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Mutagenesis Occuring Following Infection with
Herpes Simplex Virus and the Contribution of
Virus Ribonucleotide Reductase.

BY

PENNY CLARKE

A Thesis Presented for the Degree of Doctor of Philosophy

in the

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SUMMARY.

Herpes simplex virus type 2 (HSV-2) has been associated with cervical cancer for many years and certain regions of both the HSV-1 and the HSV-2 genome can induce transformation of tissue culture cells. However, no virus transforming protein has yet been identified and no region of viral DNA appears to be stably retained in transformed cells or tumours. This implies that continued expression of a virus protein is not required and has resulted in the proposal that HSV transforms cells by a "hit and run" mechanism.

HSV infection can generate chromosomal breaks together with amplification and rearrangement of cellular genes. Additionally, infection of permissive cells with UV-inactivated HSV-1 and of non-permissive cells with HSV-2 leads to an increase in the mutation frequency of the cellular hypoxanthine-guanine phosphoribosyltransferase gene. HSV-1 was also found to increase the mutagenic frequency of a plasmid-based gene located on the shuttle vector pZ189. These mutagenic effects resemble changes produced by chemical carcinogens and may represent a mechanism for transformation which would not require the retention of viral sequences.

The cause of mutations occurring in HSV-infected cells is unknown but could be connected with the destabilization of deoxyribonucleoside triphosphate (dNTP) pools seen in infected cells as studies of both prokaryotic and eukaryotic systems have stressed the importance of dNTP concentrations in mediating base substitutions and misincorporations. The HSV enzyme ribonucleotide reductase has a potential role in the destabilization of cellular dNTP pools since, unlike its cellular counterpart, it is not allosterically regulated. This is supported by results which show that a mutant cellular enzyme, which was deficient in allosteric control, raised the cellular mutation rate in transfected cells.

In order to study the effect of HSV-2 ribonucleotide reductase on mutagenesis, expression vectors were constructed which contained the large (RR1) and small (RR2) subunits of the HSV-2 ribonucleotide reductase under the control of the inducible mouse metallothionein promoter,

which responds at the transcriptional level to heavy metals such as zinc and cadmium. These constructs were used in mutagenesis assays which involved monitoring the mutation frequencies of the cellular adenine phosphoribosyltransferase gene (aprt) in Chinese hamster ovary (CHO) cells and of the plasmid-based suppressor tRNA gene (supF) in human cells. Increasing the amount of enzyme expression by zinc-induction did not increase the mutagenesis of the marker genes in either assay suggesting that virus ribonucleotide reductase expression may not be mutagenic in HSV-1 infected cells. Experiments using the HSV-1 mutant viruses tsl207 and tsl222, which are temperature sensitive (ts) for RR1 and RR2 respectively, support this finding since these viruses were as mutagenic as wild type HSV-1 at both the permissive (P) and the non-permissive (NPT) temperatures.

Although expression of the viral ribonucleotide reductase does not appear to be the cause of increased mutagenesis in infected cells, the possibility that enzyme activity is capable of producing a mutagenic effect cannot be ruled out. The mutagenesis assays described above involved only the transient expression of the viral enzyme and the lack of any mutagenic effect might be due to inefficiencies in the transfection procedure and/or insufficient expression of the viral genes. The cellular enzyme would also be present and might counteract dNTP pool imbalances brought about by viral ribonucleotide reductase expression. To address these problems, NIH3T3 and CHO cell lines were constructed using HSV-2 ribonucleotide reductase expressing plasmids that contained the G418-resistance gene (neoR). Mutations causing morphological transformation were assayed in NIH3T3 cells while in CHO cells the aprt gene was assayed. None of the G418-resistant cell lines tested were found to express RR1 or RR2 and further lines will be screened for expression before repeating the experiment.

The ribonucleotide reductase constructs were used to generate transgenic mice to determine the effect of enzyme expression in an animal model. The mice did not appear to be affected by the presence of a large number (50+) of viral ribonucleotide reductase sequences. Viral ribonucleotide reductase RNA was detected in these animals, however, it did

not appear to be inducible and protein could not be detected.

Both HSV-1 and HSV-2 increase the spontaneous mutation frequency of the plasmid based supF gene by around 6-fold which is in agreement with values obtained by others in similar studies. Of the induced mutations, roughly 30% are due to large structural changes which result in plasmids which have altered restriction maps. The remaining mutations are presumably caused by small changes and point mutations. Such changes could be examined in future studies, by sequencing.

Experiments were performed to identify the mutagenic agent in infected cells using a range of HSV-1 mutant viruses, UV-inactivated HSV-1 and incomplete HSV-1 virus particles in the pZ189 mutagenesis assay. The mutagenic effect on the supF gene was still apparent following infection with the mutant virus tsK at the NPT, with d11403 and in1814 at low multiplicities of infection and with UV-inactivated HSV-1. This suggests that viral gene expression is not required. The virus ts1204 was not mutagenic at the NPT, when cell penetration does not occur, and this observation together with the apparent lack of requirement for viral gene expression, indicates that the mutagenic effect may be caused by some part of the incoming virion. L-particles, which do not contain HSV DNA or capsid proteins, but which do enter the cell, were not found to be mutagenic, implying that the effect may be caused by one of these components. Transfected, non-infectious, cleaved HSV DNA is mutagenic in cells which suggests that the HSV DNA itself may be the mutagenic agent. HSV DNA could exert a mutagenic effect by integrating into the cellular genome causing disruption of a gene sequence or by exerting some promoter/enhancer function. Alternatively, the HSV DNA, which is nicked, could trigger an error-prone DNA repair mechanism. The sequencing of mutations occurring following HSV infection may cast light on this. Future studies might also include experiments to determine the effect of capsid proteins on mutagenesis.

ABBREVIATIONS.

A	adenine
aa	amino acid
AA	8-azaadenine
<u>aprt</u>	adenine phosphoribosyltransferase gene
ATP	adenosine-5'-triphosphate
APS	ammonium persulphate
bp	base pairs
BHK	baby hamster kidney
BSA	bovine serum albumin
C	cytosine
CAT	chloramphenicol acetyltransferase
CHO	Chinese hamster ovary
Ci	Curie
CIN	cervical intra-epithelial neoplasia
cpe	cytopathic effect
CTP	cytidine-5'-triphosphate
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	2'-deoxyribonucleoside-5'-triphosphate
<u>dl</u>	deletion
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
DW	distilled water
E	early
EBV	Epstein-Barr virus
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	sodium ethylenediamine tetra-acetic acid
EHV	equine herpesvirus
EPR	electron paramagnetic resonance
g	gram
G	guanine
GTP	guanosine-5'-triphosphate
HCMV	human cytomegalovirus
Hepes	4-(2-hydroxyethyl)-1-piperazine ethane
HPV	human papillomavirus
HRP	horse radish peroxidase

HSV	herpes simplex virus
ICP	infected cell protein
IE	immediate-early
<u>in</u>	insertion
L	late
kb, kbp	kilobase(s), kilobase pair(s)
M	molar
MDBP	major DNA binding protein
mg	milligram
moi	multiplicity of infection
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
MTR	morphological transforming region
mw	molecular weight
N	unspecified nucleotide (A, T, C or G)
nm	nanometre
NPT	non-permissive temperature
OD	optical density
ORF	open reading frame
ori	origin of DNA replication
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming unit
PRV	pseudorabies virus
PT	permissive temperature
R	purine
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RR	ribonucleotide reductase
RT	room temperature
SDS	sodium dodecyl sulphate
SV40	simian virus 40
T	thymidine
TCA	trichloroacetic acid
TEMED	N,N,N,N'-tetramethylethylene diamine
TK	thymidine kinase
Tris	tris (hydroxymethyl)aminomethane
<u>ts</u>	temperature sensitive
UTP	uridine-5'-triphosphate

UV	ultraviolet
V	volts
Vmw(X)	virus-specific polypeptide of apparent molecular weight (X)
v:v	volume : volume
VP	virion protein
VV	vaccinia virus
VZV	varicella zoster virus
wt	wild type
w:v	weight : volume
Y	pyrimidine
ug	microgram
ul	microlitre

AMINO ACIDS - SINGLE LETTER CODE.

AMINO ACID	SYMBOL
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

SECTION A: INTRODUCTION.

The aim of this introduction is to give a summary of herpes simplex virus biology followed by a more detailed account of two relevant areas of study, namely, HSV-transformation and the HSV enzyme ribonucleotide reductase.

1. THE HERPESVIRUSES.

1.1. Classification.

The family Herpesviridae comprises more than 80 species which infect a wide range of higher eukaryotic hosts (Roizman and Batterson, 1985). The family is defined by certain morphological features which are listed below:

(i) The core is an electron dense fibrillar spindle that is surrounded by the double-stranded DNA of the viral genome (Epstein, 1962; Furlong et al., 1972; Nazarian, 1974).

(ii) The capsid surrounds the core and is in the form of an icosahedron, approximately 100nm in diameter, which is composed of 162 capsomeres (Wildy et al., 1960).

(iii) The tegument is a layer of proteinaceous material between the capsid and the envelope (Schwartz and Roizman, 1969; Morgan et al., 1968; Roizman and Furlong, 1974).

(iv) The envelope surrounds the capsid and tegument. It is derived from budding through the nuclear membrane and harbours numerous virus-encoded glycoprotein spikes (Morgan et al., 1959; Wildy et al., 1960; Asher et al., 1969; Spear and Roizman, 1972).

All the herpesviruses also exhibit the ability to persist in a latent state in their hosts and they have been classified into three sub-families based on host range, duration of the reproductive cycle, cytopathology and characteristics of latent infection (Matthews, 1982; Roizman, 1982).

Alpha herpesviruses may have a wide or narrow host range in tissue culture. The reproductive cycle is short (less than 24 hours) and causes destruction of infected cells, however, latent infection is frequently established in

neuronal cells. Members of this sub-family include the human herpesviruses HSV-1, HSV-2 and VZV.

Beta herpesviruses have a narrow host range in tissue culture. Viral replication and lytic progression are slow with replication resulting in an enlargement of cells. Latent infections are usually established in secretory glands, lympho-reticular cells and kidneys. An example of a member of this sub-family is HCMV.

Gamma herpesviruses also have a limited host range, replication occurs only in T- or B-lymphocytes in vivo and latent infection is frequently established in lymphoid tissue. This sub-family includes EBV.

The herpesviruses have also been classified on the basis of characteristic repeated DNA sequences within their genomes (Roizman, 1982; Figure 1).

1.2. Pathogenicity of the Human Herpesviruses.

Herpesviruses cause a range of clinical disorders, some of which are described below:

HSV-1 causes vesicular lesions of the mouth, lips and nasal membranes, ocular keratitis and occasionally more severe symptoms such as encephalitis, particularly in immunocompromised individuals (Smith et al., 1941; Gallardo, 1943; Rawls, 1985).

HSV-2 is the agent of sexually transmitted genital herpes. It is closely related to HSV-1 and some overlaps in their clinical manifestations occur (Whitley, 1985). HSV-2 has also been implicated in the aetiology of cervical cancer. (see section A.1.5.).

HSV-1 and HSV-2 often establish latency in trigeminal and sacral ganglia respectively, with periodic recurrence of lytic infection leading to outbreaks of lesions at peripheral sites (Klein, 1982; Knox et al., 1982; Hill, 1985).

VZV is the causative agent of chickenpox (varicella), a disease resulting from primary infection normally found in children, and of shingles (herpes zoster), a localized vesicular condition occurring in adults which appears to be caused by reactivated, latent VZV (Weller, ^{et al} 1958; Gelb,

1985).

HCMV infections can cause complications in immunosuppressed individuals and in patients with acquired immune deficiency syndrome (AIDS). It is also sometimes associated with neurological damage in neonates (Alford and Britt, 1984). Infection of the vast majority of the human population, however, results only in a mild, sub-clinical condition. HCMV has been implicated in the development of cervical carcinoma (see section A.2.2.) since HCMV DNA has been detected in a small proportion of biopsies from patients with cervical intraepithelial neoplasia (Fletcher *et al.*, 1986).

EBV infects B-lymphocytes and is the causative agent of infectious mononucleosis. It is also associated with Burkitts lymphoma, nasopharyngeal carcinoma and lymphomas of immunosuppressed individuals (Neiderman *et al.*, 1976; Miller, 1985; section A.2.1.3.).

HHV6 was first isolated from immunosuppressed patients but it can infect T-cells *in vitro*. It is linked to specific childhood illness (Salahudin *et al.*, 1986).

HHV7 has been recently isolated from CD4+T cells purified from peripheral blood mononuclear cells under conditions promoting T-cell activation (Frenkel *et al.*, 1990). The role of HHV7 in human disease is, as yet, unknown.

1.3. The Structure of Herpesvirus Genomes.

The genomes of herpesviruses consist of linear duplex DNA molecules (Becker *et al.*, 1968) which have molecular weights ranging from $80-150 \times 10^6$ depending on species (Roizman and Furlong, 1974). The basic composition of the genome varies considerably; VZV has a G+C-content of 46%, pseudorabies virus (PRV) 73%, and HSV-1 68.3% (Ben-Porat and Kaplan, 1962; Kieff *et al.*, 1971; Ludwig *et al.*, 1972; McGeoch *et al.*, 1988). Members of the herpesvirus family have been grouped according to the pattern of repeated sequences in their genomes (Roizman, 1982) although these structural criteria do not correlate with the biological classification (section A.1.1.). Figure 1 shows the genome arrangement of representative members of the Herpesviridae.

Figure 1. Arrangement of sequences in herpesvirus genomes. Examples of genomes types A to F are channel catfish virus (CCV), herpesvirus saimiri (HVS), Epstein-Barr virus (EBV), varicella zoster virus (VZV), herpes simplex virus (HSV) and murine cytomegalovirus (MCMV) respectively. Lines represent unique sequences and arrowed lines show possible inversions. Open boxes are large (>1kb) repeats and shaded boxes are repeats of small reiterated sequences; arrowheads above boxes denote whether repeats are direct or inverted. The small terminal direct and internal inverted repeats of HSV (α and β sequences) are indicated. The number of isomers of each molecule are also given. The genome type according to the system of classification defined by Roizman (1982) is shown.

virus	no. of isomers	genome type
CCV	1	A
HVS	1	B
EBV	1	C
VZV	2	D
HSV	4	E
MCMV	1	F



1.3.1. Structure of the HSV-1 Genome.

The HSV DNA molecule is composed of two covalently linked segments designated long (L) and short (S). Each segment contains unique (U_S or U_L) sequences flanked by a pair of distinct inverted repeat sequences (R) of which one is terminal (TR_L or TR_S) and the other is internal (IR_L or IR_S), (Sheldrick and Berthelot, 1974). The molecule also exhibits terminal redundancy due to the presence of a short (400bp) terminal direct repeat termed the "a" sequence. One or more copies of this sequence are located internally at the joint between the L and S segments, but in the opposite orientation to the terminal "a" sequences (Wadsworth et al., 1975; Wagner and Summers, 1978). Preparations of HSV virion DNA contain equivalent amounts of 4 isomers which differ in the relative orientations of L and S segments about the joint (Delius and Clements, 1976; Wilkie, 1976). The isomers appear to be functionally identical (Davison and Wilkie, 1983), and one has been designated as the prototype for purposes of genomic map representations (Roizman et al., 1979, Figure 1). As the "a" sequence contains signals for cleavage and packaging of concatemeric DNA, the presence of internal copies provides alternative sites for these events and would account for half the isomerization events. Together with recombination of sequences dispersed throughout R_L and R_S , this arrangement provides the means to generate the 4 genome isomers. In addition to the "a" sequences and inverted repeats, HSV-1 DNA contains a number of small tandemly reiterated sequence elements that vary in copy number (McGeoch et al., 1988).

1.3.2. The Nucleotide sequence of HSV-1.

The complete nucleotide sequence of HSV-1 (strain 17) has now been determined (Davison and Wilkie, 1981; Murchie and McGeoch, 1982; McGeoch et al., 1985, 1986, 1988). Other herpesviruses whose genomes have been completely sequenced are VZV (Davison and Scott, 1986) EBV, (Baer et al., 1984), CCV (Dr. A. Davison, personal communication) and HCMV (work performed in Barrell's laboratory). The U_S region of the HSV-2 genome has also been sequenced (Dr. D. McGeoch, personal communication) and although the full genome

sequence is, as yet, unknown, it seems to closely resemble that of HSV-1 albeit with a slightly higher G+C-content (Roizman, 1983). The HSV-1 genome comprises 152260 residues although the exact length varies due to small reiterations and "a" sequence copy number. It is estimated to encode 70 unique genes (2 are repeated), 56 in U_L , 12 in U_S and 1 in each of TR_S and IR_S (McGeoch et al., 1988, Figure 2, Table 1). The genes are quite densely packed and it is clear from the close apposition and overlap of adjacent coding regions that in many cases transcriptional control elements must overlap with the polypeptide coding regions of adjacent genes.

The overall G+C-content of the genome is 68.3% and some regions are extremely G+C-rich. For example, the coding sequences of IE genes 1 and 3 have a G+C-content of 75.4% and 81.5% respectively. This is due to a large amount of G+C-rich codons and a notable bias towards a G or C in the redundant third position. High G+C codon usage, which results in restricted amino acid utilization, is thought to have arisen independently of protein functional demands (McGeoch et al., 1986; Perry et al., 1986).

1.4. The Lytic Cycle.

After entering the cell, HSV replication is co-ordinated by temporal control of gene expression. There are three groups of genes based on their kinetics and expression in the absence or presence of metabolic inhibitors of replication and translation (Jones and Roizman, 1979; Kozak and Roizman, 1974). These groups are named immediate-early (IE), early (E) and late (L, Clements et al., 1977) or alpha, beta and gamma (Honess and Roizman, 1974). Expression of these genes after infection leads to viral DNA replication, the assembly of new virus particles, and release of these particles from the cell. This process is called the lytic cycle and generally results in cell death.

1.4.1. Cell Penetration.

HSV particles adsorb to cell surfaces and penetrate by fusion of the virion envelope with the cellular membrane in a process that requires glycoprotein B (gB) which is present

Figure 2. Layout of genes in the genome of HSV-1. The HSV-1 genome is shown on four successive lines, with unique regions represented by solid lines and major repeat elements as open boxes. The sizes and orientations of proposed functional ORFs are shown by arrows. Locations of the origins of replication are indicated. In the U_L region, on the first three lines, the genes UL1 to UL56 are labelled. In the U_S region, on the bottom line, genes US1 to US12 are labelled. The location of introns in the coding regions of gene UL15 and the two copies of the IE110 gene are indicated.

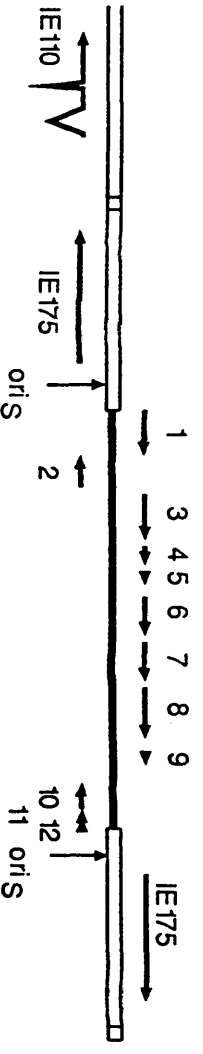
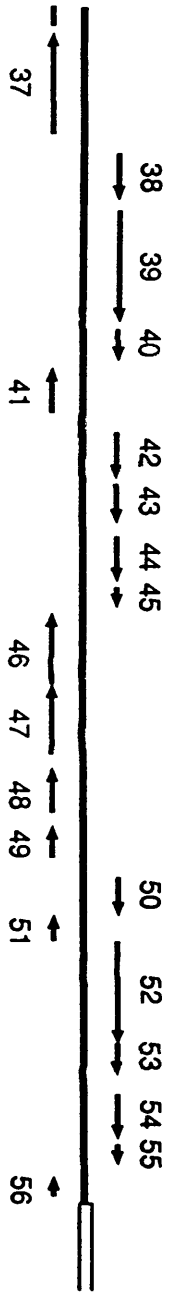
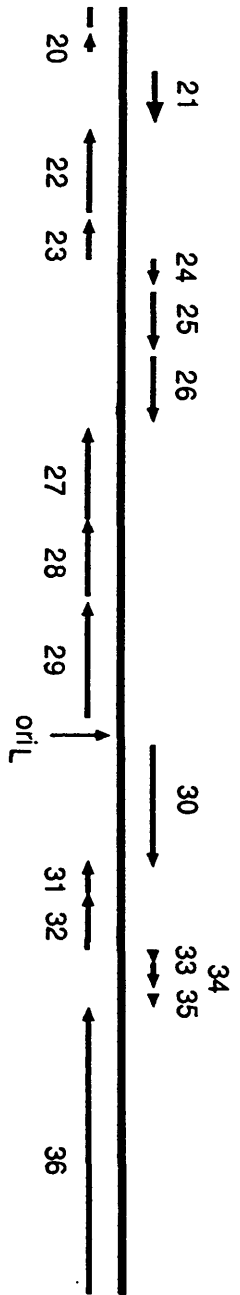
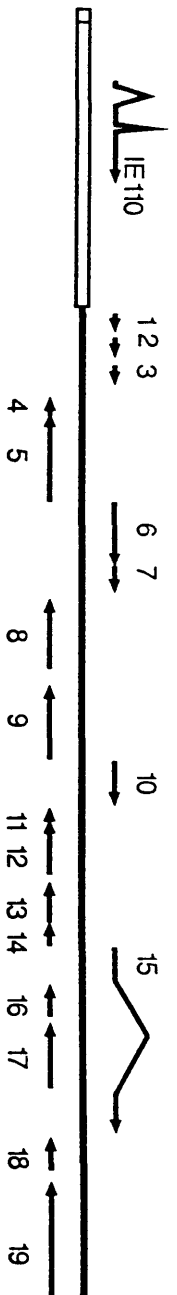


Table 1. Properties of HSV-1-encoded Proteins (taken from McGeoch et al., 1988, 1989).

HSV-1 GENE	PROTEIN AND/OR FUNCTION
IE110	IE transcriptional regulatory protein
UL1	Unknown
UL2	Uracil-DNA glycosylase
UL3	Unknown
UL4	Unknown
UL5	DNA replication: probable helicase component
UL6	Presumed virion protein: possible role in DNA packaging
UL7	Unknown
UL8	DNA replication: function unknown
UL9	DNA replication: origin-binding protein
UL10	Possible membrane-inserted protein
UL11	Unknown
UL12	Deoxyribonuclease
UL13	Predicted protein kinase
UL14	Unknown
UL15	Unknown
UL16	Unknown
UL17	Unknown
UL18	Unknown
UL19	Major capsid protein
UL20	Unknown
UL21	Unknown
UL22	Glycoprotein H
UL23	Thymidine kinase
UL24	Unknown
UL25	Virion protein
UL26	DNA packaging
UL27	Glycoprotein B
UL28	Probably structural
UL29	DNA replication: ssDNA binding protein
UL30	Replicative DNA polymerase
UL31	Unknown

Table 1 continued.

HSV-1 GENE	PROTEIN AND/OR FUNCTION
UL32	Probably structural
UL33	Structural: involved in packaging DNA
UL34	Possible virion protein
UL35	Unknown
UL36	Very large tegument protein
UL37	Unknown
UL38	Required for capsid assembly
UL39	Ribonucleotide reductase large subunit
UL40	Ribonucleotide reductase small subunit
UL41	Presumed virion protein
UL42	DNA replication: δ^s DNA binding protein
UL43	Unknown
UL44	Glycoprotein C
UL45	Unknown
UL46	Unknown. May modulate UL48 activity
UL47	Unknown. May modulate UL48 activity
UL48	Major tegument protein. Transactivates IE genes
UL49	Unknown
UL50	Deoxyuridine triphosphatase
UL51	Unknown
UL52	DNA replication: function unknown
UL53	Possible membrane protein
UL54	IE transcriptional regulatory protein
UL55	Unknown
UL56	Unknown
IE175	IE transcriptional regulatory protein
US1	IE protein: function unknown
US2	Unknown
US3	Protein kinase
US4	Glycoprotein G
US5	Unknown: possible glycoprotein
US6	Glycoprotein D
US7	Glycoprotein I
US8	Glycoprotein E

Table 1 continued.

HSV-1 PROTEIN AND/OR FUNCTION.
GENE

US9 Virion protein

US10 Virion protein

US11 Function unknown: localizes in nucleolus

US12 IE protein: function unknown

in the envelope (Sarmiento et al., 1979; Little et al., 1981). The viral capsids then migrate to the nuclear pores where they disassemble and DNA is released into the nucleus. The HSV mutant tsB7 is blocked at the stage of DNA release indicating that this process requires a viral-encoded protein (Knipe et al., 1981; Batterson et al., 1983).

1.4.2. Immediate-Early Genes.

The immediate-early (IE) genes are the first to be transcribed and unlike early (E) and late (L) genes their expression does not require de novo protein synthesis (Hones and Roizman, 1974; Clements et al., 1977). IE gene expression peaks about 2-3 hours after adsorption but IE mRNAs can still be detected in the cytoplasm at L times (Harris-Hamilton and Bachenheimer, 1985; Godowski and Knipe, 1986). During infection in the presence of cycloheximide, which blocks protein synthesis, large quantities of IE mRNAs accumulate (Preston, 1979a) and there is no transcription of E and L genes as IE polypeptides are required for the switch to E and L mRNA synthesis. There are five IE genes (Clements et al., 1979, Figure 3). IE genes 1 and 3 are located within the IR_L and IR_S respectively and there are therefore two copies of each (Watson et al., 1979; Anderson et al., 1980; Mackem and Roizman, 1980; Rixon et al., 1982). The promoter and 5'-untranslated leader sequences of IE genes 4 and 5 are located within the short repeat and are thus common to both, but the coding sequences differ since they are located in the U_S short region of the genome (Watson et al., 1981; Rixon and Clements, 1982). IE gene 2 is located in the U_L .

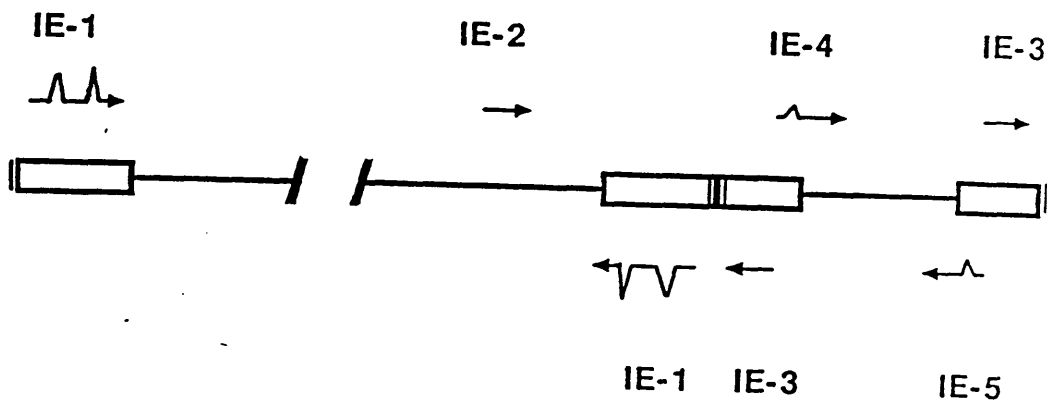
IE transcription does not require de novo protein synthesis of viral or host proteins (Kozak and Roizman, 1974; Clements et al., 1977) but it is stimulated 5-10-fold by the product of gene U_L48 , a virus-encoded transinducing factor (TIF, Mackem and Roizman, 1982a,b,c; Cordingley et al., 1983; Preston et al., 1984; Lang et al., 1984; O'Hare and Hayward, 1985b) which was later identified as the major tegument protein of the infecting virion, Vmw65, (Campbell et al., 1984). Results suggest that Vmw65 consists of two functional regions. The carboxy-terminal 78 amino acids function as an activating region (Sadawski et al., 1988; Triezenberg et al., 1988; Greaves and O'Hare, 1989), while

the amino-terminal 413 amino acids are required to form complexes with TAATGARAT motifs in a sequence specific manner (Triezenberg et al., 1988; Ace et al., 1988; Greaves and O'Hare, 1989).

The consensus A+T-rich sequence 5'-TAATGARAT-3' (where R is a purine residue) is present in one or more copies, in either orientation, upstream of all HSV-1 and HSV-2 IE genes and is essential for their stimulation although flanking elements, such as SP1 binding sites and G+A-rich elements resembling enhancer sequences, also modulate transcriptional activity (Preston et al., 1984; Mackem and Roizman, 1982a; Cordingley et al., 1983; Kristie and Roizman, 1984; Lang et al., 1984; Bzik and Preston, 1986; O'Hare and Hayward, 1987), although the G+A-rich elements were not found to mediate independent transcriptional activity. Similar results were reported by Triezenberg et al. (1988), who placed more emphasis on the importance of the G+A-rich sequences, which, they suggested, would mediate stimulation by the virion factor in the absence of the TAATGARAT motif. Further studies will be needed to resolve this conflict.

The presence of a cis-acting regulatory sequence upstream of the IE promoters was surprising as the TIF is not a DNA binding protein (Marsden et al., 1987). However, a virus induced protein/DNA complex, called the immediate-early complex (IEC), which contains the TIF, forms at, or immediately surrounding the TAATGARAT element. This complex requires 2 cellular factors for its formation and it has been suggested that the cellular factor(s) may recognize a sequence homologous to the common eukaryotic octamer element 5'-ATGCAAAT-3' (Falkner and Zachau, 1984; Parslow et al., 1984) which is found 5'- to and overlapping the TAATGARAT elements of IE genes 1 and 2. The sequence is not, however, found in the IE genes 3, 4 and 5 (Pruijn et al., 1986; O'Hare and Goding, 1988; Gerster and Roeder 1988; Preston et al., 1988). The cellular factor required for the virus-induced complex has been identified as the octamer binding transcription factor OTF1 or OCT1 (Gerster and Roeder, 1988), which is probably identical to nuclear factor III (O'Neill et al., 1988).

Many aspects of IE target sequence binding remain unresolved but the available evidence suggests that Vmw65



Gene	Polypeptide	Alternative Nomenclature
IE-1	Vmw110	ICP0
IE-2	Vmw63	ICP27
IE-3	Vmw175	ICP4
IE-4	Vmw68	ICP22
IE-5	Vmw12	ICP47

Figure 3. A representation of the HSV-1 genome showing the location and orientation of the five IE genes. The positions of the spliced regions are indicated. Also shown is a table showing the polypeptides expressed from the IE genes.

(TIF) mediates transinduction by associating with cellular factors including OTF1 and at least one other protein to form a novel transcription complex which recognizes octamer\TAATGARAT elements.

1.4.3. Immediate-Early Polypeptides.

The IE genes 1-5 code for the polypeptides Vmw110 (whose alternative nomenclature is ICPO), Vmw63 (ICP27), Vmw175 (ICP4), Vmw68 (ICP22) and Vmw12 (ICP47). The proteins are all phosphorylated and are located within the nucleus, with the exception of Vmw12 (Pereira et al., 1977; Marsden et al., 1978, 1982; Preston, 1979b; Fenwick and Walker, 1979; Ackermann et al., 1984). The functions of the polypeptides are described below:

Vmw175 is critical for HSV replication. Various conditional HSV-1 mutants containing lesions within IE gene 3 (eg. tsK) fail to synthesize E and L proteins at the NPT and cannot replicate viral DNA (Marsden et al., 1976; Preston, 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1978, 1980). The protein is continuously required for E and L gene expression (Watson and Clements, 1978, 1980; Preston, 1979a; Dixon and Schaffer, 1980) and is involved in turning off its own synthesis and that of other IE proteins (Preston 1979a; Dixon and Schaffer, 1980). Transient expression assays using cloned IE gene 3 also show that E and L genes can be activated by Vmw175 (Everett, 1984b; DeLuca and Schaffer, 1985; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a,b; Quinlan and Knipe, 1985; Mavromara-Nazos et al., 1986) and that IE genes can be repressed (DeLuca and Schaffer, 1985; Gelman and Silverstein, 1987a,b). During infection, however, the products of later genes may also be involved in the turn-off of IE gene expression (Godowski and Knipe, 1986). Transactivation and repression of HSV promoters has also been demonstrated in vitro using partially purified Vmw175 (Beard et al., 1986; Pizer et al., 1986).

Vmw175 binds specifically to the target consensus sequence 5'-ATGCTG-3' located at the transcriptional start site of its own gene, IE gene 3 (Beard et al., 1986; Faber and Wilcox, 1986; Kristie and Roizman, 1986; Muller, 1987)

and it is thought that this process mediates autoregulation (Gelman and Silverstein, 1987b; Muller, 1987; Roberts et al., 1988). A highly conserved amino-terminal region of the polypeptide is important for both transactivation and autoregulation (DeLuca and Schaffer, 1987, 1988; Paterson and Everett, 1988a,b). Paterson and Everett (1988b) propose a model whereby Vmwl75 mediates transactivation by association with cellular factors (eg. TFIID) at promoter sequences. Repression might then occur if this complex were in the vicinity of a high affinity Vmwl75 binding site which may block the progression of the transcription machinery.

Recently, Batchelor and O'Hare (1990) have shown that Vmwl75 may repress LAT promoter activity by a mechanism similar to that involved in autoregulation and have identified a putative Vmwl75 binding site. This, however, contrasts to studies performed by Zwaagstra et al. (1989) who suggested that Vmwl75 induced LAT transcription. s

Vmwl10 is a potent activator of viral and cellular genes, either by itself, or in combination either additively or synergistically, with Vmwl75 (Everett, 1984b; O'Hare and Hayward, 1985a,b; Quinlan and Knipe, 1985; Gelman and Silverstein, 1985; Mavromara-Nazos et al., 1986). As yet, no ts mutants in IE gene 1 have been isolated but a HSV-1 mutant dll403 with a large deletion in the gene gives a protein which is inactive in transient assays (Stow and Stow, 1986; Perry et al., 1986). At low moi this mutant grew inefficiently whereas at high multiplicities normal amounts of viral proteins were expressed and replication was the same as with the wt virus. It appears then, that Vmwl10 is not absolutely essential in tissue culture, but at low moi viruses that lack the polypeptide exhibit a defect that can be overcome by increasing the number of infecting viruses. Vmwl10 may therefore ensure that sufficient gene expression for lytic infection can occur at low multiplicities when Vmwl75 alone is not enough to commit the cell to the lytic cycle. Similar conclusions have been reached using other mutants of the IE 1 gene (Sacks and Schaffer, 1987).

A mutant expressing little or no Vmwl75 is unable to grow in normal cells suggesting that Vmwl10 fails to activate E gene expression in the absence of Vmwl75 during infection (DeLuca et al., 1985). The synergism between

Vmw175 and Vmw110 seems to depend on the integrity of the carboxy-terminus of the polypeptide whilst mutations in this region do not greatly affect the ability of the protein to activate by itself (Everett, 1987, 1988). Thus, two different mechanisms of activation may operate and synergy may require an association between the two proteins. As yet, there is no information regarding the DNA binding properties of Vmw110, although the coding region does contain a consensus "metal finger binding domain" (Berg, 1986) which is essential for the activity of Vmw110 in the absence of Vmw175.

Vmw63 is an essential regulatory protein. Mutants that are ts in Vmw63 overproduce Vmw175 and Vmw110 at the NPT (Sacks et al., 1985) whilst synthesizing normal levels of E proteins and allowing replication to occur. However, the mutants exhibited a severe reduction of L gene expression, notably of gB (Rice and Knipe, 1988). Vmw63 can activate expression of the promoter of the L gene encoding Vmw155 (the major capsid protein) above levels obtained with Vmw175 and Vmw110 but only when all three proteins are present, having no effect on its own or in combination with other promoters such as those of TK and gD (Everett, 1986). Vmw63 also activates and represses a variety of HSV promoters in transfection assays when Vmw110 and Vmw175 are present (Rice and Knipe, 1988; Sekulovich et al., 1988). Using viruses encoding truncated forms of Vmw63, Rice and Knipe (1990) suggested that Vmw63 has four regulatory effects during infection, stimulation of leaky L gene expression (section A.1.4.4.), induction of true L gene expression, down regulation of E and IE genes late in infection and stimulation of viral DNA replication. They also suggest that Vmw63 possesses two genetically separable transactivating functions. One stimulates, but is not required for expression of leaky L genes, while the second activity is required for true L genes.

Vmw68 may have a role in L gene expression as a deletion mutant which lacks the carboxy-terminus of the protein (Post and Roizman, 1981) exhibits poor growth and reduced expression of at least one L gene in some cell lines

(Sears et al., 1985). In addition the mutant was not neurovirulent in mice.

Vmw12 does not seem to play an important role during infection in tissue culture as viable mutants which lack the whole gene have been isolated (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

1.4.4. Early Genes.

Peak expression of E genes occurs 4-6 hours post-adsorption although there is more variation in the kinetics of their expression than for IE genes. For example, some genes, including gD, are detected at E times but are not fully expressed until after the onset of DNA replication (Gibson and Spear, 1983; Johnson and Everett, 1986a). Such genes, whose expression is moderately dependent on DNA replication have been described as early-late (EL), leaky late (beta gamma) and gamma_L (Roizman and Batterson, 1985; Wagner, 1985; Harris-Hamilton and Bachenheimer, 1985). The large subunit of ribonucleotide reductase (RR1) also has predominantly early kinetics but can still be detected in the presence of cycloheximide and in ts-Vmw175 mutants that fail to express E genes at the NPT (Preston, 1979a; DeLuca et al., 1985).

Early promoters contain upstream sequences necessary for full expression, such as a CAAT box, G+C-rich Spl sites (for TK) and A+G-rich regions (for gD). However, the TATA box of these promoters is probably the main factor involved in the response to viral products (McKnight et al., 1981; McKnight and Kingsbury, 1982; Everett, 1983, 1984a; ElKareh et al., 1985; Coen et al. 1986).

The rabbit beta globin gene was also found to be transactivated by HSV infection, but only when integrated into the viral genome (Everett, 1983, 1984a) which shows that the gene responds differently to transactivating factors depending on whether it is chromatin associated or extrachromosomal. It also suggests that E gene promoter activity is not exclusively dependent on viral-specific sequences (Smiley et al., 1987).

1.4.5. HSV Enzymes.

Many of the E proteins have enzymic activity and play roles in the metabolism or synthesis of viral DNA. For example, TK, (Kit and Dubbs, 1963; Jamieson et al., 1976; alkaline exonuclease (Morrison and Keir, 1968; Moss et al., 1979), DNA polymerase (Keir et al., 1966; Chartrand et al., 1979, 1980), ribonucleotide reductase, (Cohen, 1972; Dutia, 1983), dUTPase (Wohlrab and Franke, 1980; Preston and Fischer, 1984), uracil DNA glycosylase (Worrad and Caradonna, 1988; Mullaney et al., 1989) and protein kinase (McGeoch and Davison, 1986; Purves et al., 1986; Frame et al., 1987).

The DNA polymerase (Chartrand et al., 1980)^{TK (Jamieson et al. 1976)} and the ribonucleotide reductase (A.3.3.) are essential for growth in tissue culture whereas the dUTPase, uracil DNA glycosylase, protein kinase and alkaline exonuclease are not (Dubbs and Kitt, 1964; Fischer and Preston, 1986; Mullaney et al., 1989; Purves et al., 1987).

The HSV DNA polymerase differs from most eukaryotic polymerases by virtue of its 3'- to 5'-exonuclease (proof reading) activity (Knopf, 1979). In addition, the HSV enzyme is sensitive to phosphonoacetic acid (PAA), a pyrophosphate analogue (Leinbach et al., 1976).

1.4.6. Late genes.

L gene products, many of which are structural components of the virion, accumulate maximally by 10-16 hours post-adsorption which is roughly 2 hours after the peak of viral DNA synthesis (Munk and Sauer, 1964; Roizman, 1969; Wilkie, 1973). Indeed, it has been shown that L gene expression requires DNA replication (Swanstrom and Wagner, 1974; Honess and Roizman, 1974; Powell et al., 1975; Marsden et al., 1976; Jones and Roizman, 1979; Holland et al., 1980; Conley et al., 1981; Pederson et al., 1981).

L genes can be "leaky" L (γ_L), for example, the Vmw155 major capsid protein, whose expression is reduced but still detectable in the absence of DNA replication, or true late (γ), like US11, whose expression is very hard to detect under such conditions (Wagner, 1985; Roizman and Batterson, 1985; Johnson and Everett, 1986a, b; Johnson et al., 1986).

Unlike E promoters, which contain upstream sequences

necessary for full expression, the TATA box region (Johnson and Everett, 1986b) and sequences downstream from this (Mavromara-Nazos and Roizman, 1989), are involved in the regulation of L genes, and the E promoter of gD is regulated as a true L promoter in the absence of its upstream region when linked to an HSV origin of replication in transfection assays (Johnson et al., 1986). The L promoter regulatory sequences are either at the transcription initiation site or downstream of this (Mavromara-Nazos and Roizman, 1989).

The reason why viral replication leads to L gene induction is not understood. It is unlikely to be due, simply, to an increase in copy number of promoters, since E genes would also be expected to be dependent on DNA synthesis. Replication could possibly cause a change in the DNA template and provide a switch for L gene expression, or perhaps part of the replication machinery is needed in situ to initiate L gene transcription. However, IE gene products Vmw63 and Vmw68 seem to be involved in L gene induction (Sears et al., 1985; Sacks et al., 1985) and L gene promoters, like those of E genes, are activated by IE proteins in transfection assays (Dennis and Smiley, 1984; DeLuca et al., 1985; Mavromara-Nazos et al., 1986; Everett, 1986). It has been proposed (Mavromara-Nazos and Roizman, 1989), that the L promoter regulatory sites are blocked in the absence of viral DNA replication and that this block does not affect the upstream regulatory sequences of E and IE genes.

1.4.7. Structural Polypeptides.

HSV virions are thought to contain between 15 and 33 polypeptides (Spear and Roizman, 1972; Heine et al., 1974). These can be grouped into glycoproteins, tegument proteins and capsid proteins.

(i) Glycoproteins.

HSV encodes at least 7 glycoproteins (gB, gC, gD, gE, gG, gH and gI), (Spear, 1976; Bauke and Spear, 1979; Buckmaster et al., 1984; Spear, 1985; Frame et al., 1986; Richman et al., 1986; Ackerman et al., 1986; Longnecker et al., 1987; Johnson et al., 1988). A further ORF, whose protein is as yet unidentified, has characteristic

glycoprotein amino acid sequences (McGeoch, 1985). The glycoproteins are located within the membrane that comprises the viral envelope and are probably exposed on the surface of the virion. The glycoproteins gB, gC and gD have been implicated in cell surface adsorption (Johnson et al., 1984), gB, gD and gH play essential roles in the ability of HSV to fuse with plasma membranes and penetrate cells (Sarimento et al., 1979; Little et al., 1981; Minson et al., 1986; Desai et al., 1988; Cai et al., 1988), gD is thought to be an essential cell surface receptor-binding polypeptide (Johnson and Ligas, 1988) and gE and gI interact specifically with the Fc domain of immunoglobulin G (IgG, Bauke and Spear, 1979; Johnson et al., 1988). Thus it appears that several glycoproteins participate in the entry of HSV into cells as well as being structural proteins (Addison et al., 1984).

(ii) Tegument Proteins.

These proteins can be identified when released from virions in the presence of non-ionic detergents (Lemaster and Roizman, 1980; Roizman and Furlong, 1974; Spear, 1980). The major tegument protein is Vmw65. This protein is required for transactivation of IE genes (A.1.4.3.) and hence for lytic infection. It is, however, dispensable for the establishment and reactivation from latent infection (Steiner et al., 1990).

(iii) Capsid proteins.

Empty capsids (ie. capsids which do not contain HSV DNA) are made up of at least 5 proteins including Vmw155, the major capsid protein. Mature nucleocapsids contain 2 additional polypeptides, one of which is VP22 (p40, Gibson and Roizman, 1972, 1974; Heilman et al., 1979; Preston et al., 1983).

1.4.8. DNA Replication.

Linear herpesvirus DNA molecules circularize upon infection (Jean et al., 1977; Jacob and Roizman, 1977) by direct ligation at the termini (Jean and Ben-Porat, 1976; Davison and Wilkie, 1983; Poffenberger and Roizman, 1985). During the initial stages of replication, loops and eyes can

be seen in the DNA molecule at multiple, widely separated loci (Friedman et al., 1977). Newly replicated molecules are found in large concatemers, in a head to tail configuration, which probably arises by a rolling circle replication process (Jacob and Roizman, 1977; Ben-Porat and Tokazewski, 1977).

HSV DNA contains three origins of replication, two lie within the R_S segment (ori_S) and one within U_L (ori_L , Frenkel et al., 1976; Jean et al., 1977; Rixon and Ben-Porat, 1979; Vlazney and Frenkel, 1981; Spaete and Frenkel, 1982). The locations of origins of replication can be seen in Figure 2. Deletion of ori_L or one copy of ori_S has little or no effect on viral replication in cultured cells (Longnecker and Roizman, 1986; Polvino-Bodnar et al., 1987).

ori_S consists of a 90bp fragment containing a 45bp palindromic sequence featuring 18 centrally located A or T residues flanked by G+C-rich tracts, and is located between the 5'-ends of IE mRNAs 3 and 4/5 (Stow, 1982; Stow and McMonagle, 1983).

ori_L , located in the centre of U_L , between the genes encoding DNA polymerase and the major DNA-binding protein (MDBP), contains a 72bp palindrome which has 85% homology to ori_S (Gray and Kaerner, 1984; Quinn and McGeoch, 1985; Weller et al., 1985). Both ori_S and ori_L appear to be functionally identical in transient complementation assays (Wu et al., 1988), and it is thought that the potential ability of the origins to form cruciform structures containing a stretch of low melting point (A+T-rich) DNA may facilitate strand separation in these areas of the genome (Stow, 1985).

1.4.9. Genes Required for DNA Replication.

Seven genes, based on predicted ORFs, were found to be required for HSV DNA replication using the plasmid replication assay of Challberg (Challberg, 1986; Wu et al., 1988). Two of these genes, encoding DNA polymerase and the MDBP, were already known to be essential for viral replication by genetic analysis of mutants (Hay and Subak-Sharpe, 1976; Chartrand et al., 1979; Conley et al., 1981; Weller et al., 1983; Coen et al., 1984; Gibbs et al.,

1985).

The MDBP is an E gene product. It is a single-stranded DNA binding protein which may reduce the melting temperature at poly(dA)/poly(dT) tracts (ie. at origins of DNA replication) thereby facilitating strand separation (Powell et al., 1981). The MDBP also has a role in gene regulation since ts mutants overexpress gC and a functional MDBP is involved in the repression of IE3 and Vmw155 genes (Godowski and Knipe, 1983, 1985, 1986).

Other genes that were found to be required for replication were UL5, UL8, UL9, UL42 and UL52. The product of UL42 is a DNA binding protein (Marsden et al., 1987) and the polypeptide encoded by UL9 binds specifically to ori DNA sequences (Olivo et al., 1988; Weir et al., 1989). There is also evidence that UL5 encodes a helicase (Zhu and Weller, 1988). The remaining gene products might be expected to include a primase, a topoisomerase and accessory factors that might improve the processivity and efficiency of DNA polymerase, in analogy with prokaryotic systems (Wu et al., 1988).

1.4.10. HSV Transcript Processing.

The mechanism by which HSV DNA is transcribed and mRNAs processed is fundamentally similar to that for cellular RNA polymerase II genes. HSV mRNAs are transcribed in the nucleus by the host RNA polymerase II (Ben-Zeev and Becker, 1977; Costanzo et al., 1977). The transcripts are capped at the 5'-terminus, polyadenylated at the 3'-terminus and internally methylated (Bachenheimer and Roizman, 1972; Moss et al., 1977; Silverstein et al., 1973; Bartoski and Roizman, 1976). In vitro and in vivo analysis of HSV poly(A) site sequences has shown that efficient formation of mRNA 3'-termini requires a poly(A) signal, AAUAAA and other sequences downstream of the mature 3' end, in particular a G+U-rich element (Cole and Stacey, 1985; McLauchlan et al., 1985, 1988, Zhang and Cole, 1987). These sequence motifs are important for processing at other cellular and viral poly(A) sites (Skolnik-David et al., 1987). In addition, sequence specific complexes formed at these HSV poly(A) sites (McLauchlan et al., 1988), within which RNA processing occurs in vitro, resemble those formed at other eukaryotic

poly(A) sites (Zarkower and Wickens, 1987, 1988). The sequences required for efficient 3'-processing are not identical for all poly(A) sites (Hales et al., 1988) and thus usage of certain poly(A) sites may be a regulated event. McLauchlan et al. (1989) have identified an activity induced in HSV infected cells that increases the 3' processing specificity at a L but not an E HSV-1 poly(A) site.

Splicing of HSV mRNAs is rare (Frink et al., 1981, 1983; Watson et al., 1981; Costa et al., 1985), unlike many viruses, including HCMV and EBV, which undergo economic use of template DNA by generating multiple individual mRNAs with common 5'- and 3'-termini. HSV-1 IE mRNAs 4, 5 (Watson et al., 1981; Rixon and Clements, 1982) and 1 (Perry et al., 1986) are spliced as is UL15 (Costa et al., 1985; McGeoch et al., 1988). The LAT (section A.1.4.2.) is also spliced but only a tentative ORF has been designated (Wechsler et al., 1988). The purpose of these splicing events does not seem to be related to gene compression since the introns do not lie within overlapping reading frames. Indeed, only US10 and US11 (Rixon and McGeoch, 1984) overlap in the U_S region of the genome and there are 11 proposed overlaps in U_L (McGeoch et al., 1988). Many HSV transcripts, although not spliced, do overlap by having unique 5'- and common 3'-ends, as is the case for US10, 11 and 12 (Hall et al., 1982; Costa et al., 1983; Rixon and McGeoch, 1984).

1.4.11. HSV Polypeptide Synthesis.

One-dimensional SDS PAGE has identified about 50 novel polypeptides following infection of cells with HSV-1 (Hones and Roizman, 1973; Marsden et al., 1976). Two-dimensional PAGE increases this figure to around 230 virus-induced polypeptide species, a number presumably being related forms of the same polypeptide (Haar and Marsden, 1981).

The failure of some HSV polypeptides, synthesized in vitro, to co-migrate on SDS polyacrylamide gels with their in vivo counterparts suggests that modifications occur in vivo (Preston, 1977). There are four main types of

modification (Marsden et al., 1982);

(i) Phosphorylation. Catalyzed by the enzyme protein kinase, this reaction involves the addition of phosphate groups to a serine or threonine residue. Roughly 16 HSV-1 and 18 HSV-2 polypeptides are phosphorylated (Pereira et al., 1977; Marsden et al., 1978). In some of these phosphoproteins, namely Vmwl75, Vmw68, Vmw63 and Vmwl36, the phosphate can cycle on and off (Wilcox et al., 1980) and phosphorylation of Vmwl36 has been shown to decrease its affinity for double-stranded DNA.

(ii) Glycosylation. In this process, oligosaccharides are covalently attached to the polypeptide chain. Seven HSV-1 induced polypeptides are glycosylated (section A.1.4.7.).

(iii) Sulphation. This is a process which occurs at L times and involves the addition of inorganic sulphate to the major glycoprotein species (Hope et al., 1982; Hope and Marsden, 1983).

(iv) Proteolytic Cleavage. Several HSV polypeptides undergo specific proteolytic cleavage which does not occur in the presence of protease inhibitors. Vmwl36 gives rise to products of 100,000 and 90,000 mw which are found in the nucleus of infected cells while the polypeptide itself is predominantly cytoplasmic (McDonald, 1980). Similarly, Ingemarson and Lankinen (1987) reported that Vmwl36 is proteolytically digested to products with molecular weights of 110,000, 93,000 and 81,000.

1.4.12. DNA Packaging and Virion Assembly.

Concatemeric DNA resulting from HSV DNA replication undergoes cleavage at the "a" sequence which is present at the termini of standard molecules (Davison and Wilkie, 1981; Mocarski and Roizman, 1982; Varmuza and Smiley, 1985; Nasserri and Mocarski, 1988). The cleaved molecules are then encapsidated into virus particles in a process which also involves the "a" sequence (Kaerner et al., 1981; Vlazney and Frenkel, 1981). Cleavage of concatemeric DNA molecules into

unit length genomes is a prerequisite for, or occurs concomitantly with, packaging (Vlazny et al., 1982). Analysis of the HSV mutant tsl201 revealed that packaging of DNA requires the processing of a structural protein p40 (VP22a) to a form of lower electrophoretic mobility (Preston et al., 1983). Another mutant, tsl204, which belongs to another complementation group, was also unable to encapsidate DNA indicating that other polypeptides are involved in the process (Addison et al., 1984).

1.5. Interaction of HSV with Cellular Functions.

1.5.1. Alteration of Host Macromolecular Synthesis.

Infection of permissive cells with HSV-1 or HSV-2 results in changes to many host cell functions. Cellular DNA and RNA synthesis is inhibited (Roizman and Roane, 1964), mitosis ceases (Wildy et al., 1961) and there is a rapid shut-off of host polypeptide synthesis (Sydiskis and Roizman, 1966, 1967) accompanied by a general degradation of cellular mRNAs (Nishoika and Silverstein, 1977, 1978; Schek and Bachenheimer, 1985). In general, HSV-2 appears more efficient than HSV-1 in causing host protein synthesis inhibition (Powell and Courtney, 1975; Pereira et al., 1977; Fenwick et al., 1979; Schek and Bachenheimer, 1985).

Shut-off of host protein synthesis seems to be mediated by one or more virion components as UV-irradiated virus in the presence of actinomycin D produces the same effect. Complete shut-off, however, requires the expression of E and perhaps L genes indicating that there is a secondary shut-off event in addition to the virion-associated mechanism (Honest and Roizman, 1974; Marsden et al., 1976; Nishioka and Silverstein, 1978; Stenberg and Pizer, 1982). This secondary event may be mediated by a virus-encoded protein or might be the result of virus promoters competing with cellular promoters for host transcription factors. Mutants of HSV-1 have been isolated for virion-associated host shut-off (vhs mutants) and the gene responsible has been identified as UL41 (Read and Frenkel, 1983). Some cellular promoters, possibly including those of the genes encoding the heat shock proteins and ubiquitin, are stimulated early in infection (Notarianni and Preston, 1982;

La Thangue et al., 1984; Macnab et al., 1985; Patel et al., 1986; Latchman and Kemp, 1987), and Kemp et al. (1986) implicated the involvement of virion components, including the HSV-1 Vmw65. Such alterations in cellular gene expression during the initial stages of infection could ultimately determine the fate of an infection, whether it be lytic, latent or transforming.

1.5.2. Activation of Cellular Genes.

It has been shown that a number of cellular RNAs are induced by a mechanism which requires IE gene expression (Patel et al., 1986), while others may be induced by mechanisms that parallel activation of the IE genes by Vmw65, or simply by adsorption of the virus to the cell surface (Kemp et al., 1986).

Certain cellular promoters are activated by HSV IE gene products in transfection assays, for example the rabbit beta-globin gene promoter (Everett, 1983), but the endogenous gene is not activated by virus infection. It is thought that this may be due to a densely packed chromatin configuration making the promoter unavailable for viral transactivation (Everett, 1985). Many other cellular genes may fail to be activated on viral infection for the same reason.

Infection of human embryo fibroblasts with HSV-2 induces the synthesis of a minor cellular stress protein of 57kDa mw (La Thangue et al., 1984) and the induction of stress proteins may represent an attempt by the cell to protect itself. Alternatively, it might result from the viral induction of cellular functions which facilitate viral multiplication (Patel et al., 1986).

Heat-shock proteins are also induced in chick embryo fibroblasts (CEF) by tsK at the NPT (Notarianni and Preston, 1982) due to the presence of an abnormal Vmw175 protein (Russel et al., 1987). Natural isolates of HSV-1 frequently show mutations in the gene encoding Vmw175 (Knipe et al., 1981; Post et al., 1981). Thus, the induction of heat-shock proteins may be important in vivo. Heat-shock proteins might be induced to mediate the repression of cellular biosynthesis which is seen in heat-shocked cells (Peterson and Mitchell, 1981).

The induction of heat-shock proteins also occurs in cells infected with the DNA tumour viruses adenovirus (Nevins, 1982), polyoma virus and SV40 (Khandjian and Turler, 1983) which has led to the suggestion that the activation of such genes might be involved in transformation.

Western blotting studies have shown that TG7A (an antibody raised against affinity purified DNA binding proteins from HSV-2 infected cells) recognizes 2 polypeptides of mw 35-40,000 and 90,000 which are induced on HSV-2 infection (La Thangue and Latchman, 1988). Cellular polypeptides investigated in this way have been immunoprecipitated from HSV-2 transformed cells but not from untransformed cells, using the same monoclonal antibody and have similar molecular weights to both HSV-induced polypeptides (Macnab et al., 1985a). These proteins might be an important step in the initiation of oncogenic transformation by the virus.

1.5.3. Latency.

HSV can establish latency and coexist with its host in a non-infectious state (Stevens and Cook, 1971; Baringer and Swoveland, 1973; Galloway et al., 1979; McLennan and Darby, 1980). HSV-1 travels intra-axonally down nerves to ganglionic neurones after infection at peripheral skin sensory nerve cell sites. The latent state is characterized by the prolonged association of the viral genome in host neuronal tissue, during which time no infectious virus can be isolated (Hill, 1985).

Puga et al. (1978) were the first to detect HSV specific DNA in sensory ganglia from latently infected mice. Latent HSV genomes exist in a form other than unit length, linear DNA and it is unclear whether the viral molecules are maintained in circular or concatemeric forms (Rock and Fraser, 1983; Efstathiou et al., 1986). It does, however, appear that the latent DNA exists in an episomal rather than an integrated state (Mellerick and Fraser, 1987).

The HSV-1 genome is not completely inactive during latency and HSV-specific transcripts have been detected in ganglia of seropositive patients (Croen et al., 1987; Steiner et al., 1988; Stevens et al., 1988) and in

experimentally infected mice (Deatly et al., 1987; Puga and Notkins, 1987; Spivak and Fraser, 1987; Stevens et al., 1987; Wagner et al., 1988) and rabbits (Rock et al., 1987; Wechsler et al., 1988). At least three latency associated transcripts (LATs) of 2.0, 1.5 and 1.45kb are present during HSV-1 latency and these map to a 3.0kb region contained within the BamHI b and e fragments in the R region. These RNAs partially overlap the 3'-terminus of the IEL gene but are transcribed in the opposite direction (Rock et al., 1987; Spivak and Fraser, 1987; Stevens et al., 1987; Deatly et al., 1988; Steiner et al., 1988; Wagner et al., 1988; Wechsler et al., 1988).

The DNA of this region does not contain any convincing protein coding sequences, apart from IEL (Perry and McGeoch, 1988) and most of the transcripts present during latency are not polyadenylated (Wagner et al., 1988). The LATs may modulate latency via control of IEL gene expression, possibly by interacting with the DNA sequence of the gene or with IEL mRNA (Croen et al., 1987; Rock et al., 1987; Stevens et al., 1988) but latency competent HSV deletion variants have been isolated which lack all or part of the LATs (Javier et al., 1988, Sedarati et al., 1989, Steiner et al., 1989) suggesting that these transcripts are not essential for the latency process.

Using HSV mutants in an in vitro latency system (tissue culture cells harbouring non-replicating HSV) Russell and Preston (1986) demonstrated that only limited gene expression, probably of the IE genes, is necessary for the latent state to be established and reactivated in vitro. Vmw110 has been directly implicated in reactivation since super-infection with dll403 (a mutant containing a deletion in IEL) fails to reactivate HSV-2 in vitro (Russell et al., 1987).

Further work (Harris et al., 1989), has also implicated Vmw110 in reactivation. An adenovirus recombinant expressing an HSV-1 mutant Vmw110, containing a deletion in the carboxy terminus, reactivated latent HSV-2 in vitro whereas a similar recombinant containing a deletion in the second exon of Vmw110 did not, suggesting that Vmw110 alone is required for reactivation of latent HSV-2 in vitro. Contrary to this, Clements and Stow (1989) have shown that dll403, which has

reduced virulence in mice, is able to establish a latent infection in the sensory ganglia of mice following footpad inoculation. This latent virus can be reactivated from explanted ganglia suggesting that the IEL gene product is dispensible for establishment and maintenance of latency and reactivation from the latent state.

Vmw175 may also affect transcription from the LAT promoter (section A.1.4.3.), although the significance of this is not known.

Reactivation of latent HSV can be triggered by trauma, nerve damage or various stimuli to the neurone or dermatome (Stevens, 1975; Wildy et al., 1982; Hill, 1985). There is also some evidence from experiments using TK deletion mutants that the viral TK gene has a role in reactivation (Tenser et al., 1989; Leist et al., 1989).

1.5.4. Transformation.

The effect of HSV on cellular transformation will be discussed in the following section.

2. HSV TRANSFORMATION.

2.1. Transforming Viruses.

Both DNA and RNA tumour viruses are capable of causing tumours in animals. RNA tumour viruses are restricted to one genus (Leukovirus) and are the causative agents of natural leukaemias, carcinomas and sarcomas in a large number of animal species.

Some of the oncogenic DNA viruses will now be briefly described.

2.1.1. Transformation by SV40 and Polyoma virus.

SV40 and polyoma virus are members of the Polyomavirus genus. Both are oncogenic in rodents and transform cells in culture (Fenner et al., 1974). An infection in cells permissive for viral multiplication leads to cell death whereas in a non-productive infection, cells may become stably transformed (Sambrook, 1972). Cells transformed by SV40 or polyoma virus invariably contain a functional early region integrated into the chromosomal DNA (Sambrook et al., 1968; Basilico et al., 1980; Lania et al., 1980a) and transformation seems to be due to the addition of a specific viral oncogene rather than to the integration event itself (Lania et al., 1980b).

SV40 expresses two tumour antigens in transformed cells, large T antigen (90K) and small T antigen (19K), (Livingstone and Bradley, 1987), whereas polyoma virus expresses three, large T (90K), middle T (50K) and small T antigen (19K), (Ito, 1980). The expression of SV40 large T or polyoma middle T antigen induces cell DNA synthesis and cell division (Stoker and Dulbecco, 1969; Fried, 1970) and the integration of these viral genes into cellular DNA could result in cancer cells which would continue to divide under conditions where normal cell division is inhibited (Fried and Prives, 1986).

These antigens are discussed in more detail below.

(i) SV40 Large T Antigen.

The large T antigen of SV40 is one of the most intensely studied eukaryotic proteins because it performs a large variety of functions including ATPase activity, regulation of cellular gene transcription, control of its own synthesis and viral replication and initiation of cellular DNA synthesis (Livingston and Bradley, 1987).

Little is known about the molecular role that the large T antigen plays in transformation. An important function might be its ability to associate with cellular proteins. De Caprio et al., (1988) described the interaction of T antigen with the protein encoded by the retinoblastoma susceptibility gene (pRB) which is believed to be involved in the negative regulation of cellular proliferation (reviewed in Murphree and Benedict, 1985; Weinberg, 1988). The protein, pRB, forms complexes not only with SV40 large T antigen but also with the adenovirus E1A protein (see below) and human papilloma virus E7 protein (Whyte et al., 1988; De Caprio et al., 1988; Munger et al., 1989). Mutational analysis of the viral proteins has shown that the regions needed for interaction with pRB are also required for transformation (De Caprio et al., 1988; Whyte et al., 1989; Munger et al., 1989). This suggests that the pRB is a cellular target for transformation by these viral oncoproteins. By binding to pRB the viral proteins might interfere with its normal function thus mimicking the loss of pRB seen in tumour cell lines (Whyte et al., 1988).

Another cellular protein, p53, binds to a different region of large T antigen. Numerous reports suggest that this protein, which is overproduced in a variety of neoplastic cell types (Jenkins and Sturzbecher 1988), is an oncogene product directly involved in malignant transformation (Rovinski and Benchimol 1988) and may act as a tumour suppressor (reviewed by Levine, 1990). The large T antigen of SV40, the E1B protein of adenovirus (see below) and the papilloma E6 protein form tight complexes with p53 (reviewed by Levine, 1990) which seems to become metabolically stabilized in SV40-transformed cells leading to an enhanced level of p53 (Crawford, 1983). This, however, does not seem to be caused directly by the binding of large T antigen and p53 (Deppert and Steinmayer, 1989).

Large T antigen is sufficient for both immortalization

and transformation of primary rat cells but the efficiency of transformation caused by SV40 large T antigen is probably increased by the presence of the second tumour antigen found in SV40 transformed cells, small T (Livingstone and Bradley, 1987).

(ii) Polyoma Virus T Antigens.

Three T antigens, large, middle and small T, are expressed by integrated polyoma virus sequences in transformed rodent cell lines (Ito, 1980). Like its SV40 counterpart, polyoma virus large T antigen is able to bind to DNA, exhibits ATPase activity and is required for viral DNA replication. Unlike the SV40 protein, however, it is not able to transform cells. The transforming function of polyoma virus is located on a separate protein, middle T antigen, which is sufficient to transform established culture cells. Middle T antigen cannot, however, induce the conversion of primary cells to an "immortalized" cell line and this requires the co-operation of large T antigen (Fried and Prives, 1986). A tyrosyl-specific protein kinase activity is found associated with middle T antigen which is due to its association with the cellular proto-oncogene product pp60^{c-src} (Courtneidge and Smith, 1983, 1984). The pp60^{c-src} molecules associated with middle T antigen differ from their non-complexed counterparts by the presence of a phosphate free tyrosine at residue 527 (Cartwright et al., 1986) and a higher tyrosine kinase activity (Courtneidge, 1985). Courtneidge (1985) proposed that ~~middle T antigen might affect transformation by preventing phosphorylation of tyrosine 527 as mutants of pp60^{c-src}, with a substitution of phenylalanine for tyrosine at residue 527, readily transform cells whereas wild type c-src does not~~ preventing phosphorylation of tyrosine 527 as mutants of pp60^{c-src}, with a substitution of phenylalanine for tyrosine at residue 527, readily transform cells whereas wild type c-src does not (Cartwright et al., 1987). The prevention of phosphorylation of the tyrosine residue is thought to be caused by steric hindrance brought about by the binding of middle T antigen (Cheng et al., 1989). The amino terminal of middle T antigen is required for its capacity to transform cells (Murkland and Smith, 1987) and for its ability to associate with and activate the protein kinase activity of pp60^{c-src}. This region was further located to the first four amino acids of middle T antigen (Cook and Hassell, 1990).

* Adenovirus type 12 E1A has been shown to transform rat cells in transfection assays (Gallimore et al., 1985), however, such transformation is 30 fold less efficient than with virus transformed cells.

Small T antigen may co-operate in the transformation caused by middle T antigen (Ito, 1980).

2.1.2. Transformation by Adenovirus.

Cell lines derived from adenovirus transformed non-permissive cells maintain a subgenomic fragment from the left-hand 8-12% of the viral genome which contains genetic information sufficient for transformation of primary cells in culture (Sambrook et al., 1975). This region contains two transcriptional units ElA and ElB which are responsible for oncogenic transformation (Graham et al., 1974; Gallimore et al., 1974). In transformed cells ElA codes for two co-terminal mRNAs specifying two related proteins whereas ElB codes for one mRNA which is translated into two unrelated proteins (Branton et al., 1985).

There is a certain amount of evidence to suggest that ElA could take over the cell proliferating function of c-myc. Transformation by adenovirus types 5 and 12 is accompanied by an inhibition of expression of a number of cellular genes, including c-myc (van der Eb et al., 1989). Additional to this is the fact that the sequences responsible for transformation are also associated with the suppression of transcriptional enhancers (Velcich and Ziff, 1985; Hen et al., 1985). ElA has also been shown to interact with pRB which is believed to be involved with the negative regulation of cellular proliferation (section A.2.1.1.i).

The proteins encoded by the ElB gene region play a cooperative part in the transformation process and cannot, by themselves, induce alterations in cell growth or morphology (reviewed by Stillman, 1986). One of its encoded proteins has been shown to bind to p53 (reviewed by Levine, 1990) whereas the other protein localizes to membranes of the nuclear envelope, cytoplasm and the cell surface in transformed cells.

It has been suggested that ElA gene promotes the establishment of immortalized non-tumorigenic clones (Houwelling et al., 1980) while ElB produces the transformed phenotype. *

2.1.3. Transformation by EBV.

EBV is a human herpesvirus that is causally associated

with several malignancies including nasopharyngeal carcinoma, African Burkitts lymphoma and lymphoproliferative disorders in immunosuppressed patients (Miller, 1985). In vitro, EBV infects human B-cells and immortalizes between 10 and 100% of them (zur Hausen, 1981). The genes required to initiate and/or maintain the transformed state in vitro have not been identified. However, at least 8 viral genes are transcribed in B-cells that have been immortalized in vitro and circumstantial evidence exists that several of these gene products will be required for this process (Knutson and Sugden, 1988). Of the 8 proteins, 6 are nuclear proteins (EBNAs or Epstein-Barr virus nuclear antigens) and 2 are membrane proteins (LMPs, latent membrane proteins). BNLF-1, which is an LMP, induces established rodent cell lines to grow in an anchorage-independent fashion in agarose (Wang et al., 1985; Baichwal and Sugden, 1988). Most of these anchorage-independent clones also grow as tumours in nude mice. The product of the BNLF-1 gene contains 6 hydrophobic domains which seem to be inserted into the membrane (Liebowitz et al., 1986) and which are required to transform BALB/3T3 cells (Baichwal et al., 1989). Baichwal et al. (1989) speculated that this protein is related to cell-surface receptors due to its location at the cell surface, its rapid turnover, its presence in patches in the membrane and its being phosphorylated. If BNLF-1 is related to a receptor it is possible that it will function as a receptor for some growth factor.

EBNA1 is important in the episomal maintenance of the viral genome in transformed cells (Reisman et al., 1985). A second type of EBNA, named EBNA2, plays a continuing role in the expression of the transformed phenotype (Rickinson et al., 1987). Thus, EBV possesses more than one transforming function in the same way as SV40, polyomavirus and adenovirus.

The above sections show that SV40, polyoma virus, adenovirus and EBV all encode multiple transforming functions and that the sequences encoding these functions are retained by the transformed cell suggesting that their continued expression is important for maintenance of the transformed phenotype. In many cases the transforming

functions may exert their effects by interacting with cellular oncogenes and anti-oncogenes.

2.2. Oncogenic Herpesviruses.

The gamma herpesviruses Marek's disease virus (MDV), EBV, herpesvirus saimiri (HVS) and herpesvirus ateles (HVA) all produce tumours in animals. MDV induces malignant lymphomas in chickens, the natural host (Kato and Hirai, 1985), EBV induces lymphomas in marmosets as well as being thought to be involved in the aetiology of Burkitts lymphoma and nasopharyngeal carcinoma in man (Dambaugh et al., 1986, section A.2.1.3.) and HVS and HVA induce lymphomas on inoculation of marmosets, which are not the natural host (Melendez et al., 1970; Melendez et al., 1972). In all these cases, DNA is maintained in the tumour cells.

HSV and HCMV have been associated with cervical cancer but with these viruses the DNA is not present in the majority of samples analyzed.

(i) Herpesvirus saimiri and Herpesvirus ateles.

Herpesvirus ateles causes T-cell lymphomas of marmosets (see above) and is capable of in vitro transformation of peripheral blood T-lymphocytes (Falk et al., 1978). It appears to be similar to herpesvirus saimiri on which more work has been performed.

HVS DNA is present in transformed cells as large episomal molecules (Kaschka-Dierich et al., 1982; Medveczky et al., 1989). HVS-transformed cells are T-lymphocytes of the CD-8 class (Kiyotaki et al., 1986), and transformation appears to be caused by the left-hand end of the unique sequence (L-DNA). This region is extremely variable (Medveczky et al., 1984) and three groups of HVS (A, B and C) have been identified on the basis of DNA homology in this region. Group A and C strains can transform peripheral blood lymphocytes of common marmosets whereas group B strains cannot (Desrosiers et al., 1986). Group A and B strains have been shown to induce lymphomas in various new world primates (Wright et al., 1977). There are contradictory reports about the oncogenicity of HVS in rabbits (Daniel et al., 1974; Deinhardt et al., 1974), however, recently Medveczky et al.

(1989) found only group C viruses to be oncogenic in rabbits. This group also showed that recombinant B/C viruses, constructed by co-transfecting the L-DNA region of a C strain virus with virion DNA from a B group virus, was also tumourigenic.

Analysis of the DNA from this region of a group A virus shows that it contains a reading frame that is required for immortalization of marmoset T-lymphocytes and its protein product is termed the saimiri transforming protein (STP, Murthy et al., 1989). A group C virus did not contain a similar protein, however, two further reading frames were found in this region of DNA (Biesinger et al., 1989)

(ii) Marek's disease virus.

Several investigators have demonstrated that serial in vitro passage of virulent MDV in primary chicken embryo fibroblasts resulted in a loss of virion tumourigenicity (Churchill et al., 1969; Fukuchi et al., 1985). This loss of tumourigenicity correlates with the amplification of a 132bp repeat sequence found within the BamHI d and BamHI h fragments of the genome (Maotani et al., 1986) and was only observed with non-pathogenic strains (Fukuchi et al., 1985). A 1.8kb transcript is produced from this region in cells infected by a pathogenic strain of MDV but not in cells infected with a non-pathogenic strain which suggests that this is associated with transformation (Bradley et al., 1989).

(iii) Herpes simplex virus and Human Cytomegalovirus.

The alpha herpesvirus, HSV, and the beta herpesvirus, HCMV, may be involved in cancer of the cervix. Research into the epidemiology of cervical cancer has implicated a possible multifactorial aetiology. Cervical cancer is believed to be a sexually transmitted disease and attention has focused on sexually transmitted organisms with oncogenic potential. These include HSV-2, HPV (see below) and HCMV.

HCMV is a sexually transmissible cause of cervicitis and urethritis (Evans, 1976) and has been shown to transform human embryo lung cells (Geder et al., 1976) and human endothelial cells (Smiley et al., 1988). Also, mouse cervixes treated with UV-irradiated HCMV, showed an

increased level of development of cancer compared to untreated controls (Heggie et al., 1986). HCMV DNA, RNA and antigens have all been detected in HCMV transformed cells and in tumours although there is conflicting evidence against this (Macnab, 1987). Transformation studies have led to the identification of DNA sequences thought to be involved in initiating transformation. Nelson et al. (1983) identified a 558bp sequence within the BamHI e fragment of HCMV strain AD169 which is capable of transforming NIH3T3 cells and El Beik et al. (1986) identified a further two regions which are capable of transforming NIH3T3 and Rat-2 cells. The fragments in the latter study do not share homology with those of the previous one, however, they do show homology with the BglII c transforming fragment of HSV-2 (Jariwalla et al. 1980). Fletcher et al. (1986) detected sequences hybridizing to HCMV DNA in 2/43 biopsies taken from patients with cervical intra-epithelial neoplasia (CIN). This integrated DNA has been cloned (Fletcher and Macnab, 1989) and shows high homology with the HindIII e region of the HCMV genome. The HCMV HindIII e region contains the DNA sequences for the major IE gene which encodes a polypeptide with a strong promoter and enhancer (Boshart et al., 1985) in addition to the AD169-transforming region (Nelson et al., 1983). The major IE gene has been shown to activate transcription of other virus and cellular genes (Everett and Dunlop, 1984; Rando et al., 1987), and it is conceivable that such activation could involve cellular oncogenes during the development of transformed cells, although this has not been shown.

Early evidence of a causative role for HSV-2 in cervical cancer came from the cervical smears of women with genital HSV-2 infection which showed an increased incidence of premalignant change (Naib et al., 1966). Further seroepidemiological studies showed conflicting results, whilst some studies showed that HSV-2 infection may be an aetiological agent of cervical cancer (Nahmias et al., 1974; Aurelian et al., 1975) results from Vonka et al. (1984) disagree with this finding. The association of HSV-2 and cervical cancer is discussed in more detail below (sections A.2.2.2. and A.2.2.3.).

2.2.1. The Role of HPV in Cervical Cancer.

A good deal of evidence exists to link human papillomavirus (HPV), as well as HSV-2 and HCMV, with cervical cancer (reviewed by Baird, 1985; Macnab, 1987) and it has been proposed that the viruses may have a synergistic effect (section D.2.).

HPV-16 and HPV-18 have been detected in a high percentage of genital cancer biopsies (Durst et al., 1983; Scholl et al., 1985; Macnab et al., 1986; Murdoch et al., 1988) and in the majority of cases the HPV DNA is integrated into the cellular genome. Integration usually occurs within the viral E1 and E2 ORFs and is often accompanied by deletions in this area (Schneider-Gadicke and Schwarz, 1986). The viral early region encoding the E6 and E7 ORFs is selectively retained and expressed in cervical cancer-derived cell lines (Androphy et al., 1987; Banks et al., 1987) and transformation of primary rodent epithelial cells requires a functional E7 gene to co-operate with the activated ras (Phelps et al., 1988; Story et al., 1988, 1990) or fos (Crook et al., 1988) oncogenes. The E7 gene has also been shown to interact with pRB which is believed to be involved with the negative regulation of cellular proliferation (section A.2.1.1.i). Thus, HPV seems to transform cells in a similar manner to other DNA tumour viruses in that its DNA is integrated into the genome and there is evidence of a transforming protein which may interact with a cellular oncogene. However, since current evidence suggests that infection with oncogenic types of HPV is common, without being transforming (Macnab et al., 1986), the identification of other factors which influence the expression, persistence and progression of HPV infection may be significant (section D.2.).

2.2.2. Evidence of Retained HSV-2 Information in Cervical Carcinoma Cells.

HSV-2 DNA sequences have been detected in biopsies of cervical carcinomas, in situ (CIN), and invasive cervical cancer. Almost all studies reveal the presence of the BglII n fragment sequences in a small proportion (about 10%) of tumours (Galloway and McDougall, 1983; Park et al., 1983; Macnab et al., 1985b, 1986; Prakesh et al., 1985). The

detection of HSV DNA in cervical cancer specimens is only circumstantial evidence that HSV has an aetiological role but significance is added by the fact that it is one of the two morphological transforming regions (see below) of the HSV-2 genome which is most frequently retained.

RNA which preferentially hybridizes to the R regions of the HSV genome and a region with map units 0.07-0.40 has been demonstrated in pre-malignant cervical cells but not in matched control biopsies (McDougall et al., 1980; Egin et al., 1981). However, R regions of the HSV genome show considerable homology to regions of human DNA. Also, cellular DNA sequences in tumour cells with homology to the plasmid vector pBR322 have been found to undergo amplification (Park, 1983; Macnab et al., 1984; Cameron et al., 1985). Thus, the detection of RNA homologous to HSV DNA in cervical neoplasia does not necessarily indicate the persistence of viral sequences in the cell (reviewed by Macnab, 1987).

HSV-2 antigens have also been demonstrated in 60-90% of cases of cervical dysplasia, CIN and invasive cervical cancer (Royston and Aurelian, 1970). In addition, the major HSV-2 DNA binding protein has been detected by monospecific antisera in over 30% of cervical tumour specimens and CIN biopsies (Dreesman et al., 1980) and the large subunit of HSV-2 ribonucleotide reductase (RR1) was detected in 50% of similar tissues (Di Luca et al., 1989).

2.2.3. Morphological Transformation of Cultured Cells by HSV.

Although no direct link has been established between HSV infection and cervical cancer, the fact that abortive infections with defective HSV can transform normal rodent embryo cells (Galloway and McDougall, 1983; Cameron et al. 1985; Jariwalla et al. 1986) is well established. Three regions of the HSV genome which induce cellular transformation have been identified in vitro by transfection assays.

(i) MTRI.

Camacho and Spear (1978) identified a transforming region, present in HSV-1, which is known as MTRI

(morphological transforming region of HSV-1) and maps in XbaI f (0.29 to 0.45 map units) and BglII i (0.311 to 0.415 map units, Reyes et al. 1979). The sequences for glycoprotein B (gB) can at least be transiently expressed from this fragment. A mutagenic peptide may also be expressed from MTRI (Shillitoe et al., 1986) and this is discussed in section A.2.2.5.

(ii) MTRII.

In HSV-2, there are two regions of the genome which have been associated with the induction of morphological transformation. MTR-II, (morphological transforming region of HSV-2), maps in BglII n (0.58 to 0.62 m.u.; Reyes et al. 1979; Macnab and McDougall, 1980; Galloway and McDougall, 1981; Cameron et al. 1985; Figure 4). The minimal transforming region of this fragment has been localized to a small, putative stem-loop structure of 737bp (737TF), bounded by direct repeats, which is found in the left-hand region of BglII n (Galloway et al. 1984), within the gene encoding UL41 (Figure 4). Galloway et al. (1984) proposed that a similar structure, present in HCMV, may induce morphological transformation. The stem-loop might be important since such structures can also be formed by insertion sequences (IS) that are capable of integrating into the cellular genome. Three mechanisms of transformation were proposed (Galloway et al., 1984), activation of a cellular oncogene, function as an enhancer or action as a mutagen. The effect of this sequence on random mutagenesis remains insignificant in transfection assays (Brandt et al., 1987) and Pilon et al. (1989) have shown that transformation induced by it is not linked with mutagenesis of a cellular marker gene. The proposal that the putative stem-loop structure may function as an IS is also debatable since this 737bp sequence does not conform to the standard description of an IS; it does not appear to be capable of moving around the genome and it does not encode a transposase (Lewin, 1990). The transposase could be supplied by other regions of the genome, but this has not been shown. It should also be noted that the significance of such stem-loops is unclear since computer scanning has found many such structures elsewhere in the HSV genome (Shillitoe, 1988).

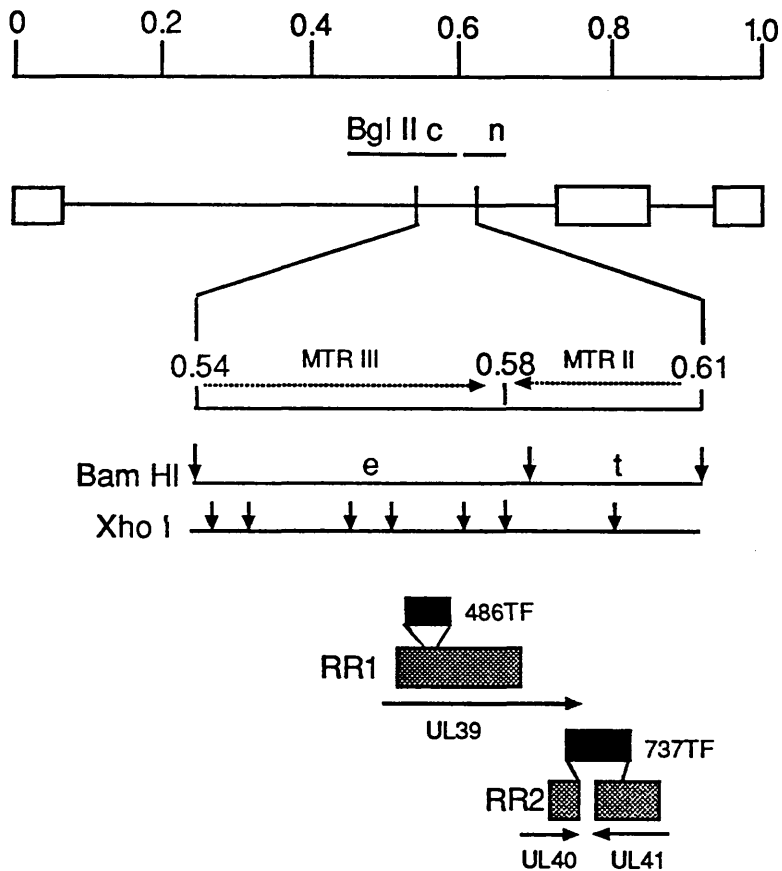


Figure 4. Genome arrangement of HSV-2 showing Bgl II ϵ (MTR III) and Bgl II η (MTR II) which encode the large (RR1) and small (RR2) subunits of ribonucleotide reductase. The positions of the genes UL39, UL40 and UL41 are indicated, as are the two transforming fragments 737TF and 486TF. 737TF is the smallest fragment of MTR II required to transform rat embryo or NIH 3T3 cells in the focus or anchorage dependent assay of Galloway *et al.* (1984). The 486TF (Jones *et al.*, 1986) is the smallest fragment of MTR III required to transform Rat 2 cells in the focus assay system of Jariwalla *et al.* (1986).

The left-hand end of MTRII also contains the coding sequences of RR2 (Figure 4, section A.3.3.) and it was proposed that expression of this protein might be involved in the induction of the transformed phenotype. However, retention and expression of these sequences were not found to correlate with transformation (Kessous-Elbaz et al., 1989).

Other experiments (Pilon et al. 1989) disagree with these findings as it was observed that the left-hand portion of BglII n caused neither transformation nor mutagenesis in NIH3T3 cells whereas the right-hand region was transforming but not mutagenic.

The topic of transformation caused by MTRII is thus very confused at present with no good evidence for a mechanism of transformation or a transforming region smaller than the original BglII n fragment.

(iii) MTRIII.

MTRIII (the third morphological transforming region of HSV), maps in HSV-2 BglII c (Peden et al. 1982). The left 64% of this fragment causes cellular immortalization whereas the remaining 30%, BamHI e (0.54 to 0.58 m.u.), acts to convert immortalized rat embryo (RE) cells to the transformed phenotype (Jariwalla et al. 1986). The minimal region of MTRIII that is capable of causing transformation has been mapped to a 486bp fragment, 486TF, located on the PstI c fragment (Jones et al. 1986). The same fragment on the HSV-1 genome can also transform cells but with much less efficiency (R. J. Jariwalla, personal communication). Sequence analysis of this region revealed the presence of short ORFs but these were found to have no upstream TATA or CAAT boxes so their significance is unclear. PstI c also contains a number of repetitive DNA elements that appear to be clustered within the 486bp sequence. There are 5 stretches of alternating purine/pyrimidines compared to only one such stretch in the corresponding region of HSV-1. Such residues may have the capacity to form Z-DNA which has been implicated in homologous recombination and destabilization of chromatin structure. The cluster of Z-DNA tracts may be a "hot-spot" for recombination and could contribute to a transforming function by exerting cis-acting sequences on

the expression of cellular genes. There are also three CCGCCC motifs (again compared to one in HSV-1), one of which occurs as an inverted repeat. This sequence is thought to be an SP1 protein binding consensus (Ishi et al. 1986), and recently three copies of the inverted sequence have been identified in the strong enhancer of HCMV (Boshart et al., 1985). Five purine- and three pyrimidine-rich elements, which resemble enhancer core sequences, are also present within the 486bp fragment, as are a number of possible stem-loop structures which could promote transposition or insertion in a similar manner to the 737bp sequence described previously (see above).

The 486bp transforming region has been shown to function as a transcriptional promoter when fused to the bacterial CAT gene (Jones, 1989) and it was suggested that the fragment may act to alter the expression of a cellular gene. A number of cellular genes have been found to be differentially expressed in cells transformed by the 486TF, namely c-H-ras and p53, which are present in higher levels. Regions of p53 were also found to have been amplified. This suggests that these genes may be involved in the transformation process possibly due to some effect of the 486TF (Jones et al., 1989). Zhu and Jones (1990) have also suggested that the 486TF, in combination with cellular factors, triggers unscheduled rounds of cellular chromosomal replication resulting in chromosomal aberrations which may ultimately lead to cellular transformation. BglII c also contains most of the coding sequences for RR1 (Figure 4, section A.3.3.), including a region with serine/threonine protein kinase activity (Chung et al., 1989, section A.3.3.4.) and although transformation can be established with the 486TF alone, the potential role of this in transformation has not been ruled out (section A.2.2.5.).

2.2.4. The "Hit and Run" Hypothesis.

In HSV-transformed cell lines the quantity of DNA present is always less than one copy per cell and varies with passage in culture (Galloway et al., 1980; Cameron et al., 1985). It seems unlikely, therefore, that sufficient HSV DNA could be retained in order to encode a protein responsible for the maintenance of the transformed state.

This does not exclude the possibility that active recombination of HSV sequences into the host cell chromosome might be important for oncogenesis (Galloway and McDougall, 1983). Short HSV sequences might be difficult to detect by Southern blotting analysis. Alternatively, HSV sequences may act in a "hit and run" mechanism where activation of a viral or cellular oncogene might occur before the HSV DNA is rapidly excised from the host cell (Skinner, 1976; Galloway and McDougall, 1983). Saavedra and Kessous-Elbaz (1985) described an experiment where co-transfection of the plasmid pAG60 (which confers resistance to the antibiotic G418) with the complete BglIII n fragment reduced the overall efficiency of colony formation in G418, compared with pAG60 alone, suggesting that this sequence is poisonous to cells. The whole BglIII n fragment seems to be required for the inhibitory effect since sub-fragments of this sequence have no effect on colony formation (Kessous-Elbaz et al., 1989).

The failure of HSV-transformed cells in tissue culture to retain viral sequences contrasts with the presence of HSV sequences in a small but significant proportion of cervical cancer and CIN biopsies. The differences observed may reflect the fact that cervical cancers are generally of epithelial origin whereas the rat cells examined in culture are predominantly fibroblastic (Macnab, 1987).

2.2.5. Possible mechanisms of HSV Oncogenesis.

(i) Activation of Cellular Genes; evidence for the trans-activation of cellular genes has been presented (section A.1.5.2.). Other ways of cellular gene activation include reduced methylation and promoter/enhancer insertion. In HSV-2 infected and HSV transformed cells, cellular DNA shows reduced methylation (J.M. Macnab, ^{et al. 1988}). This phenomenon leads to increased gene expression in experiments with mouse retroviruses (Janner et al., 1982). In addition, tumour cells often show reduced methylation when compared to normal control cells (Adams and Burdon, 1983). As for promoter/enhancer insertion causing gene activation, sequences as small as 100bp can induce a cellular gene (McKnight and Kingsbury, 1982; Everett, 1983). Such sequences would escape detection by the Southern

blotting analysis of Cameron et al. (1985). Homologies between viral and cellular DNA sequences (Peden et al., 1982; Gamez-Marquez et al., 1985; Spector et al., 1987) could allow viral integration into the host cell DNA by homologous recombination.

(ii) Activation of an Endogenous Virus; HSV-infection can activate endogenous C-type virus (mux) from a feline cell line (F81) transformed by the Moloney strain of murine sarcoma virus (Hampar et al., 1976; Boyd et al., 1980). This activation may be due to mutagenesis or integration of an HSV promoter/enhancer near to the integrated endogenous virus (Macnab, 1987). HSV-1 sequences responsible for this map in MTR-1. Boyd et al. (1980) found that the TK gene from MTR-1 is responsible but this is disputed by Shillitoe et al. (1986) who found the inducing sequences in MTR-1 to be distinct from the TK gene and also from the mutagenic BamHI g fragment (section A.2.2.5.iv) and the DNA polymerase gene.

(iii) Amplification of Cellular Genes; both HSV-1 and HSV-2 have been shown to amplify integrated SV40 sequences, in an SV40-transformed Chinese hamster cell line (Schle^{hofer} et al., 1983) in a similar manner to certain physical and chemical carcinogens (Lau, 1981). This effect seems to require the DNA polymerase (Matz et al., 1984; Heilbronn et al., 1985). Amplified and mutated cellular oncogenes have been found in many tumours suggesting that the mutagenic and amplifying properties of HSV may at least be partly responsible for its oncogenic actions.

(iv) Mutagenesis of Cellular Genes; HSV infection was first thought to be mutagenic when chromosomal aberrations, including random changes and specific gaps were reported in infected cells (Stich et al., 1964). This process does not require viral DNA synthesis (zur Hausen, 1967, 1968; Waubke, 1968) and may be due to the induction of a DNase with endonuclease activity by HSV-infection (Morrison and Keir, 1968).

An increase in the mutagenic rate of a cellular gene encoding the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) was also shown after infection of

permissive cells with UV-inactivated HSV-1 (Schlehofer and zur Hausen, 1982) and infection of non-permissive cells with HSV-2 (Pilon et al., 1985). In the latter study, the transcription rate and the size of the transcript were similar to those in uninfected cells suggesting that the mutagenic effect was due to point mutations (Pilon et al., 1986). Such mutations could have oncogenic effects if they activated oncogenes, like c-ras, or inactivated anti-oncogenes.

Hwang and Shillitoe (1990) studied the effect of HSV-1 infection on the mutagenic rate of a marker gene, supF, located on the shuttle vector plasmid pZ189 (Seidman et al., 1985, section C.2.1.1.). The mutation frequency was higher in infected cells (0.25%) than in uninfected controls (0.06%) or in cells infected with vaccinia virus (0.07%). Sequencing revealed that the mutations induced by HSV-1 were not only more frequent, but also different, in type to those occurring spontaneously. The differences included the observations that there was more than one mutation occurring in the marker genes in HSV-1 infected cells. There seemed to be a "hot spot" for HSV-1 mutations, deletion mutations occurred at a higher frequency and there were also insertions which were not present in the spontaneous mutants. These differences indicate that there might be some mechanisms resulting from HSV-1 infection which determine the type of mutations as well as increasing their frequency.

The mechanism by which HSV infection could increase the frequency of somatic mutation is unknown, but could be related to the activity of various viral proteins. The maximum increase in mutation frequency is seen 2-4 hours post-infection (Hwang and Shillitoe, 1990) implying that the effect is likely to be a function of a structural component, or an IE or E protein, particularly one which is involved in DNA metabolism, rather than late proteins. Candidate E proteins include dUTPase (Caradonna and Cheng, 1981; Preston and Fisher, 1984), uracil-DNA glycosylase (Caradonna and Cheng, 1981; Caradonna et al., 1987), alkaline exonuclease (Costa et al., 1983), thymidine kinase (McKnight, 1980), DNA polymerase (Ostrander and Cheng, 1980; Gibbs et al., 1985) or ribonucleotide reductase (Huzar and Bacchetti, 1983; Cohen, 1972).

HSV DNA polymerase and DNase activity have been proposed as having a role in mutagenesis (Galloway and McDougall, 1983, Matz et al., 1984, 1985) and viruses with mutant DNA polymerase activity have been isolated. These viruses show reduced mutagenesis of the TK gene implying that the wt DNA polymerase may be mutagenic (Hall et al., 1984, 1985). However, the HSV DNA polymerase does not appear to be linked with mutations that arise in a shuttle vector plasmid in HSV-1 infected cells (Shillitoe, personal communication).

In normal cells, intracellular dUTP levels are very low, reducing the incorporation of dUTP into DNA (Shlomai and Kornberg, 1978; Goulian et al., 1980a,b). The dUTPase is involved in the hydrolysis of dUTP to dUMP (Williams and Cheng, 1979) implying a role for an increase in dUTPase activity in preventing misincorporation of uracil into DNA. Alternatively, increased dUTPase activity in infected cells (Caradonna and Cheng, 1981) could cause an accumulation of dUMP which would be converted to dTTP by thymidylate synthetase. HSV-1 uracil-DNA glycosylase (Caradonna and Cheng, 1981; Caradonna et al., 1987) might also function in mutagenesis. Error prone editing or repair functions of this enzyme could be followed by misincorporation of dUMP and deaminated cytosine residues (Friedberg, 1985).

Perturbation of dNTP pools caused by the viral thymidine kinase or ribonucleotide reductase may also cause mutations and this will be discussed below (section A.2.2.5.).

Expression of the BamHI g fragment, which lies within HSV-1 MTRI, has been shown to increase the reversion rate of a frameshift mutation in bacterial cells by up to 39-fold (Shillitoe et al., 1986). The effect was only seen when the viral DNA was cloned in one orientation and in one reading frame and was enhanced when the promoter was induced suggesting that the mutagenic effect was dependent on the expression of RNA or a peptide encoded at the left end of BamHI g. This region lies to the right of the fragment which reactivates retroviruses (Boyd et al., 1980) and to the left of the virus DNA polymerase which has been suggested as having a role in mutagenesis (see above). The mutagenic region specifies the 3' terminal portions of 5 RNA species (Holland et al., 1984), the functions of which are unknown, and expresses a basic (21% arg nine) peptide consisting of

37 amino acids, 6 of which are encoded by the vector (Shillitoe et al., 1986). Recent work (Das and Shillitoe, 1990) has shown that immune sera directed against the peptide reacted with extracts from HSV-1 infected cells more strongly than preimmune sera, implying that this reading frame appears to be expressed in HSV-1 infected cells.

2.2.6. The Postulated Role of HSV Ribonucleotide Reductase in Mutagenesis.

A role for HSV ribonucleotide reductase in transformation was first suggested when subfragments of the HindIII a region, which encompasses UL39 and UL40, were shown to induce transformation. Supporting this observation is the fact that the HSV enzyme lacks the tight allosteric control shown by its cellular counterpart (section A.3.3.) and may be involved in the destabilization of dNTP pools in infected cells (Jamieson and Bjursell, 1976). The effect of HSV ribonucleotide reductase on mutagenesis will be discussed in more detail below (section A.3.3.5.).

3. RIBONUCLEOTIDE REDUCTASES.

3.1. Reaction Mechanism.

The enzyme ribonucleotide reductase catalyses the reduction of ribonucleoside diphosphates (NDPs) to the corresponding deoxyribonucleoside diphosphates (dNDPs). In non-growing cells, the concentrations of deoxyribonucleoside triphosphates (dNTPs) are very low and the activity of ribonucleotide reductase is thus a prerequisite for DNA synthesis and cell multiplication.

The mechanism of the reaction involves a direct replacement of the hydroxyl-group, at the 2'-position of the ribose moiety, by a hydrogen (Thelander and Reichard, 1979, Figure 5). NADPH is the ultimate hydrogen donor for the reaction but several proteins participate as hydrogen carriers. Two hydrogen donor systems have been identified so far. These are the thioredoxin system and the glutaredoxin system.

3.1.1. The Thioredoxin System.

This system was first discovered in E. coli (Laurent et al., 1964). Thioredoxin reductase consists of two, probably identical, subunits each of which has one tightly bound FAD molecule and a redox-active disulphide in its active centre (Thelander, 1968; Ronchi and Williams, 1972). It serves to reduce another protein, thioredoxin, at the expense of NADPH. The E. coli thioredoxin contains two redox-active cysteine residues (Holmgren, 1968) located on a protrusion of the active centre of the enzyme (Holmgren et al., 1975). The reduced form of thioredoxin is an efficient hydrogen donor for ribonucleotide reductase.

Mammalian enzymes specify a thioredoxin system similar to that of E. coli. Bacteriophage T4 only encodes a thioredoxin which seems to be reduced by the host cell thioredoxin reductase (Berglund et al., 1969). T4-induced thioredoxin can also be reduced by the glutathione system (see below) and appears to be a functional hybrid between these two hydrogen donor systems (Holmgren 1978).

Attempts to identify the principal hydrogen donor for

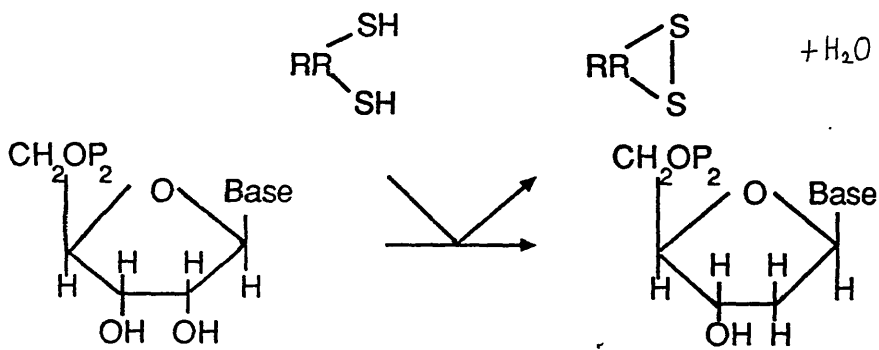


Figure 5. Reduction of a ribonucleoside diphosphate to a deoxyribonucleoside diphosphate by ribonucleotide reductase (RR). RR-(SH)₂ and RR-(S)₂ represent the reduced and oxidized forms of ribonucleotide reductase.

the HSV-1 induced ribonucleotide reductase resulted in the purification of a protein, present in both infected and mock-infected extracts, which had similar physical characteristics to the cellular thioredoxin (Darling^{et al.} 1988) suggesting that HSV-1 utilizes the cellular enzyme as a hydrogen donor. The lack of sequence evidence for the existence of a virus-induced thioredoxin supports this idea.

3.1.2. The Glutaredoxin System.

The glutaredoxin system was identified in an E. coli mutant which was unable to replicate bacteriophage T7 DNA, and failed to express any detectable level of thioredoxin without displaying a decreased capacity to reduce ribonucleotide diphosphates (NDPs) in vitro (Holmgren, 1976). In this system, glutathione reductase and NADPH reduce glutaredoxin which contains two redox-active cysteine residues (Holmgren, 1979a) and serves as a hydrogen donor for ribonucleotide reductase (Holmgren, 1979b).

The true in vivo hydrogen donor system is presently unknown, but both systems possibly substitute for each other under different growth conditions.

3.2. Classification of Ribonucleotide Reductases.

Three different classes of ribonucleotide reductases are thought to exist. Class 1 enzymes are monomeric and are found in several prokaryotes and a few eukaryotes. Class 2 enzymes are dimeric and are found mainly in eukaryotes and animal viruses. Finally, class 3 enzymes resemble those of class 2 in structure but differ from them biochemically. This class of enzyme is found in a number of gram-positive bacteria. Class 1 and 2 enzymes will be described in more detail below.

3.2.1. Monomeric Ribonucleotide Reductases.

Monomeric ribonucleotide reductases are represented by the Lactobacillus leichmanni enzyme which consists of a single polypeptide chain of 76,000 molecular weight (Panagou et al., 1972; Chen et al., 1974). The enzyme has a single regulatory site which binds the deoxyribonucleoside

triphosphate (dNTP) allosteric effectors (Singh et al., 1977). It has been proposed that the enzyme's activity is only positively regulated as no negative effectors have been found (Goulian and Beck, 1966; Vitols et al., 1967; Follman and Hogenkamp, 1971). Binding of the positive effectors dATP, dCTP, dTTP and dGTP results in the reduction of CTP, UTP, GTP and ATP respectively (Vitols et al., 1967). Chen et al. (1974) showed that the substrates can bind to the regulatory site with an affinity 100- to 1000-fold lower than that of the effectors.

It is assumed that the enzyme has a single catalytic site which binds the ribonucleoside triphosphate (NTP) substrates (Vitols et al., 1967). The activity of the class 1 enzymes requires adenosylcobalamine (AdoCbl, B12-coenzyme) which binds directly to the polypeptide chain and is dependent on effector binding to the regulatory site (Singh et al., 1977). It is thought that AdoCbl functions in a similar manner to the class 2 enzyme subunit which contains the iron centre and the tyrosyl free radical (see below; Ashley et al., 1986).

3.2.2. Oligomeric Ribonucleotide Reductases.

(i) The E. coli Enzyme.

The E. coli ribonucleotide reductase is a dimeric enzyme consisting of two non-identical subunits, B1 and B2 (Brown and Reichard, 1969a) which are encoded by the nrda and nrdb genes respectively (Fuchs and Karlstrom, 1976). The active enzyme consists of the B1 and B2 subunits weakly bound in a 1:1 stoichiometry in the presence of Mg^{2+} (Brown et al., 1967; Brown and Reichard, 1969a; Thelander, 1973).

The B1 subunit has a molecular weight of 160,000 and is a dimer of two identical polypeptides (Thelander, 1973). Each B1 subunit contains two catalytic sites which bind all four NDP substrates (von Döbeln and Reichard, 1976). Reduction of the substrates requires the presence of the active sulphhydryl groups provided by two cysteine residues also located on B1 (Thelander, 1974) and the stable free radical provided by B2 (Thelander, 1974; Ehrenberg and Reichard, 1972). The B1 subunit also contains four regulatory sites which can be separated into two types based on their

affinity for dATP binding (Brown and Reichard, 1969b). The two h sites have high affinity for dATP whereas the two l sites have low affinity for this effector. The h sites also bind ATP, dTTP and dGTP, regulating the substrate specificity of the enzyme, whilst the l sites bind only ATP in addition to dATP and regulate the overall level of enzyme activity with ATP binding activating the enzyme and dATP binding reducing its activity. Binding of an effector to the h sites results in a conformational change of the catalytic site leading to the preferential binding of the appropriate substrate. It has been proposed that in vivo there are three active and one inactive enzymic states (Thelander and Reichard, 1979). When ATP is bound to the l sites, binding of ATP, dTTP or dGTP to the h sites will reduce CDP or UDP, GDP (and ADP) or ADP (and GDP) respectively. When dATP is bound to the l site the enzyme is inactive irrespective of effector binding to the h sites.

The B2 subunit is also dimeric, consisting of two identical polypeptides. It has a molecular weight of 78,000 (Thelander, 1973). The B2 subunit has two important elements, an iron centre, which is made up of two non-heme iron atoms (Brown et al., 1969) and a stable free radical (Ehrenberg and Reichard, 1972) which is destroyed by hydroxyurea, a powerful radical scavenger. Sjöberg et al. (1977) located the radical to a tyrosine, using isotope substitution experiments, which was then identified by site-directed mutagenesis (Larsson and Sjöberg, 1986). The radical is involved in the transient transfer of the unpaired electron from the enzyme to the substrate. Sjöberg et al. (1983) proposed a scheme for this reaction (Figure 6). Firstly, the hydrogen at position C-3 of the ribose moiety is abstracted by the tyrosyl radical of B2 resulting in the formation of substrate intermediate I. The presence of the radical at C-3 destabilizes the ribose moiety which facilitates the ejection of OH^- from C-2 forming substrate intermediate II. C-2 is then reduced by thiols from B1 and recapture of the hydrogen atom at C-3 regenerates the free radical. It is unusual to find a stable free radical as an integral part of an enzyme and it is thought that it is generated and stabilized by the iron centre (Atkin et al., 1973).

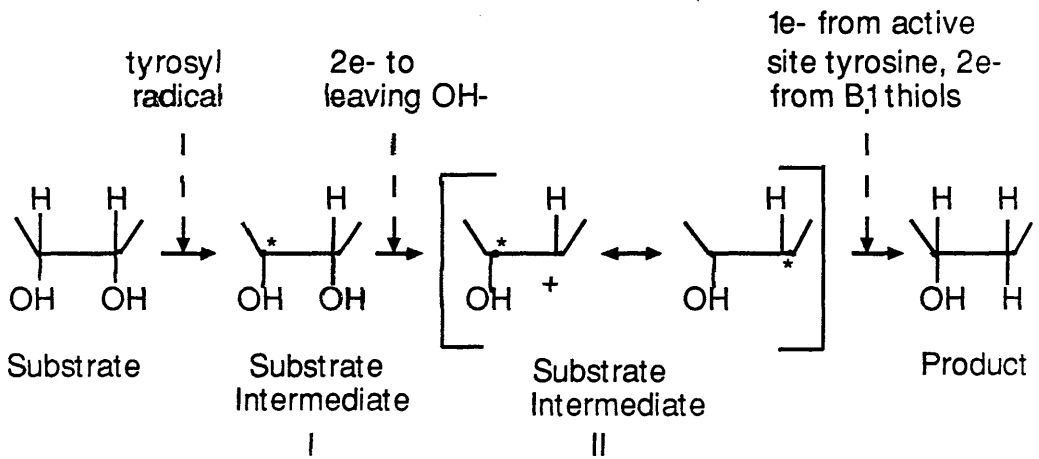


Figure 6. The Proposed Involvement of the *E. coli* Ribonucleotide Reductase radical in the reduction of a ribonucleoside diphosphate to a deoxyribonucleoside diphosphate. The diagram depicts the substitution of the 2' OH group of the substrate ribose moiety with a H. The balance of electrons (e-) and the radical (*) are shown.

Apart from being regulated at the level of enzymic activity, the E. coli ribonucleotide reductase enzyme is also regulated at the transcriptional level since the inhibition of DNA synthesis by thymine starvation increases the level of transcription of the reductase genes (Hanke and Fuchs, 1983b). This appears to be mediated by a positive regulatory protein(s) (Hanke and Fuchs, 1984). Post-transcriptional regulation also appears to exist as a report of a truncated form of B2, resulting from limited proteolysis of normal B2 by a serine protease and giving rise to an inactive enzyme, has been made (Sjoberg et al., 1987).

(ii) Bacteriophage-induced Enzymes.

Bacteriophages T2, T4, T5 and T6 induce a novel ribonucleotide reductase activity during infection of E. coli (Cohen and Barner, 1962; Biswas et al., 1965; Berglund et al., 1969; Eriksson and Berglund 1974), whereas T7 and lamda do not (Eriksson and Berglund, 1974). The T4 induced enzyme has a molecular weight of 225,000 and is composed of two subunits, T4B1 and T4B2, which form a tight complex (Berglund 1972a).

The T4B1 subunit consists of two identical polypeptides which have a molecular weight of 85,000 (Berglund, 1972a, 1975). The subunit has a single catalytic site and possibly two types of allosteric sites. One of these has a low affinity for dATP and the other has high dATP affinity and also binds ATP, dTTP and dGTP (Berglund, 1972b). The allosteric properties of the T4-induced ribonucleotide reductase are similar to those of the E. coli enzyme except for the fact that high dATP concentrations positively regulates the reduction of the pyrimidine substrates instead of inhibiting enzyme activity. The lack of negative effectors of the T4-induced enzyme may be related to the fact that T4 DNA synthesis is not turned off during infection but continues until the T4-infected cell lyses.

The T4B2 subunit also consists of two identical subunits which have a molecular weight of 34,500 (Berglund, 1972a, 1975; Cook and Greenberg, 1983). The T4B2 subunit contains two non-heme iron atoms per molecule which are essential for activity. Their optical and EPR spectra are very similar to

those of the E.coli B2 iron centre (Berglund, 1972a, 1975). The subunit also contains a tyrosine free radical (Berglund, 1972a; Sahlin et al., 1982) which is similar to that of the E. coli enzyme.

(iii) The Mammalian Enzyme.

Ribonucleotide reductase activity has been demonstrated in a number of mammalian systems including rat hepatoma (Moore, 1977), calf thymus (Engstrom et al., 1979; Eriksson et al., 1979; Thelander et al., 1980), rabbit bone marrow (Hooper, 1972, 1978) and human tissue culture cell lines (Chang and Cheng, 1979). The mammalian enzyme is again composed of two non-identical subunits, M1 and M2.

The M1 subunit consists of two identical polypeptides and its molecular weight in calf thymus is 17₀,000. The M1 dimer contains two catalytic sites and two different types of allosteric effector binding sites. The allosteric effector binding sites are similar to those of the E.coli enzyme except for two differences. Firstly, the number of sites in M1 is lower than in B1 and secondly, the M1 sites show an equal affinity for dATP binding (Thelander et al., 1980). Allosteric regulation of activity is similar to that of the E.coli enzyme (Eriksson et al., 1979) although in the case of the mammalian enzyme, control is more strict.

The M2 subunit has a molecular weight of 88,000 in the mouse and is a dimer of two polypeptides. M2 contains a non-heme iron centre, which is of the same type as the iron centre of B2 (Thelander et al., 1985). It also contains a free radical, located on a tyrosine (Graslund et al., 1982), which, like the E.coli radical, is inhibited by hydroxyurea (Engstrom et al., 1979), but to a greater extent. Kjoller-Larsen et al. (1982) and Thelander et al. (1985) suggested that this was due to different active site topologies with the mammalian site being more exposed.

Synthesis of the mouse enzyme is regulated in a cell cycle dependent manner. M1 ^{Synthetic} activity is constant throughout G1, S and G2 phases whereas M2 activity is less in G1 compared to S and G2 (Eriksson and Martin, 1981; Engstrom et al., 1985). The reduced activity of M2 in G1 is due to the de novo synthesis of the M2 protein at the start of the S phase and it has been proposed that this regulation

occurs at the transcriptional level (Eriksson et al., 1984).

(iv) Viral Induced Enzymes.

Several animal viruses including HSV-1 (Cohen, 1972), HSV-2 (Cohen et al., 1974; Huzar and Bachetti 1981; Huzar et al., 1983), PRV (Lankinen et al., 1982), EHV serotypes 1 and 3 (Cohen et al., 1977; Allen et al., 1978), EBV (Henry et al., 1978), VV (Slaubaugh et al., 1984) and VZV (Spector et al., 1987) have been reported to induce a novel ribonucleotide reductase activity in infected cells which differs from the host cell enzyme.

3.3. The HSV-Induced Enzyme.

Direct evidence for virus-encoded ribonucleotide reductase activity has been obtained for HSV-1 in that the multiple HSV-1 mutant tsG (Brown et al., 1973) expressed reduced levels of ribonucleotide reductase activity at the PT while at the NPT no activity was observed.

The HSV-induced enzyme is composed of two non-identical subunits, RR1 and RR2 (Frame et al., 1985; Cohen et al., 1985; Bachetti et al., 1986; Ingemarson and Lankinen, 1987) both of which are phosphorylated (Bachetti et al., 1984). RR1 has been shown to be a product of the UL39 gene by marker rescue of the mutant HSV-1 virus tsl207 (Nikas, 1988) and has a molecular weight of 136,000 in HSV-1 and 138,000 in HSV-2 (Marsden et al., 1978).

RR2 has a molecular weight of 38,000 in HSV-1 and 36,000 in HSV-2 and is a product of the UL40 gene (Preston, V. G. et al., 1988). It was first thought to be a component of the viral reductase when it was found to exhibit homology with the B2 subunit from E.coli (Standart et al., 1985; Sjoberg et al., 1985; McLauchlan, 1986) and was shown to be the smaller protein species (Preston V.G. et al., 1984) that precipitated with RR1 after exposure to RR1-specific antibodies (Frame et al., 1985). UL39 and UL40 mRNAs have unique 5'-ends and common 3'-ends (section A.1.4.10.). The arrangement of mRNAs at this HSV-2 genome locus is shown in Figure 7.

Conclusive evidence for the involvement of the RR2 polypeptide in enzymic activity was obtained from the HSV-1

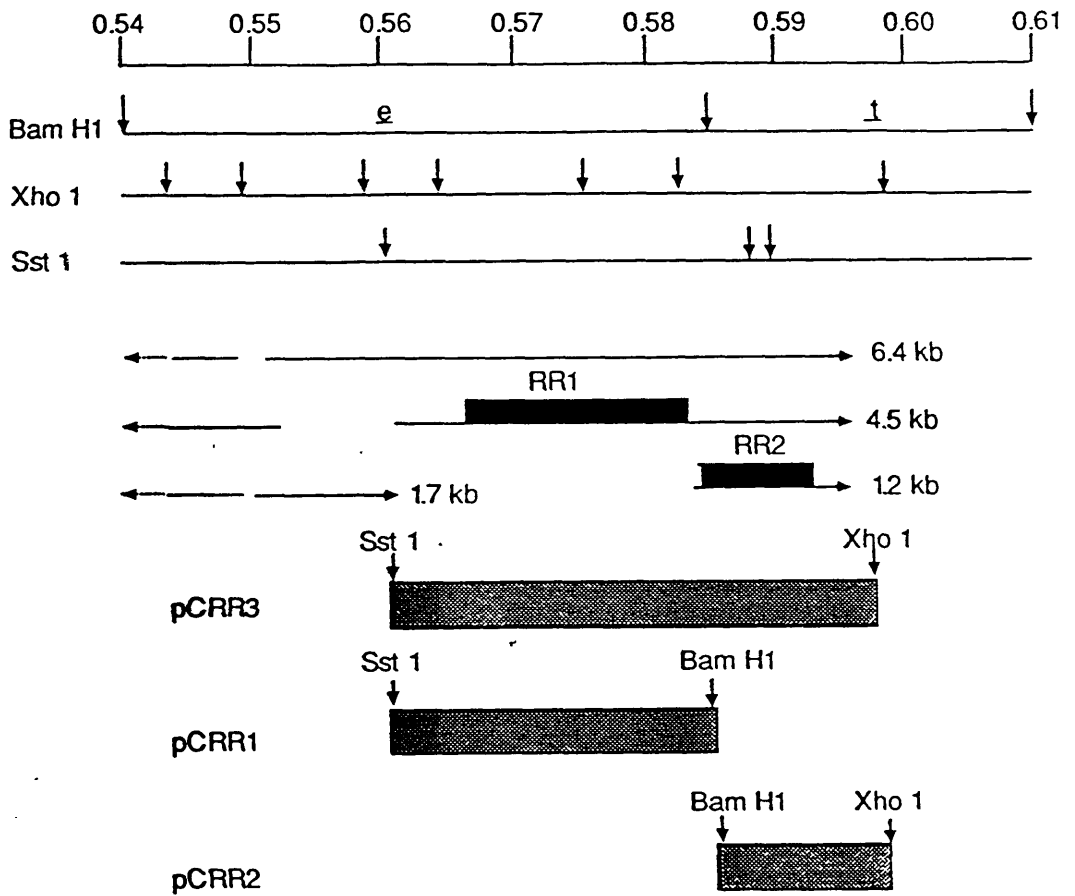


Figure 7. The Ribonucleotide reductase locus of HSV-2 showing the location of transcripts (→) and the coding regions (■) of RR1 and RR2 with respect to BamHI fragments *e* and *t* and the XhoI and Sst I sites within these fragments. The 4.5 kb and 1.2 kb transcripts encode RR1 and RR2 respectively. The other mRNAs are late transcripts (McLauchlan, 1986). Also shown (▨) are the HSV-2 fragments used for the construction of the plasmids pCRR1, pCRR2 and pCRR3 (section C.1.). The plasmid pCRR1 thus contains the sequences encoding RR1, pCRR2 contains the sequences encoding RR2 and pCRR3 contains the sequences encoding both RR1 and RR2.

mutant tsl222 which has a lesion in UL40 and induces negligible levels of ribonucleotide reductase activity at both the PT and NPT (Preston V.G. et al., 1988).

The active HSV-1 enzyme has a sedimentation coefficient of 17S which corresponds to an approximate molecular weight of 370,000 (Ingemarson and Lankinen, 1987), which lead to the suggestion that the active enzyme is a homodimeric complex of the $\alpha_2\beta_2$ structure. The complex shows tight binding (Frame et al., 1985; Ingemarson and Lankinen, 1987), similar to that of the T4-induced enzyme (Berglund 1972a).

Unlike the bacterial and mammalian enzymes, the HSV-induced ribonucleotide reductase is inhibited by Mg^{2+} or ATP (Averett et al., 1983; 1984; Huszar and Bacchetti 1981; Ponce de Leon et al., 1977). This effect is reduced in the presence of a Mg^{2+} /ATP complex, presumably because the formation of the complex lowers the concentration of free ligands.

The HSV enzymes have similar substrate binding properties. Both enzymes utilize NDP substrates which bind at a common catalytic site and which compete with the reaction products (dNDP's, Averett et al., 1983). Both enzymes are also insensitive to inhibition by dNTPs suggesting that they are not allosterically regulated (Averett et al., 1983, 1984). The same suggestion can be applied to the PRV- (Lankinen et al., 1982) and VZV-induced ribonucleotide reductase activities (Spector et al., 1987), however, the VV-induced activity is inhibited by dATP and dTTP and is thus thought to be allosterically regulated (Slaubaugh et al., 1984).

There is strong conservation of the tyrosine residue and its flanking amino acid sequences in the E.coli B2 and HSV-1 RR2 subunits and this fact along with the observed inhibition of the HSV enzyme by hydroxyurea, a radical scavenger (Averett et al., 1983; Lankinen et al., 1982), suggests that the HSV enzyme contains a tyrosine radical. The PRV-induced enzyme is also inhibited by hydroxyurea and displays an EPR spectrum resembling that of the M2 tyrosyl radical (Lankinen et al., 1982) and so also appears to contain a tyrosyl radical.

3.3.1. Regulation of HSV Ribonucleotide Reductase Synthesis.

As described above, HSV ribonucleotide reductase activity does not appear to be allosterically regulated. Synthesis of RRL and accumulation of enzymatic activity behave temporally as E events (O'Hare and Hayward, 1985a), however, studies have shown that RRL can also be synthesized under conditions that do not allow the expression of other such genes (Middleton et al., 1982). Expression from the HSV-1 RRL promoter was found to be stimulated by Vmw65 and sequence analysis has revealed the presence of three elements which show homology to the TAATGARAT element (Wymer et al., 1989). This promoter has also been shown to contain a potential binding site for the cellular "octamer motif binding factor" at position -136 (Nikas, 1988) and fragments containing this site are retarded in gels by Vmw65/cellular transcription factor complexes. These complexes have similar mobilities in gels to those of the IEC complex formed with DNA fragments containing the TAATGARAT motif of the regulatory region of IE gene promoters. The above findings thus indicate that RRL is capable of being expressed as an IE gene. The HSV-2 RRL promoter also contains an AP-1 element (Wymer et al., 1989) which may be indicative of IE gene expression since AP-1 binding factors are consistently expressed in most cell systems (Lee et al., 1987).

In contrast to RRL, the RR2 gene is not expressed in the absence of functional IE products (Nikas, 1988). However, in the presence of functional IE gene products both the RRL and RR2 mRNA levels increase compared to the levels observed under IE conditions.

3.3.2. HSV Ribonucleotide Reductase is Essential for Virus Growth.

Preston V.G. et al., (1984) suggested that HSV ribonucleotide reductase is essential for virus replication after observing that the HSV-1 mutant tsl207 failed to produce any ribonucleotide reductase activity at the NPT and that its yield was 100-fold lower than that of the wt virus at this temperature. However, three subsequent reports questioned this proposal. Firstly, hydroxyurea, which reduces ribonucleotide reductase activity to undetectable levels, only lowered the HSV-2 yield in proliferating cells 6-fold (Nutter et al., 1985). Secondly, a significant amount

of DNA replication occurred in the absence of the ribonucleotide reductase genes (Wu et al., 1988) as shown using the plasmid replication assay developed by Challberg (1986). Finally, the enzyme was shown to be dispensible in dividing cells at 34°C (Goldstein and Weller, 1988a) using the mutant HSV-1 virus hr3 which expresses the N-terminal 434 amino acids fused to the β -galactosidase (lacZ) gene.

Later studies confirmed the requirement for HSV ribonucleotide reductase in virus replication as the HSV-1 mutant tsl222 failed to grow in resting cells at the PT indicating that the enzyme is essential under these conditions (Preston V.G. et al., 1988). In exponentially growing cells, at the PT, tsl222 is dependent on the cellular enzyme for replication whilst it was suggested that at the NPT the mutant fails to replicate due to inactivation of the cellular enzyme. Goldstein and Weller (1988b) also show that growth and replication of an HSV-1 mutant, expressing less than 10% of the RR1 DNA coding region, were severely restricted in non-dividing cells or at 39.5°C.

Evidence that ribonucleotide reductase is an essential HSV function has been obtained from pathogenicity studies of the HSV-1 mutants tsl207 and tsl222. The virulence, in mice, of both these mutants was reduced by about 1 million-fold when compared to that of the parental virus (HSV-1 strain 17+), (Cameron et al., 1988). Similar results were also obtained by Jacobson et al., (1989) using a mutant containing a deletion in RR1.

3.3.3. Ribonucleotide Reductase as a Target for Antiviral Compounds.

The HSV-encoded ribonucleotide reductase is an attractive target for antiviral compounds since it is important for ^{neuro}pathogenicity, it is biochemically distinct from the mammalian enzyme and an oligopeptide, which inhibits activity, has been identified (Dutia et al., 1986; Cohen et al., 1986a; McClements et al., 1988).

This peptide, YAGAVVNDL, corresponds to the C-terminal 9 amino acids of RR2 and inhibits the enzyme by blocking RR1/RR2 interaction. Exposure of infected monolayers to the peptide, however, does not reduce the level of infective virus, presumably because it is too large to enter the

cells. Enzymic activity can be restored by reassociation of the two subunits, both in vivo and in vitro, without the requirement for any other HSV proteins (Huang et al., 1988; Darling et al., 1988). It has also been demonstrated that the two subunits exist in a state of dynamic equilibrium between the associated and disassociated forms (Dr. A. J. Darling, personal communication). These observations will prove valuable in further characterization of the enzyme and in studies with antiviral compounds.

3.3.4. The Additional Amino-terminal Domain of HSV RRL.

Comparison of the HSV ribonucleotide reductase to its counterparts in eukaryotic and prokaryotic cells or in other viruses has identified an additional amino-terminal domain in the HSV RRL, comprising 320 amino acids, which produces a 50% increase in molecular weight (Nikas et al., 1986; Swain and Galloway, 1986). Proteolytic degradation studies have associated enzymic activity with the carboxyl two-thirds of HSV RRL and further work showed that the amino-terminal 140 amino acids were not required (Lankinen et al., 1989). This lead to the suggestion that the amino-terminal domain unique to the HSV protein is functionally distinct (Ingemarson and Lankinen, 1987). Consistent with this interpretation are the following observations. Firstly, fragments covering only the promoter and/or proximal one-third of the HSV-2 RRL coding region have transforming potential (Hayashi et al., 1985; Jariwalla et al., 1980, 1986; Jones et al., 1986). Secondly, the transforming potential of HSV-1 maps at a site distant from the ICP6_(UL39) gene (Camacho and Spear, 1978) and the HSV-unique amino-terminal domain of RRL shows only 38% intertypic homology as compared to 93% for the remaining two-thirds (Nikas et al., 1986). Finally, the expression of RRL and enzymic activity behave temporally as E genes but the promoter has cis-response elements associated with the regulation of IE gene expression which may be related to the postulated distinct function of the amino-terminus.

The amino-terminal domain of HSV RRL shows less homology than the rest of the protein (Nikas et al., 1986) and Chung et al. (1989) associated this domain of HSV-2 with a protein kinase activity, which could be related to its transforming ability. Recently the

amino-terminal domain has been shown to transform immortalized Syrian hamster embryo cells, as has full-length RR1, whereas the carboxy-terminus did not (Wymer et al., 1990).

3.3.5. The Effect of HSV Ribonucleotide Reductase Activity on the Mutation Frequency in Infected Cells.

As discussed previously (section A.2.2.5.), the lack of allosteric control shown by the HSV ribonucleotide reductase may have the effect of destabilizing the dNTP pools in infected cells. The importance of dNTP concentrations in mediating base substitutions and misincorporations has been stressed in studies of both prokaryotic (Weymouth and Loeb, 1978; Fersht, 1979; Hibner and Alberts, 1980) and eukaryotic (Kunkel et al., 1982) systems and the disruption of dNTP pools in infected cells may cause an increase in somatic mutations. This, in turn, could lead to transformation if, for example, a cellular oncogene was affected. Further evidence that mutant ribonucleotide reductase activity is capable of producing an increased mutation rate within the cell was gained by Caras et al. (1988) who showed that a mutant M1 subunit, deficient in allosteric control, raised the cellular mutation rate when expressed in cells containing a normally regulated M1 subunit. No difference in dNTP pool sizes was detected, however, and this may be because the mutator phenotype is mediated by small changes which are difficult to measure due to cellular compartmentalization. In the cell lines from which the mutant M1 was isolated, the pool sizes were disrupted (Ullman et al., 1980) so it is possible that the wild-type M1 is maintaining the dNTP pool balance in the transfected cells and the mutant M1 is only exerting a localized effect within the replication complex.

There are, however, flaws in this transformation model. For instance, the ribonucleotide reductase locus of HSV-1

does not appear to transform cells and this virus has not been associated with human cancer. Since both enzymes are similar, if one caused transformation by disturbing dNTP pools, then the other would be expected to have the same effect. However, transformation appears to be such a complicated process, involving numerous factors, that other virus functions may well be required. These factors might be specific to HSV-2.

Additionally, the minimal transforming regions of HSV-2 have been mapped to small DNA fragments one of which lies within UL39 and the other which lies outside UL40 (section A.2.2.3.). However, it has been suggested that two transformation mechanisms might exist, one involving the minimal transforming regions and the other involving the ribonucleotide reductase enzyme (R.J. Jariwalla, personal communication). One aim of this thesis is to study the effect of HSV-2 ribonucleotide reductase expression on mutagenesis as a step in the initiation of cellular transformation.

SECTION B: MATERIALS AND METHODS.1. MATERIALS.1.1. Cells.

Baby hamster kidney (BHK-C13) cells (MacPherson and Stoker, 1962) were used for growth and assay of virus. Human embryo kidney cells transformed by adenovirus type 5 (293 cells, Graham and Smiley, 1977), which encode mRNAs and proteins from the early region of the viral genome (Spector *et al.*, 1980), were used for transient expression assays and for the pZ189 mutagenesis assay. These cells were supplied by Jill Macfarlen. The use of 293 cells was suggested by Dr. M. Seidman (personal communication) as they give a low background mutation frequency in the pZ189 mutagenesis assay. Chinese hamster ovary (CHO) strain D422 cells (obtained from Dr. M. Meuth) which are hemizygous for the adenine phosphoribosyl transferase gene (aprt), (Bradley and Letavonec, 1982) were used for the aprt mutagenesis assay. Mouse fibroblasts (NIH3T3 cells, obtained from Dr. J. Lang) were used in the generation of stable cell lines.

1.2. Bacterial Cells.

E. coli K12 strain DH1 (recA1, F, endA1, gyrA96, thi-1, supE44, Hanahan, 1983) were used as the host in recombinant plasmid experiments. E. coli strain MBM7070 (F-, lacZamCA 7020, lacY1, hsdR-, hsdM+, ara-D139, (araABC-leu)7679, galU, galK, rpsL, thi, Casadaban and Cohen, 1980) which was provided by Dr. M. M. Seidman, was used for analysis of plasmids in the pZ189 mutagenesis assay.

1.3. Viruses.

HSV-2 wild type (wt) strain HG52, HSV-1 (wt) strain 17+ and temperature sensitive HSV-1 mutants tsK, ts1204, ts1222 and ts1207 were obtained from stocks maintained in the Institute by Mary Murphy. The HSV-1 deletion mutant d11403 (Stow and Stow, 1986) was supplied by E. Stow. The virus in1814 (Ace *et al.*, 1989) was obtained from Dr. C. Preston.

The L-particles (HSV-1 particles containing the envelope and tegument only) were obtained from Gordon McLean. They were obtained during a method of virion purification where virus pellets were layered onto a 5-15% Ficoll 400 gradient and banded (9000rpm/2 hours/4°C). The L-particles were present in a band located above the purified virion band,

1.4. Tissue Culture Media.

(i) BHK-C13 cells were grown in Glasgow Modified Eagle's medium (Busby et al., 1964) supplemented with 100 units/ml streptomycin, 100 units/ml penicillin, 0.002% (w:v) phenol red. The growth medium used (ETC10) consisted of 80% Eagles, 10% tryptose phosphate and 10% calf serum.

(ii) 293 and NIH3T3 cells were grown in Dulbecco's medium (DMEM, Flow laboratories), a modified version of Eagles medium supplemented with 20mM glutamine, 100 units/ml streptomycin and 100 units/ml penicillin. In addition to the above ingredients, growth medium contained 10% foetal calf serum.

(iii) CHO cells were grown in α -minimal essential medium (Gibco) supplemented with 10^{-5} M thymidine, 20mM glutamine, 100 units/ml streptomycin, 100 units/ml penicillin and 5% foetal calf serum.

(iv) For washing and removal of cell monolayers the following media were used:

(a) PBS, which contained 170mM NaCl, 3.4mM KCl, 10mM NaHPO_4 , 2mM KH_2PO_4 (pH 7.2).

(b) Versene, which consisted of PBS containing 0.6mM EDTA and 0.0015% (w:v) phenol red.

(c) Trypsin, which comprised 25% (w:v) Difco trypsin dissolved in Tris-saline. The constituents of Tris-saline were 140mM NaCl, 30mM KCl, 280mM Na_2HPO_4 , 1mg/ml dextrose, 25mM Tris HCl (pH 7.4), 0.005% (w:v) phenol red supplemented with 100 units/ml penicillin and 100 units/ml streptomycin.

1.5. Bacterial Culture Media.

Bacteria were propagated in L-broth which consisted of 177mM NaCl, 1% (w:v) Difco Bactotryptone and 0.5% (w:v) yeast extract (pH 7.5 prior to sterilization). Solid medium for the growth of colonies was agar containing 1.5% (w:v) agar in L-broth. These media were supplemented where appropriate with ampicillin or kanamycin (Sigma Chemical Company).

1.6. Enzymes.

The majority of restriction enzymes were obtained from New England Biolabs or Bethesda Research Laboratories (BRL). The Klenow fragment of E. coli DNA polymerase and the calf intestine phosphatase (CIP) were as supplied by Boehringer Corporation Limited and lysozyme, proteinase K, RNase A and DNase were supplied by Sigma Chemical Company.

1.7. Radiochemicals.

5[α -³²P] deoxynucleoside triphosphates were supplied by Amersham International PLC at a specific activity of 3,000 Ci/mMol.

1.8. Chemicals and Miscellaneous Reagents.

Most chemicals of analytical grade were supplied by BDH Chemicals or Sigma Chemical Company. Koch-Light laboratories supplied acrylamide, bisacrylamide, boric acid and caesium chloride, Biorad laboratories supplied ammonium persulphate (APS), TEMED (N,N,N,N-tetramethylethylene diamine) and the reagents for Western blotting (gelatin, horse radish peroxidase conjugate and protein A). The DNA sequencing primer (5'-3') was obtained from Pharmacia. Other oligonucleotides used for cloning purposes were constructed by Dr. J. McLauchlan using a Biosearch 8600 DNA synthesizer. The dNTP's were supplied by P-L Biochemicals. Tissue culture materials were supplied by Sterilin Limited and Nunc.

1.9. Antibodies.

Rabbit antiserum 20208 was raised against a peptide, RNSQFVALMPTA (RR1 positions 959-970), (Lankinen *et al.*, 1989). The antiserum 14995 was raised against a peptide, YAGAVVNDL (RR2 positions 332-340), (Telford, 1989). Both antisera were supplied by Dr. H. Lankinen.

1.10. Plasmids.

The constructs made during the course of this study used the plasmids pBam \underline{t} and pRR1 (obtained from Dr. I. Nikas) and pUC \underline{et}_2 (obtained from S. Simpson).

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

The plasmid p175 contains the coding sequences for Vmw175 and was provided by Dr. R. Everett.

The plasmid p61, which contains the neo gene was a gift from Dr. J. Lang.

1.11. Some Commonly used Buffers and Solutions.

STET:	50mM Tris HCl (pH 8.0), 50mM EDTA, 8% (w:v) sucrose, 5% (v:v) Triton X100.
TE:	10mM Tris HCl (pH 7.5), 1mM EDTA.
TBE:	90mM Tris, 90mM boric acid, 1mM EDTA (pH 9.3).
5xLB:	100mM Tris HCl (pH 7.5), 20mM MgCl $_2$, 20mM DTT.
SSC:	150mM NaCl, 15mM sodium citrate (pH 7.5).
100xDHB:	2% (w:v) Ficoll, 2% (w:v) polyvinylpyrrolidone, 2% (w:v) BSA, (Denhardt, 1966).
HBS:	140mM NaCl, 50mM HEPES (pH 7.12), 1.5mM Na $_2$ HPO $_4$.
ILB:	150mM NaCl, 1.5mM MgCl $_2$, 10mM EDTA, 10mM Tris HCl (pH 7.9), 1% (w:v) SDS.
EB:	100mM NH $_4$ Ac, 10mM MgAc, 0.1% SDS, 1mM EDTA.
TBS:	20mM Tris, 500mM NaCl (pH 7.5).
3xBM:	10% (v:v) spacing gel buffer, 5% (v:v) mercaptoethanol, 10% (v:v) glycerol, 2.25% (w:v) SDS, bromophenol blue to colour.
PEB:	7M urea, 350mM NaCl, 10mM EDTA, 10mM Tris (pH 7.9), 1% (w:v) SDS.
NTB:	0.05M Tris/HCl (pH 7.5), 0.01M MgSO $_4$, 0.01M DTT, 50ug/ml BSA.

2. METHODS.

2.1. Cell Culture.

Working stock 293, CHO, NIH3T3 and BHK cell cultures were kept in 750ml tissue culture flasks or rotating plastic 850ml culture bottles. For expression and mutagenesis experiments, cells were harvested using a method based on cell type. For CHO and BHK cells, the monolayer was washed once with 20ml versene and once with 20ml trypsin and the detached cells were then resuspended in growth medium. 293 cells, however, were washed twice with 20mls versene before detaching the cells by striking the flask sharply.

2.2 Virus Preparation.

BHK cells in 850ml roller bottles were infected with HSV at a moi of 0.003 pfu/cell (10 mls per bottle) in 40ml growth medium. Virus-infected cells were incubated at 31°C for 3-4 days until extensive cytopathic effect (CPE) had developed. Cells were then removed by shaking them into the medium, and pelleted (1500rpm/10minutes/4°C).

The supernatant medium was further centrifuged (12,000rpm/2 hours/4°C) to concentrate cell-released virus (CRV) which was sonicated and stored at -70°C. The pelleted material from the low-speed centrifugation was sonicated in a small volume of medium for recovery of cell-associated virus (CAV). Cell debris was then removed by further low-speed centrifugation, sonicated, re-centrifuged and re-sonicated before the supernatant containing the CAV was stored at -70°C.

The CRV and CAV fractions were checked for sterility using blood agar plates and titrated on BHK monolayers.

2.3. Virus Titration.

Virus samples were serially diluted in 10-fold dilutions to a final dilution of 10^{-9} . Duplicate plates of BHK cells were then infected with 100ul of 10^{-4} to 10^{-9} dilutions. The virus was left to a ^bsorb for 1 hour at 37°C before the

inoculum was removed and the plates were overlaid with methylcellulose medium (growth medium containing 1.5% methylcellulose) and incubated for 2-3 days at 37°C. Giemsa stain was then added to the methylcellulose medium, left for 1 hour and washed off. The plaques could then be counted and the virus titre established.

2.4. Virus Growth Curves.

Tissue culture plates (30mm) were seeded at a density of 5×10^5 BHK cells per plate and incubated overnight. The following day, the cells (1×10^6 /plate) were infected with 5 pfu/cell in a total volume of 100ul. The virus was left to adsorb for 1 hour at 37°C before the medium was removed. The cells were washed twice with medium (without serum) and overlaid with 1.5ml growth medium. Plates were harvested at appropriate times by scraping the cells into the medium with rubber policemen, dispensing into black capped vials, sonicating and storing at -70°C.

Each sample was then titrated on BHK monolayers.

2.5. UV-Inactivation of Virus.

Virus was inactivated by dispensing 400ul into 35mm tissue culture plates and placing 10-20cm underneath a UV lamp for 15 minutes. This reduces the virus titre by 10^5 and there is no production of IE gene products (Dr. C. Preston, personal communication). The method was suggested by Dr. C. Preston.

2.6. Standard DNA Cloning Techniques.

(i) Restriction Enzyme Digests.

Restriction enzyme digests were performed at 37°C unless recommended otherwise by the suppliers. Plasmid DNA and restriction enzyme were incubated for 2 hours using an enzyme specific reaction buffer. For diagnostic purposes, digests were generally carried out in 30ul of buffer for 2 hours using 1 unit of enzyme for every 0.5ug of plasmid DNA. For sub-cloning DNA fragments, the enzyme units and incubation time varied according to the size and amount of

the DNA fragment and whether or not the reaction was desired to reach completion.

ii) Ligation of DNA Fragments.

DNA fragments, produced by cleavage with restriction enzymes, were ligated to a cleaved plasmid vector which contained compatible "sticky ends". Vectors and fragments were mixed in ligation buffer (LB) at a ratio of 1:10 and incubated overnight at 15°C.

In order to prevent reannealing of the linearized vector, these were treated with 25 units of CIP (calf intestine phosphatase) during the restriction enzyme digest. All traces of CIP were removed by phenol/chloroform extraction, chloroform extraction and ethanol precipitation.

(iii) Isolation of DNA Fragments.

After restriction enzyme digestion of the starting plasmid and separation of the DNA fragments using a non-denaturing agarose or acrylamide gel, specific fragments could be isolated. In the case of agarose gels, a piece of NA 45 membranefilter (Schleicher and Schuell) was inserted into the gel directly in front of the fragment so that further electrophoresis caused the fragment to become bound to the paper. Incubation of the NA 45 filter at 68°C in TE, supplemented with 1M NaCl, for 1 hour released the bound fragment. Phenol/chloroform extraction, chloroform extraction and ethanol precipitation then purified the fragment before it was re-suspended in DW.

In the case of acrylamide gels, the fragment to be isolated was cut out of the gel and shaken overnight in elution buffer (EB). The solution was then phenol/chloroform extracted, chloroform extracted and ethanol precipitated before being resuspended in DW.

(iv) Filling In DNA "Sticky ends".

After isolation of the digested DNA fragment it was incubated for 60 minutes at 37°C in a solution of 0.04M dATP, 0.04MdCTP, 0.04M dGTP, 0.04M dTTP, 10mM Tris/HCl (pH 7.5), 60mM NaCl, 10mM MgCl₂, 100ug/ml BSA and 5 units T4 DNA polymerase. The fragment was then precipitated with ethanol and dissolved in DW.

(v) Adding Restriction Enzyme Linkers to DNA fragments.

Restriction enzyme linkers were ligated to blunt-ended DNA fragments. The resulting DNA was precipitated and dissolved in DW before being digested with an excess of the relevant enzyme in order to remove any multiple copies of the linker. The digested fragment was then isolated.

2.7. Large Scale Preparation of Plasmid DNA.

Fifty μ l of a bacterial glycerol stock (section B.2.8) was used to inoculate 8ml of L-broth supplemented with 100 μ g/ml of ampicillin and the culture was shaken overnight at 37°C. Half of the resulting culture was then diluted into 400mls of L-broth containing ampicillin and incubated with shaking for a further 6 hours. Then, plasmid DNA was amplified by the addition of chloramphenicol to a concentration of 50 μ g/ml and the culture was shaken overnight at 37°C.

Isolation of plasmid DNA was essentially performed using the method of Holmes and Quigley (1981). Bacteria were recovered by centrifugation (8,000rpm/10 minutes) and were then resuspended in 25ml of STET followed by the addition of 3ml of freshly prepared lysozyme (10mg/ml, Sigma Chemical Company). The suspension was brought to the boil using a bunsen burner and was then transferred to a boiling water bath for a further 40 seconds. After centrifugation, (17,000rpm/1 hour/4°C) the nucleic acid in the supernatant was precipitated by adding an equal volume of isopropanol and standing at RT for 20 minutes. The precipitate was then pelleted by centrifugation (3,000rpm/20 minutes/4°C). The pellet was resuspended in 10.5ml of DW, 11g of caesium chloride and 0.5ml of ethidium bromide (10mg/ml) were added, and the DNA was banded at 40,000rpm for 72 hours at 12°C. Two bands of DNA could then be seen, an upper chromosomal band and a lower supercoiled plasmid band. The latter was removed with a syringe.

The ethidium bromide was removed from the plasmid DNA by multiple extractions with water-saturated butan-2-ol. The DNA solution was then made up to a volume of 10ml with water

and was precipitated overnight at -20°C using 2.5 volumes of ethanol. This precipitate was then pelleted by centrifugation (3,000rpm/20 minutes/ 4°C) and resuspended in 1ml DW. The DNA was treated with 50ug/ml RNase A for 1 hour at 65°C and then with 50ug/ml proteinase K and 0.1% (w/v) SDS for 1 hour at 37°C . The DNA was phenol/chloroform extracted, chloroform extracted and precipitated with ethanol. After washing the pellet with 70% ethanol, the DNA was lyophilized and resuspended in DW to give a final concentration of around 1mg/ml. The DNA concentration was estimated by running the DNA on an agarose gel alongside plasmid standards of known concentration.

2.8. Small Scale Preparation of Plasmid DNA ("Miniprep.").

Two ml of L-broth supplemented with ampicillin (100ug/ml) were inoculated with single plasmid-transformed bacterial colonies and were shaken overnight at 37°C . Half of the bacterial culture was saved in order to make a glycerol stock of the plasmid-transformed bacteria by mixing the 1ml culture with 1ml of sterile glycerol and storing at -20°C . The remainder was poured into a 1.5ml reaction vial and centrifuged for 15 seconds in a microfuge. The bacterial pellet was resuspended in 100ul of STET and lysozyme (10mg/ml) and heated in a boiling water bath for 45 seconds before being centrifuged for 10 minutes in a microfuge. An equal volume of isopropanol was then added to the supernatant and precipitated nucleic acids were pelleted by centrifugation for 10 minutes, washed in 70% ethanol and lyophilized. Finally, the pellet was resuspended in 50ul of DW and stored at -20°C . When DNA prepared by this technique was digested with restriction enzymes, 0.2ul of RNase A (1mg/ml) was added to remove RNA thus facilitating band visualization following gel electrophoresis.

2.9. Transformation of E.coli.

(i) Preparation of Competent DH1 Bacteria.

A 10ml culture of DH1 bacteria was shaken overnight at 37°C . Then 2mls of this were transferred to 100ml of pre-warmed media and shaken at 37°C until the OD_{600}

reached 0.3. The culture was chilled on ice for 10 minutes and the bacteria were pelleted by centrifugation (3,000rpm/10 minutes/4°C). The pellet was then resuspended in 40ml of ice-cold 100mM CaCl₂ and incubated on ice for 40 minutes. The cells were pelleted by centrifugation as before and resuspended in a total of 2ml of ice-cold 100mM CaCl₂. The bacterial cells were then kept on ice for a minimum of 1 hour before use. Maximum efficiency of DNA uptake was reached after 3 days of incubation on ice and uptake still occurred for up to 5 days.

(ii) Preparation of Competent MBM7070 Bacteria.

A 10ml overnight culture of MBM7070 bacteria was grown in LM (L-broth plus 10mM MgSO₄ and 10mM MgCl₂). Then 1ml of this culture was transferred to 100mls of LM and shaken to OD₆₀₀ 0.45. The bacteria were then treated as for DH1 bacteria (see above) except that the bacterial pellets were resuspended in ice-cold 100mM CaCl₂ supplemented with 10mM DTT (dithiotreitol) and 10mM MES (2- [N-morpholino] ethanesulphonic acid).

(iii) Transformation.

For transformation, 1-10ul of DNA was added to 100ul of competent bacteria in a 1.5ml reaction vial and held on ice for 45 minutes. The bacteria were then incubated at 42°C for 2 minutes before being spread on L-broth agar plates often containing a selective agent (eg. ampicillin) to screen for bacteria which had taken up plasmid DNA. The L-broth agar plates were incubated overnight to allow colonies to form.

2.10. Transfection of DNA into Tissue Culture Cells.

(i) The Calcium Phosphate Method.

DNA transfections were performed by the calcium phosphate procedure as developed by Graham and van der Eb (1973). Cells were seeded at 30-40% confluence 18 hours prior to transfection. Then 10-20ug of DNA was dissolved in TE to a final volume of 420ul and 60ul of 2M CaCl₂ was added dropwise to the TE/DNA solution whilst vortexing. The DNA/CaCl₂ solution was then added dropwise whilst vortexing to 480ul of 2xHBS and left at RT for 30 minutes in order for

a precipitate to form. This suspension was then added dropwise to cell monolayers and swirled into the medium (10ml). The cells were incubated at 37°C overnight before the medium was replaced with fresh medium.

Unless otherwise stated this transfection method was used in all experiments.

(ii) Electroporation.

Cells were grown to 70% confluence, harvested and washed twice with electroporation medium (272mM sucrose, 7mM sodium phosphate pH 7.4, 1mM MgCl₂). They were then resuspended in the same medium at a concentration of 0.5 to 1.0x10⁸ cells/ml. Then, 0.8ml of cell suspension was placed in the Gene Pulsar cuvette (Bio-Rad) and between 10 and 40ug of linearized plasmid was added and thoroughly mixed. The cell suspension and plasmid were incubated for 10 minutes on ice prior to electroporation. The cuvette containing cells and plasmid was then placed in the Gene Pulsar chamber, pulsed once with 400 volts and 25uFD capacitor and then returned to ice for a further 10 minutes. The cells were then plated and allowed 48 hours for recovery before selective medium was added. Cell survival was checked by plating 100 cells onto duplicate plates and seeing how many formed colonies. About 50% survival is optimum in electroporation experiments.

2.11. Electrophoresis.

(i) Minigels.

Analysis of small amounts of DNA utilized horizontal (100mmx70mmx7mm), 0.5% agarose, 20ml gels in TBE buffer plus ethidium bromide (0.5ug/ml). DNA samples were applied to the gel slots in 1xTBE, 10% (w/v) sucrose, 0.02% (w/v) bromophenol blue and xylene cyanol in a final volume of 10ul. The gel was run in TBE buffer at a constant current, 50mA for 1 hour. Following electrophoresis, the DNA was visualized by exposure to UV-light and photographed on Polaroid 667 film.

(ii) Large Agarose Gels.

Analysis and preparation of DNA restriction fragments utilized horizontal (260mmx160mmx5mm), 1% agarose, 250ml

gels in TBE buffer plus ethidium bromide (0.5ug/ml). The samples were applied as for minigels in a total volume of 30-60ul and the gel was run at 40mA for 16-18 hours or at a maximum voltage of 12V/cm. For gels which were to be used for Southern transfer of DNA 0.7% agarose gels were used.

(iii) Non-denaturing Polyacrylamide Gels.

These gels were prepared as described by Maniatis et al. (1975) and were used for analysis of DNA fragments of less than 1kb. Acrylamide stock of 30% (w/v) acrylamide (acrylamide:bisacrylamide 29:1, stored at 4°C) was diluted to 5% in 75ml of TBE. To this, 0.5ml of 10% (w/v) APS and 50ul of TEMED were added to initiate polymerization. The mixture was poured quickly into a vertical gel kit (260mmx160mmx1mm) and allowed to stand for at least 45 minutes prior to electrophoresis. Samples were prepared and loaded as for agarose gels and the gel was run at a maximum voltage of 16V/cm. The gel was stained with ethidium bromide (4ug/ml) and then the DNA was visualized as for agarose gels.

(iv) SDS Polyacrylamide Gels.

Proteins were separated on vertical 5-12.5% gradient polyacrylamide gels in the presence of 0.1% (w:v) SDS using the continuous buffer system of Laemmli (1970) modified by Marsden et al. (1976). The ratio of acrylamide to bisacrylamide was 19:1. The composition of the separating gel is shown in Table 2. The buffer used for protein separation was resolving gel buffer (1.5M Tris/HCl pH 8.9, 0.4% SDS). Proteins were loaded onto the gel via a 5% polyacrylamide stacking gel the composition of which is shown in Table 3. The buffer for stacking gels was 0.5M Tris pH6.7, 0.4% SDS.

2.12. RNA Extraction.

(i) Extraction using ILB.

During this process the cells were kept at 4°C as much as possible throughout the procedure until the phenol chloroform extraction step.

Cell monolayers were washed with PBS before 4ml of PBS

TABLE 2: SEPARATING GEL COMPOSITION.

Gel concentration	5%	12.5%
Acrylamide (30%) ml	6	15
Resolving gel Buffer ml	9	9
DW ml	21	6.6
Glycerol ml	0	5.4
10% APS ul	225	97.5
TEMED ul	15	15

TABLE 3: STACKING GEL COMPOSITION.

Acrylamide (30%) ml	4
Stacking gel Buffer ml	6
DW ml	14
10% APS ul	200
TEMED ul	10

Tables 2 and 3. The composition of separating and stacking gels used for protein separation in SDS-polyacrylamide gel electrophoresis.

was added to each plate and the cells scraped off using rubber-policemen. The cells were then centrifuged (2,000rpm/40seconds/4°C) and resuspended in fresh PBS before being re-centrifuged under similar conditions. The pellet was then resuspended in 0.3mls of isotonic lysis buffer (ILB), shaken gently and incubated on ice for 2 minutes. Nuclei were then pelleted by centrifugation (3,000rpm/5 minutes/4°C) and the supernatant was transferred to a 1.5ml reaction vial containing 0.3ml phenol extraction buffer (PEB) and mixed thoroughly. Three phenol/chloroform extractions were then carried out followed by a chloroform extraction. RNA was then precipitated twice with ethanol, washed in 70% ethanol, lyophilized and resuspended in DW. At this stage, the RNA was treated with DNase to remove any traces of DNA in the sample. This involved incubating the sample in 40mM Tris/HCl (pH 7.5) and 6mM MgCl₂ for 30minutes at 37°C. The RNA was then phenol/chloroform and chloroform extracted, precipitated, washed and lyophilized as before.

(ii) Extraction using the RNAzol method (Biogenesis).

RNA was extracted from animal tissue using this method. Tissues were first homogenized in RNAzol (2ml per 100mg tissue). Then, chloroform was added and the samples were shaken vigorously and incubated on ice, for 10 minutes, before being centrifuged (12,000rpm/15 minutes/4°C). The upper aqueous phase (containing the RNA) was transferred to a fresh tube and the RNA was precipitated with an equal volume of isopropanol for 45 minutes at -20°C. The samples were then centrifuged as before to pellet the RNA which was washed twice with 75% ethanol by vortexing and subsequent centrifugation (12,000rpm/8 minutes/4°C). The pellet was then dried and dissolved in 0.5% SDS.

The concentration of the RNA from both extraction methods was determined by spectrophotometry (OD₂₆₀ 1.0=40ug RNA).

In order to probe the RNA for specific sequences, 5ug was transferred to a nitrocellulose membrane by adding it to 150ul of 20xSSC and loading onto a well in a slot blot apparatus (Schleicher and Schuell). The resulting membrane was then air-dried and baked under vacuum at 80°C for 2 hours.

2.13. RNA:DNA Hybridization.

The nitrocellulose membrane from the slot blot apparatus or from the Northern blot was pre-hybridized and hybridized as for Southern transfer hybridizations (section B.2.17.). Following hybridization, however, the filter was washed for 4x30 minutes in 0.1xSSC and 0.1% SDS before being air-dried and autoradiographed.

2.14. Extraction of DNA.

Minced tissue or pelleted cells were put in a solution of 1% (w:v) SDS, 50mM Tris and 50mM EDTA to a total volume of 4ml. Then, 100ug of proteinase K was added and the tissues were shaken overnight at 37°C. The following day the samples were extracted twice with phenol/chloroform and twice with chloroform before the DNA was precipitated at RT with ethanol. The high molecular weight DNA precipitated as soon as the ethanol had been added and this was picked out with a pasteur pipette, washed in 75% ethanol, dried briefly in air and dissolved overnight in DW.

2.15. Southern Transfer of DNA.

This technique has been described by Southern (1975). The DNA sample was electrophoresed on an agarose gel which was then denatured (1.5M NaCl, 0.5M NaOH) for 1 hour at RT with gentle shaking. The gel was then neutralized (1M Tris/HCl pH.8, 1.5M NaCl) for 1 hour as above. A strip of Whatman 3MM paper was wrapped around a stack of gel plates and placed in a tray containing 10xSSC. The gel was placed upside-down on the Whatman 3MM paper and covered with a sheet of pre-wetted nitrocellulose membrane (Schleicher and Schuell). All air bubbles were removed from between the gel and the membrane. The nitrocellulose was then covered by two sheets of Whatman 3MM paper and a stack of paper towels (5-8cm high). The stack was completed with a glass plate and a weight (500g). DNA was transferred overnight and the membrane was then washed in 6xSSC, dried in air and baked under vacuum for 2 hours at 80°C.

2.16. Hybridization of Southern Filters (DNA:DNA Hybridization).

The baked membrane was soaked in 6xSSC and placed in a heat-sealable bag with pre-hybridization solution. Roughly 0.2ml of pre-hybridization fluid was added for each cm² of membrane. The bag was then sealed and incubated at 42°C for 2 hours with gentle shaking. The pre-hybridization fluid was then replaced with hybridization fluid and the membrane was incubated overnight at 42°C again with gentle shaking. When hybridization was complete the nitrocellulose membrane was washed in a series of solutions. Firstly it was placed in 2xSSC and 0.5% SDS at RT for 5 minutes, then 2xSSC and 0.1% SDS at RT for 15 minutes and finally it was washed twice (2 hours followed by 30 minutes) in a solution of 0.1xSSC and 0.5% SDS at 68°C. Throughout all the washes the membrane was shaken gently. After washing the membrane was air-dried and autoradiographed.

2.17. Radioactive Labelling of DNA Probes.

(i) Nick Translation.

³²P-labelled DNA was prepared essentially by the method described by Rigby et al. (1977). A 1.5ml reaction vial was set up containing 0.25ug DNA in 1x nick translation buffer (NTB), 0.1% BSA, 20nM unlabelled dNTP's (usually dATP and dCTP), 50uCi of [α -³²P]dNTP's, 0.1ug/ml DNase I and 1 unit of E. coli DNA polymerase I. This was incubated at 16°C for 60 minutes after which time the reaction was stopped by the addition of EDTA to a concentration of 0.02M. Unincorporated [α -³²P]dNTP's were then removed through a Sephadex G-50 column.

(ii) Oligolabelling with ³²P.

DNA was also labelled using an oligolabelling kit (Pharmacia). Denatured DNA (50ng) was added to a solution of 130mM KPO₄ (pH 7.7), 6.5mM MgCl₂, 1mM DTT, 32ug/ml BSA and mixed sequence hexanucleotides, 50uCi [α -³²P]dCTP _{α} and 5 ^{20nM unlabelled dUTPs} units of Klenow fragment and incubated at 37°C for 60 minutes. It was not necessary to remove unincorporated

dNTP's due to the high level of efficiency of nucleotide incorporation.

2.18. Protein Extraction and Western Blotting.

Cells were harvested by removing the medium, washing the cell surface with PBS and adding boiling mix (BM, 0.5ml/50mm plate). The cells were then left for 10 minutes before the boiling mix, containing cells, was transferred to a black capped vial, incubated in a boiling water bath for 3 minutes and stored at -70°C . The proteins were separated on an SDS-polyacrylamide gradient gel as described by Marsden et al. (1978).

Once electrophoresis was complete the proteins were transferred to a nitrocellulose membrane using a Bio-Rad blotting apparatus and blotting in 25mM Tris, 192mM glycine and 20% (v:v) methanol for 3 hours at 250mA. After blotting, the free protein binding sites in the nitrocellulose membrane were blocked by incubating twice for 30 minutes at 37°C in TBS plus 3% gelatin with gentle agitation. All subsequent washings and incubations were carried out with such agitation. The membranes were washed twice for 5 minutes at RT in TTBS (TBS plus 0.05% Tween 20) before being incubated with antisera in TTBS plus 1% gelatin for 1-2 hours at 37°C . Three washes in TTBS for 15 minutes at room temperature were then performed followed by incubation of the membrane with goat-antirabbit HRP conjugate diluted 1/3000 in TTBS plus 1% gelatin for 1 hour at RT. After washing the membrane again for 2x5 minutes in TTBS and rinsing twice in TBS, it was subjected to the HRP colour development reactions.

Sixty mg of HRP colour reagent in 20ml ice-cold methanol were mixed with 60ul ice-cold 30% $^{\text{H}_2\text{O}_2}$ and 100ml TBS and poured into a light-proof box containing the nitrocellulose membrane. This was then left until the protein bands were visualized.

2.19. Preparation of Partially Purified Extracts for HSV Ribonucleotide Reductase Assays.

Cells were infected with 5-10 pfu/cell for 7 hours at

37°C, harvested by scraping into the medium and pelleted by centrifugation (1,000rpm/5 minutes/4°C). They were then washed twice with PBS by resuspension and centrifugation and the final pellet was drained and the walls of the tube were dried. The cell pellet was resuspended in 50mM Hepes (pH 7.8) and 2mM DTT (Hepes/DTT) and frozen at -70°C (the amount of Hepes/DTT used to resuspend the cell pellet was equivalent to 150ul/90mm plate). The pellet was thawed, sonicated and centrifuged (7,500rpm/15 minutes/4°C).

Then, 0.3ml of 5% streptomycin sulphate in Hepes/DTT was added per ml of supernatant in a dropwise manner, with constant shaking, and the solution was stirred for a further 20 minutes on ice before the nucleic acids were pelleted by centrifugation (7,500rpm/15 minutes/4°C). Then, 0.82ml of saturated ammonium sulphate in Hepes/DTT was added (dropwise with constant stirring) per ml of extract and stirred on ice for 20 minutes. The extract was centrifuged (7,500/15 minutes/4°C) and the pellet was taken up in a small volume of Hepes/DTT and de-salted on a PD 10 G25 column. Fractions (0.5ml) were collected and the protein concentration was determined using Bradford assays.

2.20. Estimation of Protein Concentration.

Estimation of protein concentration was performed using the method of Bradford (1976). Ten ul of protein sample were diluted in 90ul of DW and mixed with 1ml of Bradfords reagent (0.01% Coomassie brilliant blue G, 0.0003% (w:v) SDS, 4.75% (v:v) ethanol, 8.5% (v:v) phosphoric acid) and left at RT for 10 minutes. The absorbance of the solution was then measured, at 595nm, and converted to mg of protein by comparison to a standard curve produced using known quantities of BSA.

2.21. Ribonucleotide Reductase Assays.

Reaction vials (1.5ml) containing 300ug of partially purified extract, 20ul of "special" mixture (161mM Hepes, pH8.2, 10mM DTT, 100uM CDP and 1uCi CDP) and Hepes/DTT to a total volume of 90ul were set up on ice. The solutions were mixed well and incubated at 37°C for 1/2 hour. This allows

the substrate CDP to be converted to dCDP. The samples were then boiled for 2 minutes, cooled on ice and 2mg of Crotalux adamanteus venom and $MgCl_2$ (to a concentration of 15mM) were added before further incubation for 2 hours at 37°C. This converts the dCDP reaction product to deoxycytosine (dC) which can be analyzed on the HPLC. The extracts were boiled again for 2 minutes, cooled and dC was added to a concentration of 0.1M. The tubes were then mixed and centrifuged 5 minutes before the supernatant was removed and loaded onto the HPLC.

2.22. The pZ189 Mutagenesis Assay.

The plasmid pZ189 (Seidman et al., 1985, Figure 8) carries a bacterial tyrosine suppressor tRNA gene (supF) as a mutational target and sequences that allow replication in both bacterial and mammalian cells. Subconfluent plates of 293 cells were transfected with pZ189 in combination with other plasmid DNAs or viruses using the calcium phosphate transfection method. After the transfection medium had been replaced, the cells were incubated for a 48 hour replication period, in the case of transfected cells, or 12-24 hours in the case of virus infected cells. After this period, the progeny plasmids were extracted using the Hirt extraction procedure. The plasmids were then treated with DpnI to remove any unreplicated forms. The enzyme DpnI only cuts DNA that has been methylated by the E. coli dam methylase (Hattman et al., 1978) and so all plasmids which had undergone a replication cycle in a eukaryotic system would escape digestion whereas the incoming DNA would be cleaved. MBM7070 bacteria were then transformed with the plasmids. This strain of bacteria contains a suppressible (amber) mutation in the β -galactosidase gene. Colonies containing plasmids which have a mutated supF gene are white when grown on L-broth agar plates supplemented with 20mg/ml 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (x-gal) and isopropylthio- β -D-galactoside (IPTG) ^{and ampicillin} in contrast to the blue colonies that harbour the wild type plasmid. The white colonies were replated in order to confirm their mutant phenotype. In some cases, the plasmid DNAs were sequenced. The percentage frequency of mutagenesis was calculated by

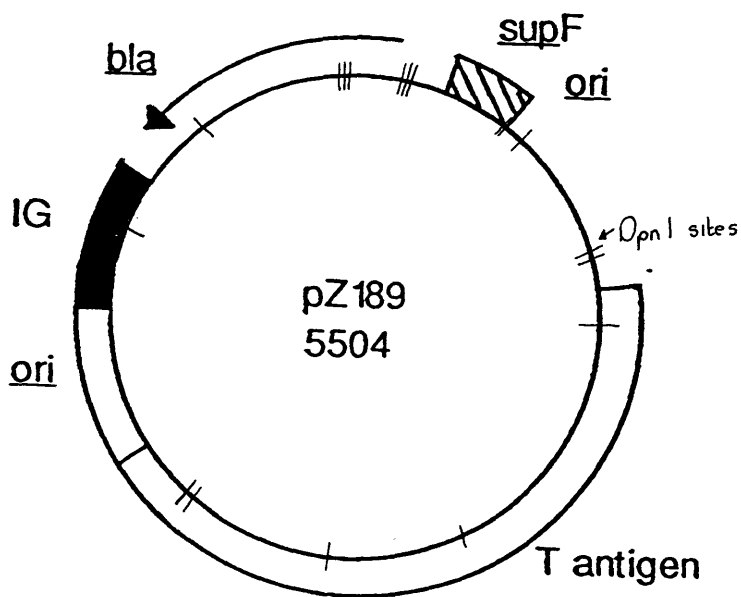


Figure 8. The plasmid pZ189 contains an ampicillin resistance gene (*bla*, →), the pBR327 origin, the origin and T antigen coding region from SV40 (double line), the tyrosine suppressor tRNA gene *supF* (hatched box) and the IG region from M13 (solid box).

the following equation:

$$\text{mutation frequency} = \frac{\text{no. white colonies} \times 100}{\text{total no. colonies}}$$

2.23. Hirt Extraction Procedure.

Medium was removed from the cells, replaced with Hirt extraction buffer (0.5% (w:v) SDS, 0.01M EDTA, pH7.5), (Hirt, 1967) and incubated at RT for 15-20 minutes. The viscous lysate was then scraped from the plates using a rubber policeman and transferred to a 1.5ml reaction vial. Then, 5M NaCl was added to a final concentration of 1M and the solution was mixed gently by inversion. The mixture was stored for a minimum of 8 hours at 4°C before being centrifuged (17,000rpm/30 minutes/4°C) to pellet the genomic DNA. RNase A was then added to the supernatant, which contains the low molecular weight plasmid DNA, at a concentration of 100ug/ml and incubated at 37°C for 3 hours. It was then phenol/chloroform extracted three times, chloroform extracted once and the DNA was precipitated with ethanol. The precipitate was washed with 70% ethanol, lyophilized and resuspended in 30ul DW.

2.24. The aprt Mutagenesis Assay.

Cultures of CHOD422 cells were mutagenized and then grown in non-selective medium for 6 days to allow expression of the aprt- phenotype. Mutagenesis in the case of this study involved transfection of the cells with plasmids designed to express HSV-2 ribonucleotide reductase. The cells were plated in medium containing 0.04mM 8-azaadenine (AA) and were incubated at 37°C for 2-3 weeks in order for the AA-resistant mutants (aprt-) to form colonies. The percentage frequency of mutagenesis was calculated by the equation:

$$\text{mutation frequency} = \frac{\text{mutant colonies} \times 100}{\text{total cells plated} \times \text{plating efficiency.}}$$

The plating efficiency was obtained by plating 100 cells in two, 50mm tissue culture plates and determining how many

proceeded to grow and form colonies.

2.25. Mutagenesis of Plasmid DNA by Hydroxylamine.

Five ug of plasmid DNA was added to a solution of 0.4M hydroxylamine, 1mM EDTA and 50mM sodium pyrophosphate and incubated at 75°C for 60 minutes. The DNA was then purified through a spin-x/G25 column (Costar).

2.26. Sequencing.

(i) Labelling the 5'-terminus of DNA restriction fragments.

The restriction fragment to be labelled was incubated with 1x bacteriophage T4 polynucleotide kinase buffer (0.5M Tris/HCl pH 7.6, 0.1M MgCl₂, 50mM dithiothreitol, 1mM spermidine and 1mM EDTA pH 8.0), 50pmoles of [γ -³²P]ATP, and 10-20 units of bacteriophage T4 polynucleotide kinase, for 30 minutes at 37°C. The reaction was then stopped with 2ul of 0.5M EDTA (pH 8.0) and the solution was extracted with phenol chloroform before being precipitated with ethanol. The DNA was then dissolved in 50ul of DW and the labelled DNA was separated from the unincorporated nucleotides using a sephadex G50 column.

(ii) Sequencing Reactions.

The nucleotide sequences of 5'-end labelled DNA fragments were determined by the methods of Maxam and Gilbert (1977, 1980). These methods are based on the specific modification of DNA bases followed by their removal and cleavage of the DNA backbone.

In outline, bases are chemically modified under conditions which confer base modification specificity. This leads to ring opening and in some cases, base elimination. Guanine residues are modified at 20 C by dimethyl sulphate (DMS) in a sodium cacodylate buffer (pH 8), adenosine and guanosine residues are modified by incubation at 37°C with piperidine formate (pH 2). Thymine and cytosine residues are modified by hydrazine at 20°C with the cytosine specific modification achieved by suppressing the modification of thymidine with the addition of NaCl to 1.5M.

Reactions with DMS and hydrazine were stopped and the

DNA precipitated by the addition of sodium acetate solution containing tRNA and ethanol. Reactions with piperidine formate were stopped by freezing at -70°C and the piperidine formate was removed by two rounds of lyophilization.

Incubation of chemically modified DNA with 1M piperidine at 90°C causes cleavage of the DNA backbone at the site of modification. Piperidine is removed by three rounds of lyophilization.

2.27 Generation of Transgenic Mice.

Transgenic mice were established essentially by the method of Hogan et al. (1986). C57xCBA hybrid female mice were hormonally super-ovulated with pregnant mares serum (51 units) and human chorionic gonadotrophin (2.5 units). They were then mated with C57xCBA hybrid males and fertilized 1-cell eggs were collected from the oviduct using a fine glass tube (120-180um diameter). Cumulus cells surrounding the eggs were removed with hyaluronidase (300 units/ml) in modified Brinsters medium (Brinster, 1972). The cells were then transferred to Brinsters medium modified by substituting the Hepes buffer (pH 7.4) with bicarbonate buffer. Then, 1-2pl of a 1-10ug/ml DNA solution consisting of DNA dissolved in 5-10mM Tris (pH7.4) and 0.1-0.2mM EDTA was injected into one of the pronuclei of a fertilized egg using an automated microinjecting system.

Around twenty eggs were transferred to both oviducts of a pseudo-pregnant female mouse (the mouse was termed pseudo-pregnant after having been mated to a sterile male). About 1 in 3 eggs survive the microinjection procedure so transferring this number of eggs should result in 7-8 offspring which is the optimum number for a mouse litter.

Mice were screened for the presence of integrated sequences by preparing DNA from the end 1cm of the tail and hybridizing this to nick-translated DNA probes using a slot blot apparatus or the Southern blotting technique.

2.28. Statistical Analysis of Data.

The probability of a mutation occurring in the marker genes of the mutagenesis assays used was assumed to be a random event, hence the data for mutation of the genes was

determined by the Poisson distribution (Mather, 1949). The Poisson distribution or "law of small probabilities" is broadly defined as a set of results that fits into the categories defined below;

- (i) The data consists of whole number observations, ie. counts.
- (ii) Each event is a small fraction of the total number at risk, ie. the event is rare.
- (iii) The events are independent of each other and occur randomly.

If a Poisson type of event has occurred r_1 and r_2 times there is an approximate significance test which gives values v such that if the smaller of r_1 and r_2 is less than or equal to v the null hypothesis that the two results are equal should be rejected.

For example; Table 8:

Mock-infected (31°C):	4 white colonies,	8912 total
HSV-1 (31°C):	17 white colonies,	5101 total

1. Standardize results ie:

Mock-infected (31°C):	4 white colonies,	8912 total
HSV-1 (31°C):	30 white colonies,	8912 total

2. $r_1=4$

$r_2=30$

$r_1+r_2=34$ $v=9$ (0.01 level of significance)

3. As r_1 is less than v the results are significantly different.

SECTION C: RESULTS.

This section outlines the procedures used and documents the results for the experiments performed. Details of the materials and methods used can be obtained from SECTION B: MATERIALS AND METHODS. The results are also discussed at the end of each set of experiments.

1. PLASMID CONSTRUCTION AND POLYPEPTIDE EXPRESSION.

An important goal of the work discussed here was to examine the role of HSV-2 ribonucleotide reductase expression on the increased somatic mutation rate seen in HSV-infected cells. One approach involved the introduction of plasmids, designed to express HSV-2 ribonucleotide reductase, into cells which were then assayed for the mutation rate of a marker gene.

Plasmids were constructed to express RR1 and RR2 in cultured cells under the control of the inducible mouse metallothionein (MT-1) promoter, such that their effect could be assessed under expressing and non-expressing conditions.

1.1. The MT-1 Promoter.

The MT-1 promoter responds at the transcriptional level to heavy metals such as zinc and cadmium (Durnam and Palmiter, 1981), to glucocorticoid hormones (Hagar and Palmiter, 1981), to an unidentified factor in the acute-phase response (Durnam et al., 1984) and to interferon (Friedman et al., 1984). The promoter contains 5 metal regulatory elements (MREs) situated upstream from the TATA box (Stuart et al., 1984, 1985). A consensus MRE sequence has been formulated from a number of metallothionein promoters including mouse, man, sheep and Drosophila and this consists of the 15bp sequence 5'-CTNTGCRCNCGGCC-3'. Several metallothionein genes have been cloned and shown to retain their expression and regulation after transfection into heterologous cells (Durnam et al., 1984; Searle et al., 1984; Hamer and Walling, 1982). The MT-1 promoter has also been shown to confer metal, steroid and/or inflammatory

response to a variety of structural genes that were transferred into cells or animals (Pavlakakis and Hamer, 1983; Brinster et al., 1982; Palmiter et al., 1982, 1983; Karin et al., 1984a,b; Richards et al., 1984). The MT-1 promoter was thus chosen to confer heavy metal inducibility to the HSV-2 reductase genes.

1.2. Construction of pCRR2.

The plasmid pCRR2 (Figure 9) contains the sequences encoding the small subunit of HSV-2 RR2 under the control of the MT-1 promoter. The starting plasmid, pUC_t (Figure 9a) contains the coding sequences of HSV-2 RR2 (Figure 7) on a BamHI/XhoI fragment. In order to clone the MT-1 promoter into this construct, an oligonucleotide was constructed which had BamHI "sticky ends" and internal KpnI and BglII restriction enzyme sites (Figure 9a). The MT-1 promoter could then be inserted on a KpnI/BglII fragment isolated from the plasmid pEE3.8_i (kindly provided by Dr. P. Searle). The oligonucleotide was constructed such that the translational ATG start codon, which was cut, by BamHI, in isolating the BamHI/XhoI subfragment, was recreated (Figure 9a). A detailed diagram of the sequences cloned in pCRR2 is shown in Figure 9b.

1.3. Construction of pCRR1.

The plasmid pCRR1 contains the coding sequences for HSV-2 RR1 under the control of the MT-1 promoter. The starting plasmid, pUCet₂ (Figure 10a) contains the sequences for RR1 on an SstI/BamHI fragment (Figure 7). Polyadenylation sequences from the HSV-2 IE gene 5 had been cloned into pUCet₂ downstream of the RR1 sequences as these were not present in the SstI/BamHI fragment.

As with pCRR2, an oligonucleotide was constructed (Figure 10a), this time with BstEII "sticky ends", containing internal KpnI and BglII restriction enzyme sites such that the MT-1 promoter could again be inserted on a KpnI/BglII fragment. The oligonucleotide was inserted into the BstEII site 247 nucleotides upstream of the translation start site (Figure 10b), however, as the SstI/BamHI

RR1-containing fragment contains 2 BstEII sites, linear molecules were first isolated after partial digestion with BstEII. The oligonucleotide was then ligated into the linearized plasmids and resultant plasmids containing the oligonucleotide inserted into the correct site were determined and isolated. The oligonucleotide was designed to take advantage of the redundant recognition sequence of BstEII by having "sticky ends" complementary to the correct insertion site. BstEII recognizes the heptanucleotide sequence GGTNACC and in the correctly situated BstEII site N=T whereas in the incorrect site N=G.

The MT-1 promoter was again ligated into the KpnI/BglII sites of the oligonucleotide, however, there is an additional BglII site elsewhere in the RR1 coding sequences and so linear plasmid molecules again had to be isolated after partial digestion with BglII before cutting with KpnI and ligating in the promoter. Restriction enzyme digests were performed on the resultant plasmids so that the ones that contained the promoter in the correct site could be isolated.

A detailed diagram of the sequences cloned in pCRR1 is shown in Figure 10b.

1.4. Construction of pCRR3.

This plasmid contains the coding sequences for both RR1 and RR2 (Figure 11). RR1 expression is under the control of the MT-1 promoter whereas the expression of RR2 is under the control of its own HSV promoter.

The starting plasmid, pRR1, (Figure 11) contains the HSV-2 ribonucleotide reductase coding sequences on a SstI/XhoI fragment (Figure 7). The MT-1 promoter was cloned into the BstEII site upstream from the RR1 coding sequences in an identical manner to pCRR1.

1.5. Detection of HSV-2 Ribonucleotide Reductase RNA in Cells Transfected with pCRR1, pCRR2 and pCRR3.

Subconfluent, 50mm plates of 293 cells were transfected with pCRR1, pCRR2, pCRR3 and pUC13 such that 2 plates were transfected with each plasmid. The transfection mixture was

Figure 9. (a) The construction of pCRR2. The starting plasmid, pUCt (obtained from Dr. I. Nikas), contains the coding sequences for HSV-2 RR2 (open box) present on a BamHI/XhoI fragment (Figure 7). This was digested with BamHI and an oligonucleotide containing internal KpnI/BglII sites was ligated into the plasmid. The oligonucleotide contains an ATG (underlined) to replace the one that was lost during BamHI/XhoI digestion in the isolation of the BamHI/XhoI fragment (Figure 7).

The resulting plasmid was then digested with KpnI and BglII, and the MT-1 promoter (solid box), present on a KpnI/BglII fragment isolated from the plasmid pEE3.8i (obtained from Dr. P. F. Searle), was ligated into this.

The location and orientation of the ampicillin resistance gene (amp^R) are indicated.

Construction of pCRR2.

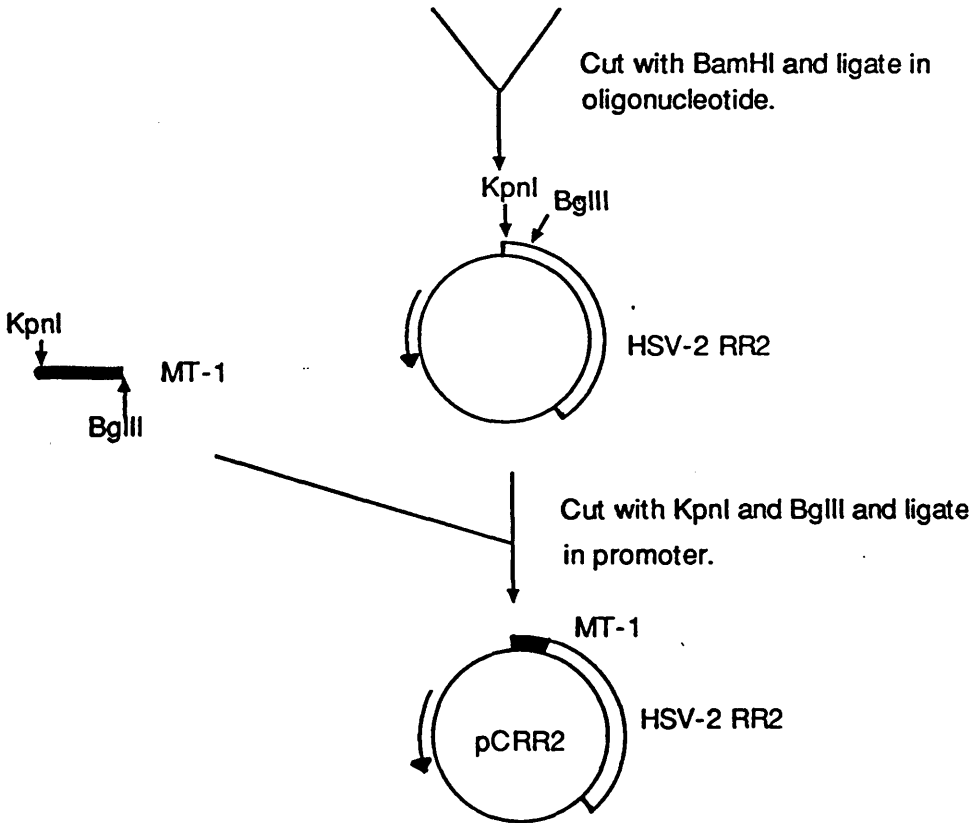
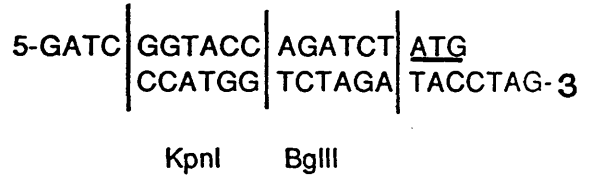
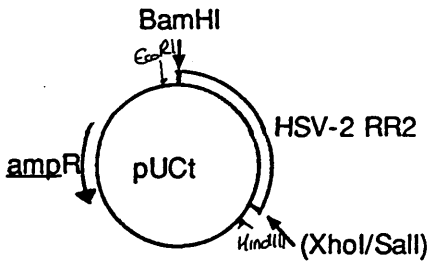


Figure 9 (b). Diagram of the sequences present in pCRR2. The MT-1 promoter is shown as a shaded box and the TATA box and cap site are indicated as are the positions of the MREs (metal regulatory elements, single lines above the promoter sequences). The HSV-2 RR2 sequences are shown as an open box and the ATG and TGA codons are marked. The ATG is provided by an oligonucleotide (Figure 9a).

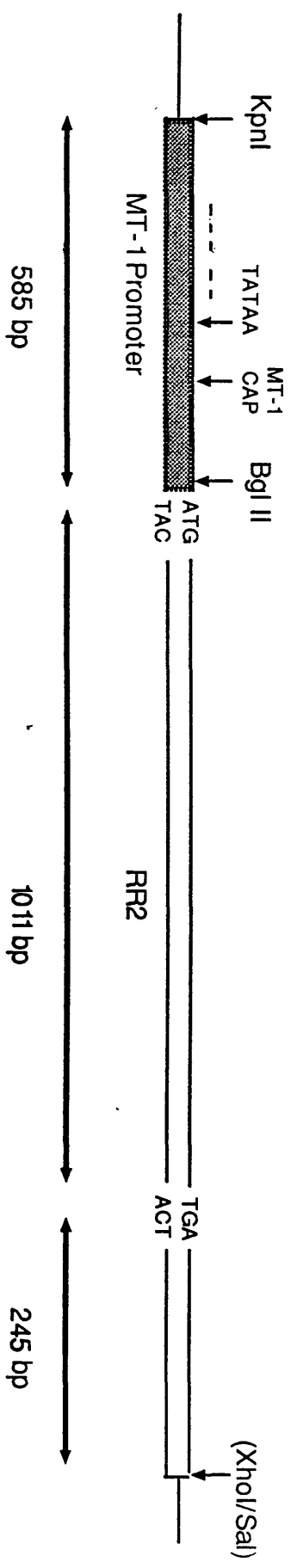


Figure 10. (a) The construction of pCRR1. The starting plasmid pUCet₂ (obtained from S. Simpson) contains the coding sequences for RR1 (open box) on an SstI/BamHI fragment (Figure 7) and the poly(A) sequences from HSV-2 IE gene 5 (hatched box). This plasmid was partially digested with BstEII and an oligonucleotide containing internal KpnI/BglII sites was ligated into the isolated linear molecules. The resultant plasmid, with the oligonucleotide inserted into the correct site, was partially digested with BglII and again linear molecules were isolated. The MT-1 promoter (solid box), located on a KpnI/BglII fragment isolated from the plasmid pEE3.8i (obtained from Dr. P. F. Searle), was ligated into the linear molecules.

The location and orientation of the ampicillin resistance gene (amp^R) are indicated.

Construction of pCRR1.

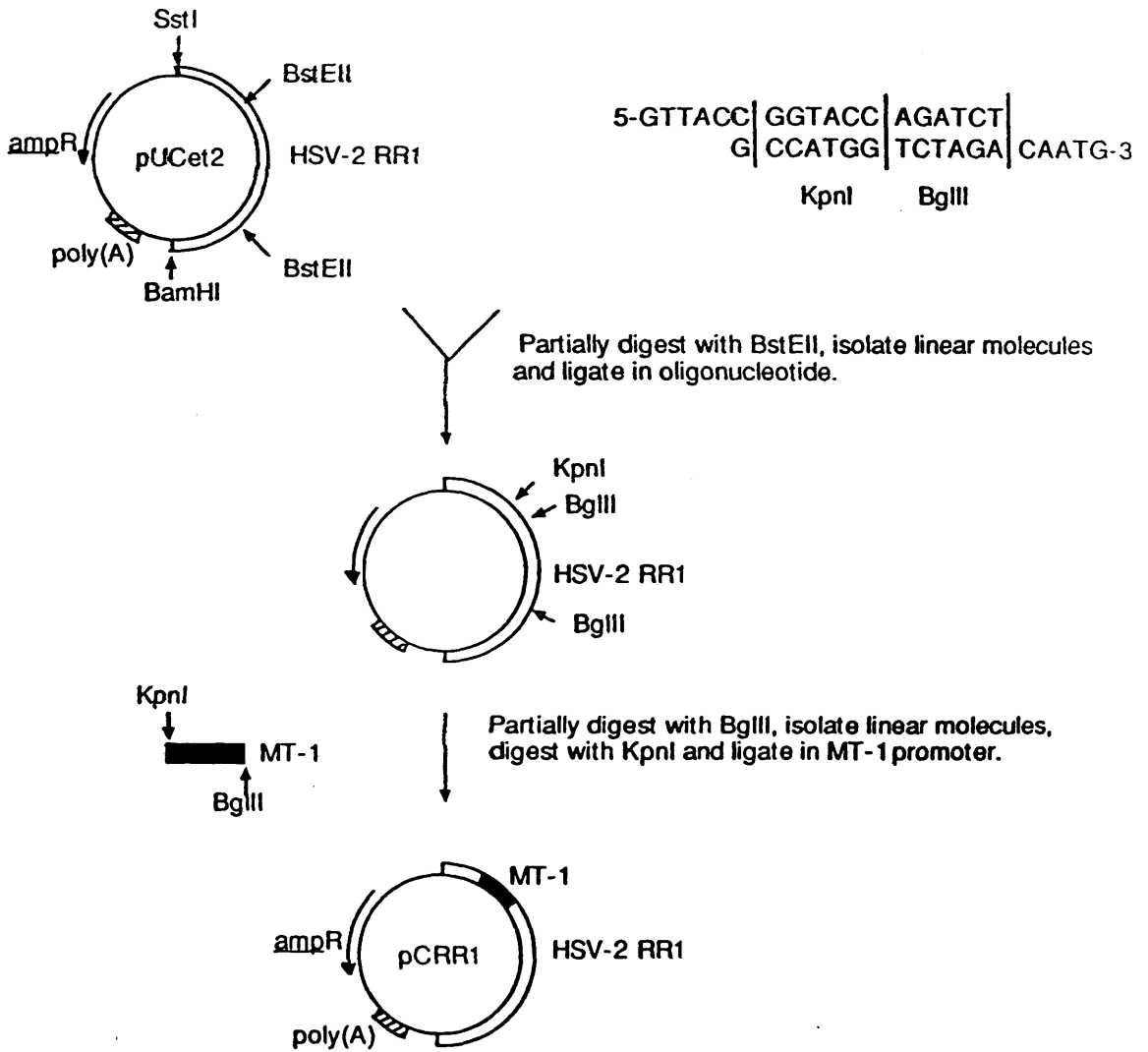


Figure 10 (b). Diagram showing the HSV-2 RRI gene sequences in pCRR1 (top) with the TATA box, the ATG and TAA codons, and the 2 BstEII sites marked.

The MT-1 promoter is shown below and its TATA box, cap site and MREs (dotted lines) are marked. The numbers below this sequence indicate the number of nucleotides a site is away from the MT-1 RNA CAP site.

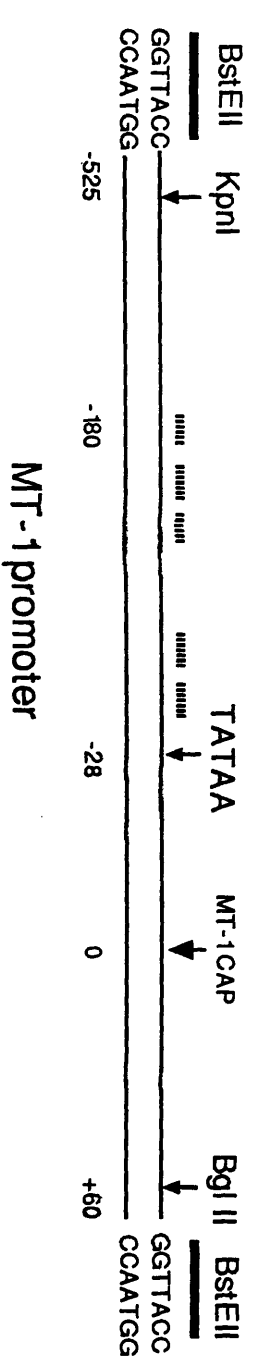
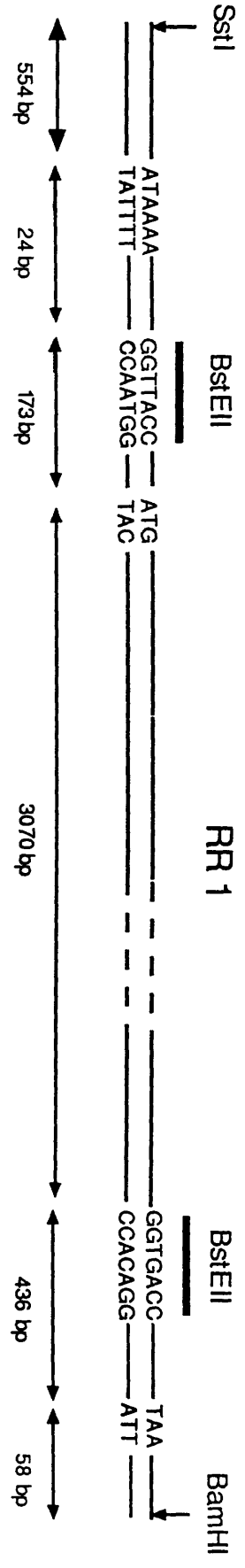
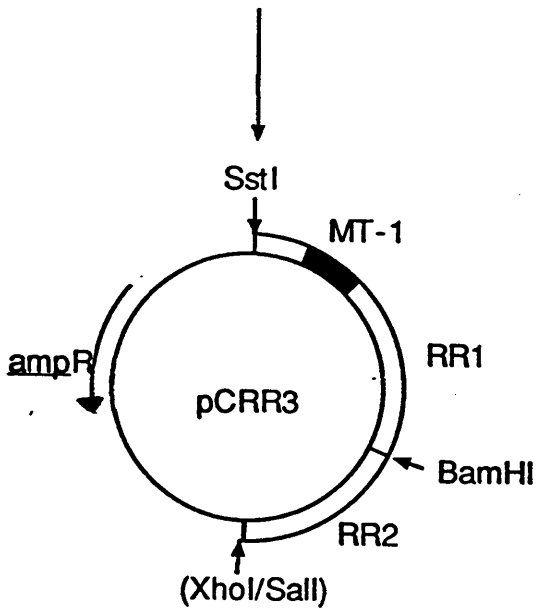
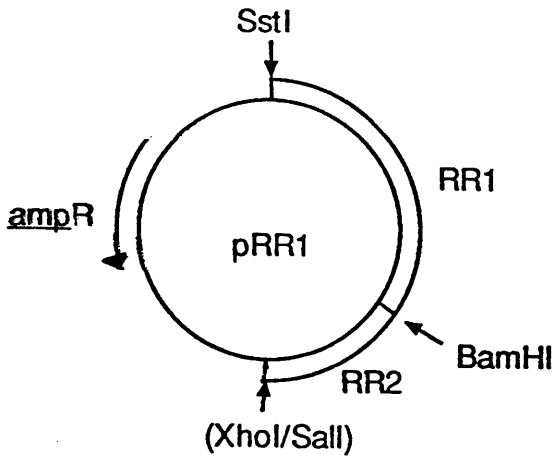


Figure 11. The plasmid pCRR3 containing the coding sequences of HSV-2 RR1 and RR2 (open box) located on an SstI/XhoI fragment (Figure 7) with RR1 under the control of the MT-1 promoter (solid box) and RR2 under the control of its own promoter.

The starting plasmid pRR1 (obtained from Dr. I. Nikas) is also shown. The cloning procedure is the same as that for the construction of pCRR1 (Figure 10).

The location and orientation of the ampicillin resistance gene (amp^R) are indicated.

Plasmids pRR1 and pCRR3.



left on the cells for some 18 hours before being removed and replaced with fresh medium. One of each set of 2 plates was then left in ordinary growth medium whilst the other plate was subjected to growth medium supplemented with 100uM zinc sulphate. The zinc sulphate was added to stimulate transcription from the MT-1 promoter and the concentration of 100uM was chosen since this had been used successfully in other studies (Searle et al., 1985). The cells were then incubated at 37°C for a further 24 hours before cytoplasmic RNA was extracted. The RNA was treated with DNase to remove all traces of DNA and then 5ug of RNA from each sample was transferred to a nitrocellulose membrane using a slot blot apparatus. The nitrocellulose membrane was incubated overnight with a nick-translated DNA probe under hybridization conditions. The probe used was a BamHI/HindIII subfragment isolated from pCRR2 (Figure 12). To check that any hybridization was due to RNA, a duplicate of each sample was treated with RNase before loading it onto the slot blot apparatus. Following hybridization the membrane was washed, air-dried and autoradiographed (Figure 13).

The amount of radioactive product was quantitated using a Joyce-Loebl scanning densitometer and the areas under the peaks of the densitometer tracing were measured by a DEC PDP 11/44 computer linked to a digitizing tablet.

The results show that pCRR2 produces HSV-2 RR2 RNA in an inducible manner with a 25-fold increase in RR2 RNA levels on induction with 100uM zinc sulphate. The hybridization is a result of the probe hybridizing to RNA since on treating the extract with RNase no hybridization was observed. In the case of pCRR3, where the coding sequence of HSV-2 RR2 was under the control of its own HSV-2 promoter HSV-2 RR2 RNA was seen in transfected cells. In most experiments, the amount of RR2 RNA observed is roughly the same in cells transfected with pCRR3 irrespective of induction, and was around 6-fold lower than that seen in induced cells transfected with pCRR2. A small amount HSV RR2 RNA was also seen in non-induced cells transfected with pCRR2. As expected, pCRR1 and pUC13 transfected cells did not produce HSV-2 RR2 RNA since these plasmids do not contain the coding sequences for this gene. A positive control consisting of 5ug of RNA isolated from cells that had been transfected

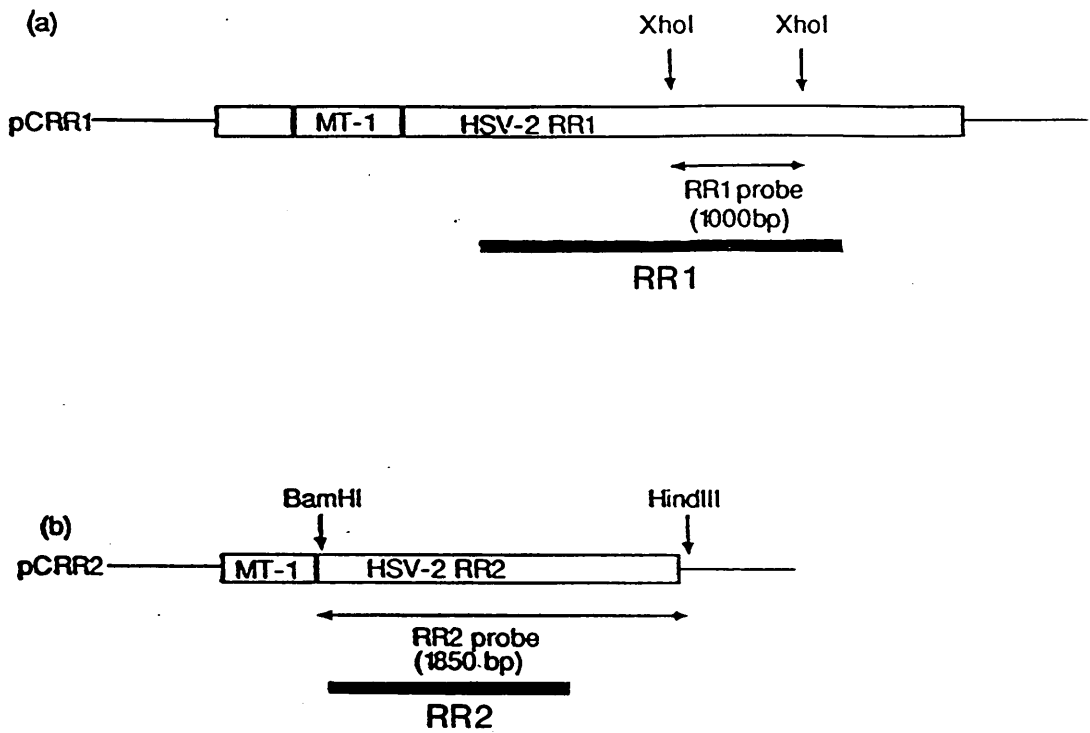


Figure 12. Linear representations of the plasmids pCRR1 (a) and pCRR2 (b) showing the regions from which the DNA probe sequences were isolated for nick-translation. The DNA probe for RR1 was a 1000bp XhoI fragment and the probe for RR2 was a 1850bp BamHI/HindIII fragment. The reading frames of RR1 and RR2 are marked as solid lines.

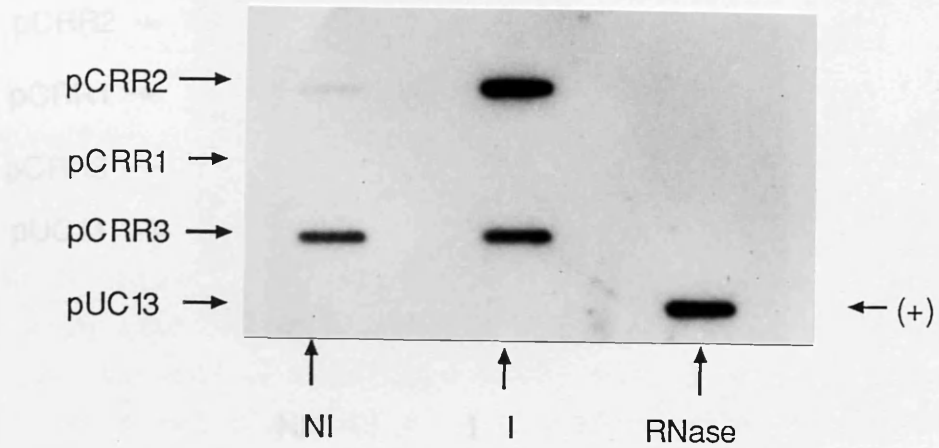
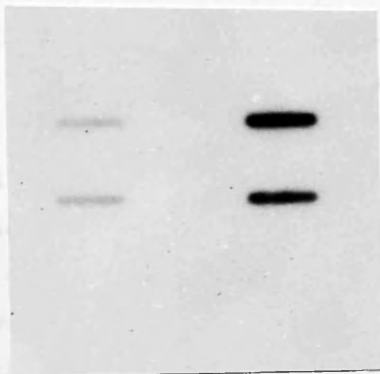


Figure 13. Slot blot analysis of the production of HSV-2 RR2 RNA in 293 cells transfected with the plasmids pCRR1, pCRR2, pCRR3 and pUC13. The amount of RR² RNA per 5ug of total cell RNA produced in cells induced with 100uM zinc sulphate (I) is compared to the amount produced in cells that were not induced (NI). RNA extracts that had been treated with RNase are also shown (RNase). The slot labelled (+) denotes RNA from pUC13 transfected cells treated with RNase and spiked with 0.005ug of unlabelled DNA probe.

pCRR2 →
pCRR1 →
pCRR3 →
pUC13 →



NI I

Figure 14. Slot blot analysis of the production of HSV-2 RR1 RNA in 293 cells transfected with the plasmids pCRR1, pCRR2, pCRR3 and pUC13. The amount of RR1 RNA/5ug of total cell RNA produced in cells induced with zinc sulphate (I) is compared to the amount produced in cells that were not induced (NI).

with pUC13 spiked with 0.005ug of probe sequence DNA is also shown on this autoradiograph.

The experiment was repeated, this time using a nick-translated DNA probe isolated from the internal XhoI fragment of the RRL gene (Figure 12). In this case, HSV-1 RRL RNA was seen in pCRR1- and pCRR3-transfected cells (Figure 14). Both these plasmids contain the HSV-2 RRL gene under the control of the MT-1 promoter. The RNA was inducible using 100uM zinc sulphate with the increase in RNA levels being 15-fold with pCRR3 and 19-fold with pCRR1 on induction. Again, there was RNA produced from the MT-1 promoter without induction. No HSV-2 RRL RNA was seen in cells transfected with pCRR2 or pUC13 neither of which contain the coding sequences for the RRL gene.

Results from other experiments mainly gave induction increases of between 15- and 25-fold for the MT-1 promoter treated with 100uM zinc sulphate.

Further experiments in this study mainly involve pCRR1 and pCRR2 as these plasmids have the HSV-2 ribonucleotide reductase genes under the control of the MT-1 promoter and are thus both inducible.

1.6. The Effect of Time on the Transient Expression of HSV-2 Ribonucleotide Reductase RNA from pCRR1 and pCRR2.

These experiments were performed in order to determine at what time following induction an increase in RNA production could be detected and for how long this increase was maintained.

Twelve, 50mm plates of subconfluent 293 cells were transfected with pCRR1. After 18 hours, the transfection mixture was washed off and replaced with fresh medium. Then 100uM zinc sulphate was added to half the plates and RNA was extracted from one induced and one non-induced plate at 0, 6, 12, 24, 36 and 48 hours after induction. After treating the RNA extracts with DNase, the RNA was compared by transferring 5ug to a nitrocellulose membrane using a slot blot apparatus and hybridizing the membrane with a nick-translated DNA probe isolated from pCRR1 (Figure 12). The membrane was autoradiographed (Figure 15) and the amount of radioactive product was assessed using a Joyce-Loebl

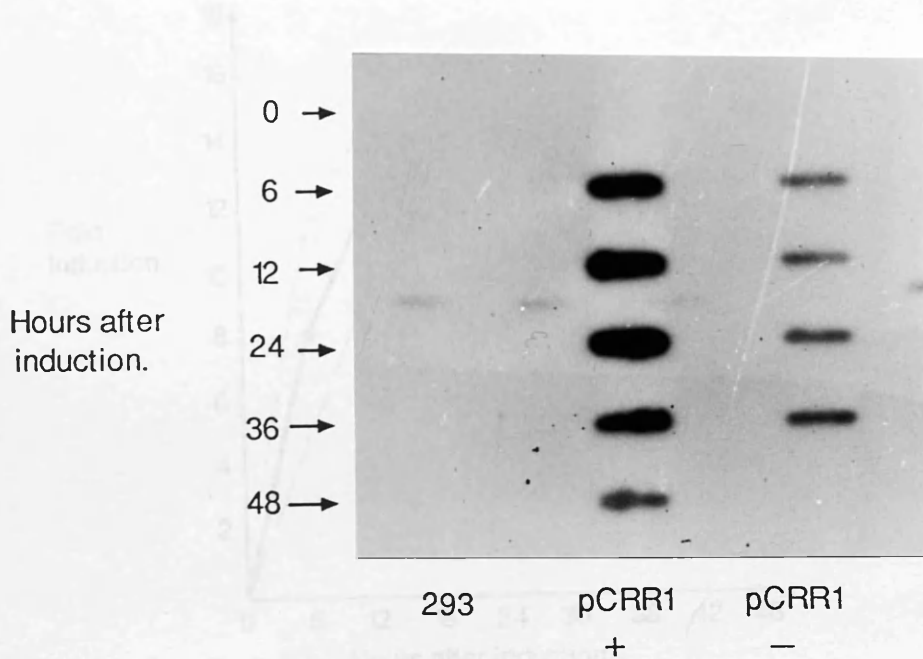


Figure 15. Slot blot analysis of the production of HSV-2 RR1 RNA with respect to time in 293 cells transfected with the plasmid pCRR1 and induced with 100uM zinc sulphate. The autoradiograph shows the RR1 RNA levels/5ug of extracted RNA at 0, 6, 12, 24, 36 and 48 hours after induction for untransfected 293 cells (293) and for cells that had been transfected with pCRR1 under induced (+) or non-induced (-) conditions.

N.B. The lack of any signal for the non-induced sample at 48 hours is surprising and may be due to an error in loading.

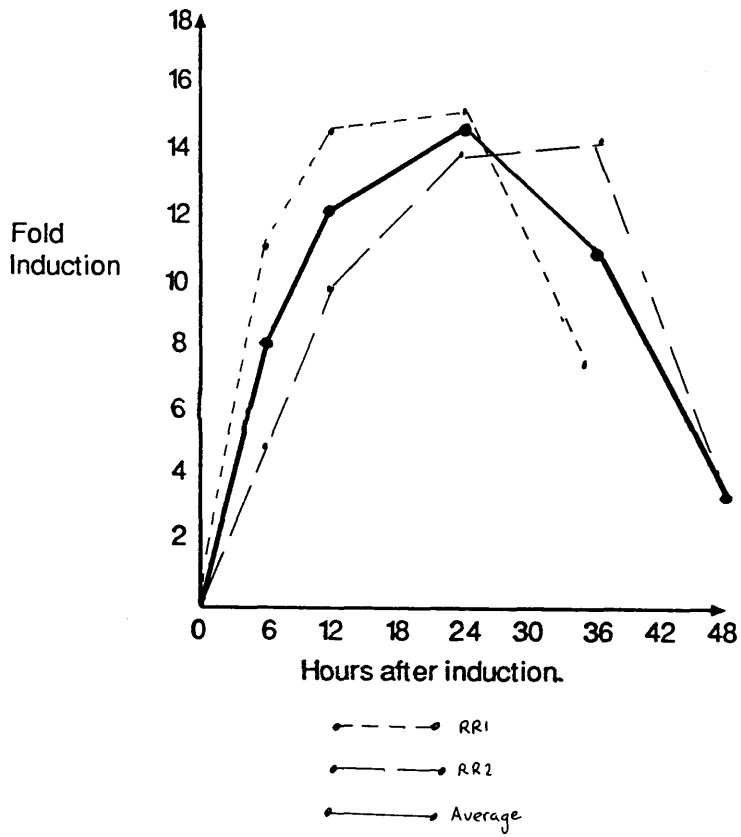
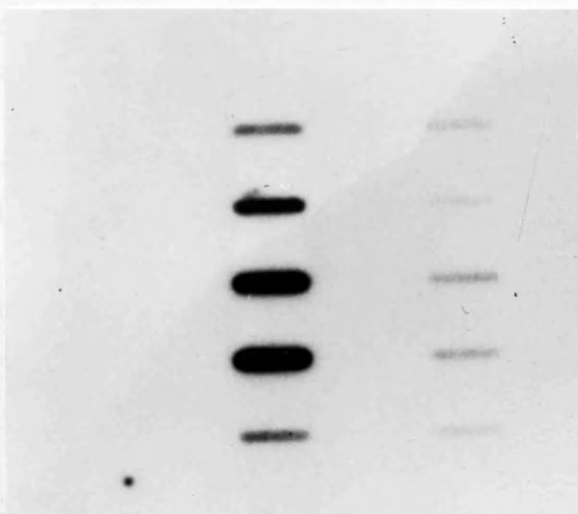


Figure 16. Graphical representation of the effect of time on the production of HSV-2 RR1 and RR2 RNA from the MT-1 promoter in pCRR1 and pCRR2 following transfection of 293 cells and induction with 100uM zinc sulphate.

Hours after
induction.

0 →
6 →
12 →
24 →
36 →
48 →



293

+

-

pCRR2

pCRR2

Figure 17 Slot blot analysis of the production of HSV-2 RR2 RNA with respect to time from 293 cells transfected with the plasmid pCRR2 and induced with 100uM zinc sulphate. The autoradiograph shows the RR2 RNA levels/5ug total cell RNA at 0, 6, 12, 24, 36 and 48 hours after induction for cells that had not been transfected (293) and for pCRR2 transfected cells under induced (+) and non-induced (-) conditions.

scanning densitometer as before (section C.1.5.).

The results show that the increase in HSV-2 RR1 RNA in cells transfected with pCRR1 was apparent 6 hours after induction of the MT-1 promoter with 100uM zinc sulphate, was maximal between 12 and 24 hours post-induction and was beginning to drop after 36 hours (Figure 16). The induced level of RR1 RNA was 15-fold greater, at its maximum level, than the non-induced level in pCRR1 transfected cells.

The experiment was repeated with pCRR2 and using a nick-translated DNA probe isolated from pCRR2 (Figure 11). Again, induction of RNA was apparent 6 hours after induction. RNA levels appear to reach maximum levels between 24 and 36 hours after induction in this experiment (Figure 17), which is slightly later than the for maximum induction level from the MT-1 promoter in other experiments. The average induction from the MT-1 promoter with respect to time is shown graphically in Figure 16.

1.7. The Effect of Zinc Concentration on the Induction of the MT-1 Promoter in pCRR1 and pCRR2.

This experiment was performed to determine the optimum concentration of zinc sulphate to use for induction of the MT-1 promoter.

Subconfluent, 50mm plates of 293 cells were transfected with pCRR2 and left for 18 hours before the transfection medium was removed. Medium containing various concentrations of zinc sulphate (0, 25, 50, 75, 100, 125 and 150uM) were added to the plates and RNA was extracted 24 hours later. The RNA was then screened for HSV-2 RR2 RNA using a slot blot apparatus as in previous experiments. The resulting filter was autoradiographed (Figure 18) and the amount of radioactive product was assessed using a Joyce-Loebl scanning densitometer as before (section C.1.5.).

The experiment was repeated with pCRR1 and the RNA was screened for HSV-2 RR1 RNA (Figure 19).

The results show that the amount of HSV-2 RR1 RNA transcribed from pCRR1 and the amount of HSV-2 RR2 RNA transcribed from pCRR2 increased as the concentration of zinc sulphate was raised (Figure 20). At a concentration of 125uM, however, the cells start to die and become detached

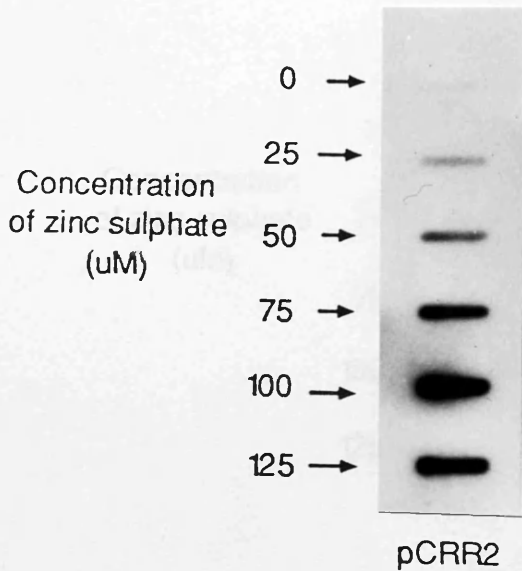


Figure 18. Slot blot analysis showing the effect of zinc concentration on the induction of the MT-1 promoter in pCRR2. 293 cells were transfected with pCRR2, induced with zinc sulphate at concentrations of 0, 25, 50, 75, 100, 125 and 150 uM and RNA harvested 24 hours later was screened for the presence of HSV-2 RR2 RNA by loading 5 ug of extracted RNA onto a slot blot apparatus and hybridizing the filter with a DNA probe.

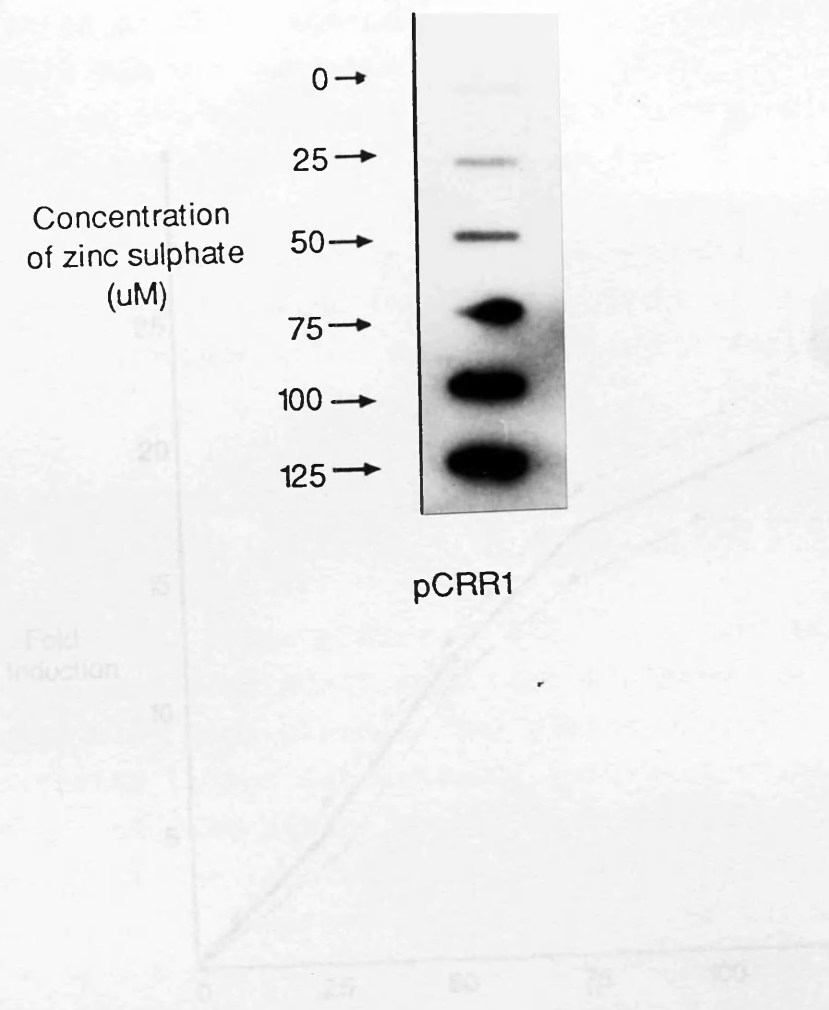


Figure 19. Slot blot analysis of the effect of zinc concentration on the induction of the MT-1 promoter in pCRR1. 293 cells were transfected with pCRR1, induced with zinc sulphate at concentrations of 0, 25, 50, 75, 100, 125 and 150 uM and RNA harvested 24 hours later was screened for the presence of HSV-2 RR1 RNA by loading 5ug of extracted RNA onto a slot blot apparatus and hybridizing the filter with a DNA probe.

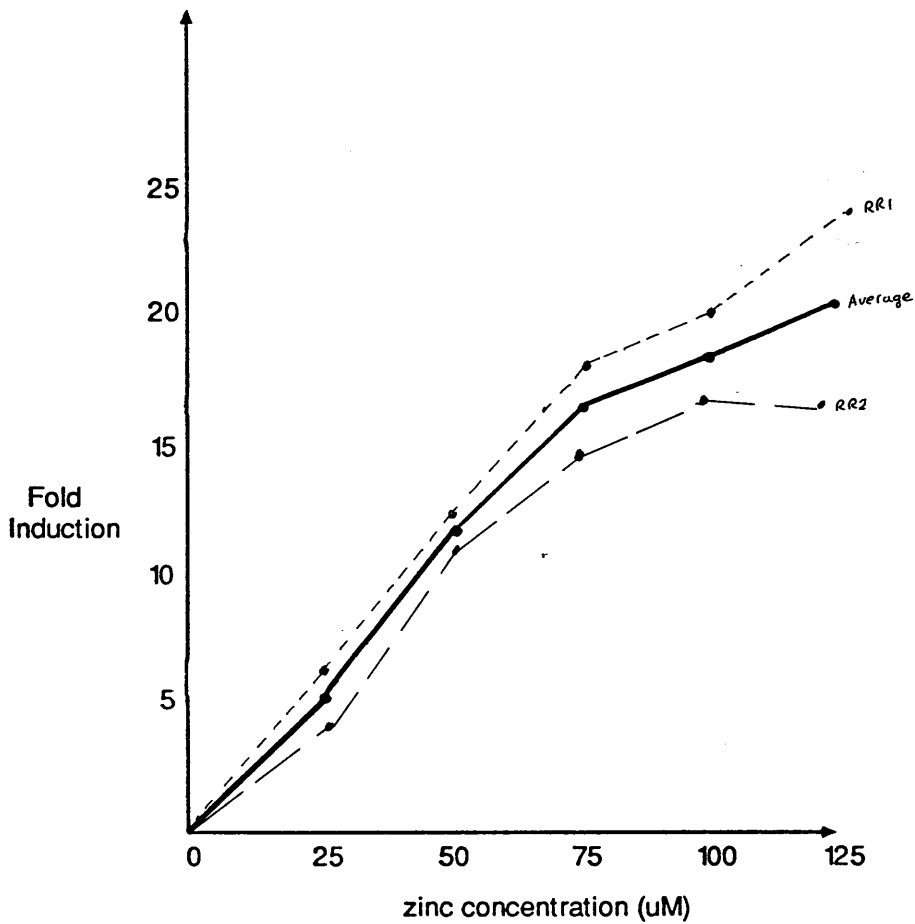


Figure 20.

Graphical representation of the effect of zinc concentration on the production of HSV-2 RR1 and RR2 RNA from the MT-1 promoter in pCRR1 and pCRR2 after being transfected into 293 cells. The effect of zinc sulphate concentrations of 0, 25, 50, 75, 100 and 125uM are shown.

from the plate and inductions are variable according to the rate of cell death. Results given here show that induction from the MT-1 promoter in pCRR1 gives the greatest value at 125uM whereas in pCRR2 the level of induction is beginning to drop at this concentration. At a zinc sulphate concentration of 150uM the cells were all detached from the plate before RNA was harvested.

Because of the cell death at 125uM zinc sulphate, 100uM zinc sulphate was chosen for subsequent transient expression experiments in order to obtain maximum induction without decreasing cell viability. In experiments where the cells were to be in contact with the zinc sulphate for more than 48 hours, the concentration was dropped still further to 75uM.

1.8. The Production of HSV-2 Ribonucleotide Reductase Proteins in Cells Transfected with pCRR1, pCRR2 and pCRR3.

Subconfluent, 100mm plates of 293 cells were transfected with pCRR1, pCRR2 and pCRR3 such that 4 plates were transfected with each plasmid. Two plates of cells were left non-transfected to act as controls. Following transfection, 2 plates out of each set of 4 were induced with 100uM zinc sulphate whilst the remaining 2 were left non-induced. After 24 hours, proteins were harvested by removing the medium, washing with PBS and then adding BM (0.5ml/plate). The extracts from the identically treated plates were then pooled, incubated in a boiling water bath for 3 minutes and stored at -70°C .

SDS-polyacrylamide protein gels were loaded in duplicate with 100ul of sample/track of each of the protein extracts. An HSV-2-infected cell extract was also loaded to act as a positive control. The gels were run overnight at 11V each and then the separated proteins were transferred to a nitrocellulose membrane by Western blotting. One membrane was then exposed to antibody directed against HSV-2 RR1 (14995) and the other to antibody directed against HSV-2 RR2 (20208). Both antibodies were kindly supplied by Dr. H. Lankinen. The proteins were then visualized using the horse radish peroxidase (HRP) technique.

The results show that antibody raised against HSV-2 RR1

Figure 21. The production of HSV-2 RR1 in 293 cells transfected with the plasmids pCRR1, pCRR2 and pCRR3. Proteins were harvested after 24 hours, separated on an SDS-polyacrylamide gel, transferred to a nitrocellulose filter by the Western blotting technique and screened for the presence of HSV-2 RR1 using the antibody 20208 (supplied by Dr. H. Lankinen). The protein produced from each plasmid under induced (100uM zinc sulphate) and non-induced conditions was compared. Also shown on the gel is a HSV-2 infected cell extract and an extract taken from non-transfected 293 cells.

M=HSV-2 (wt) infected cell extract.

1=pCRR3-transfected 293 cells, induced.

2=pCRR3-transfected 293 cells, non-induced.

3=pCRR1-transfected 293 cells, induced.

4=pCRR1-transfected 293 cells, non-induced.

5=pCRR2-transfected 293 cells, induced.

6=pCRR2-transfected 293 cells, non-induced.

7=non-transfected 293 cells.

M 1 2 3 4 5 6 7

RR1 →

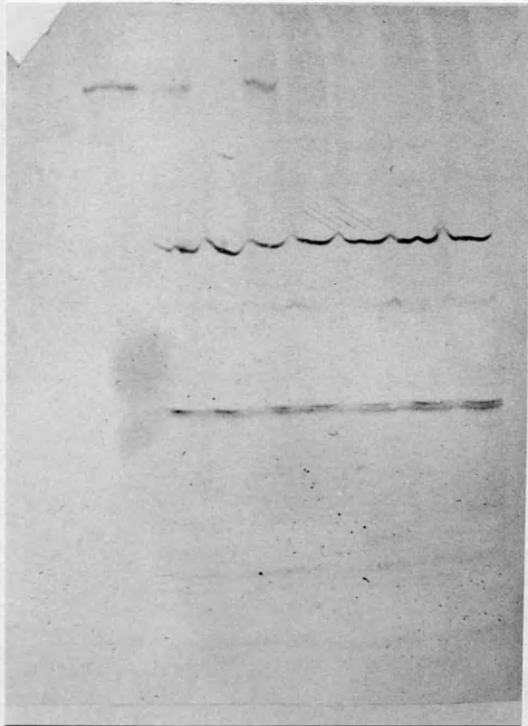


Figure 22. The production of HSV-2 RR2 in 293 cells transfected with the plasmids pCRR1, pCRR2 and pCRR3. Proteins were harvested after 24 hours, separated on an SDS-polyacrylamide gel, transferred to a nitrocellulose filter by the Western blotting technique and screened for the presence of RR2 using the antibody 14995 (supplied by Dr. H. Lankinen). The protein produced from each plasmid under induced (100uM zinc sulphate) and non-induced conditions was compared. Also shown on the gel is an HSV-2 (wt) infected cell extract and an extract taken from non-transfected 293 cells.

1=non-transfected 293 cells.

2=pCRR3-transfected 293 cells, induced.

3=pCRR3-transfected 293 cells, non-induced.

4=pCRR1-transfected 293 cells, induced.

5=pCRR1-transfected 293 cells, non-induced.

6=pCRR2-transfected 293 cells, induced.

7=pCRR3-transfected 293 cells, non-induced.

M=HSV-2 (wt) infected cell extract.

recognize a protein, presumably RR1, which had similar mobility in induced cells transfected with pCRR1, pCRR2 and in cells infected with wt HSV-2 (Figure 2). In this experiment, no HSV-2 RR1 protein was detected in non-induced cells transfected with pCRR1 or pCRR2, nor in cells (either induced or non-induced) transfected with pCRR1 or pCRR2. The antibody also recognizes several proteins in the transfected cell extracts that do not co-migrate with the protein seen in infected cells. This recognition probably represents non-specific binding.

1 2 3 4 5 6 7 M



shows a single mobility in cells infected with wt HSV-2. In cells (either induced or non-induced) transfected with pCRR1, pCRR2 or pCRR3, no RR1 protein was detected. The antibody also recognizes several proteins in the transfected cell extracts that do not co-migrate with the protein seen in infected cells. This recognition probably represents non-specific binding.

reduced to pCRR1, pCRR2 and pCRR3. The results of the plate assays were as follows: pCRR1, pCRR2 and pCRR3. The results of the plate assays were as follows: pCRR1, pCRR2 and pCRR3. The results of the plate assays were as follows: pCRR1, pCRR2 and pCRR3.

← RR2

To determine if the RR1 protein was induced in cells transfected with pCRR1, pCRR2 and pCRR3, the following experiment was performed. Cells transfected with pCRR1, pCRR2 or pCRR3 were induced with HSV-2. After 18 hours, the transfection mixture was removed and replaced with fresh medium. To 1 plate from each pair, 100µM sodium sulphate was added and all the transfected plates were incubated for 24 hours at 37°C. Partially purified HSV-2 ribonucleotide reductase extract from the infected plates was made at this time. After further incubation of the transfected plates, more partially purified HSV-2 ribonucleotide reductase extracts were made from the transfected plates. The extracts were then subjected to ribonucleotide reductase assays. The results show that in the assay used, enzyme activity was induced in cells transfected with pCRR1 and pCRR2 (Figure 3) and in HSV-2-infected cells. No activity was seen in non-induced

recognize a protein, presumably RR1, which had similar mobility in induced cells transfected with pCRR1, pCRR3 and in cells infected with wt HSV-2 (Figure 21). In this experiment, no HSV-2 RR1 protein was detected in non-induced cells transfected with pCRR1 or pCRR3, nor in cells (either induced or non-induced) transfected with pCRR2 or pUC13. The antibody also recognizes several proteins in the transfected cell extracts that do not co-migrate with the protein seen in infected cells. This recognition probably represents non-specific binding.

Antibody raised against HSV-2 RR2 recognises a single protein, presumably RR2, which had similar mobility in induced cells transfected with pCRR2 and in cells infected with HSV-2 (Figure 22). No protein was detected in non-induced cells transfected with pCRR2 or in cells (either induced or non-induced) transfected with pCRR1, pCRR3 or pUC13.

1.9. HSV-2 Ribonucleotide Reductase Activity from Cells Transfected with the Plasmids pCRR1, pCRR2 and pCRR3.

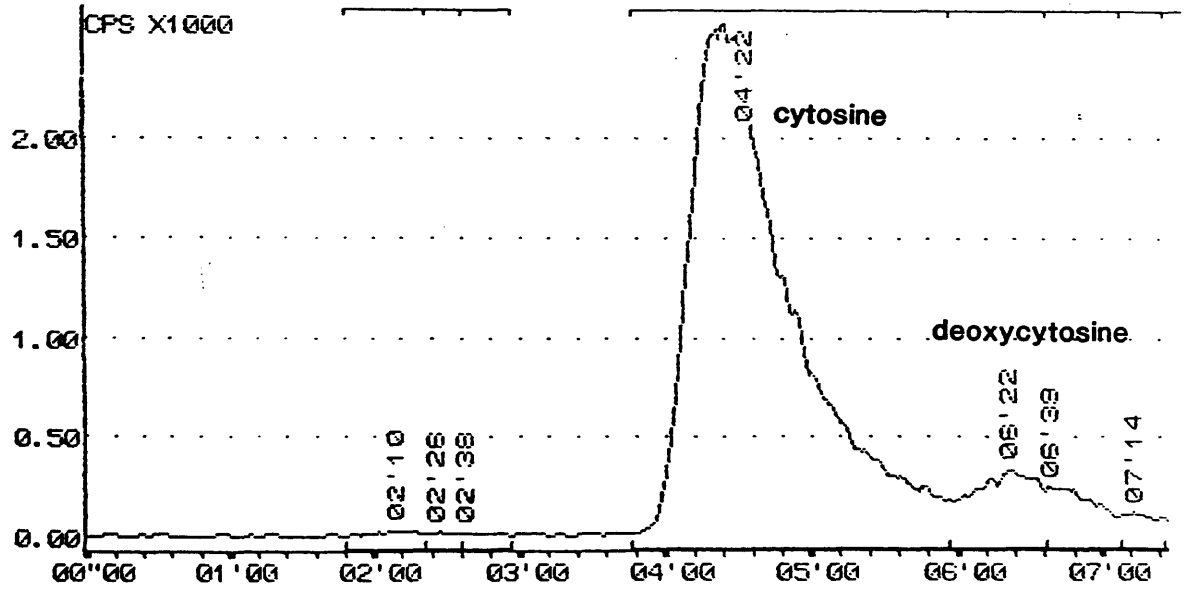
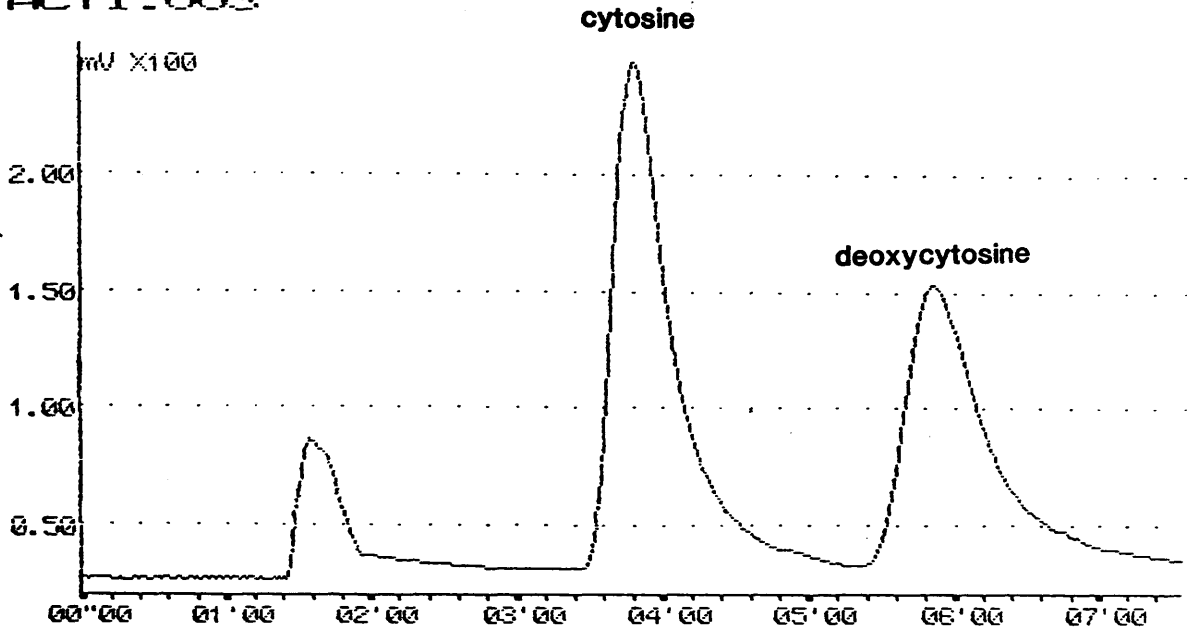
To check that the HSV-2 ribonucleotide reductase proteins produced in cells transfected with pCRR1, pCRR2 and pCRR3 were enzymatically active, activity assays were performed. Plates were transfected with one of the plasmids pUC13, pCRR1, pCRR2 and pCRR3. A further 2 plates were co-transfected with pCRR1 and pCRR2. For controls, 2 plates were left untransfected and 1 plate was infected with HSV-2. After 18 hours, the transfection mixture was removed and replaced with fresh medium. To 1 plate from each pair, 100uM zinc sulphate was added and all the transfected plates were incubated for 24 hours at 37°C. Partially purified HSV ribonucleotide reductase extract from the infected plates was made at this time. After further incubation of the transfected plates, more partially purified HSV ribonucleotide reductase extracts were made from the transfected plates. The extracts were then subjected to ribonucleotide reductase assays. The results show that in the assay used, enzyme activity was obtained from induced cells co-transfected with pCRR1 and pCRR2 (Figure 23) and in HSV-2-infected cells. No activity was seen in non-induced

Figure 23. HSV ribonucleotide reductase activity from 293 cells co-transfected with the plasmids pCRR1 and pCRR2. Partially purified enzyme extracts from the transfected cells were prepared and subjected to ribonucleotide reductase assays. The Figure shows the HPLC traces from enzyme assays performed on, (a) a pCRR1/pCRR2 co-transfected extract from cells that had been induced with zinc sulphate, (b) an HSV-2 infected cell extract and (c) untransfected 293 cells.

The top traces is the UV-absorbance and shows the position of the cytosine (substrate) and deoxycytosine (product) markers. The bottom trace is the eluted scintillation trace and the peaks of the labelled cytosine and deoxycytosine are indicated. There is no deoxycytosine peak in the untransfected extract.

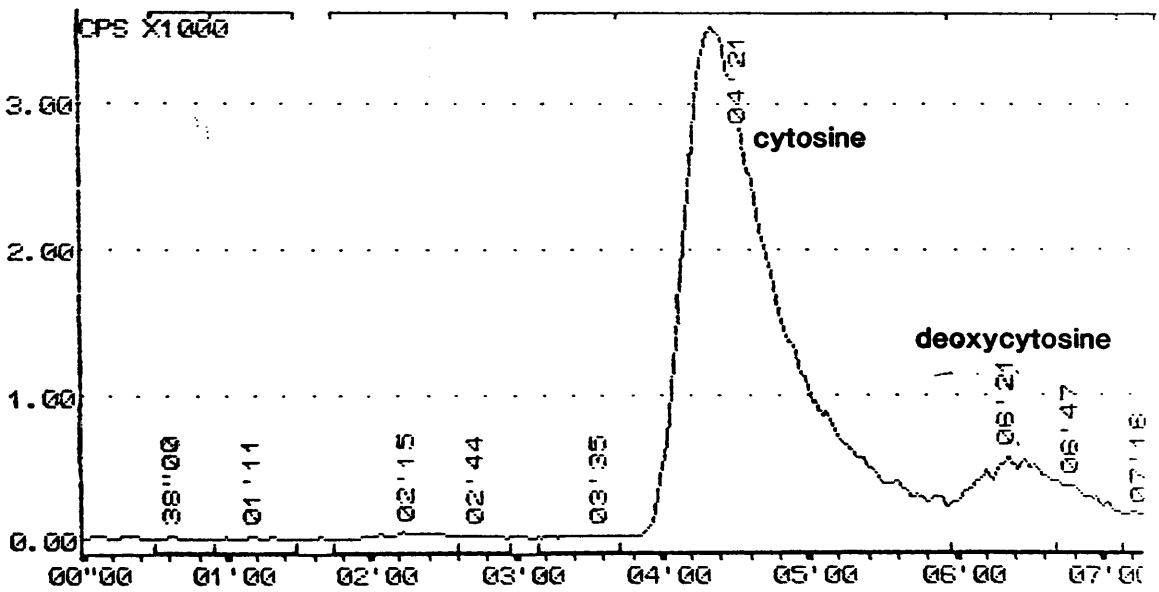
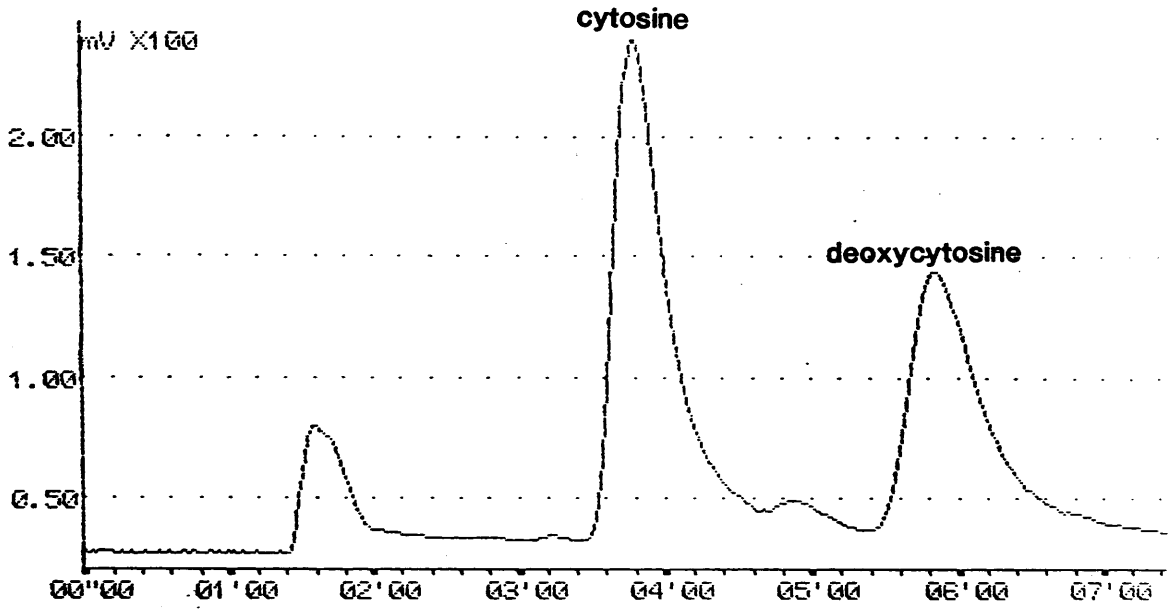
(a)

ACTI-003



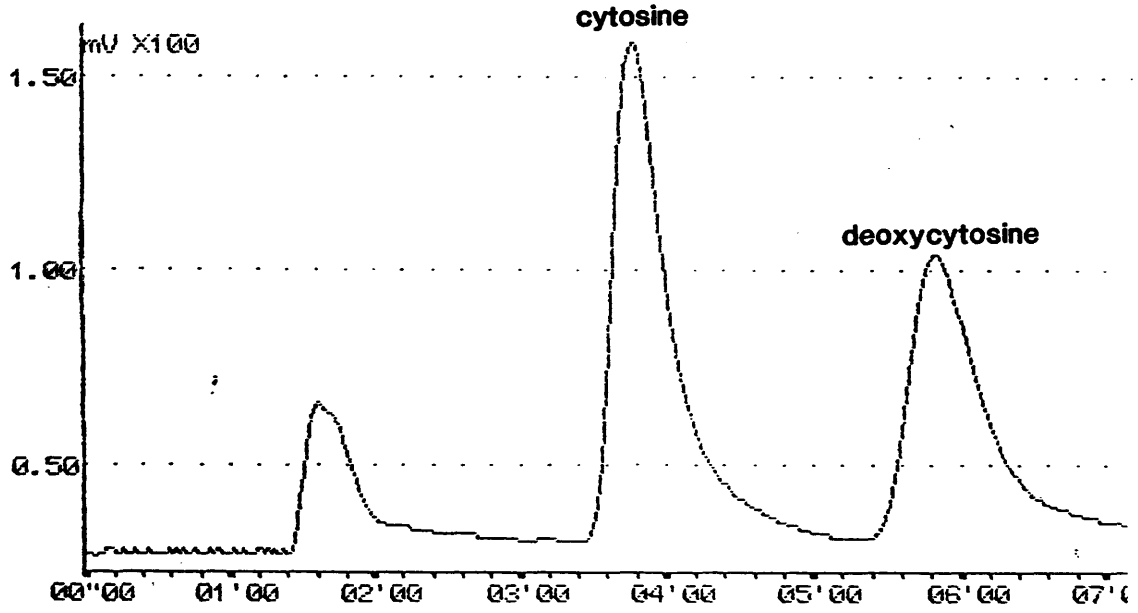
(b)

ACTI.004

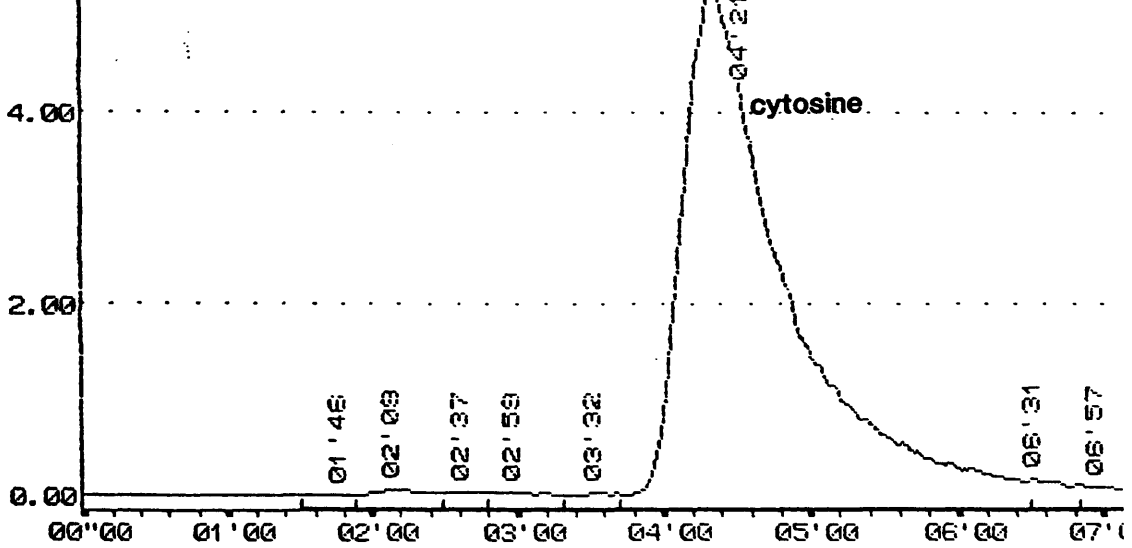


(c)

OFL



CPS X1000



cells that had been similarly transfected or in cells (either induced or non-induced) transfected with pUC13, pCRR1, pCRR2 or pCRR3 alone (data not shown), or in untransfected cells. No activity would be expected with cells transfected with pCRR1 or pCRR2 alone since the whole enzyme would not be present nor in cells transfected with pUC13 or untransfected cells since neither subunit would be present.

1.10. Discussion I.

The results presented above describe the construction of three plasmids which express the HSV-2 proteins RR1 and RR2. The plasmid pCRR1 contains the coding sequences for RR1 under the control of the inducible MT-1 promoter, pCRR2 contains the coding sequences for RR2 under the control of the MT-1 promoter and pCRR3 contains the coding sequences for RR1 and RR2. In pCRR3, RR1 is under MT-1 control and RR2 is under the control of its own HSV promoter.

Experiments to determine the optimum conditions of MT-1 stimulation found that around 100uM zinc sulphate produced the most HSV-2 ribonucleotide reductase RNA. This is in agreement with other studies (Searle et al., 1985). Concentrations of zinc sulphate above this level were found to be toxic to the cells and for longer-term exposure to zinc treatment the concentration of zinc sulphate was dropped to 75uM.

Where the HSV-2 ribonucleotide reductase genes were under the control of the MT-1 promoter, treatment with 100uM zinc sulphate solution increased the level of RNA production by 15-25-fold. Searle et al. (1985) found the MT-1 promoter to confer a similar amount of induction to the HSV TK gene; the amount of TK induction was measured at the level of enzyme activity and was found to be 18-fold.

RNA produced from the MT-1 promoter was apparent at around 6 hours post-induction, was maximal around 12-24 hours and then began to decrease. Other studies, performed in vivo have shown that induction from the MT-1 promoter is apparent much sooner, 30 minutes after induction (Durnam et al., 1984; Durnam and Palmiter, 1981), however, the fact that these experiments were performed on the endogenous gene

might account for this effect. In experiments where HSV TK gene expression from the MT-1 promoter is monitored, 22 hours^h has been used as a time period to allow protein expression (Brinster et al., 1982; Stuart et al., 1981). This is in agreement with the production of RNA and protein in this study.

HSV-2 ribonucleotide reductase RNA was present at low levels in transfected cells under conditions of non-induction. This may be due to the basal level of activity from the MT-1 promoter or to some stimulus of the promoter from the growth medium. Recent experiments suggest that a lower basal level of transcription from the MT-1 promoter can be obtained using charcoal-stripped serum, though this varies with different batches of serum (Dr. J. McLauchlan, personal communication).

HSV-2 RR2 RNA was also produced from the HSV promoter in pCRR3-transfected cells. The amount of RNA was similar in induced and non-induced cells and was around 6-fold less than the amount obtained from an induced MT-1 promoter. The production of HSV-2 RR2 RNA from the HSV promoter, which is normally activated by IE gene products, is in agreement with findings of Nikas (1988).

HSV-2 ribonucleotide reductase proteins were detected in cells that had been transfected with the plasmids. Protein was produced in detectable amounts when the relevant gene was under the control of an induced MT-1 promoter. The presence of protein in cells that were not induced cannot be ruled out as a small amount of RNA is present, however, the amounts were not detectable with this assay.

Enzyme activity was only found in induced cells that had been co-transfected with pCRR1 and pCRR2. The detection of activity would only be expected in cells transfected with pCRR3 or co-transfected with both plasmids since both subunits are required and there is no evidence that one subunit from the HSV enzyme can interact with a cellular subunit. No activity was seen in non-induced co-transfected cells or in cells that were transfected with pCRR3 (either induced or non-induced). This may be because the assay is not sensitive enough to detect this, as small amounts of HSV-2 ribonucleotide reductase RNA were found in these cells.

2. THE EFFECT OF HSV-2 RIBONUCLEOTIDE REDUCTASE EXPRESSION ON MUTAGENESIS.

The experiments in this section describe effects of HSV-2 RR1 and RR2 expression on mutagenesis. Three mutagenesis assay systems were used. One system assayed the mutagenesis of a marker gene located on a shuttle vector plasmid and the other systems assayed the mutagenesis of the cellular aprt gene.

The effects of the HSV-1 mutants tsl207 and tsl222 which have ts lesions in the genes encoding RR1 and RR2 respectively, were also assessed.

2.1. The pZ189 Mutagenesis Assay System.

2.1.1. Introduction.

Shuttle vector plasmids that can replicate in both eukaryotic and prokaryotic cells are powerful tools for the study of rearrangement and mutagenesis of DNA in mammalian cells. Plasmids carrying bacterial marker genes can be exposed to mutagenic agents in a eukaryotic environment, recovered and used to transform an appropriate bacterial host. The integrity of the marker gene can then be determined quickly and easily using standard microbiological procedures.

Studies where shuttle vectors have been cycled through mammalian cells and then screened for mutations in bacteria have, however, revealed a high frequency of spontaneous mutation (Calos et al., 1983; Razzaque et al., 1983, 1984; Lebkowski et al., 1984). The mutant plasmids contained deletions, insertions of cellular DNA and point mutations. The majority of plasmids found in one study were of the deletion/insertion type (Razzaque et al., 1983) which probably resulted from the introduction of double-stranded breaks early in infection (Razzaque et al., 1984). Additional breaks, followed by recircularization, would produce deletions while ligation of cellular DNA would produce insertions. The actual frequency of the spontaneous mutant plasmids recovered would be a function of the size of the marker gene and its proximity to sequences necessary for plasmid selection and replication. Seidman et al. (1985)

designed a plasmid, pZ189 (Figure 8), to minimize the recovery of spontaneous mutants and which also facilitated the characterization of the induced mutants. The plasmid contains the bacterial tyrosine suppressor tRNA gene (Brown *et al.*, 1979), supF, whose gene product can be quickly screened in bacteria (see below) and whose sequence can be easily determined. The tRNA gene is small (150bp) and lies between two essential functions of the plasmid; the ampicillin resistance gene and the DNA replication origin of the plasmid, which minimizes recovery of the spontaneous mutational events described above. The plasmid also contains the replication origin and early gene region from SV40 virus and replicates as efficiently in permissive cells as wild-type SV40 viral DNA.

The pZ189 mutagenesis assay (section B.2.2.3.), employs the use of the E. coli strain MBM7070 which carries the lacZ amber mutation. In the presence of IPTG, X-Gal and ampicillin, this E. coli strain forms blue colonies if it contains a plasmid with an active supF gene and white colonies if the gene is inactive.

The experiments described below (section C.2.2.2.) use the pZ189 mutagenesis assay to detect any increase in the mutation frequency of pZ189 within cells that were also transfected with plasmids expressing HSV-2 RR1 and RR2. Firstly, however, the plasmids pCRR1 and pCRR2 had to be modified for use in this assay system.

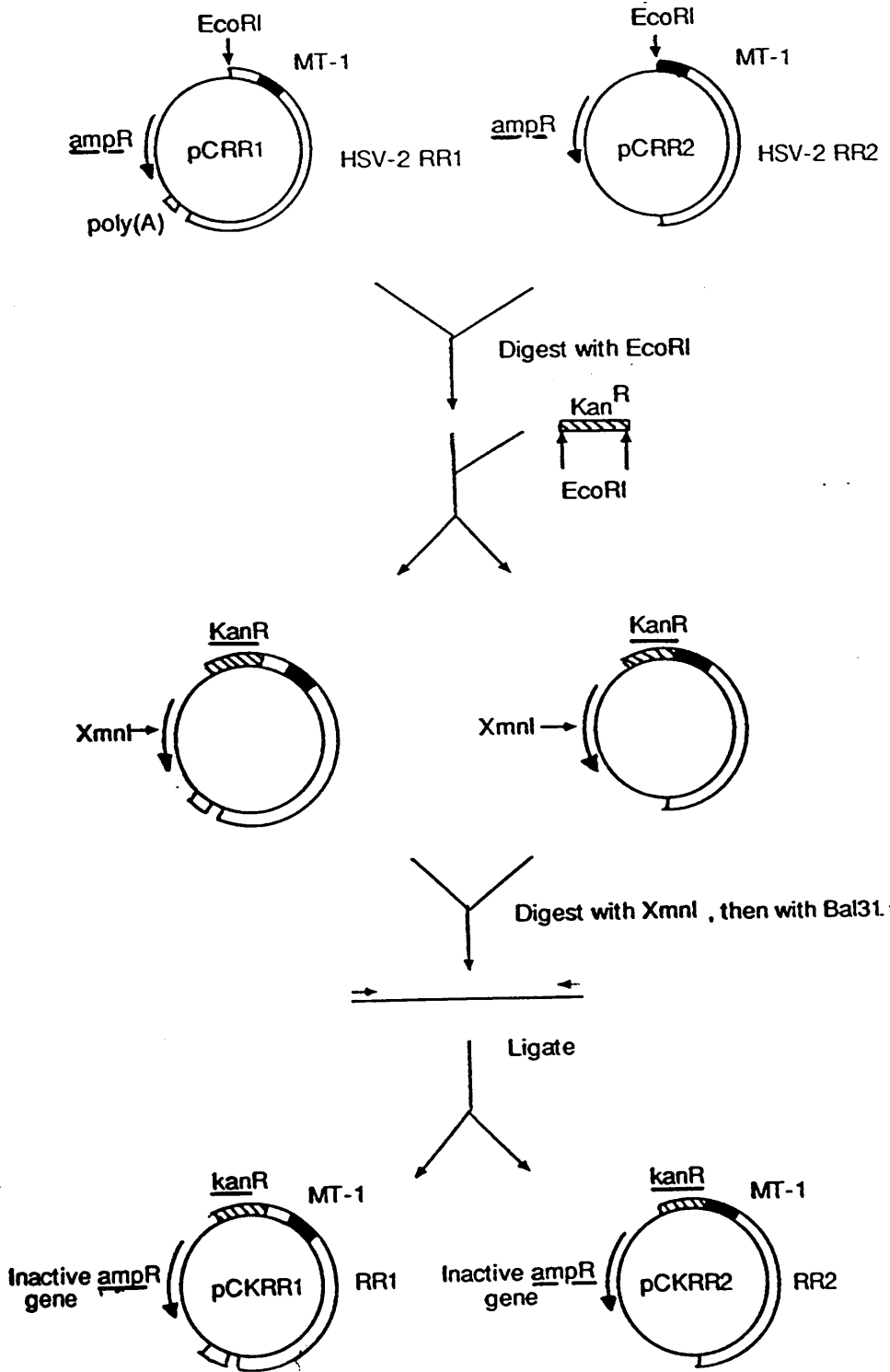
2.1.2. Modification of pCRR1 and pCRR2 for use in the pZ189 Mutagenesis Assay.

The plasmids pCRR1 and pCRR2 express the RR1 and RR2 subunits of HSV-2 ribonucleotide reductase as described previously (sections C.1.2. and C.1.3.). Unfortunately, in the pZ189 mutagenesis assay, the mutagenic frequency is obtained by counting the number of white colonies (which contain plasmids with an inactive supF gene) as opposed to blue ones (which contain plasmids with an active supF gene). Misleading data would result from this assay if pCRR1 or pCRR2 escaped the DpnI enzyme treatment (which digests DNA that has not been replicated in eukaryotic cells, section B.2.2.3.) and were introduced into the MBM7070 bacteria since these plasmids^{alone} would

Figure 24. Construction of the plasmids pCKRR1 and pCKRR2. The starting plasmids pCRR1 and pCRR2 contain the coding sequences for the HSV-2 RR1 and RR2 proteins (open boxes) under the control of the MT-1 promoter (solid box). In addition pCRR1 contains the polyadenylation (polyA) signals from the IE5 gene.

The kanamycin resistance gene (kanR, hatched box) was ligated into an EcoRI digest of these plasmids and the resulting construct were digested with XmnI, which cuts within the ampicillin resistance gene (ampR, arrow). Bal31 digestion (indicated by dotted arrows) then removed part of the ampR genes before the plasmids were religated to form the plasmids pCKRR1 and pCKRR2.

The Construction of pCKRR1 and pCKRR2.



appear as amp^R white colonies. To eliminate this effect, the amp^R gene in pCRR1 and pCRR2 was inactivated and replaced by a kanamycin resistance marker (kan^R). This meant that bacteria transformed with pCKRR1 or pCKRR2 would not grow on the mutagenesis selection plates which contain ampicillin.

The strategy for the modification of pCRR1 and pCRR2 is shown in Figure 2. The first step was to ligate in a kan^R gene. The plasmids were digested with EcoRI and the kan^R gene (located on an EcoRI fragment isolated from the plasmid pUC4K) was ligated into the linearized plasmids. The amp^R gene was then inactivated. This was achieved by digesting the plasmids with XmnI (which cuts once within the amp^R gene), isolating the linear molecules and then subjecting the linear molecules to Bal31 digestion for 30 seconds. This enzyme (Lau and Gray, 1979) catalyzes the progressive removal of nucleotides from both the 5'- and 3'-termini of double-stranded DNA and thus removes nucleotides from the ends of the linearized plasmid. Plasmids containing the kan^R gene were then selected by ligation of the Bal31-digested ends and transformation into DH1 bacteria which were grown on L-broth agar plates containing kanamycin (50ug/ml). The inactivation of the amp^R gene was checked by replica plating the kanamycin-resistant bacteria on L-broth containing ampicillin. The integrity of the remaining plasmid sequences was determined by restriction enzyme digests of mini-prep DNA and by checking that they were still capable of producing HSV-2 RR1 and RR2. The resultant plasmids were called pCKRR1 and pCKRR2.

2.1.3. The Effect of HSV-2 Ribonucleotide Reductase Expression from the Plasmids pCKRR1 and pCKRR2.

Subconfluent 90mm plates of 293 cells were co-transfected with pZ189 and one of the plasmids pCKRR1, pCKRR2, p175 and pUC13 such that 2 plates were transfected with pZ189 plus one other each plasmid. Two additional plates were transfected with pZ189, pCKRR1 and pCKRR2. Ten ug of each plasmid was used for the transfections and the total amount of DNA was kept equal (30ug) using calf thymus DNA. As controls, 2 plates were transfected with pZ189 alone and 2 plates were transfected with pZ189 that had been subjected to treatment with the chemical mutagen

hydroxylamine. This mutagen can directly cause a change in base-pairing by causing a tautomeric shift. It can react with A, C and U but the reaction with C is most common leading to the replacement of the amino group by a hydroxyamino group. This leads to the C residue behaving like a U (Singer and Kusmierck, 1982) resulting in a GC to AT transition.

After 18 hours, the transfection mixture was removed and one plate out of each pair was induced with 100uM zinc sulphate whilst the other was left in non-inducing medium. The plates were incubated for 48 hours then plasmid DNA was recovered using the Hirt extraction procedure. Unreplicated DNA was removed by treatment with DpnI and E. coli strain MBM7070 was transformed with the remaining plasmid DNA. The bacteria were spread on L-broth agar plates containing IPTG, X-Gal and ampicillin and incubated overnight at 37°C. The resulting blue and white colonies were then counted.

The experiment was repeated three times (twice in the case of p175) and the pooled data for the numbers of colonies counted is shown in Table 4. Results were assessed for their significance at the 1% level using the statistical analysis outlined in section B.2.28. The frequency of mutagenesis of pZ189 in non-induced cells co-transfected with pZ189 and pUC13 was taken as the control mutation frequency ie. the frequency to which the other mutation frequencies was compared.

The mutation frequency of pZ189 shuttled through 293 cells and screened in bacteria was found to be 0.04%. This is similar to the findings of Hwang and Shillito (1990). When ²⁹³ cells, containing pZ189, were subjected to treatment with 100uM zinc sulphate the mutation frequency was not affected implying that zinc treatment per se has no effect on mutagenesis. Co-transfection of the control plasmid pUC13 with pZ189 also did not affect the mutation frequency of pZ189 from that observed by transfecting pZ189 alone either in cells induced with zinc sulphate or in non-induced cells. This suggests that the addition of a second plasmid to the mutagenesis assay does not affect mutagenesis.

The plasmid p175 appears to have no effect on mutagenesis of the supF gene when transfected into cells together with pZ189. This plasmid contains the gene encoding

Table 4. Effect of expression of HSV-2 RR1 and RR2 from the plasmids pCKRR1 and pCKRR2 under 100uM zinc-induced (+) and non-induced (-) conditions on the mutation rate of the supF gene in the pZ189 mutagenesis assay. The Table shows the combined colony numbers from three experiments where the plasmids pCKRR1, pCKRR2, pUC13 and p175 were transfected into 293 cells. The total number of colonies counted, the number of white (mutant) colonies and the % mutagenesis are listed. As a positive control, pZ189 DNA that had been treated with the chemical mutagen hydroxylamine (pZ189*) was included in the experiment.

Transfected plasmid(s)	Induced	Colonies counted	Whites	% Mutagenesis
pZ189	-	9333	4	0.04
pZ189	+	8234	4	0.05
pZ189+pUC13	-	6786	4	0.06
pZ189+pUC13	+	8238	4	0.05
pZ189+p175	-	10002	3	0.03
pZ189+p175	+	6300	4	0.06
pZ189+pCKRR2	-	5625	4	0.09
pZ189+pCKRR2	+	4217	4	0.09
pZ189+pCKRR1	-	5339	5	0.09
pZ189+pCKRR1	+	4511	5	0.11
pZ189+pCKRR1 +pCKRR2	-	4024	4	0.10
pZ189+pCKRR1 +pCKRR2	+	3092	3	0.11
pZ189*	-	5462	16	0.30

N.B. Any mutations occurring in the T antigen region may not allow replication of the plasmid and would not score in the assay. Thus, the number of mutations in the supF gene may have been underscored.

Vmw175 and was used to determine whether any mutagenesis resulting from pCRR1- and pCRR2- transfection was specific or whether other HSV-2 genome regions were mutagenic.

Co-transfection of pCKRR1 with pZ189 raises the mutation frequency of pZ189 to 0.09% with non-induced cells and 0.11% with zinc-treated cells. These values were not found to be statistically significant compared to the level of mutagenesis in cells co-transfected with pZ189 and pUC13. A similar effect is seen in cells co-transfected with pCKRR2 and pZ189. The plasmid pCKRR2 raises the mutation frequency of pZ189 to 0.09% in both induced and non-induced cells. Again this increase is not significant.

Transfection of both pCKRR1 and pCKRR2 together with pZ189 raises the mutation frequency to 0.11% for induced cells and 0.1% for non-induced cells. Although these figures are the highest obtained in the transfection experiments involving pCKRR1 and pCKRR2 they were not found to be significantly different from the figures obtained following co-transfection of pZ189 and pUC13 using statistical analysis.

Treatment of pZ189 with a chemical mutagen (hydroxylamine) increases the mutation frequency of pZ189 by 7-fold. This value was significantly different (at the 1% level of significance) from the mutagenic frequency of the supF gene in cells co-transfected with pZ189 and pUC13.

2.2. The aprt mutagenesis assay system.

2.2.1. Introduction.

This mutagenesis assay involves the use of a cellular gene, the adenine phosphoribosyltransferase gene, (aprt) for monitoring mutagenesis.

Adenine phosphoribosyltransferase is a purine salvage pathway enzyme that catalyzes the conversion of adenine to adenosine 5'-monophosphate. The enzyme is encoded by an autosomal gene locus (Taylor et al., 1979, 1985) which is constitutively expressed throughout the cell cycle (Hordern

and Henderson, 1982). Mutants that do not express the enzyme can be readily isolated by selection with adenine analogues, such as 8-azaadenine (AA), which block the salvage pathway. Since both aprt alleles are normally expressed in Chinese hamster ovary (CHO) cells (Meuth and Arrand, 1982; Simon et al., 1982, 1983; Adair et al., 1984), direct selection of aprt-mutants from wt populations is impractical, however, a hemizygous cell line D422 has been isolated on the basis of its partial deficiency of enzyme activity (Bradley and Letovanec, 1982). This cell line permits single-step selection of fully AA-resistant (aprt-) mutants at frequencies comparable to the X-linked hypoxanthine-guanine phosphoribosyltransferase (hgpert) locus (Adair et al., 1980; Carver et al., 1980; Thompson et al., 1980; Adair and Carver, 1983). Phenotypic expression kinetics for AA-resistance are more rapid than for thioguanine-resistance (hgpert-), making the aprt locus advantageous (Carver et al., 1980; Thompson et al., 1980; Adair and Carver, 1983). The gene is also an attractive mutagenesis marker since it is less than 2.5kb in length (Lowy et al., 1980) and its sequence is known (Nalbantoglu et al., 1986).

The mutagenesis assay (section B.2.2.5.) involves exposing the CHO^{D422} cells to a mutagenic agent, growing the cells in a non-selective medium for 3-6 days to allow expression of the aprt- phenotype and then plating the cells in selective medium containing AA. Any aprt- colonies can be seen after about 2-3 weeks. The experiments described below show the effect of HSV-2 RR1 and RR2 expression from the plasmids pCRR1 and pCRR2 on the mutagenesis of the CHO^{D422} aprt gene.

2.2.2. The Effect of HSV-2 Ribonucleotide Reductase Subunit Expression from the Plasmids pCRR1 and pCRR2.

Subconfluent 90mm plates of CHO^{D422} cells were transfected with the plasmids pUC13, pCRR1 and pCRR2. One plate was also co-transfected with pCRR1 and pCRR2. Ten ug of each plasmid was used for the transfections and the total amount of DNA was kept constant (20ug) using calf thymus DNA. Control plates of CHO^{D422} cells were also used, one of which was untransfected and the other was subjected to a mock-transfection procedure with no DNA. After 18 hours, the

transfection mixture was removed and the cells from each plate were split between two, 750ml flasks. The cells in one flask from each pair were grown in medium containing 75uM zinc sulphate whereas in the other flask the cells were grown in non-inducing medium. The cells were incubated at 37°C for 6 days to allow expression of the aprt- phenotype. After this time, the cells from each flask were harvested, counted and plated in AA-selective medium at a density of 1.5×10^5 cells/90mm plate. The plates were then incubated at 37°C for 2-3 weeks until aprt- colonies appeared on the plate. These were counted and the % mutation frequency was calculated. Unfortunately, the experiment was only performed once due to many problems with contamination.

The plating efficiency for each transfection was obtained by plating 100 cells in non-selective medium in two, 50mm plates and assessing the percentage survival. Effects on mutagenesis by the pCRR1 and pCRR2 plasmids were also calculated as compared to the level obtained in non-induced cells transfected with control plasmid (pUC13) DNA. The significance of the results was assessed using the statistical test outlined in section B.2.28.

Results from this experiment are shown in Table 5. Untransfected cells gave a spontaneous mutation frequency of 1.40×10^{-4} % which is much lower than the spontaneous mutation frequency seen in the pZ189 mutagenesis assay system.

Mock-transfection of the cells did not significantly raise the mutation frequency from the level seen in untransfected cells. This agrees with Brandt et al. (1987) who showed that mock transfection had no effect on mutagenesis of the hgp_rt gene.

Zinc treatment of untransfected or mock-transfected CHOD422 cells did not significantly alter the aprt mutation frequency in agreement with the pZ189 mutagenesis assay where the mutagenic frequency of the supF gene was not affected by treating the cells with zinc sulphate (section C 2.1.3.).

Cells transfected with pUC13 DNA increased the mutation frequency of the aprt gene (2.5×10^{-4} %) compared to untransfected cells. This was not a significant increase, which also agrees with results from the pZ189 mutagenesis assay.

Table 5. Effect of expression of HSV-2 RR1 and RR2 from the plasmids pCRR1 and pCRR2 under zinc-induced (+) and non-induced (-) conditions on the mutation rate of the aprt gene. CHOD422 cells were transfected with the plasmids pUC13, pCRR1 or pCRR2 or were co-transfected with pCRR1 and pCRR2 (pCRR1/pCRR2). Plates of cells were also mock-transfected (mock) or left untreated (control). They were then grown in conditions of induction (100uM zinc sulphate) or non-induction and then plated in medium supplemented with AA. The Table shows the number of cells plated, the plating efficiency of the cells, the number of AA-resistant (mutant) colonies and the % mutagenesis for each transfection. The final column shows the enhancement of each result compared to pUC13-transfected cells. ND indicates that the enhancement calculation was not done.

Transfected Plasmid(s)	Induced	Cells Plated ($\times 10^6$)	Mutant Colonies	Plating Efficiency	% Mutagenesis ($\times 10^{-4}$)	Enhancement
control	-	1.5	2	0.95	1.4	ND
control	+	1.5	2	0.78	1.7	ND
mock	-	1.5	2	0.80	1.7	ND
mock	+	1.5	2	0.70	1.9	ND
pUC13	-	1.5	3	0.81	2.5	1.0
pUC13	+	1.5	3	0.69	2.9	1.2
PCRR1	-	1.5	4	0.80	3.3	1.3
PCRR1	+	1.5	3	0.76	2.6	1.0
PCRR2	-	1.5	5	0.79	4.3	1.7
PCRR2	+	1.5	5	0.65	5.1	2.0
PCRR1/PCRR2	-	1.5	6	0.82	4.9	2.0
PCRR1/PCRR2	+	1.5	7	0.75	6.2	2.5

Neither pCRR1- nor pCRR2-transfected cells showed a statistically significant increase in the mutation frequency of the aprt gene above that observed with pUC13 transfected cells. Similarly, inducing the plasmids with 75uM zinc sulphate solution did not produce a significant effect. Cells that had been co-transfected with pCRR1 and pCRR2 (and hence had the potential ability to express HSV-2 ribonucleotide reductase activity) gave an aprt gene mutation frequency of $4.9 \times 10^{-4}\%$ in non-induced cells and $5.1 \times 10^{-4}\%$ in induced cells. These increases were also not found to be statistically significant.

The alteration in mutation frequencies produced by the plasmids expressing RR1 and RR2 in both assays is very low and was found to be insignificant in all cases. The fact, however, that the highest mutation frequencies obtained for both assays were those of cells transfected with plasmids expressing both RR1 and RR2 may indicate some effect of enzyme activity. The low level of mutagenesis may be due to the inefficiencies of the transfection procedure, and the transient expression of RR1 and RR2 may not be sufficient to have an effect on mutagenesis via dNTP pool imbalances. The cellular ribonucleotide reductase is also present in the cells to counteract any dNTP pool changes brought about by the viral enzyme. In order to overcome these problems experiments were performed to establish cell lines that stably express the viral ribonucleotide reductase genes (section C.2.3.4.). It is also possible that other virus-associated factors, not present in transfected cells, co-operate in increasing the mutation frequency in infected cells. To further study the effect of the virus ribonucleotide reductase on the mutation frequencies in infected cells the HSV-1 mutants tsl207 and tsl222, which have temperature sensitive lesions in the genes encoding RR1 and RR2 respectively, were employed.

2.3 The Effect of Stable Expression of HSV-2 Ribonucleotide Reductase on Mutagenesis.

The generation of cell lines which stably express the HSV-2 RR1 and RR2 proteins would be useful for studying the

effect of these proteins on mutagenesis. In order to achieve this, a gene (neo^R), conferring resistance to the antibiotic G418, was cloned into pCRR1, pCRR2 and pCRR3. The plasmids were transfected into cells which were selected for G418-resistance. Such cells might be expected to contain an integrated copy of the transfected plasmids, including the genes encoding RR1 and RR2, and could be screened for mutagenesis.

2.3.1. Construction of the Plasmids pCNRR1, pCNRR2 and pCNRR3.

The plasmids pCNRR1, pCNRR2 and pCNRR3 were derived from pCRR1, pCRR2 and pCRR3 by the addition of the gene encoding G418 resistance, neo^R.

The construction of pCNRR2 and pCNRR3 was straightforward since the neo^R gene was available on a HindIII fragment isolated from the plasmid p61 (obtained from Dr. J. Lang). This HindIII fragment could easily be placed into the HindIII site located downstream of the RR2 sequences in these plasmids (Figure 9) by ligating it into a HindIII restriction enzyme digest of these plasmids. In the case of pCNRR1, the HindIII site had been used to clone the IE5 termination sequences and so was unavailable. The neo^R-containing fragment was therefore isolated and its "sticky ends" were filled in prior to the ligation of EcoRI linkers. This fragment was then ligated into the EcoRI site situated immediately upstream of the RR1 sequences in pCRR1 (Figure 10).

2.3.2. The Effect of HSV-2 Ribonucleotide Reductase Expression in the aprt Mutagenesis Assay.

The expression plasmids pCNRR1 and pCNRR2 and a control plasmid, pUC13, were transfected into CHOD422 cells using the electroporation method. A further control plasmid, p61 (which contains the neo^R gene), was also used for transfection. The plasmids pCNRR1 and pCNRR2 were co-transfected in a similar manner. The transfected cells were then plated in 50mm tissue culture plates at a density of 0.5×10^5 cells/plate and allowed to recover and express the neo^R phenotype for 3 days before being plated at a density of 1.0×10^6 /50mm tissue culture plate in growth

Days exposure	Mutation frequency after exposure to zinc sulphate			
	0	1	2	3
N7	0.6	<0.5	<0.5	0.5
1/2.2	1.7	0.5	0.5	4.5
1/2.10	3.3	<0.5	<0.5	2.0

Table 6. Mutation frequency of the aprt gene in G418-resistant cell lines derived from co-transfecting CHOD422 cells with the plasmids pCNRR1 and pCNRR2 (1/2.2 and 1/2.10). The Table shows the mutation frequencies after 0, 1, 2 and 3 days exposure to 100uM zinc sulphate. The Table also gives the mutation frequencies observed with N7 (a cell line derived from the transfection of a control plasmid, p61, containing the neoR gene). All mutation frequencies are $\times 10^{-6}$.

medium supplemented with 800ug/ml G418. The cells were then incubated for 2-3 weeks until G418-resistant colonies were visible. These colonies (which might be expected to contain an integrated copy of the transfected plasmid) were then isolated and grown up individually.

Three of the G418-resistant cell lines obtained were grown up in growth medium supplemented with 800ug/ml G418. These were N7 (derived from the p61 transfection) and 1/2.2 and 1/2.10 (which were both cell lines derived from co-transfection of pCNRR1 and pCNRR2). The cells were exposed to growth medium containing 100uM zinc sulphate for periods of 0 to 3 days. After this time, they were placed in non-selective medium for 3 days to recover and express the aprt- phenotype. The cells were then plated in medium containing 0.04mM AA and allowed to form colonies over a 2-3 week period. The number of AA-resistant colonies obtained is shown in Table 6. The cell line N7 shows a mutation frequency of 0.6×10^{-4} with no exposure to zinc treatment. On exposing the cell line to zinc sulphate, no increase in the number of AA-resistant colonies was observed indicating that zinc treatment does not affect mutagenesis. This was also found in previous experiments (sections C.2.1.3. and C.2.2.2.). With cell lines 1/2.2 and 1/2.10 there was no accumulation of mutants with increased exposure to zinc sulphate and the mutation frequencies are in line with those obtained for N7 which also suggests that these G418 resistant cell lines (obtained by the co-transfection of pCNRR1 and pCNRR2) do not increase the mutation frequency of the aprt gene.

The cell lines used in this experiment were tested for RR1 and RR2 protein expression by the Western blotting procedure. Results were negative and other cell lines will be tested for the production of these proteins and the experiment repeated.

2.3.3. The Effect of the HSV-2 Ribonucleotide Reductase Expression on the Transformation of NIH3T3 Cells.

NIH3T3 cells were also used to establish cell lines which containing integrated copies of the genes encoding HSV-2 ribonucleotide reductase.

The focus forming transformation assay in NIH3T3 cells

has been used in transformation studies involving the morphological transforming regions of HSV (Galloway and McDougall, 1981; Galloway et al., 1984). Transformation is often accompanied by the presence of an activated oncogene particularly of the ras gene family (reviewed by Weinberg, 1989). The ability of transfected ras genes to become transforming in recipient cells can occur in 2 ways. The amino acid sequence may be altered by mutation in one or more codons within functional domains of the protein necessary for transformation (Reddy et al., 1982; Taparowsky et al., 1982) or the normal proto-oncogene may be over-produced (Chang et al., 1982). The activation of ras in tumour cells involves point mutations which can result in the substitution of residues 12 (Reddy et al., 1982; Taparowsky et al., 1982) or 61 (Taparowsky et al., 1983). Activation of H-ras has also been demonstrated following in vitro modification of the proto-oncogene by chemical carcinogens, yielding mutations at codons 10 or 11 (Marshall et al., 1984; Vousden et al., 1986) or 61 (Vousden et al., 1986).

The morphological transformation of NIH3T3 cells thus appears to reflect the occurrence of point mutations within oncogenes, usually of the ras gene family. Cellular mutagenesis could, therefore, be monitored by looking for this type of phenotypic change. In the NIH3T3 cell morphological (focus forming) transformation assay, normal untransformed cells can be fairly easily distinguished from transformed cells which show dense growth with cells piling up and overlapping.

Subconfluent, 50mm tissue culture plates were transfected with pCNRR2, pCNRR3 or p61. The transfection mixture was removed after 18 hours and the cells were allowed to recover and express the neo^R phenotype for 48 hours. After this time, the cells were transferred to 750ml flasks containing growth medium supplemented with G418 (800ug/ml) to select for the neo^R gene. The cells were incubated for 2-3 weeks until G418-resistant colonies appeared which might be expected to contain integrated copies of the transfected plasmids. Transfection resulted in the generation of around 200 G418-resistant colonies, for

each plasmid, which were pooled. Each set of pooled cells was then divided into two. One half was incubated in growth medium supplemented with zinc sulphate (75uM) and G418 (800ug/ml) while the other half was incubated in medium supplemented with G418 alone. The flasks were observed for the occurrence of morphologically transformed cells over a 2-3 week period.

The only population of cells that gave rise to morphological transformants was the pCNRR3-transfected population which had not been induced with zinc sulphate. This cell population gave rise to 2 transformed foci. The fact that no transformed cells were seen when the cells were induced (and might be expected to express more enzyme activity) suggests that HSV-2 ribonucleotide reductase activity does not increase the cellular mutagenic frequency.

To assess whether the G418-resistant cell lines did indeed contain the genes encoding HSV-2 RR1 and RR2, DNA was extracted from the cells and probed for the presence of these sequences using the Southern blotting procedure. DNA (10ug) extracted from cells transfected with pCNRR3 (C-RR3 cells) was digested with EcoRI (100 units) and DNA extracted from cells that had been transfected with pCNRR2 (pC-RR2) was digested with HindIII (100 units). Lambda DNA digested with HindIII was used as size markers. The separated DNA was transferred to a nitrocellulose membrane and probed for the presence of RR2 sequences using a labelled DNA probe (Figure 12). The membrane was then autoradiographed and this is shown in Figure 25.

Digestion of C-RR2 DNA with HindIII should give rise to a DNA fragment of around 5150bp if the entire plasmid was integrated into the genome. A band of this size was seen. Similarly, in the case of C-RR3 DNA, EcoRI digestion should give a band of 10300bp if the entire plasmid has integrated. Again this band was seen. The plasmids therefore appear to have integrated into the cellular genome yet do not appear to affect mutagenesis or transformation. Like the aprt- cell lines these G418-resistant cells require to be tested for RR1 and RR2 expression.

2.4. The Effect of tsl207 and tsl222 on Mutagenesis in the pZ189 Mutagenesis Assay.

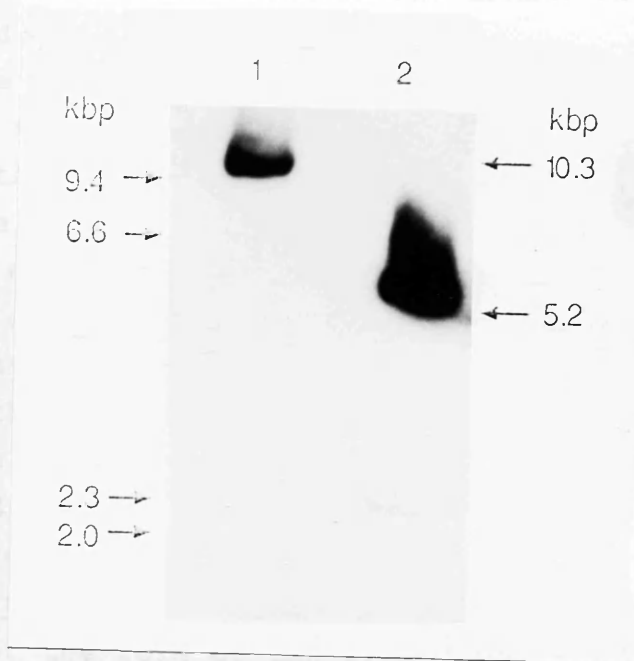


Figure 25. Detection of HSV-2 RR2 sequences in cellular DNA extracted from the cell lines C-RR3 (track 1) and C-RR2 (track 2). Ten ug of DNA from C-RR3 (digested with EcoRI) and C-RR2 (digested with HindIII) was probed with a labelled RR2 DNA probe. The sizes of the relevant DNA fragments are shown to the right of the gel and DNA size markers are on the left.

To further study the effect of HSV ribonucleotide reductase activity on mutagenesis, HSV-1 temperature sensitive mutants were used. The mutant virus tsl207 has a mutation in the gene encoding RR1 and fails to induce activity at the restricted temperature (Preston V.G. et al., 1984). The mutant phenotype is due to the substitution of an asparagine for the wt serine at RR1 position 961 (Nikas et al., in press). A comparison of the amount of enzyme activity and growth between wt HSV-1 and tsl207 is shown in Table 7. The HSV-1 mutant tsl222 has a single base pair deletion at the 3'-end of the gene encoding RR2 which alters the translational reading frame such that the codons of all but 1 of the C-terminal 15 amino acids are changed and the termination codon is removed. No enzyme activity can be detected at either 31°C (PT) or 39.5°C (NPT). The virus replicates as efficiently as wt at 31°C but is ts at 39.5°C suggesting that enzyme activity is required at high temperatures but is dispensible at lower temperatures (Preston V.G. et al., 1988, Table 7). These virus mutants have been previously discussed (section A. 3.3.2.). Use of the viruses tsl207 and tsl222 in this study allowed the assessment of the role of the viral ribonucleotide reductase in mutagenesis during infection.

For each experiment, eight, subconfluent 50mm plates of 293 cells were transfected with the plasmid pZ189. After 18 hours, the transfection mixture was removed and replaced with fresh medium. The cells were allowed to recover for 6 hours at 37°C prior to infection with the viruses tsl207, tsl222 or wt HSV-1 at 10pfu/cell. The amount of virus inoculum used was 100ul. Two plates were also mock-infected. After a sorption one plate of cells from each pair was kept at 31°C whereas the other plate was kept at 39.5°C. All medium used was also kept at the respective temperature and the plates were incubated overnight. After this time, the pZ189 plasmid was harvested and used to transform MBM7070 bacteria in the pZ189 mutagenesis assay as previously described. The experiment was repeated and the pooled results are shown in Table 8.

The results show that mock infection of 293 cells does not affect the mutation frequency of pZ189 and the level of

Virus	Temperature	% RR activity	Growth (yield after 24 hours)
wt	31°C	100	+
<u>tsl207</u>	31°C	14.7	+
<u>tsl222</u>	31°C	0	+
wt	39.5°C	80	+
<u>tsl207</u>	39.5°C	0	-
<u>tsl222</u>	39.5°C	0	-

Table 7. Ribonucleotide reductase activity and growth of tsl207 and tsl222 as compared to wt HSV-1 at 31 and 39.5°C (taken from Preston V. G. et al., 1984, 1988).

Virus	Temp. °C	Colonies counted	Whites	% mutagenesis	Increase
mock	31	8912	4	0.05	1.0
mock	39.5	4640	2	0.05	1.0
wt HSV-1	31	5101	17	0.34	6.8
wt HSV-1	39.5	4020	13	0.33	6.6
<u>tsl207</u>	31	1972	6	0.31	6.2
<u>tsl207</u>	39.5	2559	8	0.31	6.2
<u>tsl222</u>	31	3374	10	0.31	6.2
<u>tsl222</u>	39.5	2581	8	0.33	6.6

Table 8. Effect of wt HSV-1 and HSV-1 mutant viruses tsl207 and tsl222 on the mutagenesis of the supF gene in the pZ189 mutagenesis assay. The effect was studied in 293 cells at temperatures 31°C (PT) and 39.5°C (NPT). The Table shows the pooled results from two experiments and lists the total number of colonies counted, the number of white colonies obtained and the % mutagenesis. The final column shows the increase of each result compared to mock infected cells at 31°C.

mutation (0.05%) is roughly the same as for untreated cells (0.04%, section C.2.1.3.). HSV-1 infection at 31°C had the effect of increasing the mutation frequency of pZ189 to 0.34% (around 7-fold). This was statistically different at a 1% level of significance. Raising the temperature to 39.5°C did not alter the increase in mutagenesis seen in HSV-infected cells, giving a mutagenic frequency of 0.33%.

The HSV-1 mutant tsl207 increases the mutation frequency of the supF gene to 0.31% at 31°C and 39.5°C. These figures are not significantly different either from each other or from those obtained with wt virus.

Similar results were obtained with the mutant tsl222. At 31°C, this virus increases the mutation frequency of the supF gene to 0.31% at 31°C and 0.33% at 39.5°C. As with tsl207, these figures are not significantly different from each other or from those obtained with wt HSV-1. These results suggest expression of that HSV ribonucleotide reductase is not mutagenic in infected cells.

2.5. Discussion II.

2.5.1. A Comparison of the pZ189 and the aprt Mutagenesis Assays.

The pZ189 assay, studies the mutagenic frequency of a marker gene, the supF gene, located on the shuttle vector plasmid pZ189 whereas in the aprt mutagenesis assay, the aprt marker gene is chromosomal. This difference in location of the marker gene is reflected in the different spontaneous mutation frequencies of the two assay systems. In the aprt mutagenesis assay, the spontaneous mutation frequency is $1.4 \times 10^{-4}\%$ which is much lower than the spontaneous mutation frequency of the supF gene in the pZ189 assay (0.04%). This could be because pZ189 will be subject to mutations that are acquired by plasmid DNA entering cells by the transfection procedure (Calos *et al.*, 1983; Razzaque *et al.*, 1983, 1984; Lebkowski *et al.*, 1984; section C.2.1.1.). Also, the plasmid may be affected by the phenomenon that transfection of plasmid DNA is mutagenic via some mechanism of plasmid-induced DNA damage (Lau *et al.*, 1982). Plasmids are capable of integrating into the host genome (Kucherlapati, 1982) and may induce DNA endonucleolytic cleavage. Finally,

the marker gene in pZ189 may be being replicated more rapidly than the cellular marker gene. A higher rate of replication might be expected to increase the mutation frequency if mutations are occurring during replication (misincorporations) or simply because a mutation is being replicated.

Results for the effect of pUC13 transfection on mutagenesis are similar for the two systems with an increase in mutagenesis of 1.5-fold compared to the spontaneous level which was not found to be significant (section C.2.1.3.).

The two mutagenesis assays thus appear comparable in their response to transfected plasmids although they differ in the spontaneous mutation frequency of their marker genes.

2.5.2. The Effect of Expression of HSV-2 RR1 and RR2 on Mutagenesis in Transfection Experiments.

Both the pZ189 and the aprt mutagenesis assays show similar results with the transient expression of HSV-2 RR1 and RR2 from transfected plasmids. Transfection of plasmids containing the genes for HSV-2 RR1 or RR2 under the control of an inducible promoter did not significantly increase the mutation frequency of the supF gene in the pZ189 mutagenesis assay as compared to the mutation frequency obtained from transfection of the control plasmid. A significant increase in the mutation frequency of the aprt gene in the aprt assay was also not found. Induction of the RR1 or RR2 genes had no effect in either assay.

Induction of individual plasmids did not affect the mutation frequency in transfected cells as would be expected if enzyme activity was the cause of an increase in mutagenesis in HSV-infected cells and there is no evidence to suggest that the individual virus enzyme subunits can interact with the cellular M1 and M2 to produce activity. Also, even if this were possible, only RR1 would be expected to alter the mutation frequency according to the proposed mutation model of dNTP pool imbalances since it is the cellular M1 that is responsible for allosteric control of the cellular enzyme. Possibly, expression of RR1 alone in cells could affect dNTP pool sizes and hence cause mutagenesis as this subunit has the ability to bind dNDPs but the results presented here would not support that idea.

Transient expression of both RR1 and RR2 together using the pZ189 and the aprt mutagenesis assays enzyme does not significantly increase the level of mutation from that obtained with a control plasmid. Zinc-induction of the RR1 and RR2 genes also has no effect using these assays. This suggests that enzyme activity is not causing the mutagenic effect.

Although the experimental results were not significant in both the pZ189 and the aprt mutagenesis assays the highest mutation frequencies obtained were those in induced cells expressing both RR1 and RR2. These cells would be expected to have the highest levels of HSV-2 ribonucleotide reductase activity and it could be argued that the lack of a statistically significant result might be due to the inefficiencies of the transfection procedure and the transient nature of the assays. The cellular enzyme will also be present and may counteract any changes in dNTP pool sizes brought about by the viral enzyme. To avoid the problem of the inefficiency of transient transfection procedures CHO and NIH3T3 cells were transfected with the plasmids pCNRR1 and pCNRR2 (section C.2.3.1.). These plasmids contain a G418 resistance marker and allow transfected cells to be selected for integration of plasmid DNA.

The G418-resistant cell lines generated by the transfection of pCNRR2 and pCNRR3 into NIH3T3 cells did not show an increased level of mutagenesis even though these lines contained integrated plasmid sequences. Similarly, G418-resistant cell lines generated by co-transfection of the plasmids pCNRR1 and pCNRR2 did not appear to alter the mutation frequency of the aprt gene with or without exposure to zinc sulphate. The cell lines generated in both these experiments, however, may not express RR1 or RR2, hence conclusions as to the effect of prolonged enzyme expression on mutagenesis require that further cell lines be checked for expression and the experiment repeated.

The fact that the plasmid constructs do not cause mutagenesis is interesting irrespective of HSV ribonucleotide reductase activity since the plasmid pCRR1 (and pCKRR1) contains the 486TF (Jones *et al.*, 1986, section A.2.2.3.) and the putative kinase activity of the N-terminal

domain of RR1 (Chung et al., 1989, section A.3.3.5.) both of which have been implicated as having a possible role in transformation. The 486TF contains putative stem-loop structures and one proposed method of transformation was mutagenesis resulting from the fragment integrating into the genome in a similar manner to an insertion sequence. Subject to the sensitivity of the assays, results presented here would not support the suggestion that transformation brought about by the 486TF is due to random insertion into a cellular gene. The possibility that the 486TF integrates into the cellular genome at some other, more specific locus, is not, however, ruled out. The other putative stem-loop structure in the HSV-2 genome associated with transformation (737TF from BglIIIn, Galloway et al., 1984, section A.2.2.3.) has also been used in mutagenesis studies; this fragment did not increase the mutagenic frequency of a cellular marker hgprt gene (Brandt et al., 1987).

2.5.3. The Effect of wt HSV-1, tsl207 and tsl222 Infection on Mutagenesis in the pZ189 Mutagenesis Assay.

Infection of 293 cells with HSV-1 increases the mutation frequency of the supF gene in the pZ189 mutagenesis assay by around 7-fold. This figure is in agreement with other studies. Pilon et al. (1985) found that infection of non-permissive cells with HSV-2 lead to an increase in the mutation frequency of the hgprt gene of 2.5-10-fold and Hwang and Shillitoe (1990) showed that HSV-1 infection leads to an increase in mutation rate of 3.6-fold using the pZ189 mutagenesis assay.

The HSV-1 mutant tsl207, at 39.5°C and 31°C, shows the same effect on mutagenesis as the wt virus. As the levels of mutagenesis obtained with this virus are similar at the NPT (when the virus is not expressing ribonucleotide reductase activity) and at the PT (where the virus is expressing enzyme activity) this suggests that HSV-1 ribonucleotide reductase activity does not represent the mutagenic agent being assayed here. This suggestion is supported by the results obtained with tsl222. Again, no difference in mutagenesis is seen either at 39.5°C or 31°C compared to the wt virus. It is interesting to note that at 39.5°C the ts viruses do not grow (Preston V. G. et al., 1988; Preston

V.G. et al., 1984) and there is still a mutagenic effect suggesting that viral replication is not required for this process. This is supported by results from previous studies with UV-inactivated HSV-1 which was found to increase the mutagenesis of a cellular (Schelofner and zur Hausen, 1982) and a plasmid borne (Hwang and Shillitoe, 1990) marker gene.

3. EXPERIMENTS TO DETERMINE THE MUTAGENIC FACTOR IN HSV- INFECTED CELLS.

To identify the mutagenic function of HSV in infected cells, a number of experiments were performed which are described below. The experiments involve studying the mutagenic effect caused by HSV-1 and a range of HSV-1 mutant viruses, UV-inactivated HSV-1 and incomplete HSV-1 virus particles.

3.1. The Growth of HSV-1 in 293 Cells.

This experiment was performed as the growth rate of HSV-1 in 293 cells is not well documented. A one-step growth curve of HSV-1 would allow the time that the mutagenic effect is seen to be compared with the state of virus infection at that time.

Twentyfour, 30mm tissue culture plates were seeded with 5×10^5 293 cells/plate and incubated overnight at 37°C. The plates were then infected with 5 pfu/cell of HSV-1 in an inoculum of 100ul. The virus was allowed to adsorb for 1 hour before the inoculum was removed. The plates were washed with medium containing no serum, overlaid with 1.5ml growth medium and incubated at 31°C.

At 0, 2, 4, 6, 8, 12, 18 and 24 hours post-infection the virus was harvested from 2 plates by scraping the cells into the medium, dispensing into black-capped vials and sonicating for 1 minute. The samples were titrated on BHK monolayers and a one-step growth curve was established (Figure 26a). A similar one-step growth curve for HSV-1 in BHK cells was also constructed. From the two growth curves, it is apparent that the growth of HSV-1 is somewhat slower in 293 cells than in BHK cells, however, after 24 hours the yield is roughly the same with around a 15-20 fold increase in virus titre after 24 hours.

At each time point, protein was also harvested from a plate of cells by removing the growth medium, washing the cells with PBS and adding BM (0.5ml/plate). After 10-15 minutes the BM/cell mixture was transferred to a black-capped vial and incubated in a boiling water bath for 3 minutes. The samples were then run on a protein gel,

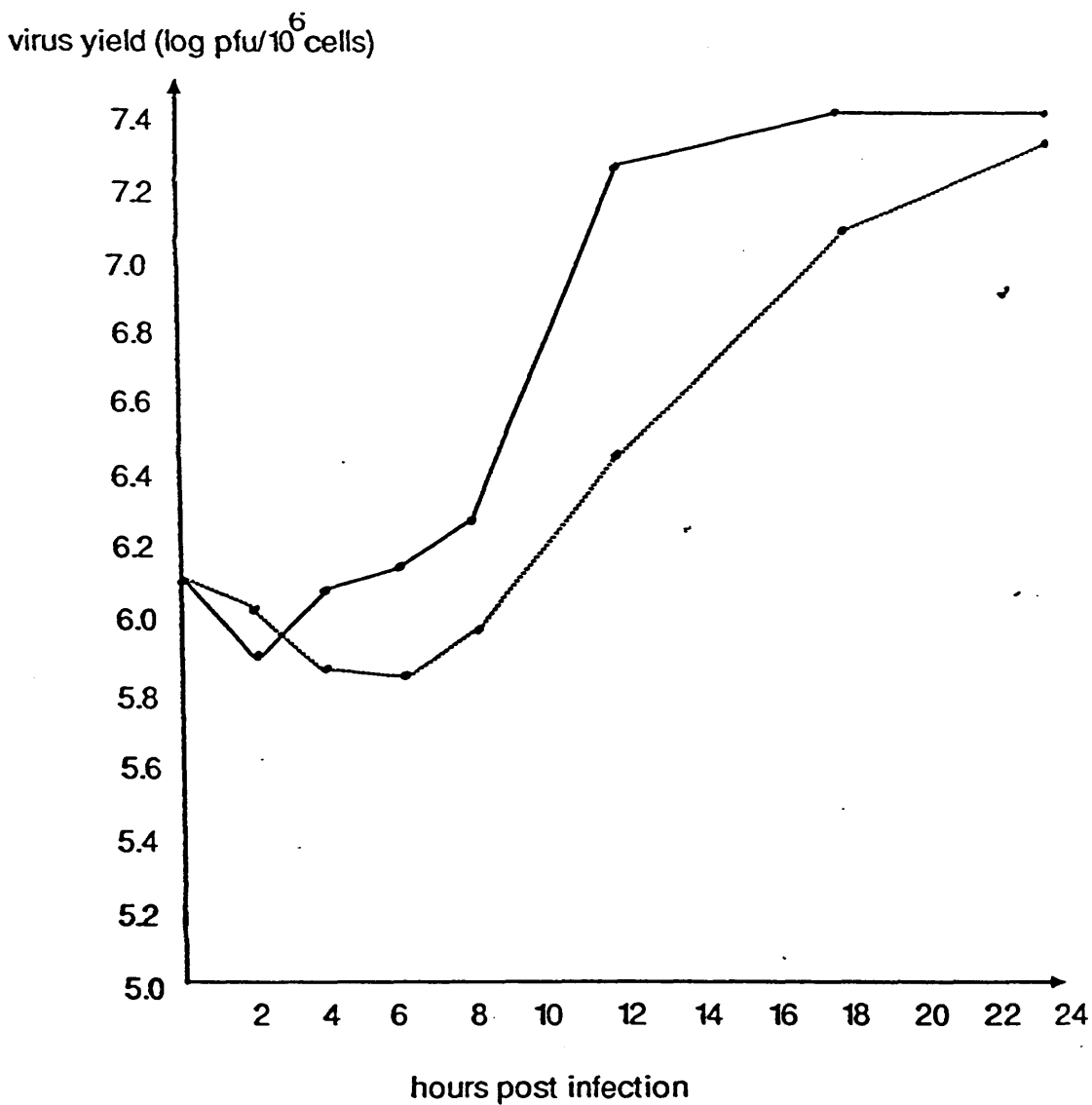


Figure 26. (a) Single step growth curve of HSV-1 in 293 (—) and BHK (---) cells infected with 5pfu/cell.

24 18 12 8 6 4 2 0 M

RR1

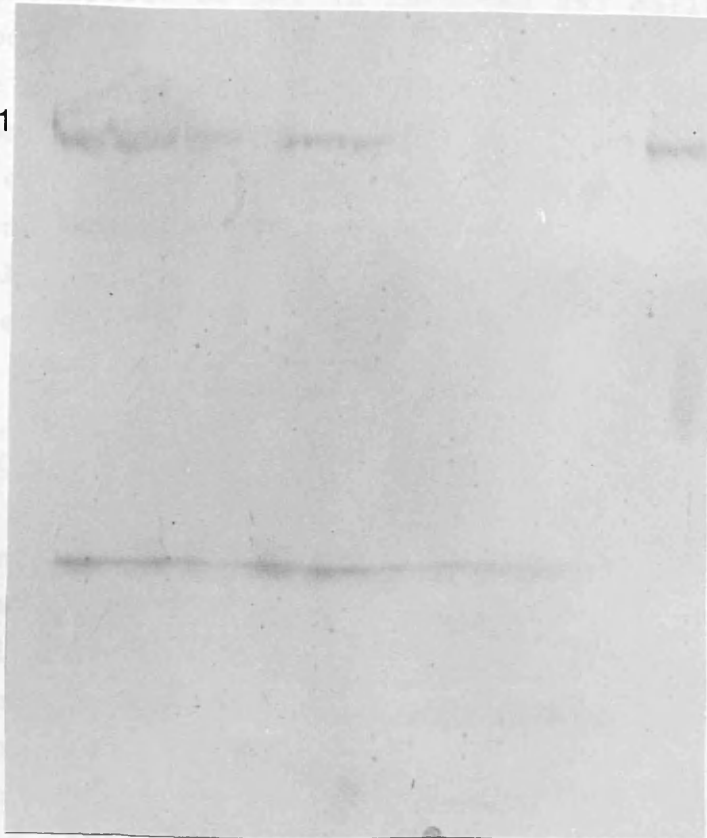


Figure 26 (b). Production of RR1 in HSV-1 infected 293 cells. The tracks labelled 0, 2, 4, 6, 8, 12, 18 and 24 indicate the number of hours post infection that the extracts were made. The track labelled M contains a positive control. The antibody used was 20208.

transferred to a nitrocellulose membrane by the Western blotting technique and probed for the presence of RR1 using the antibody 20208 (provided by Dr. H. Lankinen). The resulting membrane is shown in Figure 26b.

The production of RR1 in infected 293 cells is apparent 4 hours post-infection and its level gradually increases until the final time point at 24 hours post-infection. RR1 has predominantly early kinetics in infected cells and so would be expected to reach maximum levels 4-6 hours post-infection. However, the slower growth rate of the virus in 293 cells would account for the somewhat delayed appearance of RR1.

3.2. Time Course of the Mutagenic Effect of HSV-1 in Infected Cells.

Subconfluent 50mm tissue culture plates of 293 cells were transfected with pZ189. After 18 hours, the transfection mixture was removed and replaced with fresh medium. The cells were allowed to recover for 6 hours before half of the plates were infected with 10pfu/cell of HSV-1 in 100ul inoculum. The rest of the plates were left uninfected as controls. After ^badsorption for 1 hour, the inoculum was removed and replaced with growth medium and the cells were incubated at 37°C. At time points 0, 2, 4, 6, 8, 12, 18 and 24 hours post-infection, the pZ189 plasmid was harvested from one infected and one non-infected plate of cells and assayed as described previously. The results are shown in Table 9 and graphically in Figure 27.

The mutagenic frequency of the plasmid pZ189 in infected cells increases above the level in uninfected cells almost immediately after infection. The level of mutagenesis becomes constant after 8 hours. From the previous experiment, it was established that the production of RR1 (a predominantly E protein) is apparent 4 hours post-infection and so the mutagenic effect seems to be occurring around, or slightly before, the time of E gene expression. This is in agreement with the results from the experiments with tsl207- and tsl222-infected cells (section C.2.4) where the mutagenic effect is still present at the NPT when there is no viral replication and suggests that the mutagenic effect

Hours post Infection	Infected	Colonies	Whites	% Mutagenesis
0	HSV-1	5010	2	0.04
	mock	2996	2	0.05
2	HSV-1	2222	2	0.09
	mock	1670	1	0.06
4	HSV-1	2043	3	0.15
	mock	6664	2	0.03
6	HSV-1	1108	2	0.18
	mock	2004	1	0.05
8	HSV-1	1572	3	0.19
	mock	4286	3	0.07
12	HSV-1	1768	3	0.17
	mock	3330	2	0.06
18	HSV-1	2100	4	0.19
	mock	4996	2	0.04
24	HSV-1	1996	4	0.20
	mock	2012	1	0.05

Table 9. Time course of the mutation frequency of the supF gene in the pZ189 mutagenesis assay performed in HSV-1 infected 293 cells. The Table shows the times post infection at which the plasmid extracts were taken, the total number of colonies counted in the assay, the number of white (mutant) colonies and the % mutagenesis at each time point for HSV-1 and mock-infected cells.

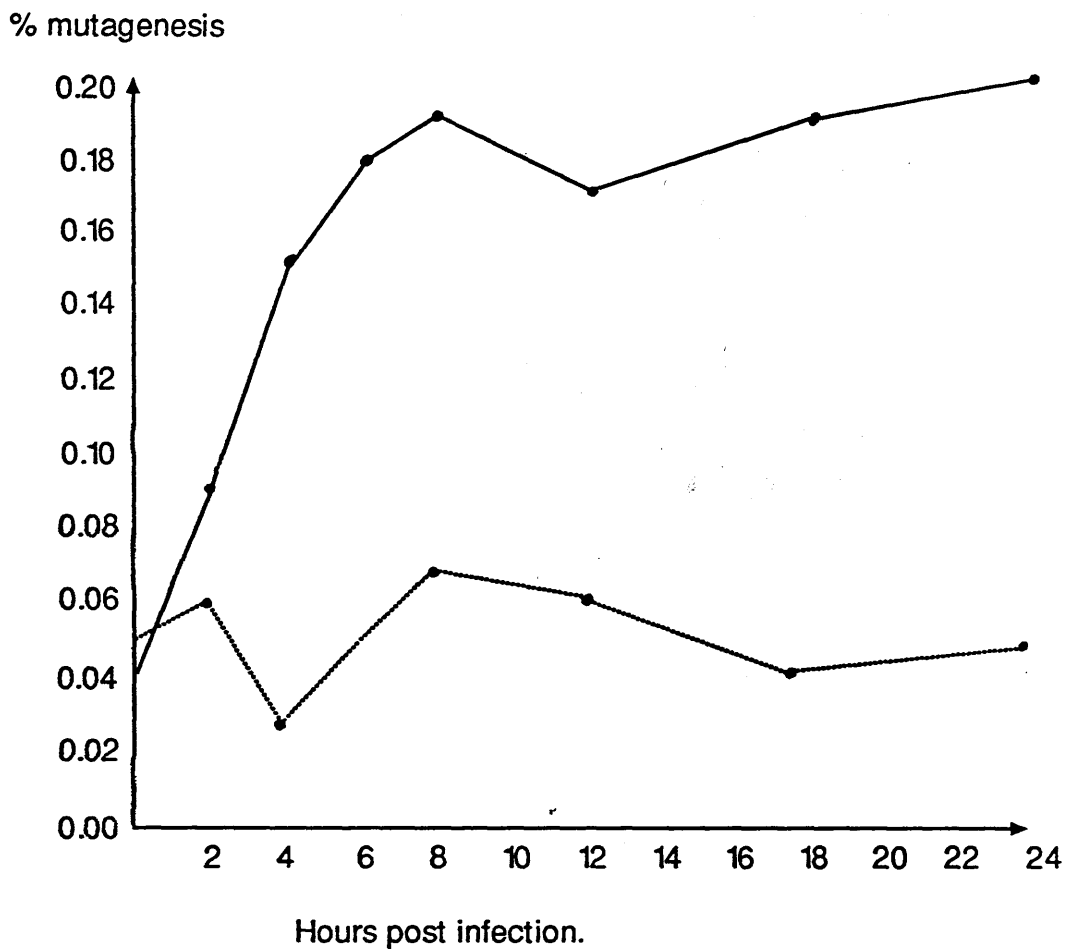


Figure 27. Effect of HSV-1 infection on the mutagenesis of the supF gene in the pZ189 mutagenesis assay. The graph shows the increase in mutagenesis with respect to time.

— HSV-1 infected cells
 mock infected cells

may be caused by a structural component or an E or IE gene function. Results of Hwang and Shillitoe (1990) also suggest that the mutagenic effect occurs around this time (2-4 hours post-infection) in COS-1 cells.

3.3. The Effect of HSV-1 Mutants and Incomplete Viruses on the Mutagenic Frequency of pZ189 in Infected Cells.

Use of HSV mutant viruses provides a further means of examining the effect of specific viral genes on mutagenesis. From the experiments described so far, the mutagenic function appears to act early in infection. Mutant viruses used in an attempt to localize this effect are described below.

(i) tsK : The virus tsK has a ts lesion in Vmw175 and produces only four functional IE polypeptides Vmw110, Vmw68, Vmw63 and Vmw12 as well as a non-functional Vmw175, and Vmw136 (Marsden et al., 1976) at the NPT.

(ii) d11403 : This virus contains a 2kb deletion within both the TR_L and IR_L copies of the Vmw110 gene. The deletion is manifest primarily at low moi and can be overcome by increasing the virus dose (Stow and Stow, 1986).

(iii) in1814 : This virus contains a 12bp insertion in the gene encoding the transinducing factor Vmw65. The insertion abolishes the ability of Vmw65 to induce IE gene expression at low multiplicities of infection and in vivo. At high multiplicities of infection the function appears to be redundant (Ace et al., 1989).

(iv) ts1204 : The virus ts1204 contains a single mutation that affects the ability of the mutant to penetrate tissue culture cells and to assemble functional capsids at the NPT (Addison et al., 1984). Analysis of virus induced polypeptides in ts1204 infected cells grown at the NPT gives results similar to those of mock-infected cells except that some inhibition of host polypeptide synthesis is observed which could be caused by a virion polypeptide (Fenwick and Walker, 1979).

(v) L-particles : These are incomplete virus particles that contain virus envelope and tegument proteins but no capsid proteins or DNA. Preparations of L-particles contain L-particles and complete virions in a ratio of 500:1 whereas

in normal virus preparations a ratio of around 1:1 is seen.

(vi) UV-inactivated virus : Virus particles were UV-inactivated according to a method suggested by Dr. C. M. Preston which results in no activation of E gene promoters i.e. no expression of IE gene products.

Subconfluent, 50mm tissue culture plates of 293 cells were transfected with 10ug of the plasmid pZ189. After 18 hours, the transfection mixture was removed and replaced with fresh growth medium. The cells were allowed to recover for 6 hours before being infected with virus. Two plates of cells were infected with the viruses HSV-1, HSV-2, UV-inactivated HSV-1, tsK, tsl204, d11403, in1814 and the incomplete virus L-particles. Two plates were mock-infected as negative controls. One of each of the plates infected (10pfu/cell) with Ad5, HSV-1, HSV-2, tsK, and tsl204 and one of the plates that had been mock-infected were incubated at 31°C after adsorption and the other plates were incubated at 39.5°C. All medium used was also kept at the relevant temperature. In the case of the virus mutants in1814 and d11403, one plate was infected with a high moi (10pfu/cell) and the other plate was infected with a low moi (0.5pfu/cell). Control plates were also set up with HSV-1 infection at low and high moi. Cells infected with L-particles and UV-inactivated HSV-1 were incubated at 31°C only. Cells infected with L-particles were infected with 10 particles per cell and cells infected with UV-inactivated HSV-1 were infected with virus that had been 10pfu/cell before UV-inactivation. After 12 hours, pZ189 was harvested from the cells and assayed as before. The experiment was repeated and the combined results are shown in Table 10. The significance of the results, compared to those obtained with mock-infected cells, was calculated using the statistical analysis outlined in section B.2.28.

Mock-infection of 293 cells has no effect on the mutagenesis of the supF marker gene in the pZ189 mutagenesis assay at either 31°C or 39.5°C with the level of mutagenesis obtained being the same as for uninfected cells.

Results for infection of the virus tsl204 also show that the procedure of infection does not produce the mutagenic

Table 10. Effect of infection with HSV-1, HSV-2 and various HSV-1 mutants and incomplete virions, under different conditions of infection, on the mutagenic rate of the supF gene in the pZ198 mutagenesis assay performed in 293 cells. The Table shows the pooled results from two experiments and lists the total number of colonies counted, the number of white (mutant) colonies and the % mutagenesis. The final column shows the increase in mutagenesis compared to mock-infected cells at 31°C.

		EXPERIMENT 1		EXPERIMENT 2	
Virus	Conditions of infect.	Colonies counted	Whites	Colonies counted	Whites
mock	31°C	2684	2	3142	1
	39.5°C	3188	1	4140	2
HSV-1	31°C	1008	2	1384	5
	39.5°C	1222	4	436	1
HSV-2	31°C	1874	6	934	3
	39.5°C	1752	4	1368	5
tsK	31°C	984	2	431	2
	39.5°C	1564	4	506	2
HSV-1/UV	31°C	996	2	846	3
ts1204	31°C	1228	4	1426	3
	39.5°C	2456	1	4420	2
L-particles	31°C	2854	3	2910	2
HSV-1	Low moi	778	1	565	2
in1814	Low moi	1668	3	990	3
	High moi	620	2	1628	4
dl1403	Low moi	686	0	728	3
	High moi	2014	5	764	3

Virus	Conditions of infection	Colonies counted	Whites	Mutagenesis (%)	Increase
mock	31°C	5826	3	0.05	-
mock	39.5°C	7328	3	0.04	0.8
HSV-1	31°C	2392	7	0.30	6.0
HSV-1	39.5°C	1658	5	0.30	6.0
HSV-2	31°C	2808	9	0.32	6.4
HSV-2	39.5°C	3120	9	0.29	5.8
<u>tsK</u>	31°C	1415	4	0.29	5.8
<u>tsK</u>	39.5°C	2070	6	0.29	5.8
UV- inactivated	31°C	1842	5	0.27	5.4
<u>tsl204</u>	31°C	2654	7	0.27	5.4
<u>tsl204</u>	39.5°C	6676	3	0.05	1.0
L-particles	31°C	5764	5	0.09	1.8
HSV-1	low moi	1314	3	0.23	4.6
<u>inl814</u>	low moi	2658	6	0.20	4.0
<u>inl814</u>	high moi	2248	6	0.27	5.4
<u>dll403</u>	low moi	1414	3	0.21	4.2
dll403	high moi	2778	8	0.29	5.8

effect seen in infected cells since at the NPT, when the virus does not enter the cell, the mutagenic frequency of the supF gene is the same as for mock-infected and uninfected cells at 0.05%. At the PT, when infection proceeds as normal, the mutation frequency is raised to 0.27% which is significantly different from the value seen in mock-infected cells and is similar to the figure obtained on HSV-1 infection (see below).

Both HSV-1 and HSV-2 have the same effect on mutagenesis in infected 293 cells, increasing the mutagenic frequency of the supF gene to around 0.3% which is 6-fold higher and significantly different from the spontaneous mutation frequency of the plasmid in mock-infected cells. This is slightly lower than the increase in mutagenesis seen in cells infected with adenovirus type 5 (0.4%). The mutagenic effect seen with HSV-1 and HSV-2 is the same at 31°C and 39.5°C. Infecting the cells with a low moi reduces the mutation frequency (to 0.23%) from that found in cells infected with a high moi (0.31%) although this was not found to be a significant reduction.

The HSV-1 mutant tsK was as mutagenic as wt HSV-1 virus at 31°C (PT) and at 39.5°C (NPT), significantly raising the mutation frequency from the level found in mock-infected cells to 0.29%. This indicates that expression of the genes Vmw110, Vmw68, Vmw63 and Vmw12 is enough to cause the mutagenic effect.

The effect can be further characterized using the mutants d11403 and in1814. At high moi, these viruses show a level of mutagenesis similar to that obtained with wt HSV-1 (0.29 with d11403 and 0.27% with in1814). At low moi, when these viruses exhibit their mutant phenotype, the mutation frequency is reduced from that seen at high moi (to 0.21% for d11403 and 0.27% with in1814). The differences between the mutation frequencies seen in cells infected with a high or low moi of d11403 or in1814 are not significant and the reduction observed at low moi is similar to the reduction observed when the moi of HSV-1 is similarly lowered. Thus, when the lower moi has been taken into account these viruses seem to be equally mutagenic in 293 cells both at low and high moi.

The effect of UV-inactivated HSV-1 also appears to be

similar to that observed with fully active virus, the mutagenic frequency of the supF gene in infected cells being 0.27%, which is significantly different from the value obtained with mock-infected cells.

L-particles are not as mutagenic as wt HSV-1 and do not significantly increase the mutation frequency in infected cells (0.09%) from that found in mock-infected cells (0.05%). A slight increase is seen which may be caused by the small proportion of complete virions in the L-particle preparations.

3.4. The Effect of HSV DNA on Mutagenesis.

The results described above suggest that viral gene expression is not required for the mutagenic effect. The introduction of HSV DNA into cells might itself be mutagenic. This possibility was investigated by transfecting viral DNA into 293 cells alongside pZ189 and observing the mutation frequency of the supF mutagenesis marker gene.

Six, subconfluent 50mm tissue culture plates of 293 cells were co-transfected with 5ug of HSV-1 DNA and 5ug of pZ189 plasmid DNA. Two, plates of cells were transfected with intact viral DNA, another 2 with BamHI-cleaved DNA and the remaining plates with XbaI-cleaved DNA. Two plates of 293 cells were also transfected with pZ189 alone. The cells were incubated overnight at 37°C before the transfection mixture was removed and replaced with fresh growth medium. The cells were then incubated for a further 24 hours at 37°C before pZ189 was harvested from 1 plate of cells from each transfection, digested with DpnI and used to transform MBM7070 bacteria. The resulting blue and white colonies were counted. The remaining cells were incubated at 37°C for a further 48 hours in order to establish whether or not the transfected DNA was infectious. The experiment was repeated and the combined results are shown in Table 11. The results were assessed for their significance using the statistical test described in section B.2.28.

HSV-1 DNA is mutagenic in transfected cells. Uncleaved HSV-1 DNA significantly increases the mutation frequency of the supF marker gene to 0.25% from the spontaneous mutation frequency of 0.06%.

Transfected DNA	Colonies counted	Whites	% Mutagenesis	Increase
HSV-1+pZ189	3492	8	0.23	4.6
HSV-1(BamHI) +pZ189	3032	8	0.26	5.2
HSV-1(XbaI) +pZ189	2256	5	0.22	4.4
pZ189	5834	3	0.05	-

Table 11. Effect of transfection of intact HSV-1 DNA and HSV-1 DNA cleaved with BamHI (Bam) and XbaI (Xba) on the mutagenesis of the supF gene in the pZ189 mutagenesis assay. The Table shows the pooled results from two experiments and lists the total number of colonies counted, the number of white (mutant) colonies and the % mutagenesis. Also shown is the increase in mutagenesis shown by each transfection compared to the spontaneous mutation frequency of pZ189.

Transfected DNA	Infectious
HSV-1	+
HSV-1 (BamHI)	-
HSV-1 (XbaI)	-

Table 12. The effect of transfection of intact HSV-1 DNA and HSV-1 DNA cleaved with BamHI and XbaI on infection in FG cells. Extensive CPE after 48 hours is indicated by (+) and no CPE by (-).

HSV-1 DNA cleaved with BamHI or XbaI, similarly increases the mutation frequency to 0.28% and 0.23% respectively. These cleaved DNAs were not found to be infectious whereas the intact viral DNA produced extensive CPE after 48 hours (Table 12).

3.5. Determination of the Type of Mutations occurring following HSV-1 Infection.

To establish the nature of the mutations induced by HSV-1 in the supF gene of pZ189^(Seidman et al. 1985), restriction enzyme digests were performed on mutant plasmid DNA obtained in the pZ189 mutagenesis assay.

Mutant (white) colonies arising in the assay were picked and retransformed into MBM7070 bacteria to check their phenotype. Overnight cultures were then set up. DNA was harvested from these using a large scale preparation method. The plasmid DNAs were then digested with the enzymes BamHI and HindIII before being electrophoresed on an agarose gel. An example of such a gel is shown in Figure 28. The mutant plasmid DNAs were run alongside a wt plasmid that had been similarly digested, and DNA marker digests. The DNA marker digests used were pAT (digested with HinfI) and lambda DNA (digested with EcoRI and HindIII). This method of analysis allowed the detection of large structural changes and rearrangements in the plasmids. The results showed that roughly 30% (7 out of 20) of mutations occurring in pZ189 following the use of HSV-1 in the pZ189 mutagenesis assay were due to such changes which is somewhat lower than the figure of 56% that has been reported by Hwang and Shillitoe (1990). *

To determine the nature of the mutations in the supF gene of plasmids that did not show altered gel mobility, future work will include the sequencing of these DNAs. In an attempt to initiate this work an 850bp EcoRI/BglII fragment containing the supF gene of wt pZ189 has been sequenced and found to be in agreement with the known sequence (M. Seidman, personal communication).

3.6. Discussion III.

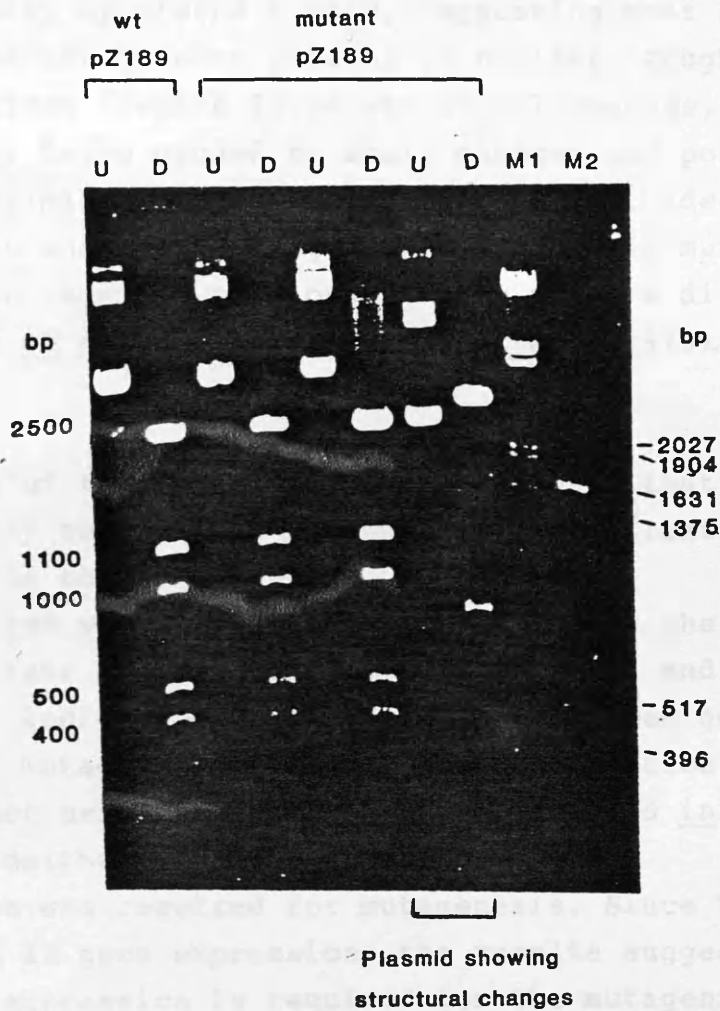


Figure 28. An agarose gel showing Bam HI/Hind III digests of the plasmid pZ189 isolated from white (mutant) and blue (wt) colonies obtained in the pZ189 mutagenesis assay following HSV-1 infection. The gel shows digested (D) and undigested (U) DNA from 3 mutant and 1 wt plasmid. The tracks labelled M1 and M2 show the positions of DNA markers. The sizes of the predicted fragments from Bam HI/Hind III digestion of wt pZ189 are marked (on the left), as are the sizes of the DNA markers (on the right).

The experiments described in this section attempt to identify the mutagenic factor in HSV-infection. Both HSV-1 and HSV-2 are equally mutagenic in cells, raising the spontaneous mutation frequency of the supF gene in the pZ189 mutagenesis assay by around 6-fold, suggesting that the mechanism of action of both viruses is similar. Roughly 30% of these mutations involve large structural changes, the rest presumably being caused by small changes and point mutations. Preliminary results also suggest that adenovirus is mutagenic in the pZ189 mutagenesis assay. This mutagenic effect has been shown in previous studies using a different assay (Marengo et al., 1981; Paraskeva et al., 1975).

The timing of the mutagenic effect suggests that an IE or E protein may be involved and that viral replication is not required for the effect.

The virus tsK was mutagenic at the PT and at the NPT, when only the four IE genes Vmw110, Vmw68, Vmw63 and Vmw12 are expressed, indicating that expression of these genes is sufficient for mutagenesis. Further characterization of the mutagenic effect using the mutants d11403 and in1814 revealed that neither Vmw110 expression or Vmw65 transactivation was required for mutagenesis. Since Vmw65 transactivates IE gene expression, the results suggest that no viral gene expression is required for the mutagenic effect. Results using UV-inactivated virus support this, as UV-inactivated virus, which does not express IE proteins (Dr. C. Preston, personal communication), is mutagenic.

Use of the virus ts1204, which fails to penetrate cells at the NPT, shows that penetration is required for mutagenesis and so the mutagenic effect appears to be part of the incoming virion, perhaps a structural component or the viral DNA itself. L-particles, which do not contain viral DNA or capsid proteins were found to be significantly less mutagenic than wt virus suggesting that the mutagenic effect may be caused by one of these components.

Both intact and cleaved, non-infectious HSV-1 DNA is capable of increasing the mutagenic frequency in transfected cells. This finding along with the observation that viral gene expression is not required raise the possibility that

the viral DNA itself may be the mutagenic agent. The mechanism by which HSV DNA could induce mutations is discussed in section D.1.1.

No attempt has been made in this study to assess the effect of capsid proteins on mutagenesis and further work might include such studies.

It would also be useful to assay for mutagenesis after transfection with restriction fragments of the HSV genome in order to try to localize the mutagenic effect.

4. THE GENERATION OF TRANSGENIC MICE CONTAINING THE GENES ENCODING RR1 AND RR2.

The production of transgenic animals provides a further way to study the effect of the HSV-2 ribonucleotide reductase genes in eukaryotic cells. Transgenic animals provide a good system for the expression of exogenous genes since the introduced DNA is stably integrated into the host cell genome (Brinster *et al.*, 1981; Constantini and Lacey, 1981; Gordon and Ruddle, 1981) and as integration normally occurs at the one-cell stage (Constantini and Lacey, 1981) all the cells should contain copies of the microinjected sequence. Transgenic animals containing the genes encoding HSV-2 RR1 and RR2 would also allow the effect of these genes to be assessed in an animal model system.

4.1. Generation of Transgenic Lines 47-8 and 58-1.

Eggs from two C57xCBA hybrid female mice (47 and 58) were microinjected with a KpnI/HindIII fragment isolated from pCRR3. This fragment contains both the genes encoding the HSV-2 enzyme ribonucleotide reductase (Figure 29). The gene encoding RR1 is under the control of the mouse MT-1 promoter whereas the gene encoding RR2 is under the control of its own HSV-2 promoter.

4.1.1. Transgenic Line 47-8.

Microinjection of eggs from mouse 47 resulted in the production of 11 offspring. Three of these mice were found to contain the HSV-2 ribonucleotide reductase genes by preliminary screening by Dr. P. F. Searle, however, only 1 transferred the genes to the next generation. This female mouse was termed 47-8. Mouse 47-8 was mated with a C57xCBA hybrid male resulting in 8 offspring, 4 of which were positive for the RR1 and RR2 genes. Unfortunately the 47-8 transgenic mother died after giving birth and attempts to foster the offspring were only successful with 2 of the litter. The surviving 2 mice, one of which was positive for the presence of the viral reductase genes, were mated together producing offspring of which 1 in 2 were transgenic.

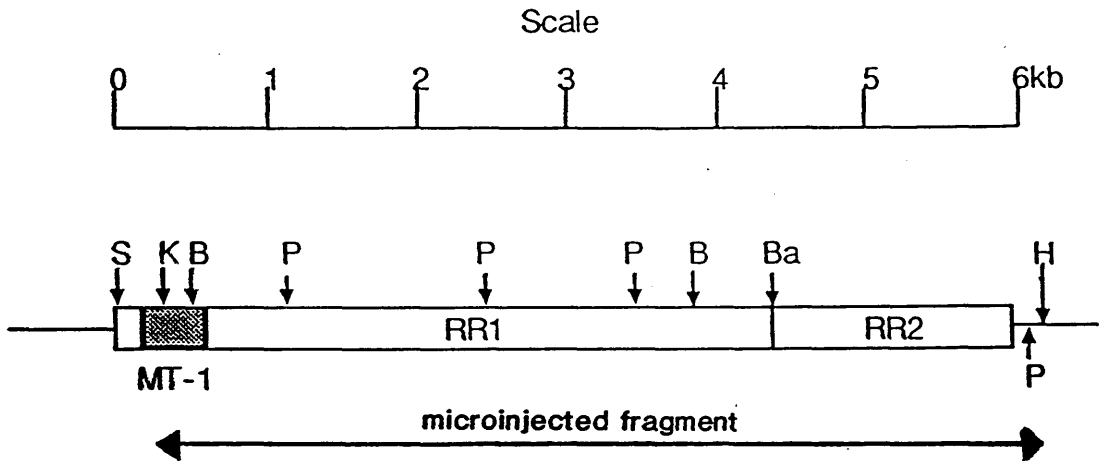



Figure 29. Diagram of the microinjected fragment containing the HSV-2 RR1 and RR2 genes with RR1 being under the control of the MT-1 promoter () The restriction enzyme sites for SstI (S), KpnI (K), BglIII (B), PstI (P), BamHI (Ba) and HindIII (H) are marked.

4.1.2. Transgenic Line 58-1.

Microinjection of eggs from mouse 58 resulted in the production of 3 offspring, 1 of which was positive for the HSV-2 ribonucleotide reductase genes. This positive male mouse was mated with a C57xCBA female and half the offspring from this mating were transgenic for the genes encoding RR1 and RR2.

4.2. Detection of HSV-2 Ribonucleotide Reductase ^{transcription} in Transgenic Lines 47-8 and 58-1.

Two mice that had been found positive on preliminary screening for the presence of HSV-2 ribonucleotide reductase sequences (47-8.29 and 58-1.27) were subjected to an injection of zinc sulphate (0.1ml of a 3mM ZnSO₄ solution/10g of mouse) and were then left for a period of 6 hours. This treatment should stimulate ^{transcription} of the RR1 gene from the MT-1 promoter (Dr. P. Searle, personal communication). The mice were then sacrificed and the tissues were removed and homogenized in 4mls RNazol. RNA was then prepared by the RNazol method. Isolated RNA was treated with DNase in order to remove any contaminating DNA.

DNA was also prepared from tail, kidney and liver tissue in order to check that the mice were indeed positive for the viral genes and so that the integrity of the genes could be studied in more detail since only slot-blot analysis had been performed.

4.2.1. Detection of HSV-2 Ribonucleotide Reductase DNA in Transgenic Lines 47-8 and 58-1.

Three separate aliquots of 10ug of DNA prepared from tail tissue of mice that had been identified as containing HSV-2 ribonucleotide reductase sequences (47-8.29 and 58-1.27) were cut with 100 units of the restriction enzymes PstI, BamHI and BglII/BamHI. The digests were incubated at 37°C overnight. The DNA was then separated on a 0.7% agarose gel alongside BamHI-digested samples from a non-transgenic control mouse. Four aliquots of 10ug of control tail tissue DNA were cut with BamHI and to these, unlabelled RR2-probe DNA was added after digestion so that a control sample

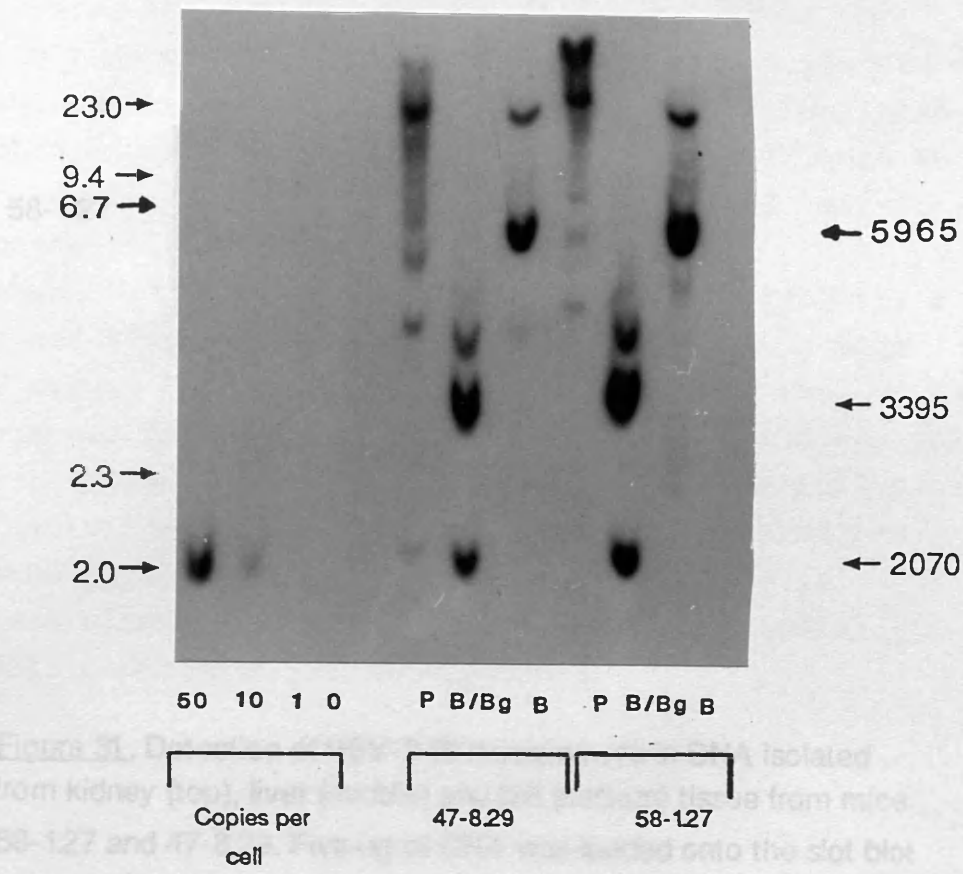


Figure 30. Detection of HSV-2 RR sequences in cellular DNA extracted from the tails of transgenic mice 58-127 and 47-8.29. The tracks show 10ug of DNA digested with the restriction enzymes PstI (P), BamHI (B) and BamHI/BglIII (B/Bg) and probed with DNA probes isolated from the RR1 and RR2 genes (Figure 12) The sizes of the relevant fragments (bp) are marked to the right of the gel and DNA size markers (kb) are marked to the left. The 4 left-most tracks show DNA from a control mouse digested with BamHI and "spiked" with RR2 probe DNA equivalent to 0, 1, 10 and 50 copies per cell.

contained probe DNA approximately 10⁵ copies/cell. A marker probe was also tested with HindIII and was found to be present in all samples. After running the gel the DNA was transferred to a nitrocellulose membrane by the standard blotting procedure. The resulting membrane was probed with the labeled DNA probes.

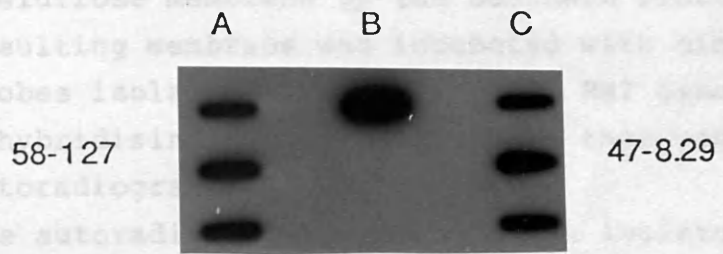


Figure 31. Detection of HSV-2 RR1 sequences in DNA isolated from kidney (top), liver (middle) and tail (bottom) tissue from mice 58-127 and 47-8.29. Five ug of DNA was loaded onto the slot blot apparatus and screened for the presence of RR1 sequences with a DNA probe (Figure 12). Column B contains 0.005ug of probe sequence DNA.

The gel shows that the PstI assay did not detect any copies of the gene in any of the tissues tested with this assay. The gel also shows dilutions of probe DNA representing 1, 10 and 50 copies/cell showing an estimation of the number of copies of the RR1 gene present in the tissues tested. These results appear to be consistent with a copy number of greater than 50 copies per cell.

4.2.2. Are the RR1 gene sequences present in all the tissues?

contained probe DNA equivalent to 50, 10, 1 and 0 copies/cell. A marker track containing lambda DNA digested with HindIII was run alongside the experimental samples. After running the gel the DNA was transferred to a nitrocellulose membrane by the Southern blotting procedure. The resulting membrane was incubated with nick-translated DNA probes isolated from the RR1 and RR2 genes (Figure 12) under hybridizing conditions. It was then washed, air-dried and autoradiographed (Figure 30).

The autoradiograph shows that DNA isolated from the mice 58-1.27 and 47-8.29 and cleaved with BamHI gives a major band of around 6000bp. This band corresponds to the microinjected fragment (5965bp) containing the RR1 and RR2 genes. The appearance of a band representing the full size of the microinjected fragment suggests that the DNA has integrated in a head to tail configuration which is consistent with the results of other groups (Brinster et al., 1981; Constatini and Lacey, 1981).

Cutting the DNA with BamHI and BglIII ^h could give 3 bands assuming that the DNA has integrated in a head-to-tail configuration, 1 band of 3395bp, 1 of 2070bp and 1 of 500bp. The 2 larger bands from this digest are present on the autoradiograph. The smaller band is not present and would have run off the bottom of this gel.

The gel shows that the PstI enzyme did not cut properly and so no conclusions can be drawn from the digest with this enzyme.

The gel also shows dilutions of probe DNA representing 0, 1, 10 and 50 copies/cell allowing an estimation of the number of copies of the HSV-2 ribonucleotide reductase sequences present per cell in the transgenic mice. These genes appear to be present in a copy number of greater than 50 copies per cell.

4.2.2. Are the HSV-2 Ribonucleotide Reductase Sequences Present in all the Mouse Tissues?

To check that HSV-2 ribonucleotide reductase DNA was present in all the mouse tissues, 5ug of the DNA prepared from liver, kidney and tail was loaded onto a slot blot apparatus and screened for the presence of the RR1 gene using a nick-translated DNA probe as before. The resulting membrane (Figure 31) shows that HSV-2 DNA appears to be present in all the tissues screened. This was to be expected since the microinjected DNA generally integrates at the one-cell stage (Constantini and Lacey, 1981) and so all the cells of the mouse would be expected to contain HSV-2 ribonucleotide reductase sequences.

4.2.3. Detection of HSV-2 Ribonucleotide Reductase RNA in Transgenic Lines 47-8 and 58-1.

Five ug of RNA isolated from two mice that had been shown to contain HSV-2 ribonucleotide reductase DNA sequences (47-8.29 and 58-1.27, see above) and subjected to injections of zinc sulphate (0.1ml of a 3mM ZnSO₄ solution/10g of mouse), were bound to a nitrocellulose membrane using a slot blot apparatus. RNA isolated from a mouse that contained the HSV-2 sequences but which had not been subjected to zinc-induction (58-1.15) and RNA isolated from a mouse that had not been microinjected (control) was also bound to the nitrocellulose membrane. In all the mice, RNA was isolated from a number of tissues. In order to ensure that any hybridization was due to RNA and not to any contaminating DNA, 5ug of each isolated RNA was treated with RNase A before loading onto the slot blot kit. The membrane was hybridized overnight to a nick-translated DNA probe isolated from the HSV-2 RR1 gene (Figure 12). It was then washed, air dried and autoradiographed (Figure 32). The experiment was repeated using a probe isolated from RR2 (Figure 33).

The results for the expression of RR1 RNA in the transgenic mice show that RR1 RNA can be detected in all the tissues screened from mice that contain the HSV-2 ribonucleotide reductase sequences. The amount of RR1 RNA appears to be the same whether or not the animals are subjected to zinc-induction even though the RR1 gene is under the control of the MT-1 promoter. No HSV-2 RR1 RNA is detected in the mice that did not contain HSV-2 sequences.

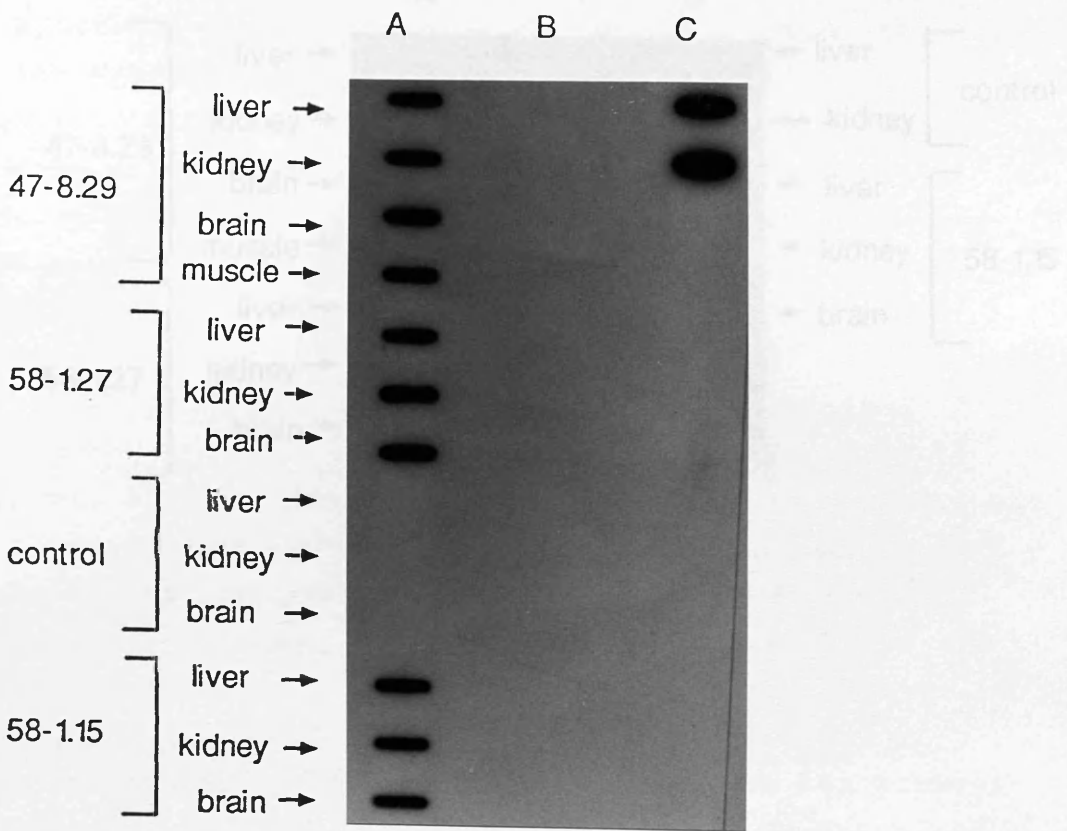


Figure 32. Detection of HSV-2 RR1 RNA in RNA isolated from the transgenic mice 58-1.27, 58-1.15 and 47-8.29. Mice 58-1.27 and 47-8.29 were treated with zinc sulphate before the DNA was extracted whereas mouse 58-1.15 had not been similarly treated. Five μ g of RNA isolated from the tissues indicated was loaded onto the slot blot apparatus (column A) and probed with a DNA probe (Figure 12) RNA extracted from a control mouse is also shown. Column B shows identical samples treated with RNase before being loaded. Column C shows control mouse RNA "spiked" with 0.0025 and 0.005 μ g of probe DNA.

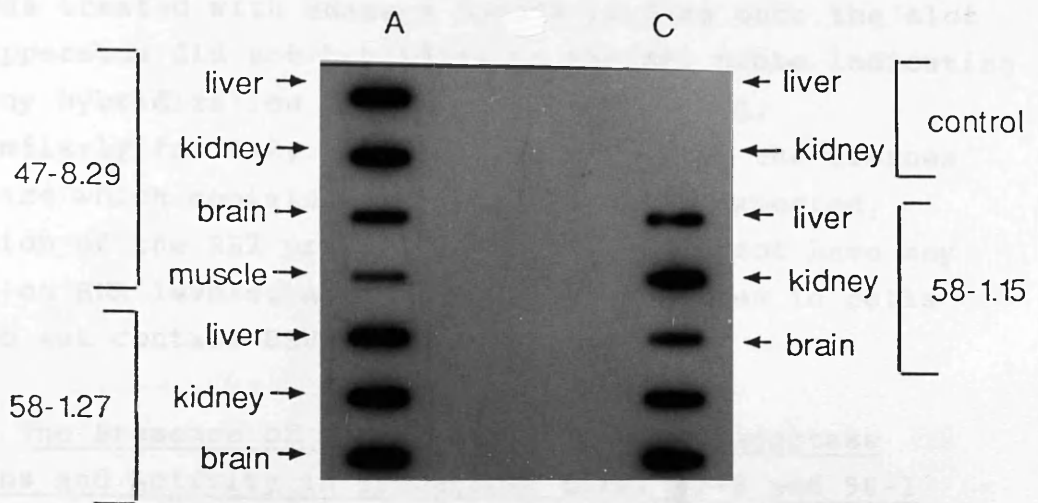


Figure 33 Detection of HSV-2 RR2 RNA in RNA isolated from the transgenic mice 58-1.27, 58-1.15 and 47-8.29. Mice 58-1.27 and 47-8.29 were treated with zinc sulphate before the RNA was extracted whereas mouse 58-1.15 had not been similarly treated. Five ug of RNA from the tissues indicated was loaded onto the slot blot apparatus (columns A and C) and probed with an RR2 DNA probe (figure 12). RNA from a control mouse is also shown. The 2 unlabelled slots show control mouse RNA "spiked" with probe DNA.

There was some variation in the amount of HSV RNA in the extracts loaded but this may be due to some form of differential expression or ^{to different efficiencies of RNA} isolation between tissues. RNA that was treated with RNase A before loading onto the slot blot apparatus did not hybridize to the RR1 probe indicating that any hybridization occurring was due to RNA.

Similarly for RR2, RNA is apparent in all the tissues from mice which contain HSV-2 sequences. As expected, induction of the RR2 promoter with zinc does not have any effect on RNA levels. Again, no RR2 RNA is seen in cells that do not contain HSV-2 sequences.

4.2.4. The Presence of HSV-2 Ribonucleotide Reductase Proteins and Activity in Transgenic Lines 47-8 and 58-1

Liver, kidney, intestine and brains from 3 mice that had been shown to contain the HSV-2 ribonucleotide reductase sequences were homogenized in 1ml BM using a Wheaton 1ml Dounce tissue grinder. The mice were offspring of the transgenic mouse 58-1. One mouse (58-1.55), which was positive for ribonucleotide reductase DNA, had been subjected to zinc treatment for 8 hours before its tissues were removed, another mouse (58-1.3) which also contained these sequences had not been treated with zinc and the final mouse did not contain HSV-2 DNA.

The samples were boiled and stored at -70°C before separating the protein on a gel. The proteins were transferred to a nitrocellulose membrane using the Western blotting technique, incubated with antibodies directed against RR1 (20208) and RR2 (14995) and visualized by the HRP method.

The resulting membrane did not show the presence of HSV-2 RR1 or RR2 in any of the samples. Ribonucleotide reductase activity assays, performed on partially purified extracts from tissues, were also negative.

4.3. Discussion IV.

The results in this section describe the generation of two lines of transgenic mice, 47-8 and 58-1, which contain the genes encoding the HSV-2 proteins RR1 and RR2. The HSV-2 sequences are present at over 50 copies per cell and are

* Many problems were encountered in the generation of transgenic mice. The litter sizes were smaller than normal and some of the offspring had developmental abnormalities. It is possible that the HSV ribonucleotide reductase sequences are causing problems in the developing embryos and it may be that the mice that have survived the microinjection may have survived because the reductase genes were not being expressed.

integrated in a head-to-tail array which is consistent with studies involving other microinjected sequences (Brinster et al., 1981; Constantini and Lacey, 1981). The transgenic mice transmit the RR1 and RR2 genes to their offspring in the expected ratio for a dominant gene, ie. one out of two offspring contains the HSV-2 sequences if one parent is transgenic. The DNA is contained in all tissues of the transgenic mice indicating that it integrated at the one-cell stage which is normal for microinjected sequences (Constantini and Lacey, 1981).

RNA is present in all the screened tissues in the transgenic mice. The amount of RNA present does not appear to be affected by induction with zinc even though the RR1 gene is under the control of the MT-1 promoter. This may be because integration into the genome affects stimulation of the MT-1 promoter rendering it insensitive to induction by zinc.

Although RNA appears to be present in the mouse tissues no evidence of protein expression is apparent either by screening cell extracts with antibodies or by activity assays. This could be because the protein is not being made in enough quantity to be detected by these methods (detection of protein may not be as sensitive as detection of RNA) or that the protein is not being made at all due to some block in the translational process of the ribonucleotide reductase RNAs. This lack of protein expression in transgenic animals that express RNA has also been seen with papilloma virus sequences and it has been suggested that translation may not occur due to the lack of introns within the virus genes (Dr. P. Searle, personal communication). If protein is being expressed, be it in small amounts, this appears to have little effect on the mice. *

Although there is some doubt about the expression of the RR1 and RR2 proteins in the transgenic mice, the presence of HSV-2 ribonucleotide reductase DNA is not in dispute. This DNA contains the 486TF which has been shown to cause transformation of tissue culture cells (Jariwalla et al., 1986) and the region encoding the N-terminal domain of RR1 which may have protein kinase activity (Chung et al., 1989), neither of which appears to cause ill-effects in the mice.

SECTION D: FINAL DISCUSSION.1.1. The Mutagenic Effect of HSV-Infection.

Many tumour viruses can also act as mutagens. This has been determined by studying the frequency of ^{mutations in} the cellular hgp_{rt} gene in both SV40- (Marshak et al., 1975) and adenovirus- (Marengo et al., 1981; Paraskeva et al., 1983) infected cells. In addition, mutations in loci encoding glutamine and methionine synthesis and drug resistance have been reported after infection with SV40 (Marshak et al., 1975; Thiele et al., 1976; Varshaver et al., 1977). The DNA viruses VZV (Aula, 1963), adenovirus type 12 (Stich et al., 1964) and papillomavirus (Prunerias et al., 1974) have also been shown to induce chromosomal aberrations in infected cells.

HSV has been found to cause chromosomal breaks (Hampar and Ellison 1963; Stich et al., 1964), and amplification (Schlehofer et al., 1983) and rearrangement (Bejcek and Conley, 1986) of chromosomal DNA, as well as increasing the frequency of ^{mutations in} cellular- and plasmid-based marker genes by around 2-10-fold (Schlehofer and zur Hausen, 1982; Pilon et al., 1985; Hwang and Shillitoe, 1990; section A.2.2.5.). Such mutagenic effects can all be produced by chemical carcinogens which lead to the proposal that many mutagens also function as carcinogens (McCann et al., 1975). HSV-2 may have a role in the development of cervical cancer and certain ^{regions} of both the HSV-1 and HSV-2 genomes can transform tissue culture cells (section A.2.) thus the study of HSV mutagenesis is important, since it may reveal a mechanism of transformation.

The mechanism by which HSV causes cellular mutations is unknown, however, its occurrence early in infection (Hwang and Shillitoe, 1990, section C.3.2.), implies that a structural component, or the expression of an IE or E protein may be involved. There is some evidence of a mutagenic peptide which is encoded within HSV-1 MTRI (Shillitoe et al., 1986, section A.2.2.5.), and which may have a counterpart in HSV-2 MTRII (Das and Shillitoe, 1990), however, further work is needed to determine the significance of this.

E proteins, particularly those involved in DNA metabolism, are also candidates for causing mutations in infected cells (section A.2.2.5.) and the work presented here studies the role of the HSV-2 enzyme ribonucleotide reductase in this process.

The HSV-2 enzyme ribonucleotide reductase was first linked with transformation after subfragments of the HindIII a region, which spans the RR1 and RR2 genes, were found to be transforming in tissue culture cells (section A.2.2.3.). Further work on this ^{region} of the genome, however, located two transforming regions, MTRII and MTRIII (section A 2.2.3.), neither of which is capable of encoding the entire enzyme. The minimal transforming regions of MTRII and MTRIII have now been shown to be two small regions of DNA which have the potential to form stem-loop structures and thus resemble insertion sequences. The first of these, 486TF (Jones et al., 1976), maps within UL39 (which encodes RR1, Nikas, 1989) and the second, 737TF (Galloway et al., 1984), maps downstream of UL40 (which encodes RR2, Preston V. G. et al., 1988). The fact that these two regions of DNA are transforming but are unable to encode the ribonucleotide reductase proteins suggests that transformation of tissue culture cells does not require enzyme activity. However, the possibility that another mechanism of transformation exists, involving HSV-2 ribonucleotide reductase activity, is still under discussion (Dr. R. J. Jariwalla, personal communication).

HSV-2 ribonucleotide reductase activity has the potential to cause mutations within cells since it does not appear to be subject to the tight allosteric control shown by its cellular counterpart (Averett et al., 1983, 1984) and may play a role in the destabilization of dNTP pools in HSV-infected cells (Jamieson and Bjursell, 1976). Such imbalances in dNTP pools have been shown to be mutagenic in both prokaryotic (Weymouth and Loeb, 1978; Fersht, 1979; Hibner and Alberts, 1980) and eukaryotic (Kunkel et al., 1982) systems.

The initial experiments described in this thesis were designed to evaluate the role of the HSV-2 enzyme ribonucleotide reductase in the increased mutagenic frequency seen in infected cells. Plasmids expressing HSV-2

RR1 and RR2 under the control of the inducible MT-1 promoter were not found to significantly increase the ^{frequency of mutations} ~~mutagenicity~~ of marker genes in the mutagenesis assays used (section C.2.). Results from the transfection experiments were consistently negative when the plasmids were transfected separately (when no activity would be expected) or together (when activity would be expected) under both non-induced and induced conditions.

Also presented, are results assessing the mutagenic frequency of a marker gene in cells infected with the HSV-1 mutant viruses tsl207 (Preston V. G. et al., 1984) and tsl222 (Preston V. G. et al., 1988), which have

lesions in RR1 and RR2 respectively, and which do not express enzyme activity at the NPT. These viruses were found to be as mutagenic as wt HSV-1 at both the PT and the NPT (section C.2.4.).

These two sets of data imply that ribonucleotide reductase activity is not the cause of increased mutagenesis in HSV-infected cells.

In the transfection experiments referred to above, the highest mutation frequencies were obtained in induced cells that had been co-transfected with plasmids expressing RR1 and RR2 in both the pZ189 and the aprt mutagenesis assays. These conditions would be expected to give the highest levels of enzyme activity. The lack of statistical significance of this result might be explained by the inefficiencies of the transfection procedure, the low level of induction from the MT-1 promoter and the presence in the transfected cell of an endogenous ribonucleotide reductase activity (section C.2.2.2.), and it may be that a greater level of expression may indeed have some effect on mutagenesis. Caras et al. (1988) showed that a plasmid expressing a mutant M1 subunit that was lacking in allosteric control conveyed a mutator phenotype when transfected into mammalian cells. In this case, however, cells were primarily selected for the stable expression of a second plasmid-based gene which would indicate the integration of the plasmid into the host cell DNA. These cells would be expected to give higher levels of expression than those obtained using a transient transfection procedure. In order to increase the amount of viral

ribonucleotide reductase expression CHO D422 and NIH3T3 cells were transfected with the plasmids pCNRR1 and pCNRR2 (section C.2.2.1.) which contained the G418-resistance gene neoR. These cells were selected for resistance to G418 which would imply integration of the plasmid. The selected cells, did not show increased mutagenesis, however, they were later found not to express the viral ribonucleotide reductase proteins and more lines will now be tested before the experiments are repeated.

The possibility still remains that HSV ribonucleotide reductase activity might raise the ^{of mutations} frequency in transfected cells although it does not appear to be required to induce the mutator phenotype seen in HSV-infected cells, since the viruses tsl207 and tsl222, which do not express this enzyme at the NPT, are mutagenic (section C.2.4.).

It is interesting to see that cells transfected with sequences encoding RRL which encompass both the 486TF (Jones et al., 1986; section A.2.2.3.) and the region encoding the putative kinase activity (Chung et al., 1989), do not show an increase in ^{mutation rate} and transgenic mice containing these sequences do not show any ill-effects. These regions have been implicated as having a role in transformation. The 486bp fragment contains a number of repetitive DNA elements which have been implicated in homologous recombination, destabilization of chromatin structure and promoter/enhancer function (Jones et al., 1986, section A.2.2.3.). It can also act as a promoter for the bacterial CAT gene and it was suggested that the 486TF might act to alter the expression of cellular genes by insertion of cis-acting regulatory elements into the cellular genome (Jones, 1989). Transformation caused by other DNA viruses often appears to involve interaction with cellular oncogenes and anti-oncogenes (section A.2.1.) and two cellular genes, p53 and c-H-ras, were subsequently found to be differentially expressed in cells transformed by the 486TF DNA fragment (Jones et al., 1989). It is ^{possible} that their increase in expression was due to some effect of insertion and/or enhancement by the 486TF. The fact that this sequence does not appear to cause mutagenesis is similar to the results obtained by Brandt et al. (1987) who showed that the 737bp transforming region (Galloway et al., 1984, section

A.2.2.3.) was not mutagenic in transfected cells. These transforming fragments have the potential to form putative stem-loop structures, thus resembling insertion sequences, and may be capable of insertion into the cellular genome (Galloway *et al.*, 1984). Such integration may not, however, occur in a random manner, in which case the increase in frequency of ^{mutations in} marker genes would be no indication of integration of the fragment at a specific site elsewhere in the genome.

The evidence that the 486bp and the 737bp transforming DNA fragments are similar to insertion sequences is slim, only involving the fact that they have the potential to form stem-loop structures and the possibility that they can integrate into the cellular genome in a similar manner to these DNA elements. There is no evidence that they are mobile or that they encode a transposing function, which is the normal requirement for such DNA elements. It is possible that the transposing function may be supplied by other regions of the HSV or cellular genome, but there is no evidence for this.

Since ribonucleotide reductase activity appears not to be the mutagenic factor in HSV-infected cells, experiments were performed to determine the nature of this effect. The mutagenic effect was found to occur early in infection (section C.3.2.) in agreement with the results of Hwang and Shillitoe (1990), and experiments using a range of mutant viruses (tsK, inl814 and dll403) suggested that no virus gene expression was required (section C.3.3.). This observation is consistent with the finding that UV-inactivated HSV is also mutagenic (Hwang and Shillitoe, 1990; Schlehofer and zur Hausen, 1982; section C.3.3.).

Increased mutagenesis does, however, appear to require virus penetration since tsl204 is not mutagenic at the NPT when it does not enter cells (section C.3.3.). Infection with HSV-1 L-particles, which do not contain viral DNA or capsid proteins (Dr. F. Rixon, personal communication, section C.3.3.), are also not mutagenic implying that the mutagenic agent is caused by one of these factors (section C.3.3.). The observation that both intact, and non-infectious, cleaved DNA was found to be mutagenic in transfected cells suggests that it may be HSV DNA that is

the mutagenic agent (section C.3.4.). Experiments using HSV capsid proteins to try to increase cellular mutagenesis were not performed and it would be worthwhile to try this in future studies.

HSV DNA could cause mutations in host cell DNA by the integration of small regions of DNA into the cellular genome which may disrupt the integrity of cellular genes. Small DNA fragments might easily go undetected using the Southern blotting analysis of Cameron et al. (1985) who was unable to find stably integrated sequences in the genome of transformed cells. Such DNA fragments might also contain promoter/enhancer elements which could affect the expression of cellular genes. Studies by Pilon et al. (1986) and Hwang and Shillitoe (1990), however, suggest that the majority of mutations in the hgprt gene within HSV-2 infected cells are due to point mutations rather than integration of viral DNA sequences. The same effect is also apparent with adenovirus type 2-induced mutagenesis (Raphs et al., 1982). These observations point to a mechanism of mutagenesis seemingly independent of the integration of specific viral sequences and may explain why many viruses appear to be mutagenic.

An alternative mutational mechanism might involve the induction of cellular DNA repair. Several investigators have debated whether HSV has single stranded nicks or gaps in its DNA (Kieff et al., 1971; Wilkie, 1973; Gordin et al., 1973, Hirsch and Vonka 1974) which could act as sites of DNA repair. Kolber (1975) observed repair replication of viral DNA in an in vitro system containing nuclei isolated from HSV-2 infected human cells, Hyman et al. (1977) described the in vitro repair of isolated HSV-1 DNA and Nishiyama and Rapp (1981) found that native DNA which accumulated in infected cells was repairable without requiring incision implying that it did contain nicks or gaps and was thus capable of inducing cellular DNA repair. Some repair synthesis of cellular DNA has also been detected in HSV-2 infected human cells under conditions restrictive for viral DNA replication (Kucera and Edwards, 1979). This appeared to occur early in infection (Nishiyama and Rapp, 1981) and by 14 hours post-infection cells suffer extensive strand breaking. These results are consistent with those of Hwang and Shillitoe (1990) who showed that HSV infection produced

mainly point mutations early in infection but that larger mutations, including insertions and deletions, were seen at later times. Most types of repairable DNA damage is both mutagenic and carcinogenic (Hanawalt et al., 1979) and hence the mutagenic effect of HSV DNA could act to promote transformation of cells giving rise to alterations that eventually result in tumour development.

1.2. The Involvement of HSV and HPV in Cervical Cancer.

A variety of clinical observations and epidemiological studies have suggested that several factors and/or sexually transmitted infections could play a role in the development of cervical cancer (Kessler, 1986). In particular, the viruses, HSV-2 and HPV (types 16 and 18) have been associated with this neoplasm.

Many studies have shown that regions of the HSV-2 genome can immortalize primary rat cells and transform immortalized cells in culture (section A.2.2.3.) and in vitro studies have also indicated that HPV may promote immortalization of primary cell cultures (Pirisi et al., 1987) or neoplastic transformation of already initiated cells (Yasomoto et al., 1986). Additionally, as described earlier (section A.2.2.1.), HPV16 and HPV18 have been detected in a high proportion of cervical cancer biopsies (Durst et al., 1983; Schull et al., 1985; Macnab et al., 1986; Murdoch et al., 1988) and in the majority of these cases HPV DNA is integrated into the cell genome (Schneider-Gadicke and Schwarz, 1986). Integration appears to involve different regions of the genome but the E6 and E7 ORFs never seem to be disrupted (Lehn et al., 1988). The viral E6 and E7 proteins are selectively retained and expressed in cervical cancer derived cell lines (Androphy et al., 1987; Banks et al., 1987) and transformation of primary rodent epithelial cells requires a functional E7 gene (Phelps et al., 1988; Story et al., 1988, 1990; Crook et al., 1988). The E7 gene has also been shown to interact with pRB (section A.2.2.1.). Thus, HPV seems to transform cells in a similar manner to other DNA tumour viruses (section A.2.1.) in that its DNA is integrated into the genome and there is evidence for a transforming protein. In the case of HSV-2, although the

BglII_n fragment has been detected in a small proportion (10%) of tumours (Galloway and McDougall, 1983, Park et al., 1983; Macnab et al., 1985b, 1986; Prakesh et al., 1985; Di Luca et al., 1989), there is no evidence of a transforming protein. In one study (Di Luca et al., 1989), HSV-2 RRI was detected in around 50% of cervical carcinoma tissues but this protein has not been shown to be required to cause any form of transformation and was used mainly as a marker for the presence of BamHI e sequences since protein detection appeared to be more sensitive than DNA detection.

The fact that HPV sequences are present in a high proportion of cervical cancer biopsies and the evidence of a transforming protein makes this virus a likely candidate for cervical cell transformation, however, the high prevalence (around 50%) of HPV sequences in normal cervical tissue (Tidy et al., 1988) suggests that other factors must be involved. The observation that HPV specific RNA is only detected in a proportion of the ne^opl^astic tissues containing HPV DNA agrees with this suggestion and lead Lehn et al. (1988) to postulate that HPV may only induce neoplastic transformation by synergistic interaction with other carcinogens including radiation, cigarette smoking and other oncogenic viruses.

The proposal that HPV-infection works as a promoter of cervical carcinogenesis and that among the initiating factors herpes simplex virus plays a key role was also suggested by zur Hausen (1982). Evidence that this could indeed be the case was obtained by Iwasaka et al. (1988). They showed that HPV-16 or HPV-18 DNA transfected into Syrian hamster embryo cells produced morphologically transformed foci but that these were not tumorigenic in nude mice whereas transfection of AE cells (obtained by transfection of the immortalizing sequences of HSV-2 DNA), however, produced morphologically transformed foci that were tumorigenic. Additionally, examination of cervical carcinoma tissues for both HPV and HSV DNA revealed that all the genital neoplasias that contained HSV-2 BamHI_e or BglII_n sequences (around 15%) were also positive for HPV DNA (Di Luca et al., 1987, 1989). In the later study no HSV or HPV DNA could be detected in healthy tissue which is contradictory to results from previous studies (Tidy et al.,

1988). The idea of one virus augmenting the transforming capacities of another has been shown in vitro for HCMV which enhances bovine papilloma virus transformation (Goldstein et al., 1987) and may increase HPV gene expression and transformation efficiency (Kowalik et al., 1990). It is thus quite plausible that HSV has a similar effect on HPV. HSV-1 TIF and ICP0 have also been shown to activate HPV 18 gene expression implying that the action of enhancement may not be limited to one factor, for example a DNA sequence, but may take effect from a variety of virus functions including gene expression.

In the majority of neoplastic tissue studied the various parameters considered as risk factors for cervical carcinoma have been examined independently. More recently, however, Di Luca et al., (1989) showed the simultaneous presence of two such risk factors (HSV-2 BamHI e and HPV DNA) which supports the proposal of Lehn et al. (1988, see above) accounting for the fact that a large proportion of people whose cervical biopsies contain HPV DNA do not have cancer. It seems likely that a number of initiating factors might function to enhance the transforming potential of HPV. These factors, which have also been tentatively linked with cervical cancer, include smoking (Trevathan et al., 1984) and oral contraceptive use (Vessey et al., 1983), as well as infection by other viruses including HSV. The effect of HSV on the enhancement of HPV-transformation may not be limited to one factor. HSV TIF and ICP0 activate HPV gene expression and the BamHI e fragment also appears to have a role in HPV-transformation (Di Luca et al., 1989). BamHI e has itself been linked to the morphological transformation of immortalized tissue culture cells (Jariwalla et al., 1986), and its possible involvement in HPV-transformation agrees with the proposal of Lehn et al. (1988) who suggested that the synergistic action of two carcinogens was required for the transformation of cervical cells. If this is the case a potential role in HPV transformation for the mutagenic potential of HSV must also be considered since, as previously discussed (section D.1.), many mutagens also act as carcinogens (McCann et al., 1975).

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