gene product (like HFEM), they would be avirulent when inoculated by routes other than intracranial if the UL55/UL56 effect is not limited to tree shrews, although results by Rosen *et al.*, (1986) indicate that it may be. It is worth remembering that most of these variants also underproduce Vmw63 and it would perhaps be difficult to dissociate the effects of that underproduction and the presence or absence of UL55 or UL56. There is a HSV-1 variant, constructed during the course of this project, called 1703PA in which

Vmw63 production is equivalent to wild-type and genes UL55 and UL56 are deleted. The use of this variant as a control for the experiment described above would enable the effects of reduced production of Vmw63 and the absence of the products of genes UL55 and UL56 to be dissociated. The HFEM revertant exhibits wild-type pathogenicity in tree shrews indicating that either production of Vmw63 has returned to normal levels or that UL56 is responsible for HFEMs virulence characteristics. Spivack & Fraser, (1988a) attribute the defect in Vmw63 production in HFEM infected cells to a mutation in IE2 hence it is likely that the latter is the case.

The above authors also document the latency characteristics of HFEM which, like 1703, has only one copy of LAT and, also like 1703, establishes, maintains and reactivates from latency with a similar frequency to wild-type. The information gained from the latency studies of both HFEM and 1703 indicate that UL55 and UL56 are dispensable for that function and that wild-type amounts of Vmw63, Vmw110 and the LAT gene product are not required.

As well as supporting the restriction endonuclease data obtained by MacLean & Brown, (1987a) which concluded that the deletion in 1703 affected genes UL55, UL56, IE1 and one copy of LAT, sequencing has demonstrated that a *Bam*H1 site, shown to be present during the initial analysis of 1703, was now missing. The deletion in *Hpa*1m' had been extended during the cloning and sequencing of *Hpa*1m', the 1703 DNA fragment in which the deletion end-points were located. Spontaneous deletion of HSV-1 DNA also took place during the cloning of the long origin of replication of HSV-1 into a bacterial vector system, where deletions of >100 base pairs were recorded (Spaete & Frenkel, 1982). The deleting region was found to consist of a long perfect palindrome with an AT rich region at its centre when it was subsequently cloned

into a yeast vector system. The sequence arrangement between the end points of the deletion and the *Bam*H1 site is not particularly unusual for HSV-1 DNA and the extended region of the genome (between the *Bam*H1 site and the sequenced deletion end points) does not affect the three imperfect repeat elements located in the intron (Perry *et al.*, 1986). The deletion in the sequenced *Hpa*1m' fragment was 7784 base pairs in length and it is thought that the deletion in 1703 is about 7500 base pairs, closer to that estimated by MacLean & Brown, (1987a).

As this fragment was used to construct the recombinant virus containing the polyadenylation signal, 1703PA, the net result of the extension of the deletion in *Hpa*1m' was relevant. On the UL55 side of the deletion, it is not known for certain if the deletion had been extended since there were no markers available. MacLean & Brown (1987a) estimated 500 base pairs between the deletion end points and the 3' termination signals of IE2. Sequencing put this at 555 base pairs indicating that it is unlikely that the deletion has been extended. The deletion extends slightly more into the first intron of the IR_L copy of IE1 which is detectable since the *Bam*H1 site can be used as a marker. There is no apparent reason why the extension should have any effect on the properties of 1703.

A wild-type recombinant of 1703 was constructed by co-transfecting *Bgl*IIf' from 1703 into 17^+ DNA. The reason for not choosing the already cloned *Hpa*1m' fragment for this procedure was that there was only about 55 base pairs between the deletion end-point and one end of *Hpa*1m', which would result in inefficient recombination. The recombinant 1750, generated from the *Bgl*IIf'/17^+ co-transfection experiment produced IE2 gene products in similar amounts to those produced by 1703, being reduced during immediate-early times of infection and returning to wild-type levels during early and late times

thereby determining that the deletion was responsible for the underproduction of Vmw63. 1750 produced about twice the amount of Vmw63 at immediate-early times of infection compared to 1703 in the Western blot analysis of two different preparations of 1750 and 1703 polypeptides indicating that the production of slightly more Vmw63 by 1750 was not a gel or loading artifact. It is known that there is plaque variation among HSV isolates, for example, the isolation of a HSV-2 strain HG52 plaque which had heightened neurovirulence (Taha *et al.*, 1988) demonstrated that individual plaque isolates of HSV-2 strain HG52 differed with respect to neurovirulence, but had identical genome structures. Viruses with divergent genome structures in a population of HSV-2 strain HG52 showing deletions in R_L have been isolated with significant frequency (up to 24%); (Harland & Brown,1985,1988; Brown & Harland,1987). Although there is less variation among HSV-1 strain 17^+ isolates (MacLean & Brown, 1987a,b), it is possible that 1750 is a 'healthier' isolate than 1703 with respect to Vmw63 production. Alternatively, the increased amounts of Vmw63 synthesized by 1750 may be due to experimental variation. The analysis of 1750 had therefore demonstrated that underproduction of Vmw63 by 1703 was not due to a mutation in a region of the genome outwith the deletion.

The suggestion that Vmw63 synthesis in 1703 infected cells was controlled by the production of a transcript initiating from the intact promoter of the IR_L copy of IE1 was supported by the exclusion of any other mechanism for the pattern of production of Vmw63 as discussed above. Mechanisms by which gene expression is controlled by antisense nucleic acids are discussed in section 1.10.

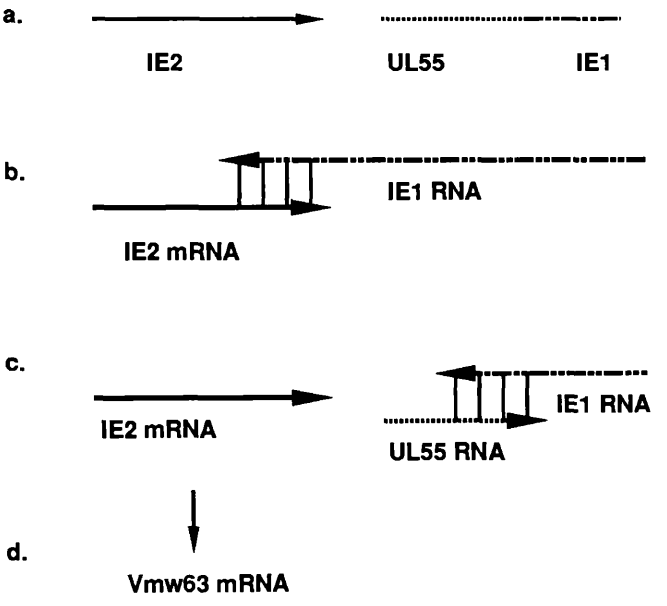
The construction of 1703PA, which is composed of a 1703 DNA backbone with an HSV-2 polyadenylation signal placed in the correct orientation to terminate the synthesis of a potential antisense transcript

before reaching IE2 coding sequences, and the characterization of 1703PA IE2 gene products, has shown that the synthesis of IE2 mRNA and Vmw63 has returned to wild-type levels during all transcriptional conditions. The production of transcripts from the deleted IR_L copy of IE1, whose promoters remain intact, was polyadenylated at the inserted novel polyadenylation signal and could no longer affect the synthesis or processing of IE2 mRNA. The detection of the small, novel transcript initiating from the IE1 promoter and polyadenylating at the inserted polyadenylation signal has conclusively proven that the promoter was capable of initiating the synthesis of a transcript antisense to IE2 mRNA. This information demonstrated that an antisense transcript controlled the synthesis of IE2 gene products in 1703 infected cells, but did not identify the mechanism by which this takes place. It is possible to classify the antisense transcript into any of the three categories identified by Inouye, (1988), (section 1.10) and further work is required to establish which is the correct class.

The initial view of the mechanism of inhibition of IE2 mRNA production by the antisense transcript was that the transcript initiating from the IE1 promoter was transcribed until a polyadenylation signal was encountered whose orientation resulted in its termination. UL51 is the closest suitable polyadenylation signal and the resultant transcript would be about 15kb in length, possibly unstable, and as well as being antisense to IE2 mRNA, would be antisense to the RNA products of the adjacent genes UL53, UL52 and UL51 which encode a possible membrane protein, part of the primase-helicase complex required for viral DNA synthesis and a protein of unknown function, respectively. Under immediate-early conditions, the production of RNA/RNA hybrid molecules (figure 4.1) would render the complexed RNA unavailable for further processing, and the complexes may become the target of double

Figure 4.1 Antisense RNA.

a. The genes IE2, UL55 and IE1 and their structures in the IR_L/U_L region of the 1703 genome. The termination signals of both UL55 and IE1 have been deleted as have sequences between both points. These genes therefore form one stretch of DNA bounded by promoter sequences. **b**. During immediate-early times of infection, the promoters of both IE1 and IE2 are stimulated resulting in transcripts being generated from both. IE1 RNA is complementary to IE2 RNA and the antisense theory depends on both hybridizing (class 1 and 2) or interfering (class3) resulting in IE2 RNA not being available for translation. **c**. At times other than immediate-early, RNA is synthesized from the UL55 promoter which then forms a RNA duplex with IE1 RNA resulting in IE2 mRNA being translated and the production of wild-type quantities of Vmw63 (**d**).


a.
IE2
UL55
IE1
b.
IE1 RNA
IE2 mRNA
c.
IE1 RNA
IE2 mRNA
UL55 RNA
d.
Vmw63 mRNA

stranded RNA degrading enzymes. This is an example of class 1 antisense RNA.

Class 2 antisense RNAs are characterized by hybridizing to a region of the transcript distant from the translation initiation signals of the target RNA, changing its conformation and resulting in the mRNA not being translated. Hybridization of the antisense transcript synthesized from the IE1 promoter to a region of the IE2 transcript outwith translational initiation sites may result in the absence of IE2 gene products during immediate-early conditions of infection due to an alteration in configuration of the IE2 mRNA molecule resulting in it not being translated.

Transcript mapping of IE2 mRNA produced by 1703 during immediate-early times of infection demonstrated that small amounts of IE2 mRNA present in the nucleus are transported to the cytoplasm, a process that depends of the polyadenylation of the 3' end of the message. This indicates that, as discussed above, the block in Vmw63 synthesis occurs at the transcriptional level. Thus, the antisense transcript can also be assigned to class 3, the only class of antisense RNAs which function at the level of transcription. An antisense RNA that functions at the level of transcription is one which controls *crp* production (Okamoto & Freundlich, 1986). This is thought to function by the creation of a RNA polymerase conformational terminator (termed *rho*) resulting in the termination of transcription. An additional mechanism whereby class 3 antisense transcripts function to control transcription of the target gene is transcriptional interference.

An example of transcriptional interference is the avian retrovirus which controls the synthesis of c-myc mRNA by transcriptional overlap interference. Integration of the avian retrovirus, avian leucosis virus (ALV) into the host DNA results in the viral coding sequences being

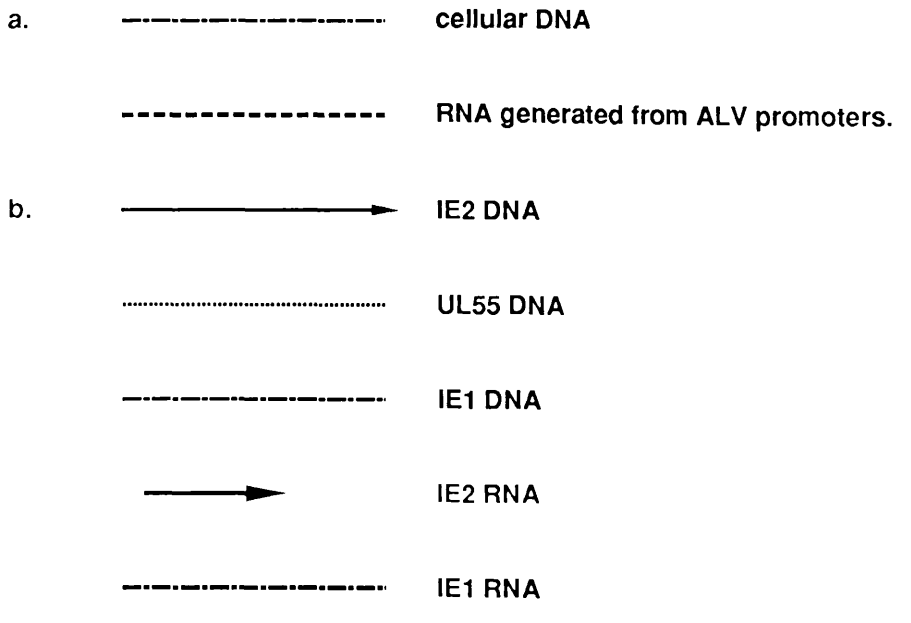
flanked by two long inverted repeats (LTR) containing signals for both initiation and termination of transcription. Figure 4.2a demonstrates this. The observation that c-myc transcripts in avian induced lymphomas contained U5, the LTR promoter element, and that provirus integrated 5' of the c-myc gene contained deletions including the 5' LTR led to the theory that transcription from the 5' LTR controlled the initiation of transcription from the 3' LTR.

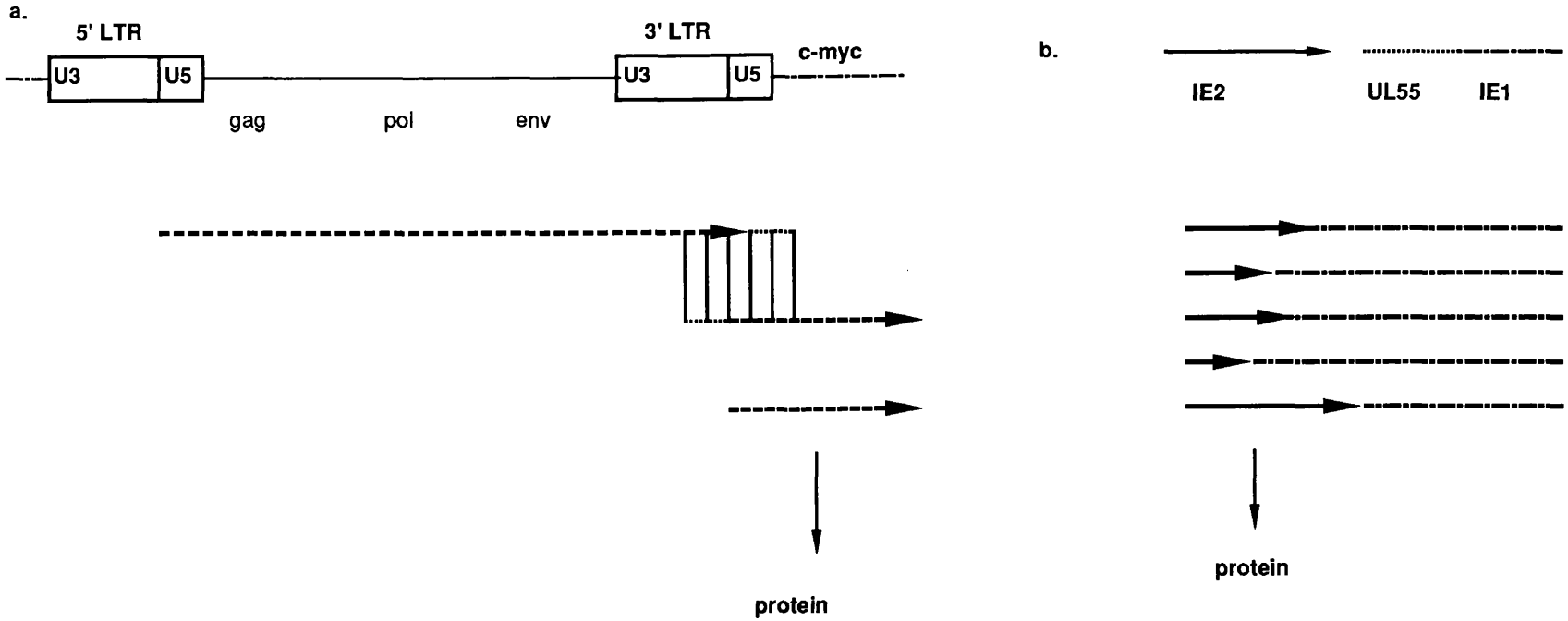
Cullen *et al.*,(1984) studied the mechanism by which this occurs and found that the 3' end of the normal viral transcript overlapped the 5' end of transcripts initiating from the 3' LTR (Figure 4.2a). The deletion of the 5' LTR, or the insertion of a termination signal in the viral open reading frame, resulted in the 3' LTR initiating transcription of a reporter gene (in this case the preproinsulin gene). This conclusively demonstrated that transcripts initiating from the 5' LTR interfered with those initiating from the 3' LTR resulting in it being unable to initiate transcription, probably because RNA polymerase could not bind the promoter region of the 3' LTR. A similar situation exists in the HSV-1 genome (figure 1.3) in which the termination signals and open reading frame of many genes overlap, for example, UL4/UL5, and UL13/UL14. Transcriptional interference has not been established as a control mechanism for either of these sets of genes.

Transcriptional interference resulting in the underproduction of Vmw63 in 1703 infected cells would not be due to transcriptional interference as described, but to the interference of two RNA polymerase molecules transcribing mRNA from opposite DNA strands (figure 4.2b) resulting in the production of truncated and mainly non-polyadenylated IE2 RNA. Transcript mapping of 1703 IE2 mRNA produced under immediate-early conditions always gave full length molecules indicating that truncated forms may be unstable. In any case,

Figure 4.2 Transcriptional interference.

a. The avian leucosis virus (ALV) integrates into cellular DNA, sometimes upstream of the oncogene c-myc. This can result in leukaemogenisis and in most cases of this, the 5' LTR is deleted. LTR is the long terminal repeat, U3 and U5 are termination and promoter signals respectively. ALV genes gag, pol and env are indicated. Dotted lines indicate cellular DNA. Normally transcripts generated from the 5' LTR interfere with those initiating from the 3' LTR and c-myc is not transcribed, a phenomenum known as transcriptional interference. If however the 5' LTR is deleted, RNA polymerase is free to initiate transcription from the 3' LTR and c-myc protein is produced. b. Transcriptional interference in 1703. During immediate-early times of infection, RNA initiating from the IE1 promoter interferes with that being synthesized from the IE2 promoter resulting in premature termination of IE2 mRNA. Transactivation of the UL55 promoter at other times of infection generates RNA which interferes with that initiating from the IE1 promoter resulting in restored production of Vmw63 at these times of infection.




a.
5' LTR
3' LTR
c-myc
U3
U5
U3
U5
gag
pol
env
protein
b.
IE2
UL55
IE1
protein

the probe only detects 790 base pairs of the 3' end of IE2 and RNA molecules shorter than about 2600 base pairs (the IE2 transcript is about 3250 base pairs in length) would not be detectable by this method. That the probe detected IE2 mRNA at all under immediate-early conditions may be due to the formation of a quantity of polyadenylated IE2 mRNA which is translated normally. This indicates that the position at which transcriptional interference occurs may be variable along the length of the IE2 molecule. Although transcriptional interference as described by Cullen *et al.*, (1984) does not involve antisense RNA, the principle of interference of two RNA polymerase molecules is pertinent to describing the 1703 antisense transcript as class 3.

Vmw63 is synthesized in reduced amounts by 1703 during immediate-early conditions of infection as demonstrated by S1 nuclease mapping and Western blot analysis, for which there are two possible explanations. The first relies on findings by Sandri-Golden *et al.*, (1987) which demonstrates that the promoter for the antisense transcript may have to be 10x stronger than that for the sense transcript for inhibition of the sense transcript to occur. The transactivator for IE2 and IE1 during the immediate-early experiments reported in the results section of this thesis in which cycloheximide was used as an inhibitor of protein synthesis was the Vmw65/cellular factor complex. Both genes have been shown to have an equivalent requirement for Vmw65 (Ace *et al.*,1989) demonstrating that both may be transactivated to the same extent. However in the case of 1703, the environment of the antisense transcript is different from that in Sandri-Golden's experiments in that the antisense transcript is synthesized from the same DNA template as the sense transcript and the coding regions of both are in close proximity. It is therefore possible that in an infected cell environment,

•It is also possible that at early and late times of infection increased levels of IE2 transactivators (eg. Vmw110 and Vmw175) generate higher levels of IE2 mRNA thereby overcoming the block imposed by the antisense transcript.

the IE1 promoter is capable of synthesizing enough antisense transcript to see this effect (class 1). The hybridization of the antisense transcript to a region of IE2 mRNA outwith the IE2 transcriptional start sites (class 2) may be inefficient resulting in the production of small quantities of Vmw63 during immediate-early times of infection.

The production of IE2 gene products returns to wild-type levels during conditions other than immediate-early which can be attributed to the transactivation of UL55 during early and late conditions of infection as follows; **1**. The transcription of UL55 during early and late conditions of infection leads to the production of UL55 mRNA/IE1 mRNA duplex molecules (class 1), **2**. The same process results in the IE1 antisense transcript hybridizing to UL55 RNA changing its conformation (class 2), and **3**. RNA polymerase transcribing UL55 interferes with the RNA polymerase molecule transcribing the IE1 gene (class 3). All of these allow wild-type quantities of IE2 mRNA to be produced by freeing IE2 mRNA from constraints imposed by the antisense transcript.

Future prospects.

Criteria for defining regulation by natural antisense RNAs are threefold (Krystal, 1992). **1**. The complementary RNA must be shown to coexist within the cell. This can be done by strand specific hybridization techniques, for example, S1 nuclease mapping using strand specific probes, **2**. Evidence for the interaction of the two complementary RNAs should be provided, that is, the detection of the duplex molecules and **3**. A function for the interaction of the complementary molecules should be demonstrated. The complementary RNA strand was never detected in this study, except after termination by the insertion of the polyadenylation signal, and the presence of RNA hybrids was not

investigated. As mentioned in the introduction, the detection of the antisense transcript was an aim of this project, however at that time it was thought that the transcript was about 15kb in length and the strand specific probe used corresponded to a region of HSV-1 DNA 5' to the IE2 open reading frame. No antisense transcripts were detected with this probe. In light of later evidence it became apparent that the transcript was probably not as long as 15kb, and further work could involve the use of more suitable strand specific probes to detect the antisense transcript. Duplex RNA molecules in the nucleus of 1703 infected cells could be detected by the use of single stranded RNA degrading enzymes to digest a preparation of 1703 immediate-early RNA, leaving double stranded RNA (the sense/antisense RNA duplex) intact, and the subsequent S1 nuclease mapping or Northern blot analysis of the products. A function for the complementary molecules has been established and is the inhibition of IE2 mRNA synthesis during immediate-early times of infection.

The role of LAT in HSV-1 latently infected cells is controversial and there is evidence that LAT may function to control latency in an antisense manner. The use of the criteria described above to examine the interaction of LAT and its possible target, IE1 mRNA, may give further information concerning this possibility.

REFERENCES.

ABMAYR, S.M., WORKMAN, J.L. and ROEDER R.G. (1988). The pseudorabies immediate-early protein stimulates *in vitro* transcription by facilitating TFIID: promoter interactions. *Genes & Development* **2**, 542-553.

ACE, C.I., MCKEE, T.A., RYAN, J.M., CAMERON, J.M. and PRESTON, C.M. (1989). Construction and characterization of a herpes simplex type 1 mutant unable to transinduce immediate-early infection. *Journal of Virology* **63**, 2260-2269.

ACKERMAN, M., CHOU, J., SARIMENTO, M., LERNER, RA., ROIZMAN, B. (1986). Identification by antibody to a synthetic peptide of a protein specified by a specific gene located in the terminal repeats of the L component of the herpes simplex virus genome. *Journal of Virology* **58**, 843-850.

ADDISON, C., RIXON, F.J. and PRESTON, V.G. (1990). Herpes simplex virus type 1 UL28 gene product is important for the formation of mature capsids. *Journal of Virology* **71**, 2377-2384.

AIBA, H. (1985). Transcription of the *Eschericia coli* adenylate cyclase gene is negatively regulated by cAMP-cAMP receptor protein. *Journal of Biological Chemistry* **260**, 3063-3070.

BACCHETTI, S., EVELEGH, M.J. and MUIRHEAD, B. (1986). Identification and separation of the two subunits of herpes simplex virus ribonucleotide reductase. *Journal of Virology* **57**, 1177-1181.

BARINGER, J.R. and SWOVELAND, P. (1973). Recovery of herpes simplex virus from human sacral ganglia. *New England Journal of Medicine* **288**, 648-650.

BARKER, D.E. and ROIZMAN, B. (1992). The unique sequence of the herpes simplex virus L component contains an additional translated open reading frame designated UL49.5. *Journal of Virology* **66**, 562-566.

BASTION, F.O., RABSON, A.S., YEE, C.L. and TRALKA, T.S. (1972). Herpesvirus hominus: isolation from the human trigeminal ganglion. *Science* **178**, 306-307.

BATTERSON, W. and ROIZMAN, B. (1983). Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. *Journal of Virology* **46**, 371-377.

BATTERSON, W., FURLONG, D., ROIXMAN, B. (1983). Molecular genetics of herpes simplex virus VIII. Further characterization of a *ts* mutant defective in the release of viral DNA and in other stages of viral reproductive cycle. *Journal of Virology* **45**, 397-407.

BECKER, Y., HADAR, J., TABOR, E., BEN-HUR, T., RAIBSTEIN, I., ROSEN, A. and DARAI, G. (1986). A sequence in *Hpa* 1-P fragment of herpes simplex virus-1 DNA determines intraperitoneal pathogenicity in mice. *Virology* **149** 255-259.

BEN-ZEEVE, A. and BECKER, Y. (1977). Requirement of host cell RNA polymerase II in the replication of HSV in α-amanitin sensitive and resistant cell lines. *Virology* **76**, 246-253.

BIGGIN, M.D., GIBSON, T.J. and HUANG, G.F. (1983). Buffer gradient gels and ^{35}S label as an aid to rapid sequence determination. *Proceedings of the National Academy of Sciences* USA **80**, 3963-3965.

BIRNBOIM, H.C. and DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513-1523.

BLOCK, T.M., SPIVACK, J.G., STEINER, I., DESHMANE, S., MACINTOSH, M.T., LIRETTE, R.P. and FRASER, N.W. (1990). A herpes simplex virus type 1 latency associated transcript mutant reactivates with normal kinetics from latent infection. *Journal of Virology* **64**, 3417-3462.

BLOCK, T., JORDAN, R., FARKAS, D.M. and HUGHES, R.G. (1991). Inhibition of transient gene expression with plasmids encoding herpes simplex type 1 UL55 and alpha genes. *Journal of General Virology* **72**, 131-141.

BROWN, S.M. and HARLAND, J. (1987). Three mutants of herpes simplex virus type 2: One lacking the genes US10, US11 and US12 and two in which R_S has been extended by 6Kb to 0.91 map units with the loss of U_S sequences between 0.94 and U_S/TR_S junction. *Journal of General Virology* **68**, 1-18.

BROWN, S.M., RITCHIE, D.A. and SUBAK-SHARPE, J.H. (1973). Genetic studies with herpes simplex virus type 1. The isolation of temperature sensitive mutants, their arrangement into complementation groups and

recombination analysis leading to a linkage map. *Journal of General Virology* **18**, 329-346.

BROWN, S.M., HARLAND, J. and SUBAK-SHARPE, J.H. (1984). Isolation of restriction endonuclease site deletion mutants of herpes simplex virus. *Journal of General Virology* **65**, 1053-1068.

BUCKMASTER, E.A., GOMPELS, U., MINSON, A. (1984). Characterization and physical mapping of an HSV-1 glycoprotein of approximately $115x10^3$ molecular weight. *Virology* **139**, 408-413.

BUSBY, D.W.G., HOUSE, W. and MACDONALD, J.R. (1964). In: Virological techniques; Churchill, London.

BZIK, D.J., FOX, B.A., DELUCA, N.A. and PERSON, S. (1984). Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. *Virology* **133**, 301-314.

CAI, W., GU, B., PERSON, S. (1988). Role of glycoprotein B of herpes simplex type 1 in viral entry and cell fusion. *Journal of Virology* **62**, 2596-2604.

CAI, W. and SCHAFFER P.A. (1989). Herpes simplex type 1 ICP0 plays a critical role in the *de novo* synthesis of infectious virus following transfection of viral DNA. *Journal of Virology* **63**, 4579-4589.

CAMERON, J.M., MCDOUGALL, I., MARSDEN, H.S., PRESTON, V.G., RYAN, D.M. and SUBAK-SHARPE, J.H. (1988). Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus in mice and a valid antiviral target. *Journal of General Virology* **69**, 2607-2612.

CAMPADELLI-FIUME, G., ARSENAKSIS, M., FARABEGOLI, F., ROIZMAN, B.(1988). Entry of herpes simplex virus type 1 in BT cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. *Journal of Virology* **62**, 159-167.

CAMPBELL, M.E.M., PALFREYMAN, J.W. and PRESTON, C.M. (1984). Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate-early transcription. *Journal of Molecular Biology* **180**, 1-19.

CASPARY, L., SCHINDING, B., DUNDAROV, S. and FALKE, J. (1980). Infection of susceptible and resistant mouse strains with herpes simplex virus type 1 and 2. *Archives of Virology* **65**, 219-227.

CHALLBERG, M.D. (1986). A method for identifying the viral genes required for herpesvirus DNA replication. *Proceedings of the National Academy of Sciences USA* **83**, 9094-9098.

CHARTRAND, P., CRUMPACKER, C.S., SCHAFFER, P.A. and WILKIE, N.M. (1980). Physical and genetic analysis of the herpes simplex virus DNA polymerase locus. *Virology* **103**, 311-326.

CHOU, J. and ROIZMAN, B. (1985). Isomerization of herpes simplex virus 1 genome: Identification of *cis*-acting and recombination sites within the domain of the 'a' sequence. *Cell* **41**, 803-811.

CHOU, J. and ROIZMAN, B. (1986). The terminal 'a' sequence of the herpes simplex virus genome contains the promoter of a gene located in the repeat sequences of the L component. *Journal of Virology* **57**, 629-637.

CHOU, J. and ROIZMAN, B. (1989). Characterization of the DNA sequence-common and sequence-specific proteins binding to cis-acting sites for the cleavage of the terminal 'a' sequence of the herpes simplex virus 1 genome. *Journal of Virology* **63** 1059-1069.

CHOU, J. and ROIZMAN, B. (1990). The herpes simplex virus 1 gene for ICP 34.5, which maps in the inverted repeats, is conserved in several limited passage isolates but not in strain 17 syn$^+$. *Journal of Virology* **64**, 1014-1020.

CHOU, J., KERN, E.R., WHITELY, R.J., and ROIZMAN, B. (1990). Mapping of herpes simplex virus 1 neurovirulence to γ1 34.5, a gene non-essential for growth in tissue culture. *Science* **250**, 1262-1266.

CHOUSTERMAN, S., LACASA, M. and SHELDRAKE, B. (1979). Physical maps of channel catfish virus genome: location of sites for restriction endonucleases *Eco*R1, *Hind*III, *Hpa*1 and *Xba*1. *Journal of Virology* **31** 73-85.

CHUNG, C.T. and MILLER, R.H. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. *Nucleic Acids Research* **16** No 8, page 3580.

CLEMENTS, G.B. and SUBAK-SHARPE, J.H. (1983). Recovery of herpes simplex virus type 1 *ts* mutants from the dorsal root ganglia of mice. *Progress in Brain Research* **59**, 203-208.

CLEMENTS, G.B. and SUBAK-SHARPE, J.H. (1988). Herpes simplex virus type 2 establishes latency in the mouse footpad. *Journal of General Virology* **69**, 375-383.

CLEMENTS, G.B. and STOW, N.D. (1989). A herpes simplex virus type 1 mutant containing a deletion within immediate-early gene 1 is latency competent in mice. *Journal of General Virology* **70**, 2501-2506.

CLEMENTS, J.B., CORTINI, R. and WILKIE, N.M. (1976). Analysis of herpesvirus DNA substructure by means of restriction endonucleases. *Journal of General Virology* **30** 243-256.

CLEMENTS, J.B., WATSON, R.J. and WILKIE, N.M. (1977). Temporal regulation of herpes simplex virus type 1: location of transcripts in the viral genome. *Cell* **12**, 275-285.

COHEN, G.H. (1972). Ribonucleotide reductase activity of synchronised KB cells infected with herpes simplex virus. *Journal of Virology* **9**, 408-418.

CONLEY, A.F., KNIPE, D.M., JONES, P.C. and ROIZMAN, B. (1981). Molecular genetics of herpes simplex virus VII: Characterization of a temperature sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of α polypeptides. *Journal of Virology* **37**, 191-206.

CONSTANZO, F., CAMPADELLI-FIUME, G., FOA-TOMAS, L. and CASSAI, E. (1977). Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase II. *Journal of Virology* **21**, 996-1001.

CORDINGLEY, M., CAMPBELL, M. and PRESTON, C.M. (1983). Functional analysis of a HSV-1 promoter: identification of far upstream regulatory sequences. *Nucleic Acids Research* **11**, 2347-2365.

CROEN, K.D., OSTROVE, J.M., DRAGOVIC, L.J. and STRAWS, S.E. (1987). Latent HSV in human trigeminal ganglia: detection of an immediate-early gene 'antisense' transcript by *in situ* hybridization. *New England Journal of Medicine* **317**, 1427-1432.

CRUTE, J.W., TSURUMI, T. and ZHU, L. (1989). Herpes simplex virus 1 helicase-primase: a complex of three herpes encoded gene products. *Proceedings of the National Academy of Sciences USA* **86**, 2186-2189.

CULLEN, B., LOMEDICO, P. and JU, G. (1984). Transcriptional interference in avian retroviruses-implications for the promoter insertion model of leukaemogenisis. *Nature* **307**, 241-245.

DALZIEL, R.G. and MARSDEN, H.S. (1984). Identification of two herpes simplex virus type 1 induced proteins (21K and 22K) which interact

specifically with the 'a' sequence of herpes simplex virus DNA. *Journal of General Virology* **65**, 1467-1475.

DAVISON, A.J. (1981). Detailed structural aspects of the herpes simplex virus genome. PhD thesis, University of Glasgow.

DAVISON, A.J. (1984). Structure of the genome termini of varicella-zoster virus. *Journal of General Virology* **65**, 1969-1977.

DAVISON, A.J. and TAYLOR, P. (1987). Genetic relationships between varicella-zoster virus and Epstein-Barr virus. *Journal of General Virology* **68**, 1067-1079.

DAVISON, A.J. and WILKIE, N.M. (1981). Nucleotide sequence of the joint between the L and S segments of herpes simplex virus types 1 and 2. *Journal of General Virology* **55**, 315-331.

DAVISON, A.J. and WILKIE, N.M. (1983a). Inversion of the two segments of the herpes simplex virus genome in intertypic recombinants. *Journal of General Virology* **64**, 1-18.

DAVISON, A.J. and WILKIE, N.M. (1983b). Either orientation of the L segment of the herpes simplex virus genome may participate in the production of viable intertypic recombinants. *Journal of General Virology* **64**, 247-250.

DEATLY, A.M., SPIVACK, J.G., LAVI, E. and FRASER, N.W. (1987). RNA from an immediate-early region of the type 1 herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice. *Proceedings of the National Academy of Sciences* **84** 3204-3208.

DEBROY, C., PEDERSON, N. and PERSON, S. (1985). Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion. *Virology* **145**, 36-48.

DEISS, L.P. and FRENKEL, N. (1986). The herpes simplex amplicon: Cleavage of concatemeric DNA is linked to packaging and involves the amplification of the terminally reiterated 'a' sequence. *Journal of Virology* **57**, 933-941.

DEISS, L.P., CHOU, J. and FRENKEL, N. (1986). Functional domains within the 'a' sequence involved in the cleavage-packaging of herpes simplex virus DNA. *Journal of Virology* **59**, 605-618.

DESAI, P.J., SCHAFFER, P.A. and MINSON, A.C. (1988). Excretion of non-infectious virus particles lacking glycoprotein H by a temperature

sensitive mutant of herpes simplex virus type 1; evidence that gH is essential for virus infectivity. *Journal of General Virology* **69**, 1147-1156.

DESHMANE, S.L. and FRASER, N.W. (1989). During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. *Journal of Virology* **63**, 943-947.

DIX, R.D., MCKENDALL, R.R. and BARINGER, J.R. (1983). Comparative neurovirulence of herpes simplex virus type 1 strains after peripheral or intracranial inoculation of BALB/c mice. *Infection and Immunity* **40**, 103-112.

DOBSON, A.T., SENDERATI, F., DEVI-RAO, G., FLANAGAN, W.M., FARRELL, M.J., STEVENS, J.G., WAGNER, E.K. and FELDMAN, L.T. (1989). Identification of the LAT promoter by expression of rabbit beta-globin mRNA in mouse sensory nerve ganglia latently infected with a recombinant herpes simplex virus. *Journal of Virology* **63**, 3844-3851.

DOLAN, A., MCKIE, E., MACLEAN, A.R., and MCGEOCH, D.J. (1992). Status of the ICP 34.5 gene in herpes simplex virus type 1 strain 17^{+}. In press.

DOWDLE, W.R., NAHMIAS, A.J., HARWELL, R.W., and PAULS, F.P. (1967). Association of antigenic types of herpes simplex hominus with site of viral recovery. *Journal of Immunology* **99**, 974-980.

DULBECCO, R. and VOGT, M. (1954). Plaque formation and isolation of pure lines with poliomyelitus. *Journal of Experimental Medicine* **99**, 167-182.

DUTIA, B.M. (1983). Ribonucleotide reductase induced by herpes simplex virus has a virus specific constituent. *Journal of General Virology* **65**, 513-521.

EFSTATHIOU, S., MINSON, A.C., FIELD, H.J., ANDERSON, J.R. and WILDY, P. (1986). Detection of HSV specific DNA sequences in latently infected mice and in humans. *Journal of Virology* **57** 446-455.

EGLIN, R.P., SHARP, F., MACLEAN, A.B., MACNAB, J.C.M., CLEMENTS, J.B. and WILKIE, N.M. (1981). Detection of RNA complementary to herpes simplex virus DNA in human cervical squamous cell neoplasms. *Cancer Research* **41**, 3597-3604.

ELIAS, P. and LEHMAN, I.R. (1988). Interaction of the origin binding protein with an origin of replication of herpes simplex virus type 1. *Proceedings of the National Academy of Sciences USA* **85**, 2959-2963.

ELIAS, P., O'DONNELL, M.E., MOCARSKI, E.S. and LEHMAN, I.R. (1986). A DNA binding protein specific for an origin of replication of herpes simplex type 1. *Proceedings of the National Academy of Sciences* **83**, 6322-6326.

ELSHIEKH, N.A., HARRIS-HAMILTON, E. and BACHENHEIMER, S.L. (1991). Differential dependence of herpes simplex virus immediate-early gene expression on *de novo* infected cell protein synthesis. *Journal of Virology* **65**, 6430-6437.

ESTRIDGE, J.K., KEMP, L.M., LA THANGUE, N.B., MANN B.S., TYMS, A.S. and LATCHMAN, D.S. (1989). The herpes simplex type 1 immediate-early polypeptide ICP27 is obligately required for the accumulation of a cellular protein during viral infection. *Virology* **168**, 67-72.

EVERETT, R.D. (1984a). A detailed analysis of an HSV-1 early promoter: sequences involved in transactivation by immediate-early gene products are not early gene specific. *Nucleic Acids Research* **12**, 3037-3056.

EVERETT, R.D. (1984b). Transactivation of transcription by herpes virus gene products: requirement for two herpes simplex virus type 1 immediate-early polypeptides for maximum activity. *The EMBO Journal* **3** 3135-3141.

EVERETT, R.D. (1985). Activation of cellular promoters during herpes simplex virus infection of biochemically transformed cells. *The EMBO Journal* 4, 1973-1980.

EVERETT, R.D. (1986). The products of herpes simplex virus type 1 (HSV-1) immediate-early genes 1, 2 and 3 can activate HSV-1 gene expression in *trans*. *Journal of General Virology* **67**, 2507-2513.

EVERETT, R.D. (1987). The regulation of transcription of viral and cellular genes by herpesvirus immediate-early gene products. *Anticancer Research* **7**, 589-604.

EVERETT, R.D. (1988a). Promoter sequence and cell type can dramatically affect the efficiencies of transcriptional activation induced by herpes simplex type 1 and its immediate-early gene products Vmw175 and Vmw110. *Journal of Molecular Biology* **203** 739-751.

EVERETT, R.D. (1991). Construction and characterization of herpes simplex type 1 viruses without introns in immediate-early gene 1. *Journal of General Virology* **72** 651-659.

FARRELL, M.J., DOBSON, A.T. and FELDMAN, L.T. (1991). Herpes simplex virus latency associated transcript is a stable intron. *Proceedings of the National Academy of Sciences USA* **88** 790-794.

FENNER, F. (1976). Classification and nomenclature of viruses. *Intervirology* **7**, 1-115.

FENWICK, M.L. and EVERETT, R.D. (1990). Inactivation of the shut-off gene (UL41) of HSV types 1 and 2. *Journal of General Virology* **71**, 2961-2967.

FIELD, H.J. and COEN, D.M. (1986). Pathogenicity of herpes simplex virus mutants containing drug resistant mutations in the viral DNA polymerase gene. *Journal of Virology* **60** 286-289.

FIELD, H.J. and WILDY, P. (1978). The pathogenicity of thymidine kinase deficient mutants of herpes simplex virus in mice. *Journal of Hygiene* **81** 267-277.

FINELLI, P.F. (1975). Herpes simplex virus and human nervous system: current concepts and review. *Military Medicine* **140**, 765-771.

FRAME, M.C., MARSDEN, H.S. and DUTIA, B.M. (1985). The ribonucleotide reductase induced by herpes simplex type 1 involves minimally a complex of two polypeptides (136K and 38K0). *Journal of General Virology* **66**, 1581-1587.

FRAME, M.C., MARSDEN, H.S. and MCGEOCH, D.J. (1986). Novel herpes simplex virus type 1 glycoproteins identified by antiserum against a synthetic oligopeptide from the predicted product of gene US4. *Journal of General Virology* **67**, 745-751.

FRENKEL, N., SCHIRMER, E.C., WYATT, L.S., KATSAFENAS, G., ROFLEMN, E DANOVICH, R.M. and JUNE, C.M. (1990). Isolation of a new herpesvirus from human CD4+ T cells. *Proceedings of the National Academy of Sciences USA* **87**, 748-752.

FRINK, R.J., EISENBERGR, F., COHEN, G. and WAGNER, E.K. (1983). Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein G. *Journal of Virology* **45**, 634-647.

GAFFNEY, D.F., MCLAUCHLAN, J., WHITTON, J.L. and CLEMENTS, J.B. (1985). A molecular system for the assay of transcription regulatory elements: the sequence TAATGARAT is required for herpes simplex virus immediate-early activation. *Nucleic Acids Research* **13** 7847-7897.

GILDEN, D.M., WROBLEWSKA, Z., KINDT, V., WARREN, K.G. and WOLENSKY, J.S. (1978). Varicella-zoster virus infection of human brain cells and ganglion cells in tissue culture. *Archives of Virology* **56**, 105-117.

GIRALDO, G., BATH, E., KOURILSKY, F.M., HENLE, G., MIKE, V., HURAUX, J.M., ANDERSON, H.K., GHARBI, M.R., KAYLWAZI, S.K. and PUISSANT, A. (1975). Antibody patterns to herpesviruses in Kaposi's sarcoma: serological association of European Kaposi's sarcoma with cytomegalovirus. *International Journal of Cancer* **15**, 839-848.

GLEB, L.D. (1985). Varicella-zoster virus. In: Virology pp591-627, B.N. Fields (Ed) Raven Press, New York.

GODOWSKI, P.J. and KNIPE, D.M. (1986). Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. *Proceedings of the National Academy of Sciences* USA **83**, 256-260.

GOLDSTEIN, D.J. and WELLER, S.K. (1988). Herpes simplex type 1 induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: Isolation and characterization of a *lac*Z insertion mutant. *Journal of Virology* **62**, 196-205.

GOMPELS, V.A., CRAXTON, M.A. and HONESS, R.W. (1988). Conservation of gene organization in the lymphotrophic herpesviruses Herpesvirus Saimiri and Epstein-Barr virus. *Journal of Virology* **62**, 757-767.

GOODMAN, J.L. and STEVENS, J.G. (1986). Passage of herpes simplex type 1 on chick embryo fibroblasts confers virulence for chick embryos. *Virus Research* **5**, 191-200.

GOTTESMAN, S. (1984). Bacterial regulation: global regulatory networks. *Annual Review of Genetics* **18**, 415-441.

GOTTLIEB, J., MARCY, A.I., COEN, D.M. and CHALLBERG, M.D. (1990). The herpes simplex virus type 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. *Journal of Virology*.**64**, 5967-5987.

HARDWICKE, M.A., VAUGHAN, P.J., SEKULOVICH, R.E., O'CONNOR, R. and SANDRI-GOLDEN, R.M. (1989). The regions important for the activator and repressor functions of herpes simplex virus type 1 α protein ICP27 map to the C-terminal half of the molecule. *Journal of Virology* **63**, 4590-4620.

HARLAND, J. and BROWN, S.M. (1985). Isolation and characterization of deletion mutants of herpes simplex virus type 2 (strain HG52). *Journal of General Virology* **66**, 1305-1321.

HARLAND, J and BROWN, S.M. (1988). Generation of a herpes simplex virus type 2 variant devoid of Xba 1 sites: removal of the 0.91 map coordinate site results in impaired synthesis of glycoprotein G2. *Journal of General Virology* **69**, 113-124.

HARLAND, J. and BROWN, S.M. (1989). A herpes simplex virus type 2 variant in which a deletion across the L-S junction is replaced by single or multiple reiterations of extraneous DNA. *Journal of General Virology* **70** 2121-2137.

HARRIS, R.A. and PRESTON, C.M. (1991). Establishment of latency *in vitro* by the herpes simplex type 1 mutant *in1814*. *Journal of General Virology* **72**, 907-913.

HAYWARD, G.S., JACOB, R.J., WADSWORTH, S.C. and ROIZMAN, B. (1975). Anatomy of herpes simples virus DNA: evidence for four populations of molecules that differ in relative orientation of their long and short components. *Proceedings of the National Academy of Sciences* USA **72**, 4243-4247.

HILL, T.J., FIELD, H.J. and ROOME, A.P.C. (1972). Intra-axonal location of herpes simplex virus particles. *Journal of General Virology* **15** 253-255.

HO, D.Y. and MOCARSKI, E.S. (1989). Herpes simplex virus RNA (LAT) is not required for latent infection in the mouse. *Proceedings of the National Academy of Sciences* USA **86**, 7596-7600.

HOLLAND, L.E., ANDERSON, K.P., SHIPMAN, J.R. and WAGNER, E.K. (1980). Viral DNA synthesis is required for the efficient expression of specific herpes simplex virus type 1 mRNA species. *Virology* **101**, 10-24.

HOMA, F.L., OTAL, T.M., GLORIOSO, J.C. and LEVINE, M. (1986). Transcriptional control signals of a herpes simplex virus type 1 late (γ2) gene lie within bases -34 to +124 relative to the 5' terminus of the mRNA. *Molecular and Cellular Biology* **6**, 3652-3666.

HOMA, F.L., GLORIOSO, J.C. and LEVINE, M. (1988). A specific 15 base pair TATA box promoter element is required for the expression of a herpes simplex type 1 late gene. *Genes & Development* **2** 40-53.

HONESS, R.W. (1984). Herpes simplex and the 'herpes complex': diverse observations and a unifying hypothesis. *Journal of General Virology* **65**, 2077-2107.

HONESS, R.W. and ROIZMAN, B. (1973). Proteins specified by herpes simplex virus XI. Identification and relative molar rates of synthesis of structural and non-structural herpesvirus polypeptides in infected cells. *Journal of Virology* **12**, 1346-1365.

HONESS, R.W. and ROIZMAN, B. (1974). Regulation of herpes virus macromolecular synthesis 1. Cascade of regulation of the synthesis of three groups of viral proteins. *Journal of Virology* **14**, 8-19.

HONESS, R.W. and ROIZMAN, B. (1975). Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. *Proceedings of the National Academy of Sciences USA* **72**, 1276-1280.

HONESS, R.W. and WATSON, D.H. (1977). Unity and diversity in the herpesviruses. *Journal of General Virology* **37**, 15-37.

HUBENTHAL-VOSS, J., STARR, L. and ROIZMAN, B. (1987). The herpes simplex virus origins of DNA synthesis in the S component are each contained within a transcriptional open reading frame. *Journal of Virology* **61**, 3349-3355.

IMBALZANO, A.N., COEN, D.M. and DELUCA, N.A. (1991). Herpes simplex virus transactivator ICP4 operationally substitutes for the cellular transcription factor Sp1 for efficient expression of the viral thymidine kinase gene. *Journal of Virology* **65**, 565-574.

INOUYE, M. (1988). Antisense RNA: Its function and applications in gene regulation- A review. *Gene* **72**, 25-34.

JACOB, R.J., MORSE, L.S. and ROIZMAN, B. (1979). Anatomy of herpes simplex virus DNA XII: Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *Journal of Virology* **29**, 448-457.

JAMIESON, A.T. and SUBAK-SHARPE, J.H. (1974). Biochemical studies on the herpes virus specific deoxypyrimidine kinase activity. *Journal of General Virology* **24**, 481-492.

JAMIESON, A.T., GENTRY, G.A. and SUBAK-SHARPE, J.H. (1974). Induction of both thymidine and deoxycytidine kinase activity by herpesviruses. *Journal of General Virology* **24**, 465-480.

JAQUEMONT, B., VERRIER, B., EPSTEIN, A.L. and MACHUCA, I. (1984). Expression of immediate-early genes in herpes simplex type 1 infected XC cells: Lack of ICP22 (68K) polypeptide. *Journal of General Virology* **65**, 1331-1340.

JAVIER, R.T., SEDERATI, F. and STEVENS, J.G. (1986). Two avirulent herpes simplex viruses interact in the animal to generate virulent recombinants and a lethal infection. *Science* **234**, 746-748.

JOHNSON, D.C. and SPEAR, P.G. (1984). Evidence for translational regulation of herpes simplex virus type 1 glycoprotein D expression. *Journal of Virology* **51**, 389-394.

JOHNSON, D.C. and LIGAS, M.W. (1988). Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus specific cell surface receptors. *Journal of Virology* **62**, 4605-4612.

JOHNSON, P.A. and EVERETT, R.D. (1986). The control of herpes simplex type 1 late gene expression: a TATA box/cap site region is sufficient for fully efficient regulation and activity. *Nucleic Acids Research* **14**, 8247-8264.

JOHNSON, P.A., MACLEAN, C., MARSDEN, H.S., DALZIEL, R.G. and EVERETT, R.D. (1986). The product of the gene US11 of herpes simplex virus type 1 is expressed as a true late gene. *Journal of General Virology* **67**, 871-883.

JONGENEEL, C.V. and BACHENHEIMER, S.L. (1981). Structure of replicating herpes simplex virus DNA. *Journal of Virology* **39**, 656-660.

JUNEJO, F. (1991). Characterization of three deletion variants of herpes simplex virus type 1 (HSV-1): sequence, latency and virulence analysis. PhD thesis, University of Glasgow.

JUNEJO, F., MACLEAN, A.R. and BROWN, S.M. (1991). Sequence analysis of the herpes simplex type 1 strain 17 variants 1704, 1705 and 1706 with respect to their origin and effect on the latency-associated transcript sequence. *Journal of General Virology* **72**, 2311-2315.

KAERNER, H.C., SCHROEDER, C.H., OTT-HARTMAN, A., KUMEL, C. and KUCHNER, H. (1983). Genetic variability of herpes simplex virus: development of a pathogenic variant during passaging of a non-pathogenic HSV-1 strain in the mouse brain. *Journal of Virology* **46**, 83-93.

KATSUKI, M., SATO, M., KIMURA, M.,YOKOYAMA, M., KOBAYASKI, K. and NOMURA, T. (1988). Conversion of normal behaviour to shiverer by myelin basic protein in transgenic mice. *Science* **241**, 593-595.

KAWAMURA, M., HAYASHI, M., FURUICHI, T., NONOYAMA, M., ISOGAI, E. and NAMIOKA, S. (1991). The inhibitory effects of oligonucleotides, complementary to Mareks disease virus mRNA transcribed from the *Bam*H1-H region, on the proliferation of transformed lymphoblastoid cells, MDCC-MSB1. *Journal of General Virology* **72**, 1105-1111.

KESSLER, I. (1977). Venereal factors in human cervical cancer. *Cancer* **39**, 1912-1919.

KIBLER, P.K., DUNCAN, J., KEITH, B.D., HUPEL, T. and SMILEY, J.R. (1991). Regulation of herpes simplex virus true late gene expression: sequences downstream from the US11 TATA box inhibit expression from an unreplicated template. *Journal of Virology* **65**, 6749-6760.

KOFF, A. and TEGTMEYER, P. (1988). Characterization of the major recognition sequences for a herpes simplex virus type 1 origin binding protein. *Journal of Virology* **62**, 4096-4103.

KOHL, S. and LOO, L.S. (1980). Ontogeny of murine cellular cytotoxicity to herpes simplex virus infection. *Infection and Immunity* **30**, 847-850.

KRAUSE, P.R., CROEN, K.D., STRAUS, S.E. and OSTROVE, J.M. (1988). Deletion and preliminary characterization of herpes simplex virus type 1 transcripts in latently infected trigeminal ganglia. *Journal of Virology* **62**, 4189-4823.

KRINKE, L. and WULFF, D.L. (1987). *oop* RNA, produced from multicopy plasmids, inhibits λ c11 gene expression through an RNAse III-dependant mechanism. *Genes & Development* **1** 1005-1013.

KRYSTAL, G.W. (1992). Regulation of eukaryotic gene expression by naturally occuring antisense RNA. In: Gene regulation: Biology of antisense RNA and DNA. pp11-19. R.P. Erikson and J.G. Izant (Eds). Raven Press Ltd, New York.

KRYSTAL, G., BIRRER, M., WAY, J., NAU, M., SAUSVILLE, E., THOMPSON, C., MINNA, J. and BATTEY, J. (1988). Multiple mechanisms for the transcriptional regulation of the *myc* gene family in small-cell lung cancer. *Molecular and Cellular Biology* **8**, 3373-3381.

KUMAR, A. and LINDBERG, U. (1972). Characterization of nucleoprotein and messenger RNA from KB cells. *Proceedings of the National Academy of Sciences* USA **69**, 681-685.

LANGELAND, N., HOLMSEN, H., LILLEHAUG, J.R. and HAARR, L. (1987). Evidence that neomycin inhibits binding of herpes simplex virus type 1 to the cellular receptor. *Journal of Virology* **61**, 3388-3393.

LANGELAND, N., MOORE, L.J., HOLMSEN, H. and HAARR, L. (1988). Interaction of polylysine with the cellular receptor for herpes simplex virus type 1. *Journal of General Virology* **69**, 1137-1145.

LEIB, D.A., COEN, D.M., BOGARD, C.L., HICKS, K.A., KNIPE, D.M., TYLER, K.L. and SCHAFFER, P.A. (1989a). Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. *Journal of Virology* **63**, 759-768.

LEIB, D.A., BOGARD, C.L., KOSZ-VNENCHAKI, M., HICKS, K.A., COEN, D.M., KNIPE, D.M. and SCHAFFER, P.A. (1989b). A deletion mutant in the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. *Journal of Virology* **63**, 2893-2900.

LITTLE, S.P. and SCHAFFER, P.A. (1981). Expression of the syncitial (*syn*) phenotype in HSV-1, strain KOS: Genetic and phenotypic studies of mutants in two *syn* loci. *Virology* **112**, 686-702.

LOCKSHON, D. and GALLOWAY, D.A. (1986). Cloning and characterization of oriL2, a large palindromic DNA replication origin of herpes simplex type 2. *Journal of Virology* **62**, 513-521.

LOGAN, J., FALCK-PEDERSEN, E., DARNELL, J.E. and SHENK,T. (1987). A poly(A) addition site and a downstream region are required for efficient cessation of transcription by RNA polymerase II in the mouse β^{maj}-globin gene. *Proceedings of the National Academy of Sciences* **84**, 8306-8310.

LONGNECKER, R. and ROIZMAN, B. (1986). Generation of an inverting herpes simplex virus type 1 mutant lacking the L-S junction 'a' sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E, and the alpha 47 gene. *Journal of Virology* **58**, 583-591.

LONSDALE, D.M. (1979). A rapid technique for distinguishing herpes simplex type 1 from type 2 by restriction technology. *Lancet* **1**, 849-852.

LOPEZ, C. (1975). Genetics of natural resistance to herpesvirus infection in mice. *Nature* London **258**, 152-153.

LUI, F. and ROIZMAN, B. (1991). The herpes simplex virus 1 gene encoding a protease also contains within its coding domain the gene encoding the more abundant substrate. *Journal of Virology* **65**, 5149-5156.

MCCARTHY, A.M., MCMAHON, L. and SCHAFFER, P.A. (1989). Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. *Journal of Virology* **63**, 18-27.

MCGEOCH, D.J. (1987). The genome of herpes simplex virus: structure, replication and evolution. *Journal of Cell Science* **7** (supplement), 5194-5222.

MCGEOCH, D.J. (1989). The genome of the human herpes virus: contents, relationships and evolution. *Annual Review of Microbiology* **43**, 235-265.

MCGEOCH, D.J., DOLAN, A., DONALD, S. and RIXON, F.J. (1985). Sequence determination and genetic content of the short unique region of herpes simplex virus type 1. *Journal of Molecular Biology* **181**, 1-13.

MCGEOCH, D.J., DOLAN, A., DONALD, S. and BRAVER, D.H.K. (1986). Complete DNA sequence of the short repeat region of the genome of herpes simplex virus type 1. *Nucleic Acids Research* **14**, 1727-1744.

MCGEOCH, D.J., DALRYMPLE, M.A., DAVISON, A.J., DOLAN, A., FRAME, M.C., MCNAB, D., PERRY, L.J., SCOTT, J.E. and TAYLOR, P. (1988a). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *Journal of General Virology* **69**, 1531-1574.

MCGEOCH, D.J., DALRYMPLE, M.A., DOLAN, A., PERRY, L.J., TAYLOR, P. and CHALLBERG, M.D. (1988b). Structure of herpes simplex virus type 1 genes required for replication of virus DNA. *Journal of Virology* **62**, 444-453.

MCGEOCH, D.J., CUNNINGHAM, C., MCINTYRE, G. and DOLAN, A. (1991). Comparative sequence analysis of the long repeat and adjoining parts of the long unique regions of the genome of herpes simplex virus type 1 and 2. *Journal of General Virology* **72**, 3057-3075.

MCKNIGHT, J.L. (1980). The nucleotide sequence and transcript map of herpes simplex virus thymidine kinase gene. *Nucleic Acids Research* **8**, 5949-5960.

MCKNIGHT, J.L. and TIJAN, R. (1986). Transcriptional selectivity of genes in mammalian cells. *Cell* **46**, 795-805.

MCKNIGHT, J.L., KRISTIE, T.M. and ROIZMAN, B. (1987). Binding of the virion protein mediates α gene induction in herpes simplex virus type 1 infected cells to its *cis* sites requires cellular proteins. *Proceedings of the National Academy of Sciences* USA **84**, 7061-7065.

MCLAUCHLAN, J. and CLEMENTS, J.B. (1982). A 3' co-terminus of two early herpes simplex virus type 1 mRNAs. *Nucleic Acids Research* **2**, 502-512.

MCLAUCHLAN, J. and CLEMENTS, J.B. (1983a). Organization of the herpes simplex virus type 1 transcription unit encoding two early proteins with molecular weights of 140000 and 40000. *Journal of General Virology* **64**, 997-1006.

MCLAUCHLAN, J. and CLEMENTS, J.B. (1983b). DNA sequence homology between two colinear loci on the herpes simplex virus genome which have different transforming abilities. *The EMBO Journal* **2**, 1953-1961.

MCLAUCHLAN, J., GAFFNEY, D., WHITTON, J.L. and CLEMENTS, J.B. (1985). The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNA 3' termini. *Nucleic Acids Research* **4**, 1347-1368.

MCMAHON, L. and SCHAFFER, P.A. (1990). The repressing and enhancing functions of the herpes simplex virus regulatory protein ICP27 map to C-terminal regions and are required to modulate gene expression very early in infection. *Journal of Virology* **64**, 3471-3485.

MACDONALD, D. (1980). Herpes simplex virus induced early proteins. PhD thesis, University of Glasgow.

MACKEM, S. and ROIZMAN, B. (1982a). Structural features of the α gene 4, 0 and 27 promoter regulatory sequences which confer α regulation on chimeric thymidine kinase genes. *Journal of Virology* **44**, 939-949.

MACKEM, S. and ROIZMAN, B. (1982b). Differentiation between promoter regulatory regions of herpes simplex virus 1: the functional domains and

sequence of a moveable α regulator. *Proceedings of the National Academy of Sciences* USA **79**, 4917-4921.

MACLEAN, A.R. (1988). *Xba*1 site loss mutants and deletion/duplication variants of herpes simplex virus type 1: isolation, characterization and recombination studies. PhD thesis, University of Glasgow.

MACLEAN, A.R. and BROWN, S.M. (1987a). A herpes simplex virus type 1 variant which fails to synthesize immediate-early polypeptide Vmw63. *Journal of General Virology* **68**, 1339-1350.

MACLEAN, A.R. and BROWN, S.M. (1987b). Deletion and duplication variants around the long repeats of herpes simplex virus type 1 strain 17. *Journal of General Virology* **68**, 3019-3031.

MACLEAN, A.R., UL-FAREED, M., ROBERTSON, L., HARLAND, J and BROWN, S.M. (1991b). Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence-related sequences in Glasgow strain 17^+ between immediate-early gene 1 and the 'a' sequence. *Journal of General Virology* **72**, 631-639.

MACLEAN, C.A., RIXON, F.J. and MARSDEN, H.S. (1987). The products of gene US11 of herpes simplex virus type 1 are DNA binding and localize to the nucleoli of infected cells. *Journal of General Virology* **68**, 1921-1937.

MACLEAN, C.A., EFSTATHIOU, S., ELLIOT, M.L., JAMIESON, F.E. and MCGEOCH, D.J. (1991). Investigation of herpes simplex type 1 genes encoding multiply inserted membrane proteins. *Journal of General Virology* **72**, 897-906.

MACPHERSON, I. and STOKER, M. (1962). Polyoma transformation of hamster clones-and investigation of genetic factors affecting cell competence. *Virology* **16**, 147-151.

MANIATIS, T., FRITSCH, E.F. and SAMBROOK, J. (1982). Molecular Cloning. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

MANSERVIGI, R., SPEAR, P.G. and BUCHAN, A. (1977). Cell fusion induced by herpes simplex virus is promoted and suppressed by different glycoproteins. *Proceedings of the National Academy of Sciences* USA **74**, 3913-3917.

MARSDEN, H.S., CROMBIE, I.K. and SUBAK-SHARPE, J.H. (1976). Control of protein synthesis in herpesvirus infected cells: analysis of polypeptides

induced by wild-type and sixteen temperature sensitive mutants of HSV-1 strain 17^+. *Journal of General Virology* **31**, 347-372.

MARSDEN, H.S., STOW, N.D., PRESTON, V.G., TIMBURY, M.C. and WILKIE N.M. (1978). Physical mapping of herpes virus induced polypeptides. *Journal of Virology* **28**, 624-642.

MARSDEN, H.S., LANG, J., DAVISON, A.J., HOPE, R.G. and MACDONALD, D.M. (1982). Genomic location and lack of phosphorylation of the HSV immediate-early polypeptide IE12. *Journal of General Virology* **62**, 17-27.

MATSUKATA, H. and TOMIZAWA, J. (1986). Control of primer formation for ColE1 plasmid replication: conformational change in the primer transcript. *Cell* **44**, 125-136.

MATTHEWS, R.E.F. (1982). Classification and nomenclature of viruses. *Intervirology* **17**, 1-200.

MAVROMARA-NAZOS, P. and ROIZMAN, B. (1987). Activation of herpes simplex virus type 1 γ2 genes by viral DNA replication. *Virology* **161**, 593-598.

MELLERICK, D.M. and FRASER, N.W. (1987). Physical state of the herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. *Virology* **158**, 265-275.

MESSING, J. (1983). New M13 vectors for cloning. *Methods in Enzymology* **101**, 20.

MOCARSKI, E.S. and ROIZMAN, B. (1981). Site-specific inversion sequence of herpes simplex virus genome: domain and structural features. *Proceedings of the National Academy of Sciences* USA **78**, 7047-7051.

MOCARSKI, E.S. and ROIZMAN, B. (1982). The structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. *Cell* **31**, 89-97.

MULLANEY, J., MOSS, H.W. and MCGEOCH, D.J. (1989). Gene UL2 of herpes simplex virus type 1 encodes a uricil-DNA glycosylase. *Journal of General Virology* **70**, 449-454.

MURCHIE, M.J. and MCGEOCH, D.J. (1982). DNA sequence of an immediate-early gene region of the herpes simplex type 1 genome. (map coordinates 0.950-0.978). *Journal of General Virology* **62**, 1-15.

NAHMIAS, A.J. (1972). Herpesviruses from fish to man- A search for pathobiologic unity. *Pathobiology Annual* **2**, 153-182.

NAIB, Z.M., NAHMIAS, A.J. and JOSEY, W.E. (1966). Cytology and histopathology of cervical infection. *Cancer* **19**, 1026-1031.

NASSERI, M. and MOCARSKI, E.S. (1988). The cleavage recognition signal is contained within sequences surrounding and 'a'-'a' junction in herpes simplex virus DNA. *Virology* **167**, 25-30.

NEPVEU, A. and MARCU, K.B. (1986). Intragenic pausing and antisense transcription in the murine c-myc locus. *The EMBO Journal* **5**, 2859-2965.

NII, S., MORGAN, C. and ROSE, H.M. (1968). Electron microscopy of herpes simplex virus II. Sequence of development. *Journal of Virology* **2**, 517-536.

OKAMOTO, K. and FREUNDLICH, M. (1986). Mechanism for the autogenous control of the *crp* operon: transcriptional inhibition by a complementary RNA transcript. *Proceedings of the National Academy of Sciences* USA **83**, 5000-5004.

OLIVIO, P.D., NELSON, N.J. and CHALLBERG, M.D. (1988). Herpes simplex virus DNA replication: the UL9 gene encodes an origin binding protein. *Proceedings of the National Academy of Sciences* USA **85**, 5414-5418.

PARK, M., KITCHENER, M.C. and MACNAB, J.C.M. (1983). Detection of herpes simplex type 2 DNA sequences in human cervical carcinoma tissue. *The EMBO Journal* **2**, 1029-1034.

PERRY, L.J. and MCGEOCH, D.J. (1988). The DNA sequence of the long repeat region and adjoining parts of the long unique region in the genome of herpes simplex virus type 1. *Journal of General Virology* **69**, 2831-2846.

PERRY, L.J., RIXON, F.J., EVERETT, R.D., FRAME, M.C.and MCGEOCH, D.J. (1986). Characterization of the IE110 gene of herpes simplex type 1. *Journal of General Virology* **67**, 2365-2380.

POFFENBERGER, K.L. and ROIZMAN, B. (1985). A non-inverting genome of a viable herpes simplex virus type 1. Presence of head to tail linkages in packaged genomes and requirements for circularization after infection. *Journal of Virology* **53**, 589-595.

POFFENBERGER, K.L., TABARES, E. and ROIZMAN, B. (1983). Characterization of a viable non-inverting herpes simplex virus type 1 genome derived by insertion and deletion of sequences at the junction of L and S. *Proceedings of the National Academy of Sciences* USA **80**, 2690-2694.

POLVINO-BODNAR, J., ORBERG, P.K. and SCHAFFER, P.A. (1987). Herpes simplex type 1 oriL is not required for virus replication or for the establishment and reactivation of latent infection in mice. *Journal of Virology* **61**, 3528-3535.

POST, L.E. and ROIZMAN, B. (1981). A generalised technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. *Cell* **25**, 227-232.

POST, L.E., MACKEM, S. and ROIZMAN, B. (1981). Regulation of α genes of herpes simplex virus: expression of chimeric genes by fusion of thymidine kinase with α gene promoters. *Cell* **24**, 555-565.

PRESTON, C.M. (1979a). Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus and the temperature sensitive mutant *ts*K. *Journal of Virology* **29**, 275-284.

PRESTON, C.M. (1979b). Abnormal properties of an immediate-early polypeptide in cells infected with the herpes simplex virus type 1 mutant *ts*K. *Journal of Virology* **32**, 357-369.

PRESTON, C.M., CORDINGLEY, M.G. and STOW, N.D. (1984). Analysis of DNA sequences which regulate the transcription of a herpes simplex virus immediate-early gene. *Journal of Virology* **50**, 708-716.

PRESTON, C.M., FRAME, M.C. and CAMPBELL, M.E.M. (1988). A complex formed between cell components and a HSV structural polypeptide binds to a viral immediate-early gene regulatory DNA sequence. *Cell* **52**, 425-434.

PRESTON, V.G. and FISCHER, F.B. (1984). Identification of the herpes simplex virus type 1 gene coding the dUTPase. *Virology* **138**, 58-68.

PRESTON, V.G., COATES, J.A.V. and RIXON, F.J. (1983). Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. *Journal of Virology* **45**, 1056-1064.

PRESTON, V.G., PALFREYMAN, J.W. and DUTIA, B.M. (1984). Identification of a herpes simplex virus type 1 polypeptide which is a component of

the virus-induced ribonucleotide reductase. *Journal of Virology* **65**, 1457-1466.

PRESTON, V.G., DARLING, A.J. and MCDOUGALL, I. (1988). The herpes simplex virus type 1 temperature sensitive mutant ts1222 has a single base pair deletion in the small subunit of ribonucleotide reductase. *Virology* **167**, 458-467.

QUINN, J.P. and MCGEOCH, D.J. (1985). DNA sequence of the region in the genome of herpes simplex virus type 1 containing the gene for DNA polymerase and the major DNA binding protein. *Nucleic Acids Research* **13**, 8143-8163.

RAAB-TRAUB, N., DAMBAUGH, T., and KIEFF, E. (1980). DNA of Epstein-Barr virus. *Cell* **22**, 257-267.

RAPP, F. (1980). Persistence and transmission of cytomegalovirus- In: virus-host interactions: viral invasion, persistence and disease. Volume 16 pp192-232. Eds H. Fraenkel-Conrat and R.R.Wagner. Plenum Publishing Corporation, New York.

RESNICK, J., BOYD, B.A. and HAFFEY, M.L. (1989). DNA binding by the herpes simplex virus type 1 ICP4 protein is necessary for efficient down regulation of the ICP0 promoter. *Journal of Virology* **63**, 2497-2503.

RICE, S.A. and KNIPE, D.M. (1988). Gene specific transactivation by herpes simplex virus type 1 alpha protein ICP27. *Journal of Virology* **62**, 3814-3823.

RICE, S.A. and KNIPE, D.M. (1990). Genetic evidence for two distinct transactivation functions of the herpes simplex virus α protein ICP27. *Journal of Virology* **64**, 1704-1715.

RIFKIND, D. (1966). The activation of varicella-zoster virus infections by immunosuppressive therapy. *Journal of Laboratory and Clinical Medicine* **68**, 463-474.

RIXON, F.J. and CLEMENTS, J.B. (1982). Detailed structural analysis of two spliced herpes simplex type 1 immediate-early mRNAs. *Nucleic Acids Research* **10**, 2241-2256.

RIXON, F.J. and MCGEOCH, D.J. (1984). A 3' co-terminal family of mRNAs from the herpes simplex type 1 short region: Two overlapping reading frames encode unrelated polypeptides one of which has a highly reiterated amino acid sequence. *Nucleic Acids Research* **12**, 2473-2487.

RIXON, F.J., CAMPBELL, M.E. and CLEMENTS J.B. (1982). The immediate-early mRNA that encodes the regulatory polypeptide Vmw175 of herpes simplex virus type 1 is unspliced. *The EMBO Journal* **1** 1273-1277.

RIXON, F.J., CAMPBELL, M.E. and CLEMENTS, J.B. (1984). A tandemly reiterated DNA sequence in the long repeat region of herpes simplex virus type 1 found in close proximity to immediate-early mRNA. *Journal of Virology* **52**, 715-718.

RIXON, F.J., ADDISON, C. and MCLAUCHLAN, J. (1992). Assembly of enveloped tegument structures (L-particles) can occur independently of virion maturation in herpes simplex virus type 1 infected cells. *Journal of General Virology* **73**, 277-284.

ROCK, D.L. and FRASER, N.W. (1983). Detection of the herpes simplex virus type 1 genome in central nervous system of latently infected mice. *Nature*, London **302**, 523-525.

ROCK, D.L. and FRASER, N.W. (1985). Latent herpes simplex virus type 1 DNA contains two copies of the virion DNA joint region. *Journal of Virology* **55**, 849-852.

ROCK, D.L., NESBURN, A.B., GHIASI, H., ONG, J., LEVIES, T.L., LOKENSGARD, J.R. and WECHSLER, S.L. (1987). Detection of latency related viral RNAs in trigeminal ganglia of rabbits latently infected with HSV-1. *Journal of Virology* **61**, 3820-3826.

ROFFMAN, E., ALBERT, J.P., GOFF, J.P. and FRENKEL, N. (1990). Putative site for the aquisition of human herpes virus 6 virion tegument. *Journal of Virology* **64**, 6308-6313.

ROIZMAN, B. (1979a). The structure and isomerization of herpes simplex viral genomes. *Cell* **16**, 481-494.

ROIZMAN, B. (1979b). The organization of herpes simplex virus genomes. *Annual Reviews in Genetics* **13**, 25-57.

ROIZMAN, B. (1982). The family herpesviridae: general description, taxonomy and classification. In 'The herpesviruses' volume 1. pp1-23. Ed: B Roizman. Plenum Press, New York and London.

ROIZMAN, B. and FURLONG, D. (1974). The replication of herpesviruses. In 'Comprehensive Virology' volume 3, pp229-403. Eds: H. Fraenkel-Conrat and R.R. Wagner. Plenum Press. New York and London.

ROIZMAN, B. and SEARS, A.E. (1987). An enquiry into the mechanism of herpes simplex virus latency. *Annual Review of Microbiology* **41**, 543-571.

ROLLER, R.J. and ROIZMAN, B. (1990). The herpes simplex virus US11 open reading frame encodes a sequence specific RNA binding protein. *Journal of Virology* **64**, 3463-3470.

ROSEN, A and DARAI,G. (1985). Mapping of the deletion in the genome of HSV-1 HFEM responsible for its avirulent phenotype. *Medical Microbiology and Immunology* **173**, 329-343.

ROSEN, A., ERNST, F., KOCH, H.G., GELDERBLON, H., DARAI, G., HADAR, J., TABAR, E., BEN-HUR, T. and BECKER, Y. (1986). Replacement of the deletion in the genome (0.762-0.789 m.u.) of avirulent HSV-1 HFEM strain using cloned *Mlu*1 DNA fragment (0.7615-0.796 m.u.) of virulent intertypic recombinant. *Virus Research* **5**, 157-175.

RUSSELL, J. and PRESTON, C.M. (1986). An *in vitro* latency system for herpes simplex virus type 2. *Journal of General Virology* **67**, 397-403.

SACKS, W.R. and SCHAFFER, P.A. (1987). Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. *Journal of Virology* **61** 829-839.

SACKS, W.R., GREENE, C.C., ASCHMAN, D.P. and SCHAFFER, P.A. (1985). Herpes simplex virus type 1 ICP27 is an essential regulatory protein. *Journal of Virology* **55**, 796-805.

SALAHUDDIN, S.Z., ABLASKI, D.V., MARKHAM, P.D., JOSEPHS, S.F., STURZENEGGER, S., KAPLAN, M., HALLIGAN, G., BIBERFELD, P., WONG-STAAL, F., KRAMARSKY, B. and GALLO, R.C. 1986. Isolation of a new virus HBLV, in patients with lymphoproliferative disorders. *Science* **234**, 199-208.

SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T. (1989). Molecular cloning. Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.

SANDERS, P.G., WILKIE, N.M. and DAVISON, A.J. (1982). Thymidine kinase deletion mutants of herpes simplex virus type 1. *Journal of General Virology* **63**, 277-295.

SANDRI-GOLDIN, R.M., SEKULOVICH, R.E. and LEARY, K. (1987). The α protein ICP0 does not appear to play a major role in the regulation of herpes simplex virus gene expression in tissue culture. *Nucleic Acids Research* **15**, 905-918.

SANGER, F., NICKLEN, S. and COULSON, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences* USA **74**, 5463-5467.

SEARS, A.E., HALLIBURTON, I.W., MEIGNIER, B., SILVER, S. and ROIZMAN, B. (1985). Herpes simplex virus type 1 mutant deleted in the α22 gene: growth and gene expression in permissive and restricted cells and establishment of latency in mice. *Journal of Virology* **55**, 338-346.

SEKULOVICH, R.E., LEARY, K. and SANDRI-GOLDEN, R.M. (1988). The herpes simplex type 1 α protein ICP27 can act as a *trans*-repressor or a *trans*-activator in combination with ICP4 and ICP0. *Journal of Virology* **62**, 4510-4522.

SHELDRAKE, P. and BERTHLOT, N. (1974). Inverted repetitions in the chromosome of herpes simplex virus. *Cold Spring Harbour symposium on quantitative biology* USA **39**, 667-678.

SILVER, S. and ROIZMAN, B. (1985). γ2 thymidine kinase chimeras are identically transcribed but regulated as γ2 genes in herpes simplex virus genomes and as β genes in cell genomes. *Molecular and Cellular Biology* **5**, 518-528.

SMITH, C.C., AURELIAN, L., REDDY, M.P., MILLER, P.S. and TS'O, P.O.P. (1986). Antiviral effect of an oligo (nucleoside methylphosphonate) complementary to the splice junction of herpes simplex virus type 1 immediate-early pre-mRNAs 4 and 5. *Proceedings of the National Academy of Sciences* USA **83**, 2787-2791.

SMITH, J.L., SEKULOVICH, R.E., HARDWICKE, M.A. and SANDRI-GOLDEN, R.M. (1991). Mutations in the activation region of herpes simplex virus regulatory protein ICP 27 can be *trans* dominant. *Journal of Virology* **65** 3656-3666.

SPAETE, R.R. and FRENKEL, N. (1982). The herpes simplex virus amplicon: a new eukaryotic defective virus cloning amplifying vector. *Cell* **30** 295-304.

SPIVACK, J.G. and FRASER, N.W. (1987). Detection of herpes simplex virus type 1 transcripts during latent infection in mice. *Journal of Virology* **61**, 3841-3847.

SPIVACK, J.G. and FRASER, N.W. (1988a). Expression of herpes simplex type 1 (HSV-1) latency associated transcripts and transcripts affected by

the deletion in avirulent mutant HFEM: Evidence for a new class of HSV-1 genes. *Journal of Virology* **62** 3281-3287.

SPIVACK, J.G. and FRASER, N.W. (1988b). Expression of herpes simplex virus type 1 latency associated transcripts in the trigeminal ganglia of mice during acute infection an reactivation of latent infection. *Journal of Virology* **62**, 1479-1485.

STAMINGER, T., HONESS, R.W., YOUNG, D.F., DODERMER, W., BLAIR, E.D. and FLECKENSTEIN, B. (1987). Organization of terminal reiterations in the virion DNA of herpesvirus saimiri. *Journal of General Virology* **68**, 1049-1066.

STEINER, I., SPIVACK, J.G., LIRETTE, R.P., BROWN, S.M., MACLEAN, A.R., SUBAK-SHARPE, J.H. and FRASER, N.W. (1989). Herpes simplex type 1 latency associated transcripts are evidently not essential for latent infection. *The EMBO Journal* **8** No 2, 505-511.

STEVENS, J.G. (1989). Human herpesviruses: a consideration of the latent infection. *Microbiological Reviews* **53**, 318-332.

STEVENS, J.G. and COOK, M.L. (1971). Latent herpes simplex virus in spinal ganglia of mice. *Science* **173**, 843-847.

STEVENS, J.G., NASBURN, N.B. and COOK, M.L. (1972). Latent herpes simplex virus from trigeminal ganglia of rabbits with eye infection . *Nature New Biology* **235**, 216-217.

STEVENS, J.G., WAGNER, E.K., DEVI-RAO, G.B., COOK, M.L. and FELDMAN, L. (1987). RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. *Science* **235**, 1056-1059.

STOW, N.D. (1985). Mutagenesis of a herpes simplex virus origin of DNA replication and its effect on viral interference. *Journal of General Virology* **66**, 31-42.

STOW, N.D. and WILKIE, N.B. (1976). An improved technique for obtaining enhanced infectivity with herpes simplex virus DNA. *Journal of General Virology* **33**, 447-458.

STOW, N.D. and DAVISON, A.J. (1986). Identification of a varicella-zoster origin of DNA replication and its activation by herpes simplex type 1 gene products. *Journal of General Virology* **67**, 1613-1623.

STOW, N.D. and MCMONAGLE, E.C. (1983). Characterization of the TRS/IRS origin of DNA replication of herpes simplex virus type 1. *Virology* **130**, 427-438.

STOW, N.D. and STOW, E.C. (1986). Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate-early polypeptide Vmw110. *Journal of General Virology* **61**, 2571-2585.

STOW, N.D., MCMONAGLE, E.C. and DAVISON, A.J. (1983). Fragments from both termini of the herpes simplex virus type 1 genome contains signals for the encapsidation of viral DNA. *Nucleic Acids Research* **11**, 8205-8220.

SU, L. and KNIPE, D.M. (1989). Herpes simplex virus α protein ICP17 can inhibit or augment viral gene transactivation. *Virology* **170**, 496-504.

SUSSKIND, M.M. and YOUDERIAN, P. (1983). Bacteriophage P22 antisense repressor and control. In: Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg R.A. (Eds).Lambda II. Cold Spring Harbour Laboratory, Cold Spring Harbour NY, pp347-363.

SZILAGI, J.F. and CUNNINGHAM, C. (1991). Identification and characterization of novel non-infectious herpes simplex virus related particle. *Journal of General Virology* **72**, 661-668.

TAHA, M.Y., BROWN, S.M. and CLEMENTS, G.B. (1988). Neurovirulence of individual plaque stocks of herpes simplex virus type 2 strain HG52. *Archives of Virology* **103**, 15-25.

TAHA, M.Y., CLEMENTS, G.B. and BROWN, S.M. (1989a). A variant of herpes simplex type 2 strain HG52 with a 1.5kb deletion between 0 to 0.02 and 0.81 to 0.83 map units is non-neurovirulent for mice. *Journal of General Virology* **70**, 705-716.

TAHA, M.Y., CLEMENTS, G.B. and BROWN, S.M. (1989b). The herpes simplex virus type 2 (HG52) variant has a 1488bp deletion which eliminates neurovirulence in mice. *Journal of General Virology* **70**, 3073-3078.

TENSER, R.B. and DUNSTAN, M.E. (1979). Herpes simplex virus thymidine kinase expression in the infection of the trigeminal ganglion. *Virology* **99**, 417-422.

THOMPSON, R.L. and STEVENS, J.G. (1983). Replication at the body temperature selects a neurovirulent HSV-2. *Infection and Immunity* **40**, 855-857.

THOMPSON, R.L., DEVI-RAO, G.B., STEVENS, J.G. and WAGNER, E.K. (1985). Rescue of an HSV-1 neurovirulence function with a cloned DNA fragment. *Journal of Virology* **55**, 504-508.

THOMPSON, R.L., CASH, M.L., DEVI-RAO, G.B., WAGNER, E.K. and STEVENS, J.G. (1986). Functional and molecular analysis of the avirulent wild type herpes simplex virus type 1 strain KOS. *Journal of Virology* **58**, 203-211.

THOMPSON, R.L., ROGER, S.K. and ZEUHUSEN, M.A. (1989). Herpes simplex virus neurovirulence and productive infection of neural cells is associated with a function which maps between 0.82 and 0.832 map units on the HSV genome. *Virology* **172**, 435-450.

TOGNON, M., FURLONG, D., CONLEY, A.J. and ROIZMAN, B. (1981). Molecular characterization of herpes simplex virus V. Characterization of a mutant defective in ability to form plaques at low temperatures and in a viral function which prevents accumulation of coreless capsids and nuclear pores late in infection. *Journal of Virology* **40**, 870-880.

TOMIZAWA, J. (1987). Regulation of ColE1 DNA replication by antisense RNA. In: Inouye M. and Dudock B.S. (Eds), Molecular Biology of RNA: New Perspectives. Academic Press, New York, 1987 pp249-256.

TOSIC, M., ROACH, A., DE RIVAZ, J.C., DOLIVIO, M. and MATTIEU, J.M. (1990). Post transcriptional events are responsible for low expression of myelin basic protein in myelin deficient mice: role of natural antisense RNA. *The EMBO Journal* **9** No 2, 401-406.

TOWBIN, H., STAEHELIN, T. and GORDON, J. (1979). Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences* USA **76**, 4350-4354.

TRIESENBERG, S.J., LA MARCO, S.L. and MCKNIGHT, S.L. (1988). Evidence of DNA:protein interactions that mediate HSV-1 immediate-early gene activation by VP16. *Genes & Development* **2** 730-742.

TROUSDALE, M.D., STEINER, I., SPIVACK, J.G., DESHMANE, S.L., BROWN, S.M., MACLEAN, A.R., SUBAK-SHARPE, J.H. and FRASER, N.W. (1991). *In vitro* and *in vivo* reactivation of a herpes simplex virus type 1 latency associated transcript in a rabbit eye model. *Journal of Virology* **65**, 6989-6993.

TWIGG, A.J. and SHERRATT, D. (1980). Trans complementable-copy number mutants of plasmid ColE1. *Nature*, London **283**, 216-218.

UMENE, K. (1986). Conversion of a fraction of the unique sequence to part of the inverted repeats on the S component of the herpes simplex type 1 genome. *Journal of General Virology* **67**, 1035-1048.

UMENE, K. (1987). Transition of a heterozygous to a homozygous state of a pair of loci in the inverted repeat sequence of the L component of the herpes simplex virus type 1 genome. *Journal of Virology* **61**, 1187-1192.

VARAMUZA, S.L. and SMILEY, J.R. (1985). Signals for site specific cleavage events at sites distal to the recognition sequence. *Cell* **41**, 793-802.

VLAZNEY, D.A., KWONG, A. and FRENKEL, N. (1982). Site specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full length DNA. *Proceedings of the National Academy of Sciences* USA **79**, 1423-1427.

WADSWORTH, S., JACOB, R.T. and ROIZMAN, B. (1975). Anatomy of herpes simplex virus DNA II. Size, composition and arrangement of inverted terminal repetitions. *Journal of Virology* **15**, 1487-1497.

WAGNER, E.K. and ROIZMAN, B. (1969). Ribonucleic acid synthesis in cells infected with herpes simplex virus type 1. Patterns of ribonucleic acid synthesis in productively infected cells. *Journal of Virology* **4**, 36-46.

WAGNER, M.J. and SUMMERS, W.C. (1978). Structure of the joint region and the termini of herpes simplex virus type 1. *Journal of Virology* **27**, 374-387.

WATSON, R.J. and CLEMENTS, J.B. (1978). Characterization of transcription-deletion temperature sensitive mutants of herpes simplex virus type 1. *Virology* **91**, 364-379.

WATSON, R.J., PRESTON, C.M. and CLEMENTS, J.B. (1979). Separation and characterization of herpes simplex virus type 1 immediate-early mRNAs. *Journal of Virology* **31**, 42-52.

WATSON, R.J., UMENE, K. and ENQUIST, L.W. (1981a). Reiterated sequences within the intron of an immediate-early gene of herpes simplex virus type 1. *Nucleic Acids Research* **9**, 4189-4199.

WEIR, H.M. and STOW, N.D. (1990). Two binding sites for the herpes simplex virus type 1 UL9 protein are required for efficient activity of the oriS replication origin. *Journal of General Virology* **71**, 1379-1385.

WEIR, H.M., CALDER, J.M. and STOW, N.D. (1989). Binding of herpes simplex virus type 1 UL9 gene product to an origin of viral DNA replication. *Nucleic Acids Research* **17**, 1409-1425.

WELLER, T.H. (1971). The cytomegaloviruses: ubiquitous agents with proven clinical manifestations. *New England Journal of Medicine* **285**, 267-274.

WELLER, S.K., SPADORO, A., SCHAFFER, J.E., MURRAY, A.W., MAXAM, A.M. and SCHAFFER, P.A. (1985). Cloning, sequencing and functional analysis of oriL, a herpes simplex type 1 origin of DNA synthesis. *Molecular and Cellular Biology* **5**, 930-942.

WELLER, S.K., SEGHETOLESAMI, M.R., STAO, L., ROWSE, D. and CARMICHEAL, E.P. (1990). The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a *lacZ* insertion mutant. *Journal of General Virology* **71**, 2941-2952.

WECHSLER, S.L., NESBURN, A.B., WATSON, R., SLANINA, S. and GHIASI, H. (1988). Fine mapping of the latency related gene of herpes simplex virus type 1: Alternative splicing produces distinct latency associated RNAs containing open reading frames. *Journal of Virology* **62**, 4051-4058.

WHITELAW. E and PROUDFOOT, N. (1986). α-Thalessaemia caused by a poly(A) site mutation reveals that transcriptional termination is linked to 3' end processing in the human α2 globin gene. *The EMBO Journal* **5** no11, 2915-2922.

WHITELY, R.J. (1985). Epidemiology of herpes simplex virus. In: The Herpesviruses, volume 3. Plenum Press, New York and London.

WHITTON, J.L. and CLEMENTS, J.B. (1984). Replication origins and a sequence involved in coordinate induction of the immediate-early gene family are conserved in an intergenic region of herpes simplex virus. *Nucleic Acids Research* **12**, 2061-2079.

WHITTON, J.L., RIXON, R.J., EASTON, A.J. and CLEMENTS, J.B. (1983). Immediate-early mRNA of herpes simplex viruses type 1 and 2 is unspliced: conserved sequences around the 5' and 3'termini correspond to transcriptional regulatory signals. *Nucleic Acids Research* **11**, 6271-6287.

WIGDAHL, B.L., ISOM, H.C. and RAPP, F. (1981). Repression and activation of the genome of herpes simplex virus in human cells. *Proceedings of the National Academy of Science* USA **78**, 6522-6526.

WIGDAHL, B.L., SCHECK, A.C., DECLERQ, E. and RAPP, F. (1982). High efficiency latency and activation of herpes simplex virus in human cells. *Science* **217**, 1145-1146.

WIGDAHL, B.L., ZTEGLER, R.J., STEVE, M. and RAPP, F. (1983). Herpes simplex virus latency and reactivation in isolated rat sensory neurons. *Virology* **127**, 159-167.

WIGDAHL, B.L., SMITH, C.A., TRAGLIA, H.M. and RAPP, F. (1984). Herpes simplex virus latency in isolated human neurons. *Proceedings of the National Academy of Sciences* USA **81**, 6217-6221.

WILCOX, C.L. and JOHNSON, E.M. (1988). Characterization of nerve growth factor dependant herpes simplex virus associated latency in neurons *in vitro*. *Journal of Virology* **62**, 393-399.

WILCOX, C.L., SMITH, R.L., FREED, C.R. and JOHNSON, E.M. (1990). Nerve growth factor dependance of herpes simplex virus latency in peripheral sympathetic and sensory neurons *in vitro*. *Journal of Neuroscience* **104**, 1268-1275.

WILKIE, N.M. (1973). The synthesis and substructure of herpesvirus DNA: The distribution of alkali-labile single-stranded interruptions in HSV-1 DNA. *Journal of General Virology* **21**, 453-467.

WILDY, P., RUSSEL, W.C. and HORNE, R.W. (1960). The morphology of herpesviruses. *Virology* **12**, 204-222.

WILDY, P., FIELD, H.J. and NASH, A.A. (1982). Classical herpes latency revisited. In: 'Virus persistence' 33rd symposium of the Society for General Microbiology pp144-167. Eds: B.W.J. Mahy, A.C. Minson and G.K. Darby. Cambridge University Press, Cambridge.

WOLLERT, K., EL ANSERI, T., ROSEN-WOLFF, A. and FALKE, D. (1991). The region 0.7165-0.796mu of the HSV-1 genome determines suppression of humoral antibody formation against herpes simplex virus. *Archives of Virology* **116**, 175-184.

WONG, S.W. and SCHAFFER, P.A. (1991). Elements in the transcriptional regulatory region flanking herpes simplex virus type 1 *oriS* stimulate origin function. *Journal of Virology* **65**, 2601-2611.

WU, C.A., NELSON, N.J., MCGEOCH, D.J. and CHALLBERG, M.D. (1988). Identification of herpes simplex virus type 1 genes required for origin dependant DNA synthesis. *Journal of Virology* **62**, 435-443.

WU, M., HYMAN, R.W. and DAVIDSON, N. (1979). Electron microscopic mapping of proteins bound to herpes simplex virus DNA. *Nucleic Acids Research* **6**, 3427-3411.

WU, T.H., LIAO, S.M., MCCLURE, W.R. and SUSSLAND, M.M. (1987). Control of gene expression in bacteriophage P22 by a small antisense RNA. II Characterization of mutants defective in repression. *Genes & Development.* **1** 204-212.

WUDUNN, D. and SPEAR, P.G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulphate. *Journal of Virology* **63**, 52-58.

WYATT, L.S., RODRIGUEZ, W.J., BALACHANDRIN, N., and FRENKEL, N. (1991). Human herpesviruses 7: Antigenic properties and prevalence in children and adults. *Journal of Virology* **65**, 6260-6265.

YAMANISHI, K., OKUNO, T., SHIRAKI, K., TAKAHISHI, M., KONDO, T., ASANO, Y. and KURATA, T. (1988). Identification of human herpesvirus-6 as a causal agent for exanthum subitum. *Lancet* **1**, 1065-1067.

ZARKOWER, D., STEPHENSON, P., SHEETS, M. and WICKENS, M. (1986). The AAUAAA sequence is required for both cleavage and for polyadenylation of simian virus 40 pre-mRNA *in vitro. Molecular and Cellular Biology* **6**, 2317-2323.

ZUR HAUSEN, H. (1982). Human genital cancer: synergism between two virus infections. *Lancet* **2**, 1370-1372.

ZWAAGSTRA, J., GHIASI, H., NESBURN, A.B. and WECHSLER, S.L. (1989). *In vitro* promoter activity associated with the latency associated transcript gene of herpes simplex virus type 1. *Journal of General Virology* **70**, 2163-2169.

ZWAAGSTRA, J.C., GHIASI, H., SLANINA, S.M., NESBURN, A.B., WHEATLY, S.C., LILLYCROP, K., WOOD, J., LATCHMAN, D.S., PATEL, K. and WECHSLER, S.L. (1990). Activity of herpes simplex type 1 latency associated transcript (LAT) promoter in neuron derived cells: evidence for neuron specificity and for a large LAT transcript. *Journal of Virology* **63**, 5019-5028.