HERPES SIMPLEX VIRUS RIBONUCLEOTIDE REDUCTASE:

STRUCTURAL FEATURES AND TRANSCRIPTIONAL REGULATION

by

IOANNIS NIKAS

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Institute of Virology
Church Street
GLASGOW G11 5JR

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Dedicated to
my parents
George and Diatsenta Nika

SUMMARY

Ribonucleotide reductase (EC 1.17.4.1.) catalyses the direct reduction of all four ribonucleotides to the corresponding deoxyribonucleotides, this reaction being the first unique step in the de novo pathway of DNA biosynthesis. The herpes simplex virus type 1 (HSV-1)-induced enzyme is composed of two non-identical subunits, termed large (RR1) and small (RR2), which are dimers of the Vmw136 (RR1) and Vmw38 (RR2) polypeptides respectively. These polypeptides are specified by two early, unspliced and 3' co-terminal mRNAs with sizes of 5.0kb (RR1 mRNA) and 1.2kb (RR2 mRNA). The work presented in this thesis has been primarily directed at obtaining the predicted amino acid sequence of the HSV-1 RR1 polypeptide. The HSV-1 RR1 and RR2 amino acid sequences were analysed for conserved structural and functional features by comparisons to equivalent polypeptides of herpesviral and cellular origin. Other studies have identified the nucleotide changes in a portion of the RR1 gene of the HSV-1 temperature-sensitive (ts) mutant ts1207 and have examined the transcriptional regulation of RR1 and RR2 mRNA expression.

The Nucleotide and Predicted Amino Acid Sequence of the HSV-1 RR1 Polypeptide.

The nucleotide sequence of the HSV-1 DNA region encoding the RR1 polypeptide was obtained with the M13 dideoxy/chain termination method in combination with a 'shotgun' cloning approach. The sequencing data predicted that the RR1 DNA coding region is an open reading frame (ORF) of 3414 nucleotides which encodes a polypeptide of 1137 amino acids in length. In contrast to the remainder of the RR1 polypeptide, the N-terminal region contains unique amino acid composition features and seven sets of tandemly repeated amino acid sequences. A hypothetical scheme of evolutionary events leading to the formation of this region has been postulated. Further, as this region appears not be directly involved in enzymatic activity, a possible function has been

suggested on the basis of two potential nuclear localisation signals.

Amino Acid Conservation between Herpesvirus and Cellular Ribonucleotide Reductases.

Analysis of amino acid conservation between the HSV-1 RR1 and RR2 polypeptides with identified or proposed large (RR_L) and small (RR_S) subunit polypeptides of herpesviral or cellular origin was performed using computer programs.

a) Comparisons of the HSV-1 RR1 polypeptide with homologue RR_L polypeptides. Comparison of the HSV-1 RR1 polypeptide with the equivalent herpes simplex virus type 2 (HSV-2) polypeptide revealed that they are essentially colinear with the exception of the N-terminal regions where a number of insertions or deletions were predicted. Other analyses revealed that the RR1 N-terminal region was absent from other RR_L polypeptides while the colinear parts exhibited clustered homology.

b) Comparisons of the HSV-1 RR2 polypeptide with homologue herpesviral RR_S polypeptides. Comparisons of the HSV-1 RR2 polypeptide with homologue herpesviral RR_S polypeptides revealed the existence of clustered homology. The Escherichia coli (E. coli) tyrosine residue, on which the (essential for function) stable free radical has been localised, is conserved in all the RR_S polypeptides examined.

These comparisons strongly indicate that the herpesviral RR_L and RR_S polypeptides examined are the constituents of the ribonucleotide reductase activities specified by these viruses.

Conserved Structural and Potential Functional Features of the Herpesviral and Cellular Ribonucleotide Reductases.

To identify more precisely regions of clustered homology and to determine potential functional features of the RR_L and RR_S polypeptide sequences, these were aligned with the consensus template alignment program and secondary structure predictions were obtained.

a) The RR_L alignment. The consensus secondary structure predictions identified four RR_L Regions. Region 1 is present only in the HSV-1 and HSV-2 RR1 polypeptides and the majority of sequences within this region are represented by the RR1 N-terminal region. The remaining regions are present in all the polypeptides and contain sixteen blocks of clustered homology. A block in Region 4, with a sequence of GxGxxG (where G=glycine, x=any amino acid) has a predicted secondary structure of β-strand/turn/α-helix. As this structure approximates to the structure of nucleotide binding sites, it is likely to represent the RR_L nucleotide binding site. One of the blocks in Region 3 and an adjacent leucine residue are proposed, on the basis of homology to the primary and secondary structures of the adenylate kinase enzyme, to participate in the formation of the RR_L nucleotide binding site. Only two homologous blocks are retained in a human cytomegalovirus (HCMV) ORF which would correspond by genome location to HSV-1 RR1; the first of these contains a sequence similar to the proposed RR_L binding site.

b) The RR_S alignment. This alignment demonstrated that the E. coli tyrosine residue is conserved in all the RR_S polypeptides with the exception of a second HCMV ORF, which corresponds by genome location to HSV-1 RR2. Six blocks of clustered amino acid homology and a number of conserved histidine, aspartic acid and glutamic acid residues where identified; the latter could represent ligands of the enzyme's non-heme iron atoms which stabilise the free radical. None of the homologous blocks within RR_S, except for three amino acids of a carboxy-terminal block, are present in the HCMV ORF which would correspond by genome location to HSV-1 RR2.

The Nucleotide Changes within an HSV-1 Mutant ts1207 DNA Fragment Encoding Part of the RR1 Polypeptide.

The HSV-1 mutant ts1207 fails to specify any detectable ribonucleotide reductase activity at the non-permissive temperature because the RR1 and RR2 subunits fail to associate. Marker rescue experiments demonstrated that the ts1207 lesion lies within a DNA fragment encoding part of the

RR1 polypeptide. Sequencing studies within this fragment identified two nucleotide changes from which the second results in a change of a serine residue in the wild-type RR1 to an asparagine residue in the mutant RR1. The region where the second mutation lies appears not to be directly involved in subunit association.

The Transcriptional Regulation of the HSV-2 RR1 and RR2 Gene Expression.

The HSV-2 ribonucleotide reductase activity is specified by two transcripts, termed RR1 and RR2 mRNA, which are similarly arranged with their HSV-1 counterparts. To study the transcriptional regulation of the RR1 and RR2 mRNAs a vector was constructed containing the HSV-2 DNA sequences specifying these mRNAs. Transient expression assays demonstrated that the constitutive RR1 mRNA levels were readily detectable, whereas, after induction by HSV-1 infection or *trans*-activation with HSV-1 immediate early (IE) polypeptides moderate increases were observed. Analysis of the RR1 gene promoter (P1) sequences identified a number of potential *cis*-acting transcription elements one of which is identical to the binding site of the 'octamer motif binding factor'. It is proposed that, in the absence of HSV *trans*-activating functions, the constitutive RR1 mRNA levels are due to the interaction of cellular transcription factors with the P1 sequences. The basal RR2 mRNA levels were barely detectable, but in the presence of HSV-1 *trans*-activating functions a significant increase was observed. These results in conjunction with other data allow the proposal of a scheme for the transcriptional regulation of the HSV ribonucleotide reductase gene expression.

ABBREVIATIONS

A	adenine
Å	Angstrom
aa	amino acid(s)
ADP	adenosine diphospate
AE	alkaline exonuclease
AK	adenylate kinase
APS	ammonium persulphate
ATP	adenosine triphosphate
bp	base pairs
BHK	baby hamster kidney
BSA	bovine serum albumin
BU	5-bromouracil
BUdR	5'-bromodeoxyuridine
C	cytosine
Ci	Curie(s)
CAT	chloramphenicol acetyltransferase
CDP	cytidine diphosphate
C-terminus	carboxy terminus of a polypeptide
dADP	deoxyadenosine diphosphate
dATP	deoxyadenosine triphosphate
dCDP	deoxycytidine diphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
dGDP	deoxyguanosine diphosphate
dGTP	deoxyguanosine triphosphate
DNase	deoxyribonuclease
dNTP(s)	deoxyribonucleoside triphosphate(s)
DTT	dithiothreitol
dTTP	thymidine triphosphate
E	early
EBV	Epstein-Barr virus
EDTA	sodium ethylenediamine tetra-acidic acid
EPR	electron paramagnetic resonance
E. coli	Escherichia coli
G	guanine

g	gram(s)
g(D)	glycoprotein (D)
G+C	guanosine and cytidine
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HCMV	human cytomegalovirus
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HSV	herpes simplex virus
IE	immediate early
Ig	immunoglobulin
ILB	isotonic lysis buffer
IPTG	isopropyl-D-thiogalactoside
kb, kbp	kilobase(s), kilobase pair(s)
Km	Michaelis constant
Kd	dissociation constant
l	litre
L	late
lacZ	β-galactose
MCS	multiple cloning site
MDBP	major DNA-binding protein
min	minute
ml	millilitre
mM	millimolar
mmol	millimole
m.o.i.	multiplicity of infection
mol. wt.	molecular weight
N	unspecified nucleotide (A, G, C or T)
NDP(s)	ribonucleoside diphosphate(s)
ng	nanogram
nm	nanometre
NDP(s)	ribonucleoside diphosphate(s)
NPT	non-permissive temperature
N-terminus	amino terminus of a polypeptide
NTP(s)	ribonucleoside triphosphate(s)
nuc	nucleotide(s)
OD	optical density
^{32}P	radiolabelled phosphate
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis

PEB	phenol extraction buffer
p.f.u.	plaque forming unit
p.i.	post infection
PRV	pseudorabies virus
PT	permissive temperature
R	purine moiety
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SV40	simian virus 40
T	thymine
TEMED	N,N,N',N'-tetramethylethylene diamine
TK	thymidine kinase
ts	temperature-sensitive
U	uracil
UV	ultraviolet
V	volt(s)
VV	vaccinia virus
v/v	volume/volume
VZV	varicella-zoster virus
W	watts
w/v	weight per volume
Xgal	5-bromo 4-chloro 3-indoyl - β D galactopyranoside
Y	pyrimidine moiety
ug	microgram
ul	microlitre

AMINO ACID SYMBOLS

AMINO ACID	THREE LETTER SYMBOL	SINGLE LETTER SYMBOL
Alanine	**Ala**	A
Arginine	**Arg**	R
Asparagine	**Asn**	N
Aspartic acid	**Asp**	D
Cysteine	**Cys**	C
Glutamine	**Gln**	Q
Glutamic acid	**Glu**	E
Glycine	**Gly**	G
Histidine	**His**	H
Isoleucine	**Ile**	I
Leucine	**Leu**	L
Lysine	**Lys**	K
Methionine	**Met**	M
Phenylalanine	**Phe**	F
Proline	**Pro**	P
Serine	**Ser**	S
Threonine	**Thr**	T
Tryptophan	**Trp**	W
Tyrosine	**Tyr**	Y
Valine	**Val**	V

INTRODUCTION

This thesis reports the study of the enzyme ribonucleotide reductase specified by herpes simplex virus (HSV), the objective being to elucidate its structure and transcriptional regulation. The aim of this Introduction is to provide a brief background to the biology of herpes simplex virus type 1 (HSV-1), the virus upon which most of this study was carried out. Particular reference will be made to (i) the HSV-1 transcriptional program, (ii) the enzyme functions specified by HSV-1, (iii) the structure and function of the ribonucleotide reductases from prokaryotic, eukaryotic and viral origin, with the aim of putting into context (iv) a detailed account of the HSV-1-specified ribonucleotide reductase.

SECTION A.

THE HERPESVIRUSES.

1. Classification of Herpesviruses.

The family of Herpetoviridae comprises at least 80 different members. Of these, six have been isolated from humans and the remainder from a wide variety of eukaryotic hosts (Roizman and Batterson, 1985). Membership of this family is based on four morphological features of the virion:

i) The core, which contains the double-stranded viral DNA in the form of a ring-like structure. Passing through the centre of this structure is a proteinaceous, fibrillar spindle, thought to be embedded in the wall of the capsid (Furlong et al., 1972; Nazerian, 1974).

ii) The capsid, which surrounds the core, is icosahedral in shape and consists of 162 capsomeres (12 pentamers and 150 hexamers; Wildy et al., 1960).

iii) The tegument, which is a rather ill-defined layer of proteinaceous material between the capsid and the envelope of the virion (Roizman and Furlong, 1974). The thickness of this layer varies considerably among the herpesviruses (McCombs et al., 1971).

iv) The envelope, the outermost structure of the virion (Wildy et al., 1960), which is derived by budding through the inner nuclear membrane (Darlington and Moss, 1968). The envelope contains numerous protruding spikes, estimated to be approximately 8nm in length (Wildy et al., 1960).

Viruses within the family of Herpetoviridae can be classified on the basis of their biology and their genome structure. On the basis of their biology, they have been divided into three subfamilies, the alpha-, beta-, and gammaherpesvirinae, using the criteria of host range, duration of reproductive cycle, cytopathology and characteristics of latent infection (Roizman et al., 1978; Roizman, 1978).

Certain Alphaherpesvirinae have a variable host range both in vivo, in experimental animals, and in vitro. They have a short reproductive cycle, spread rapidly during infection and frequently establish latent infections in ganglia. This group includes herpes simplex virus types 1 and 2, equid herpesvirus type 1 (EHV-1; Randall et al., 1953), pseudorabies virus (PRV; Gustafsohn, 1970), bovine mammilitis virus (BMV; Martin et al., 1966), varicella-zoster virus (VZV; Weller, 1953) and channel catfish virus (CCV; Wolf and Darlington, 1971; Chousterman et al., 1979).

Betaherpesvirinae are typified by a narrow host range, a long reproductive cycle, spread slowly in culture and may become latent in secretory glands and lymphoreticular cells. Murine cytomegalovirus (MCMV; Smith, 1954) and human cytomegalovirus (HCMV; Smith, 1956) are members of this group.

Gammaherpesvirinae, include Epstein-Barr virus (EBV; Epstein et al., 1965), Marek's disease virus (MDV; Marek, 1907; Churchill and Biggs, 1967), herpesvirus saimiri (HVS; Melendez et al., 1968) and herpesvirus ateles (HVA;

Melendez et al., 1972). They vary in duration of reproductive cycle and in cytopathology but have a host range limited in vitro to lymphoblastoid cells, in which they may also give rise to latent infection.

2. Biology and Pathogenicity of Herpesviruses.

Herpesviruses are known to infect numerous eukaryotic orders, from fungi (Kazama and Schornstein, 1972) to man, who is host to HSV-1, HSV-2, VZV, HCMV and EBV. Recently, a novel herpesvirus, HHV-6, originally termed human B-lymphotropic virus, has been isolated from immunocompromised patients (Josephs et al., 1986; Salahuddin et al., 1986).

HSV-1 is the agent of 'cold sores', facial lesions primarily around the mouth (Fiddian et al., 1983), but the virus can infect many organs. Primary infection may be asymptomatic or lead to development of a number of clinical symptoms such as fever, sore throat, oedema, localised lymphadenopathy and malaise (Whitley, 1985). Following primary infection, the virus establishes a latent state in the trigeminal ganglia and may be periodically reactivated to produce recurrent disease. This is evidenced by the identical restriction enzyme pattern of viral DNA isolated from primary and recurrent lesions (Lonsdale et al., 1979).

HSV-2 is the primary cause of genital herpes, a vener ally-transmitted disease (Kessler, 1977). HSV-2 genital infections exhibit periods of exacerbation and remission due to reactivation of latent virus in the sacral ganglia (Baringer, 1974). This virus has also been associated with cervical carcinoma, however, HSV-2 DNA sequences are present in only a small percentage of cervical carcinomas (Park et al., 1983; Macnab et al., 1985), whilst human papilloma virus DNA can be detected in up to 80% of cervical carcinomas examined (Macnab et al., 1986).

VZV is the causative agent of chickenpox (varicella) in children. In common with HSV-1 and HSV-2, VZV can establish a latent state and reactivate later in life to cause shingles (herpes zoster; Weller, 1976).

HCMV usually infects salivary glands but may also be found in other tissues, usually resulting in a mild or

subclinical disease although congenital neonatal infection can result in mental retardation (Alford and Britt, 1985). Blood transfusions or organ transplants in immunosuppressed individuals frequently result in outbreaks of HCMV infection (Ho, 1982).

EBV is the causative agent of infectious mononucleosis (glandular fever) and is involved in the aetiology of Burkitt's lymphoma (Epstein et al., 1964) and in nasopharyngeal carcinoma (zur Hausen et al., 1970).

SECTION B.

STRUCTURE AND REPLICATION OF HSV-1 DNA.

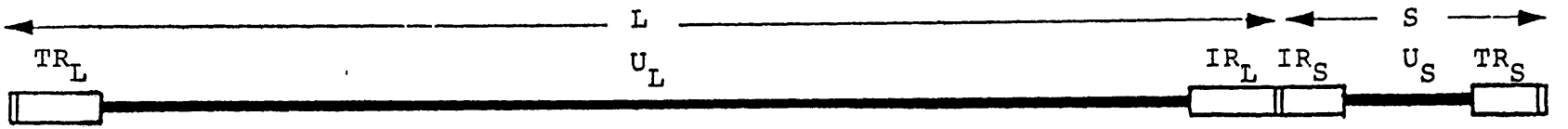
3. Structure of the HSV-1 Genome.

a) Arrangement of DNA sequence elements.

HSV-1 strain 17^+ is the third virus with a genome larger than 10^5 base pairs (bp) whose complete nucleotide sequence has been determined (Davison and Wilkie, 1981; Preston and McGeoch, 1981; Murchie and McGeoch, 1982; Dalrymple et al., 1985; Quinn and McGeoch, 1985; McGeoch et al., 1985; Davison and Scott, 1986a; McGeoch and Davison, 1986a and b; McGeoch et al., 1986a and b; Nikas et al., 1986; Perry et al., 1986; McGeoch et al., 1988; Perry and McGeoch, 1988). Its genome is a linear duplex DNA molecule of 152,260bp and has a mean G+C content of 68.3% (McGeoch et al., 1988). HSV DNA can be fragmented by treatment with alkali or formamide suggesting that it contains single-stranded nicks (Kieff et al., 1971; Wilkie, 1973; Ecker and Hyman, 1981).

The HSV genome consists of two distinct segments, designated Long (L) and Short (S), which are covalently linked at the junction (Fig. 1; Sheldrick and Berthelot, 1974). Each segment consists of a unique region (U_L and U_S) which is flanked by a pair of inverted repeats (R_L and R_S). The R_L and R_S sequences are distinct and, depending on whether they are located at the genomic termini (T) or internally (I) at the junction, they are designated TR_L, IR_L and IR_S and TR_S. The sizes of these regions in bp are: U_L, 107,943; U_S, 12,978; R_L, 9,215; R_S, 6,676 (McGeoch et al., 1988). The HSV genome possesses a direct terminal repeat which is also present in one or more inverted copies at the junction (see Page 6).

Preparations of HSV DNA contain approximately equimolar amounts of four isomers which differ in the relative orientation of the L and S segments about the junction (Delius and Clements, 1976; Clements, 1976;


L
S
TR_L
U_L
IR_L
IR_S
U_S
TR_S

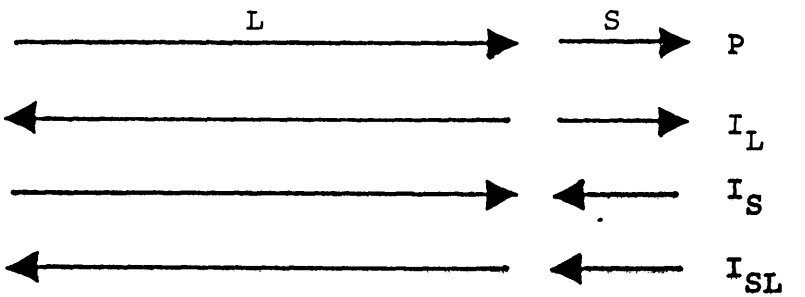

L
S
P
I_L
I_S
I_SL

Figure 1. The genome structure of HSV-1 DNA. Boxed areas represent repetitive sequences and single lines represent the unique segments. L, the Long segment; S, the Short segment; U_L, the long unique sequence; U_S, the short unique sequence; TR_L and IR_L, the terminal and internal repeats of the L segment; IR_S and TR_S, the internal and terminal repeats of the S segment. The four possible isomers of the HSV-1 genome are depicted below. P, prototype orientation; I_L, inversion of the L segment; I_S, inversion of the S segment; I_{SL}, inversion of both the L and S segments.

Wilkie and Cortini, 1976; Cortini and Wilkie, 1978). One of the four isomers, chosen arbitrarily, is designated prototype (P) and the remainder are designated as I_S, I_L, and I_{SL} (see Fig. 1; reviewed in Roizman, 1979). In this thesis, genome representations are given in the P orientation.

b) Organisation of HSV-1 genes.

It is estimated that the HSV-1 genome contains 72 genes which encode 70 distinct polypeptides (Fig. 2; reviewed in McGeoch et al., 1988). Two genes, whose products have been well characterised (see Page 17), map entirely within the R_L and R_S repeats and are therefore each represented twice. The U_L region contains 56 genes, designated UL1 to UL56 and the U_S region contains 12 genes, designated US1 to US12. In general, HSV-1 genes are densely arranged and in certain cases, such as US10 and US11 or in the regions between UL5 and UL14 and between UL30 and UL33, their coding regions overlap (Rixon and McGeoch, 1984; McGeoch et al., 1988). Each gene has its own promoter, and a feature of their arrangement is that they specify families of overlapping and unspliced transcripts with common 3' termini.

c) The a sequence.

The HSV molecule possesses a direct terminal redundancy, termed the a sequence, which is present in the inverted orientation at the L/S junction (Fig. 3; Grafstrom et al., 1974 and 1975; Wadsworth et al., 1976; Wagner and Summers, 1978). The a sequence comprises a direct terminal repeat of 17bp to 21bp (DR1 or reiteration I), adjacent to which are two unique sequences (Ub and Uc). The latter are separated by two direct repeat elements of 12bp and 37bp (DR2 and DR4). Overall, the structure of the a sequence can be represented by:

$$DR1 - Ub - (DR2)_n - (DR4)_m - Uc - DR1$$

where n represents 1 to at least 22 copies and m represents 1 to 3 copies (Davison and Wilkie, 1981; Mocarski and Roizman, 1981 and 1982a). The a sequences vary in length both inter- and intra-strain (Wagner and Summers, 1978;

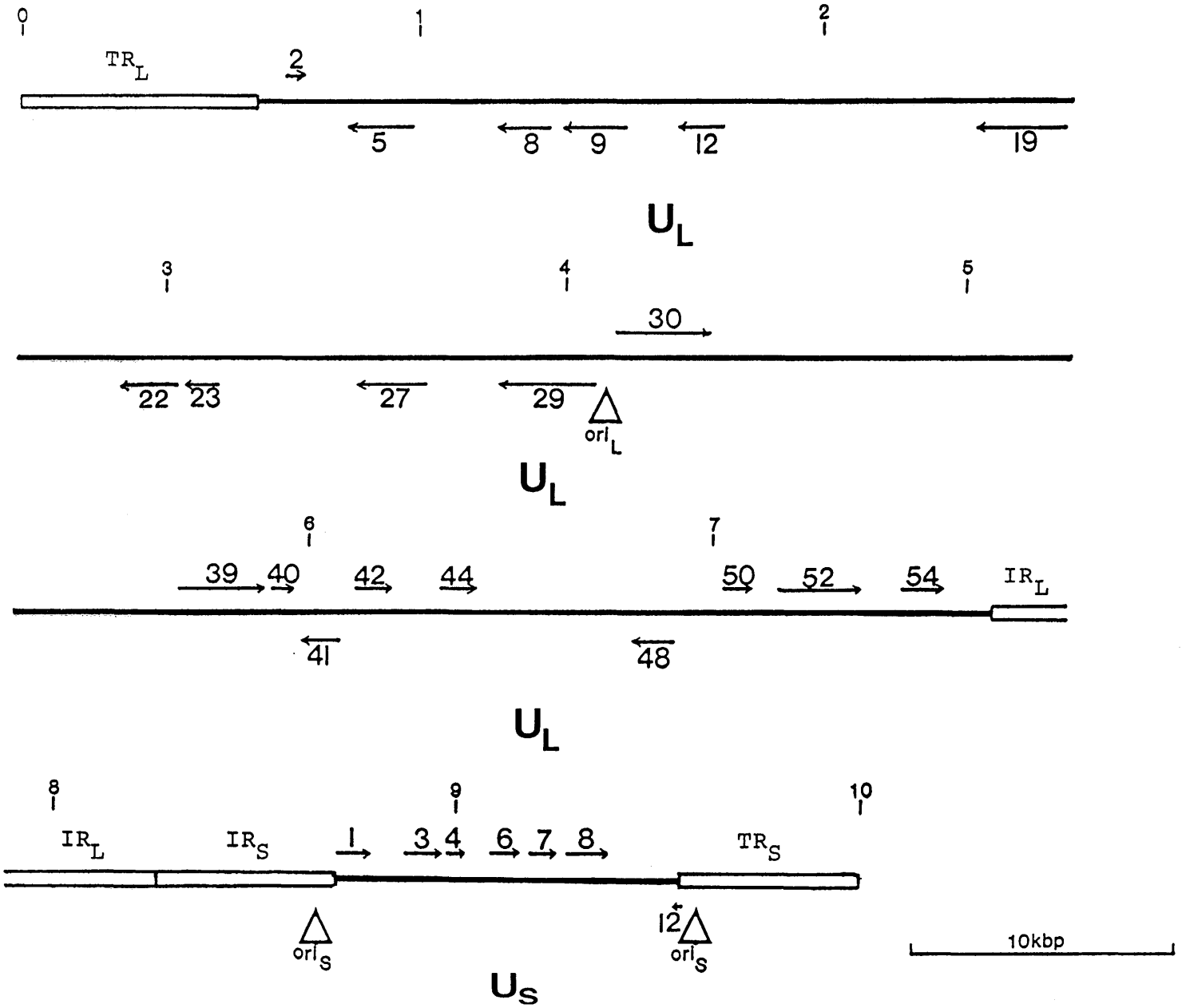

0
1
2
TR_L
2
5
8
9
12
19
U_L
3
4
5
30
22
23
27
29
ori_L
U_L
6
7
39
40
42
44
50
52
54
IR_L
41
48
U_L
8
9
10
IR_L
IR_S
1
3
4
6
7
8
TR_S
ori_S
12
ori_S
10kbp
U_S

Figure 2. The locations of HSV-1 genes, mapping within the unique U_L and U_S sequences, which encode proteins with known functions or properties. Arrowed lines above or below the genome represent genes transcribed rightwards or leftwards respectively. The three origins of viral DNA replication (see text) are indicated (△).

UL2, uracil-DNA glycosylase (J. Mullaney personal communication); UL5, UL8, UL9, UL42, and UL52, DNA replication (Wu et al., 1988; McGeoch et al., 1988); UL12, alkaline exonuclease (Preston and Cordingley, 1982; McGeoch et al., 1986b); UL19, major capsid protein Vmw155 (Costa et al., 1984; Davison and Scott, 1986a); UL22, glycoprotein H (McGeoch and Davison, 1986a); UL23, thymidine kinase (McKnight, 1980; Wagner et al., 1981); UL27, glycoprotein B (Bzik et al., 1984; Pellet et al., 1985); UL29, major DNA-binding protein, DNA replication (Conley et al., 1981; Quinn and McGeoch, 1985); UL30, DNA polymerase (Chartrand et al., 1979; Quinn and McGeoch, 1985); UL39, large subunit of ribonucleotide reductase (Preston, V.G. et al., 1984; Nikas et al., 1986; this thesis); UL40, small subunit of ribonucleotide reductase (McLauchlan and Clements, 1983a; Preston, V.G. et al., 1988); UL41, host virion shut-off (Kwong and Frenkel, 1987; Kwong et al., 1988); UL44, glycoprotein C (Frink et al., 1983); UL48, major tegument protein Vmw65, transcriptional activator (Campbell et al., 1984; Dalrymple et al., 1985); UL50, deoxyuridine triphosphatase (Preston and Fischer, 1984); UL54, immediate early protein (see Page 17; Everett, 1986).

US1, immediate early protein (see Page 17; McGeoch et al., 1985); US3, protein kinase (McGeoch and Davison, 1986b; Frame et al., 1987); US4, glycoprotein G (McGeoch et al., 1985; Frame et al., 1986a); US6, glycoprotein D (Watson et al., 1982); US7, glycoprotein I (McGeoch et al., 1985); US8, glycoprotein E (McGeoch et al., 1985); US12, immediate early protein (see Page 17;Murchie and McGeoch, 1982).

Davison and Wilkie, 1981). In HSV-1 strain 17^+ the a sequence is approximately 400bp and contains one DR4, whereas, in HSV-2 strain HG52 it is approximately 250bp and contains one DR2 and one DR4 (Davison and Wilkie, 1981).

The a sequence is present as a single copy at the S terminus, but at the L terminus and the L/S junction more than one copy may be present (Wagner and Summers, 1978; Locker and Frenkel, 1979). The DR1 elements at the genomic termini are incomplete; the L terminal DR1 contains 18bp and a single 3' nucleotide extension (total length 18.5bp) while the S terminal DR1 contains the remaining 1.5bp (Mocarski and Roizman, 1982a). Therefore, together these partial sequences form a complete DR1 element.

The a sequences appear to play a significant role in genome circularisation, inversion, cleavage and packaging.

i) Circularisation. After penetration of the infected cell nucleus, HSV DNA circularises rapidly (Davison and Wilkie, 1983; Poffenberger and Roizman, 1985). This process appears to occur by direct ligation of the complementary a sequences at the termini (Davison and Wilkie, 1983) rather than by annealing of the exposed terminal single-stranded ends (reviewed in Roizman, 1979). The former is supported by the ability of HSV-1/HSV-2 intertypic recombinants to circularise although they contain heterotypic a terminal sequences (Davison and Wilkie, 1983). Circularisation takes place in the absence of HSV protein synthesis indicating that ligation of the termini is mediated either by a host function or a virion factor (Poffenberger and Roizman, 1985).

ii) Inversion. Inversion of the L and S segments of the HSV genome results in the generation of four HSV genome isomers which, at least in tissue culture, appear to be functionally equivalent (Jenkins and Roizman, 1986). The necessary signal for this process appears to reside within the a sequences; insertion of a fragment bearing the a sequence into the thymidine kinase (TK) gene of HSV-1 promoted additional inversions resulting in the formation of 12 isomers (Mocarski et al., 1980; Mocarski and Roizman,

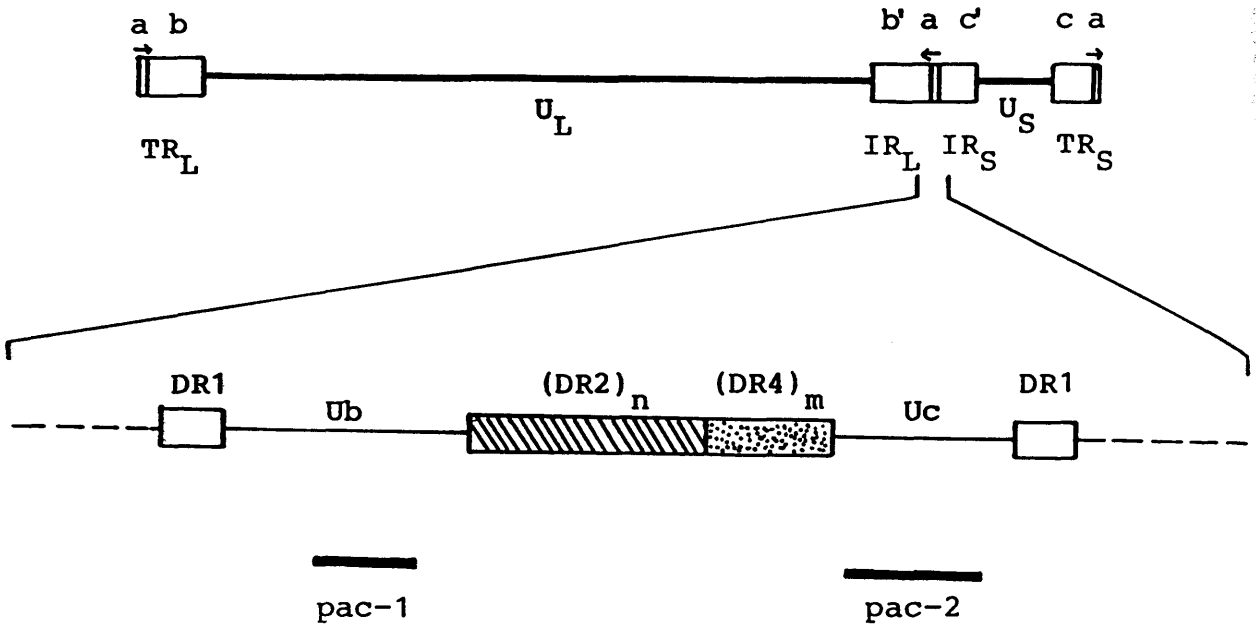


Figure 3. The location of the HSV-1 a sequence on the P genomic arrangement. The a sequence is present as a direct repeat at the genomic termini, and in inverted orientation at the L/S junction. An expansion of the a sequence at the L/S junction is shown below. Unique and repeated sequence elements within the a sequence are: DR1, a 17-21bp repeat present as a direct repeat at the ends of the a sequence; Ub, a unique sequence located toward the b sequnce; DR2, a 12bp repeat present as 1 to at least 22 copies; DR4, a 37bp repeat present as 1 to 3 copies; Uc, a unique sequence located toward the c sequence. The black boxes labelled pac-1 and pac-2 indicate unique a DNA sequences conserved in a number of herpesviruses.

1982b). Inverting DNA fragments must be flanked by homotypic and inverted a sequences relative to those at the termini or the junction. Davison and Wilkie (1983) demonstrated that an HSV-1 strain 17^+ recombinant (RE4), possessing an inverted HSV-2 a sequence at the junction, failed to invert and was predominantly frozen in the I_S arrangement. Likewise, DNA fragments flanked by a sequences in the same orientation failed to invert (Mocarski et al., 1980; Mocarski and Roizman, 1981). Deletion analysis within the a sequences demonstrated that only the DR2 and DR4 elements are required for genomic inversion (Chou and Roizman, 1985).

Site-specific recombination need not be postulated for the generation of the four genomic isomers. Genomic inversion could result partly from the use of alternative packaging phases of the precursor concatemer, which could account for half of the genome isomerisation events, and partly from the summed recombinational activity of sequences primarily dispersed throughout R_L and R_S (Varmuza and Smiley, 1985). The HSV genome contains short segments of tandemly reiterated sequences which vary in length between 5bp and 54bp and have a high G+C content (Rixon et al., 1984; Whitton and Clements, 1984a and b; Perry et al., 1986). These sequences could be highly recombinogenic and homologous recombination could occur between any members of the same sequence family (Rixon et al., 1984).

Further supportive evidence that recombination leading to segment inversion is not site-specific is obtained from a number of reports. An HSV-1 mutant lacking the a sequences at the junction and predominantly frozen in the I_S arrangement generated a minor population of I_{SL} molecules most probably due to recombination between the remaining b sequences of the junction (see Fig. 3; Longnecker and Roizman, 1986). Likewise, an HSV-1 mutant containing a second copy of a U_L fragment inserted into the TK gene induced inversion (Pogue-Geile and Spear, 1986) as did a fragment from the c sequences albeit at a low frequency (Varmuza and Smiley, 1985).

iii) Cleavage and packaging. The a sequence is involved in the cleavage of replicated concatemeric DNA into unit length linear molecules and their subsequent packaging into virus particles; although plasmid constructs containing an HSV-1 origin of replication are able to replicate they are packaged, as defective particles or 'amplicons', only when they contain an a sequence (Stow et al., 1983; Deiss and Frenkel, 1986). The necessary signals for these processes appear to reside within the Ub and Uc sequences (see Fig. 3; Deiss et al., 1986). 'Amplicons' containing deletions within the Uc region fail to become packaged while those containing deletions within the Ub region are packaged only when a wild-type a sequence is acquired from the helper virus. The Ub region contains a 28bp sequence which is highly conserved in HSV-1, HSV-2 (Davison and Wilkie, 1981), VZV and PRV (Davison and Rixon, 1985). Deiss and Frenkel (1986) termed this sequence pac-1 and identified a less homologous sequence, pac-2, within the Uc region (see Fig. 3). Although the function of these conserved sequences is at present unknown, these authors proposed that they might play a role in the cleavage and packaging processes.

d) Proteins interacting with the a sequence.

The processes in which the a sequence is involved appear to require the interaction of specific HSV-induced and/or host cell proteins. This is evidenced by the requirement of at least one trans-acting viral component for inversion (Mockarski and Roizman, 1982b) and the occurence of genomic circularisation in the absence of de novo protein synthesis (Poffenberger and Roizman, 1985). Dalziel and Marsden (1984), using competition binding assays with HSV-1 DNA fragments containing the a sequence, identified two polypeptides with sizes of 21,000 and 22,000 mol. wt. (21K, 22K) which consistently interacted with these fragments. It was therefore suggested that these polypeptides bind to the a sequence either directly or indirectly (Dalziel and Marsden, 1984). MacLean et al. (1987) demonstrated, by means of antisera raised against oligopeptides corresponding to regions of the predicted primary structure of gene US11 (McGeoch et al., 1985), that

the latter specified two polypeptides with sizes identical to those interacting with the a sequence. Although these authors were unable to isolate substantial quantities of proteins interacting with a sequences to test with anti-US11-peptide sera, they demonstrated that the US11 gene products are DNA binding proteins. The a sequence-interacting 21K and 22K proteins of Dalziel and Marsden (1984) may therefore correspond to the products of US11 (McLean et al., 1987). The relationship between the 21K and 22K proteins is not clear.

Immune electron microscopy showed that the US11 gene products were localised in the nucleoli of infected cells (MacLean et al., 1987). However, viral DNA is located in the nucleoplasm and is specifically excluded from the nucleoli (Rixon et al., 1983; Randall and Dinwoodie, 1986). Therefore, it appears that these proteins may not be involved in DNA replication and maturation in vivo. This suggestion is supported by the viability in tissue culture of HSV mutants, which lack the US11 gene (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987). Recently, Meigner et al. (1988) reported that one of these mutants was able to establish latency in mice.

4. HSV DNA Replication.

a) Cis-acting signals for DNA replication.

The mechanism by which HSV DNA replicates is largely unknown. Jacob et al. (1979) proposed that after infection HSV DNA circularises and replication proceeds by a rolling circle mechanism. In support of this suggestion is the fact that newly replicated viral DNA possesses fewer detectable termini indicating that it consists of head-to-tail concatemers (Jacob et al., 1979; Jongeneel and Bachenheimer, 1981). Furthermore, electron microscopic studies using cells with replicating viral DNA detected circular unit length genome molecules (Shlomai et al., 1976; Friedmann et al., 1977; Jacob and Roizman, 1977).

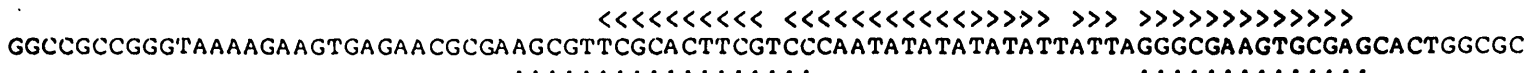
Approximate locations for HSV origins of replication were initially characterised using defective viral genomes

obtained following passage at high multiplicities of infection (m.o.i.; Kaerner et al., 1979; Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982). These defective genomes consisted of tandem repetitions of sequences derived from the standard genome and were divided into two classes. Both classes contained sequences from the end of the TR_S repeat. Class I contained in addition sequences from the S segment while class II contained sequences from near the centre of the L segment. The ability of these defective genomes to replicate and package in the presence of wild-type helper virus indicated the presence of cis-acting signals for DNA synthesis and processing. Thus, it was suggested that the HSV genome contains i) two potential origins of replication, one in each R_S, termed ori_S and present in class I defective genomes and ii) a potential origin of replication in U_L, termed ori_L and present in class II defective genomes (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982).

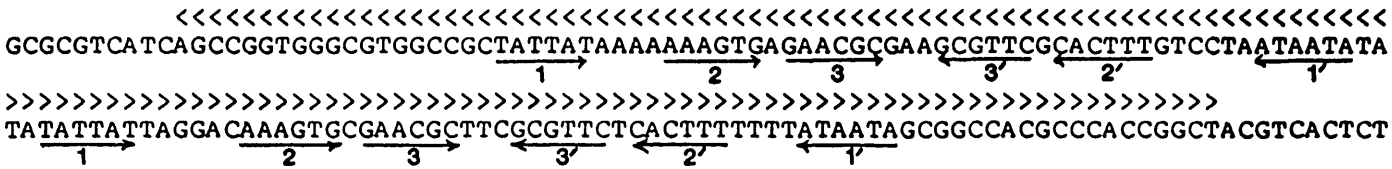
i) Ori_S. Stow (1982) developed an assay system for determining functional origins of replication in which the amplification of plasmid DNA sequences containing inserted HSV fragments was estimated in the presence of HSV-1 functions supplied in trans. Using this assay system, ori_S was positioned in a 90bp R_S region (see Fig. 2), located between the 5' termini of IE mRNAs 3 and 4/5 (for a description of these mRNAs see Page 17; Rixon and Clements, 1982). This region comprised a 45bp almost perfect palindromic sequence, at the centre of which is positioned an 18bp A+T-rich tract, and contained all the necessary cis-acting sequences for replication (Fig. 4a; Murchie and McGeoch, 1982; Stow and McMonagle, 1983). The A+T-rich segment appears to be important for replication since deletion of it completely abolished origin activity (Stow, 1985).

Within the HSV-2 strain HG52 R_S, Whitton and Clements (1984a) identified two copies of a sequence highly homologous to HSV-1 ori_S; this sequence is present as a 137bp direct repeat and it is the proposed HSV-2 ori_S. In VZV a functional ori_S was identified, cloned copies of which could be activated by VZV replication functions

(a) HSV-1 ori_S sequence



(b) HSV-1 ori_L sequence



(c) HSV-1 ori_L/ori_S comparison

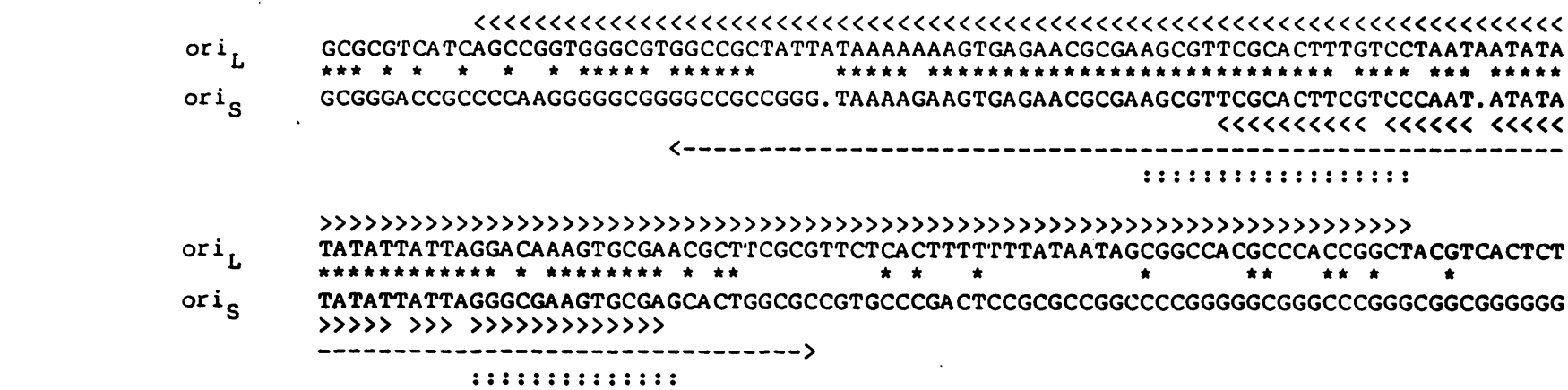


Figure 4. Structure of the HSV-1 origins of replication.

a) The ori_S sequence. The arms of the palindrome are indicated by (<) or (>) and the binding sites of the UL9 protein are indicated by (:).

b) The ori_L sequence. Palindromic sequences are indicated as above. Numbered arrowed lines indicate regions of interrupted and inverted nucleotide repetitions.

c) Comparison of the ori_S and ori_L sequences. Asterisks indicate conservation and dots indicate gaps introduced into the sequences for optimal alignment. Dashed lines represent the sequences essential for ori_S activity and the palindromic sequences and binding sites of the UL9 protein are indicated as above.

supplied in trans (Stow and Davison, 1986); in addition, the cloned VZV ori_S could be activated by HSV-1 infection although at a reduced efficiency (Stow and Davison, 1986). The VZV ori_S contains a tract of alternating A+T residues located at the centre of an almost perfect palindrome of 45bp and displays limited homology to the HSV-1 equivalent.

ii) Ori_L. The HSV ori_L is positioned between the 5' termini of the mRNAs encoding the major DNA-binding protein (MDBP) and the DNA polymerase (genes UL29 and UL30 respectively in Fig. 2), and its sequence has been determined for HSV-1 strains 17^+ (Quinn and McGeoch, 1985), KOS (Weller et al., 1985) and the Angelotti class II defective of HSV-1 (Gray and Kaerner, 1984). Ori_L sequences consist of an A+T-rich region positioned at the centre of a perfect palindrome with arms each of 72bp; each arm contains three regions of interrupted and inverted repetitions (Fig. 4b; Weller et al., 1985). Comparison between ori_S and ori_L sequences revealed that the whole of the ori_S palindrome is quite similar to the central portion of the ori_L palindrome (Fig. 4c). The latter was shown to be a functional origin and deletions within the palindrome abolished activity (Weller et al., 1985).

HSV-2 contains a perfect palindrome of 136bp which is highly homologous to that of HSV-1 ori_L (Lockshon and Galloway, 1986). On the contrary, VZV does not appear to have an HSV-1 ori_L homologue sequence, at least in the equivalent HSV-1 genomic position (Stow and Davison, 1986).

Recently, it was shown that an HSV-1 deletion mutant lacking most of the ori_L sequences, and therefore rendering ori_L non-functional in the presence of helper virus, was able to replicate in vitro (Polvino-Bodnar et al., 1987). Further, an HSV-1 recombinant that contains ori_L and a single copy of ori_S was viable in tissue culture (Longnecker and Roizman, 1986). These observations demonstrate that two out of three HSV origins are sufficient for replication, and this raises the question of the role of a third origin. It is possible that different origins are preferentially used during different stages of lytic infection (reviewed in McGeoch, 1987).

b) HSV-1 genes required for replication.

A number of HSV-1-induced enzymes have been shown by genetics to be essential for viral DNA replication in cultured cells and these are described in Section D. Recently, Challberg (1986) developed a method for identifying viral genes required for DNA replication. This method was based on the observation that transfected HSV DNA could provide in *trans* the functions to support amplification of an ori_S-containing plasmid introduced concurrently. Thus, it was shown that a set of plasmids representing most of the HSV genome could substitute efficiently for intact virus DNA in supplying replication functions and that the DNA polymerase and MDBP genes were essential components of this system. Subsequent subcloning and inactivation by restriction endonuclease digestion identified, in conjuction with DNA sequencing data, five additional essential genes (Wu *et al.*, 1988; McGeoch *et al.*, 1988); these are UL5, UL8, UL9, UL42 and UL52 (see Fig. 2).

The products of genes UL9 and UL42 have been characterised. Gene UL9 encodes a protein, identified previously by Elias *et al.* (1986), which binds to ori_S. This protein is present in nuclear extracts of HSV-1-infected cells and generates a DNase I footprint of 18bp which maps to the left of the palindrome centre (Elias *et al.*, 1986); however, it has recently been shown that this protein has an additional binding site to the right of the palindrome centre (see Fig. 4a; Olivo *et al.*, 1988; H. Weir, personal communication). It is notable that the DNase I protected region to the left of the palindrome centre includes an 11bp sequence which is highly conserved between HSV-1 ori_L, ori_S and VZV ori_S (Stow and Davison, 1986). Gene UL42 encodes the 65K DNA-binding protein ($65K_{DBP}$; Marsden *et al.*, 1987; Parris *et al.*, 1988). This protein is the major species in high-salt eluates from double-stranded DNA cellulose columns used to fractionate nuclear extracts of HSV-1-infected cells (Marsden *et al.*, 1987). Recently, a temperature-sensitive (*ts*) HSV-1 mutant with a mutation lying most probably in gene UL42 failed to induce synthesis of viral DNA at the non-permissive temperature (NPT) and was unable to replicate an

oriS-containing plasmid (Marchetti et al., 1988). Further, this mutant at the NPT failed to induce synthesis of the true-late class of HSV-1 mRNAs (for a description of these mRNAs see Page 17). The functions of the other three essential genes are currently being investigated.

SECTION C.

PROCESSES INVOLVED IN PRODUCTIVE HSV INFECTIONS.

5. Effects of HSV on Host Cell Metabolism.

Productive infection with HSV virus significantly impairs several host functions. Following infection, host DNA synthesis ceases (Aurelian and Roizman, 1964; Roizman and Roane, 1964) and host RNA synthesis decreases (Hay et al., 1966; Flanagan, 1967; Pizer and Beard, 1976). However, certain host cell RNAs continue to be synthesised at late times post infection (p.i.; Stringer et al., 1977) and the promoters of cellular genes, integrated into the genome of biochemically transformed cell lines, are activated after HSV infection (Everett, 1985). The steady-state levels of pre-existing host polyadenylated mRNAs decrease by 4h p.i., probably due to deadenylation (Nishioka and Silverstein, 1977 and 1978; Nakai et al., 1982; Mayman and Nishioka, 1985).

Infection of cells with HSV results, also, in inhibition of host protein synthesis with the exception of the heat shock or stress proteins whose production is increased (Notarianni and Preston, 1982; LaThangue et al., 1984). Host protein shut-off appears to be more rapid and efficient with HSV-2 than HSV-1, although HSV-2 strain HG52 appears to be an exception to this generalisation (Powell and Courtney, 1975; Pereira et al., 1977). Host protein shut-off has been divided into 'early' and 'delayed' stages.

The 'early' stage occurs after HSV infection and is mediated by a virion component(s) as it can be achieved either by UV-irradiated virus or in the presence of drugs which inhibit incoming virus expression (Fenwick and Walker, 1978; Nishioka and Silverstein, 1978). This stage appears to be associated with disaggregation of infected-cell polyribosomes which reassemble to form new ones exhibiting an altered size distribution (Sydiskis and Roizman, 1967 and 1968). The majority, but not all, of the mRNA associated with new polyribosomes is virally-encoded (Stringer et al.,

1977). Further, the 'early' shut-off appears to be associated with host mRNA degradation and with a reduction in the half-life of immediate-early and, possibly, early and late HSV mRNAs (for a description of these HSV mRNAs see below; Schek and Bachenheimer, 1985; Kwong and Frenkel, 1987; Strom and Frenkel, 1987; Kwong et al., 1988). A number of HSV-1 mutants defective in virion-associated host shut-off (vhs) have been isolated (Read and Frenkel, 1983). In one mutant, the mutation(s) affecting both inhibition of host protein synthesis and stability of host and viral mRNAs, most likely maps within gene UL41 (see Fig. 2; Kwong et al., 1988). In certain cell lines, host cell mRNA degradation occurs during 'delayed' shut-off of host polypeptide synthesis (Nishioka and Silverstein, 1978).

The 'delayed' stage of the shut-off process completes the inhibition of host protein synthesis and requires viral gene expression (Nishioka and Silverstein, 1978; Read and Frenkel, 1983). The gene(s) involved in 'delayed' shut-off can function in the absence of the virion component(s) responsible for the 'early' shut-off (Read and Frenkel, 1983).

6. Temporal Regulation of HSV Gene Expression.

The HSV virion is uncoated in the cytoplasm of the infected cell and subsequently the viral DNA is transported to the nucleus (Hummeler et al., 1969). In the nucleus, mRNA is transcribed from viral DNA by a process which appears to involve cellular RNA polymerase II; this is evidenced by the sensitivity to α-amanitin (Alwine et al., 1974; Ben-Zeev et al., 1976), a fungal toxin which specifically inhibits RNA polymerase II, and by the absence of any novel RNA-polymerasing activity in HSV-1-infected cells (Lowe, 1978). The sensitivity against the drug persists throughout all stages of infection (Costanzo et al., 1977). Subsequently, HSV mRNA is transported to the cytoplasm where it becomes associated with polyribosomes, and is translated (Wagner and Roizman, 1969). HSV gene expression is temporally regulated and can be sub-divided

into three broad stages, immediate early (IE), early (E) and late (L) gene expression (Swanstrom and Wagner, 1974; Clements et al., 1977; Jones and Roizman, 1979).

a) Immediate early gene expression.

i) Immediate early genes. IE or α genes are the subset of genes which are transcribed by an unmodified cellular RNA polymerase II (Costanzo et al., 1977) in the absence of de novo protein synthesis (Kozak and Roizman, 1974; Roizman et al., 1974). IE mRNAs are usually detectable by 1h p.i. and their accumulation peaks at 2h to 3h p.i., although, some of them persist in the cytoplasm at late times of infection (Harris-Hamilton and Bachenheimer, 1985; Godowski and Knipe, 1986).

IE transcription is limited to restricted regions of the genome (Fig. 5; Clements et al., 1977 and 1979; Anderson et al., 1980; Easton and Clements, 1980). Five IE genes have been identified, IE genes 1 to 5, which encode polypeptides Vmw110 (ICP0), Vmw63 (ICP27), Vmw175 (ICP4), Vmw68 (ICP22) and Vmw12 (ICP47) respectively (the 'Chicago' nomenclature is given in brackets). The genome locations and sizes of the IE mRNAs encoded by the IE genes have been determined. IE mRNAs 1 and 3 map entirely within TR_L/IR_L and IR_S/TR_S respectively, and are therefore encoded by diploid genes (Rixon et al., 1982). IE mRNAs 4 and 5 span the IR_S/U_S and TR_S/U_S regions and share identical promoter and 5' untranslated leader sequences (Watson et al., 1981; Rixon and Clements, 1982); these are encoded by genes US1 and US12 respectively. Finally, IE mRNA 2 maps entirely within U_L (Whitton and Clements, 1983) and is encoded by gene UL52. Three of the five IE transcripts, IE mRNAs 1, 4, and 5, are spliced. IE mRNAs 4 and 5 have a single splice located entirely within the R_S repeats (Watson et al., 1981a; Rixon and Clements, 1982; Whitton and Clements 1984b) and upstream of the polypeptide coding regions (Murchie and McGeoch, 1982; Whitton and Clements, 1984b; Rixon and McGeoch, 1984). HSV-1 IE mRNA 1 contains two introns with sizes of 765 nucleotides (nuc) and 135nuc, both of which are

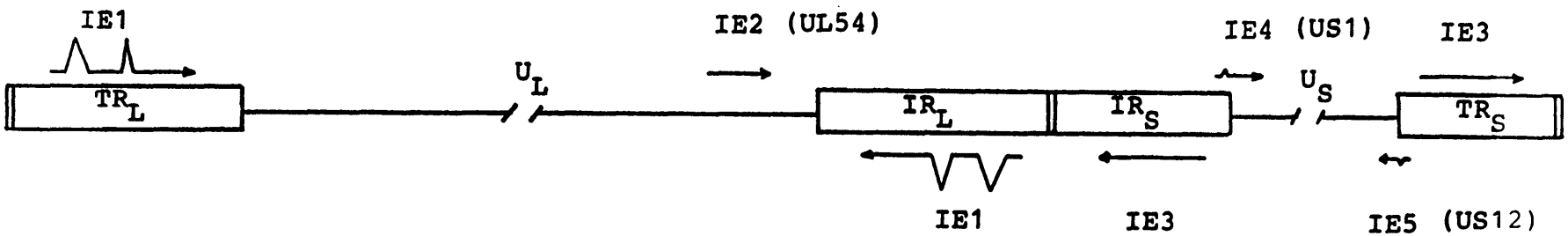

IE1
IE2 (UL54)
IE4 (US1)
IE3
TR_L
U_L
IR_L
IR_S
U_S
TR_S
IE1
IE3
IE5 (US12)

Figure 5. A representation of the HSV-1 genome showing the location and orientation of the five IE genes. The designation of the IE genes mapping in the U_L or U_S segments is given in brackets (see Fig. 2). The IE genes are depicted as arrows and splices are indicated as (Λ) and (V).

located in the translated portion of the gene (Perry et al., 1986).

ii) IE gene regulatory sequences. The 5'-upstream regions of HSV IE genes contain sequence elements that clearly differentiate them from the respective regions of E and L genes. A chimeric gene comprising the upstream control signals and 50bp of leader sequence from IE mRNA 3 when fused to an early HSV-1 TK gene, behaved as an IE gene either when rescued into a TK^- virus or inserted into biochemically transformed cell lines (Post et al., 1981). Similar results were obtained when the TK gene was fused to the upstream region of IE mRNA 2 (Mackem and Roizman, 1982a).

Deletion studies within the IE gene 3 upstream region identified a promoter region, spanning positions +26 to -108 (+1=mRNA start site), which is sufficient for transcription initiation in the absence of upstream promoter sequences (Mackem and Roizman, 1982a; Cordingley et al., 1983). This region contains elements found in most eukaryotic promoters such as the TATA box and two G+C-rich tracts separated by an A+T-rich stretch; however, the 5'-CCAAT-3' homology, which is usually present at position -80 relative to the mRNA start site in many eukaryotic promoters (Benoist and Chambon, 1980; Efstratiadis et al., 1980), is not present. Further, they identified a regulatory region spanning positions -174 to -331. In all IE genes examined so far this region induces IE gene transcription in a process mediated by a virion component (see Page 19). Deletion studies within the regulatory region (Cordingley et al., 1983; Preston, C.M. et al., 1984; Bzik and Preston, 1986) and DNA sequence comparisons of the upstream regions of IE mRNA 5' termini (Mackem and Roizman, 1982b; Whitton and Clements, 1984a) demonstrated that the target sequence for the trans-inducing virion component is an A+T-rich sequence with a core consensus of 5'-TAATGARAT-3' (R=purine). Gaffney et al. (1985) demonstrated that when a 20bp synthetic oligonucleotide, containing a TAATGARAT element, was fused to a promoter driving the chloramphenicol acetyltransferase (CAT) gene, it increased CAT activity

after infection with the HSV-1 tsK mutant; the latter has a mutation in IE gene 3 and does not synthesise E or L gene products at the NPT (Preston, 1979). The observed stimulation of expression was dependent on the number of TAATGARAT copies, since increasing the copy number had an additive effect (Gaffney et al., 1985). However, it appears that flanking sequences, although not essential, are important for full induction of IE gene transcription. Bzik and Preston (1986) demonstrated that an adjacent G+A-rich element is required for full activity which, although it was unable to confer full activity on its own, could activate an otherwise non-functional homologue of TAATGARAT (Bzik and Preston, 1986).

The regulatory region of IE gene 3 has an enhancer-like activity comparable to that of the SV40 enhancer, since it can stimulate expression of target promoters in an orientation-independent manner and at a distance from the target promoter (Lang et al., 1984; Preston and Tannahill, 1984). In contrast to a typical enhancer, the HSV-1 IE gene 3 enhancer activity is abolished when placed downstream from a gene and, also, is further stimulated by a virion component (see below; Lang et al., 1984; Preston and Tannahill, 1984). Deletion analysis of the upstream regulatory region of HSV-1 IE gene 3 demonstrated that the enhancer-like activity and the responsiveness to the trans-inducing virion component reside in distinct sequence elements (Bzik and Preston, 1986). Elements important for enhancer-like activity are: i) two copies of the hexanucleotide 5'-GGGCGG-3' which can bind the mammalian Spl transcription factor (Jones and Tijan, 1985) and, ii) a region containing both a 5'-GCGGAAAC-3' motif, which resembles the simian virus 40 (SV40) enhancer core (Weiher et al., 1983), and a 5'-CGGAAGCGGAA-3' motif, which resembles the adenovirus E1A and polyoma virus enhancer cores (Hearing and Shenk, 1983; Herbomel et al., 1984).

iii) Characterisation of the virion factor which induces IE gene expression. Experiments designed to identify the mechanism by which IE gene expression is induced demonstrated that a component of the virus particle

could stimulate IE gene expression (Post et al., 1981; Mackem and Roizman, 1982b; Cordingley et al., 1983; Preston, C.M. et al., 1984). Campbell et al. (1984) identified the virion component as the major tegument protein Vmw65, which is distinct from the $65K_{DBP}$ (see Page 13), and its primary structure was determined by Dalrymple et al. (1985).

DNA-binding studies with Vmw65 indicated that it did not directly bind either calf thymus (Marsden et al., 1987) or HSV DNA (Preston, C.M. et al., 1988). However, Vmw65 has been shown to be the only viral constituent of a complex, termed IEC, which contains cellular factors and binds specifically to TAATGARAT motifs via one of the cellular factors (Preston, C.M. et al., 1988). It has been proposed that one of these factors is a protein related to nuclear factor III (NF-III; C. Ace, personal communication). NF-III facilitates the binding of a complex, involved in initiation of adenovirus 2 DNA replication, to core sequences of the adenovirus 2 origin (Pruijn et al., 1986). The NF-III binding site, 5'-TATGATAATGAG-3' (Pruijn et al., 1986), is quite similar to the TAATGARAT motif. Interestingly, it has been shown that both of these motifs are equally competitive for binding of a cellular factor present in mock-infected HeLa cell nuclear extracts (O'Hare and Goding, 1988).

iv) Control of HSV IE gene expression. As discussed previously, IE gene transcription does not require de novo protein synthesis. However, a number of HSV-1-induced polypeptides appear to be involved in the control of IE gene expression (Fig. 6). First, is the virion component Vmw65 which, as already mentioned, positively regulates IE gene transcription. Second, is Vmw175 which is involved both in positive and negative regulation of IE gene expression. Polypeptide profiles of HSV ts-Vmw175 mutant viruses demonstrated that IE gene products were overexpressed, and blocking of IE mRNA translation with cycloheximide led to production of large amounts of IE mRNAs (Preston, 1979; Dixon and Schaffer, 1980; Watson and Clements, 1978 and 1980). The same results were obtained from transient expression assays where increased amounts of Vmw175 could repress both the basal and activated levels of transcription

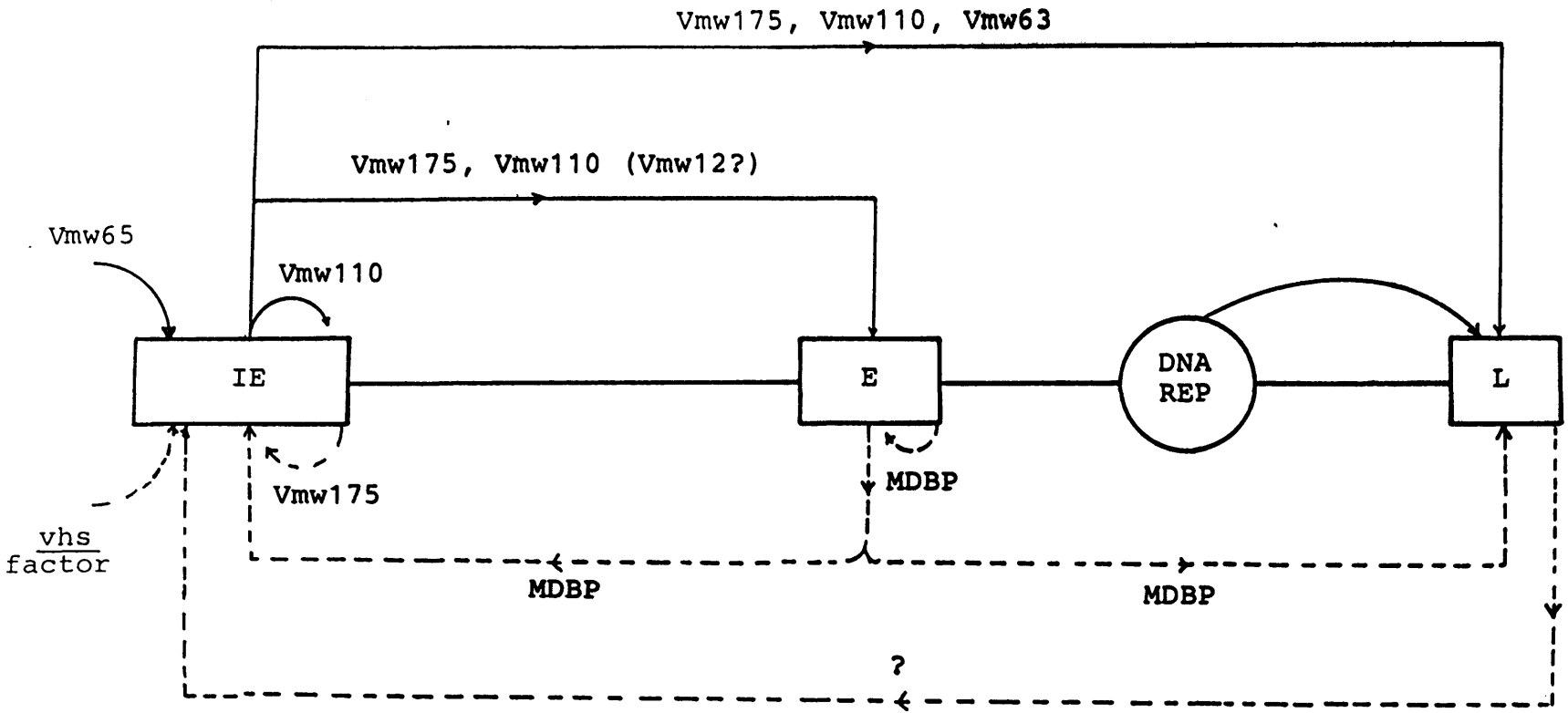

Vmw175, Vmw110, Vmw63
Vmw175, Vmw110 (Vmw12?)
Vmw65
Vmw110
IE
E
DNA
REP
L
Vmw175
MDBP
vhs
factor
MDBP
MDBP
?

Figure 6. The HSV-1 functions thought to be involved in the regulation of immediate early (IE), early (E) and late (L) gene expression. The solid lines represent positive regulation and dashed lines indicate negative regulation. The positive regulators include Vmw65, Vmw110, Vmw175, Vmw63 and DNA replication (DNA REP). The negative regulators include the virion-associated host shutoff (vhs) factor, Vmw175, and the major DNA-binding protein (MDBP). The precise roles of Vmw12 (O' Hare and Hayward, 1985a) and a L function(s) (DeLuca et al., 1984) require further investigation and this is depicted by (?).

repress both the basal and activated levels of transcription from IE gene promoters (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986 and 1987). It appears that the only exception to this generalisation is the HSV-1 IE gene 2 promoter which, in HeLa cells, was active in the presence of increased amounts of Vmw175 (Gelman and Silverstein, 1987). Reduced levels of Vmw175, however, have been shown to activate IE gene promoters (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986 and 1987). Third, is Vmw110 which has been shown to activate IE promoters in transfection assays (O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986 and 1987). In addition to these polypeptides, MDBP (Godowski and Knipe, 1986) and the product of gene UL41 (Strom and Frenkel, 1987; Kwong et al., 1988) appear to have a role in IE gene regulation since mutations in these genes increase the half-life of HSV IE mRNAs.

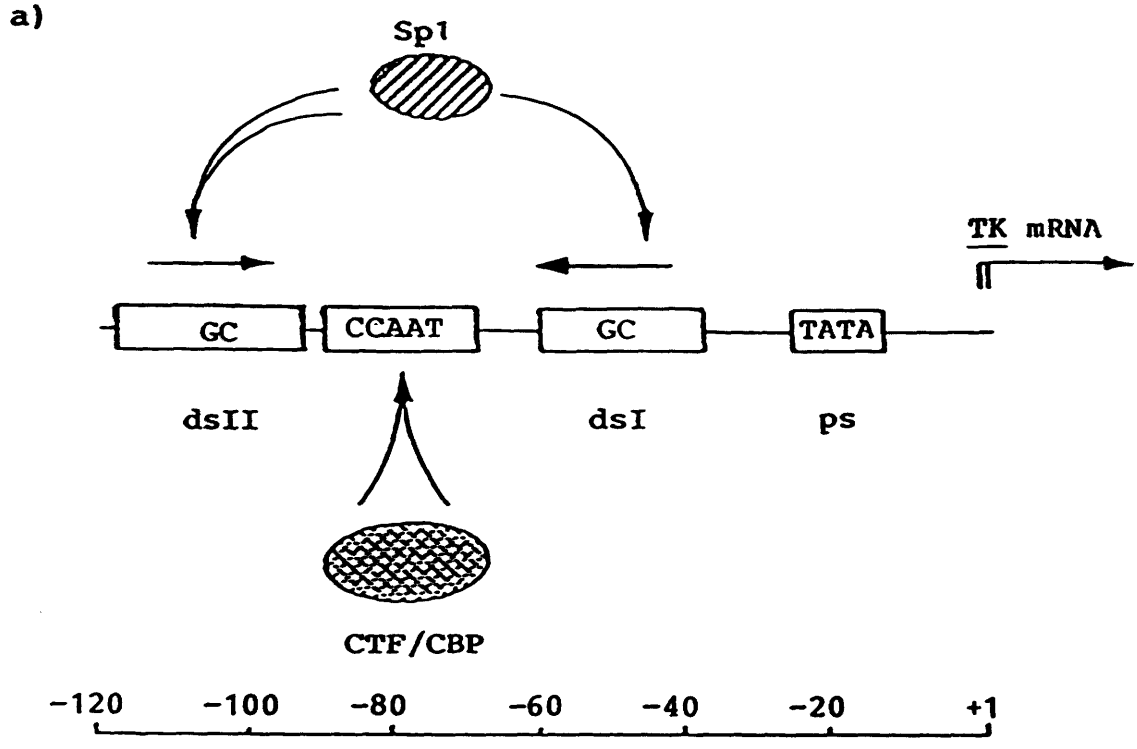
b) Early gene expression.

i) Early genes. E or β genes are defined as that subset of viral genes which are expressed in the presence of functional IE gene products and prior to the onset of DNA replication (Wagner, 1972; Swanstrom et al., 1975; Clements et al., 1977). Early transcripts are detectable at 2h p.i. and reach their maximum levels at 4h to 6h p.i., after which, the levels of individual transcripts either decrease or remain unaltered (McLauchlan and Clements, 1982; Harris-Hamilton and Bachenheimer, 1985). However, some variation in their kinetics of appearance is observed. For example, the polypeptide of the ribonucleotide reductase large subunit (Vmw136), although classified as an E polypeptide, can be detected under IE conditions (see Results and Discussion, Section E). On the other hand, synthesis of glycoprotein D (gD) mRNA requires DNA replication to reach maximum expression although moderately high levels are detected early in infection (Gibson and Spear, 1983). E transcripts have been shown to map across the entire length of the genome (Clements et al., 1977).

ii) E gene promoter sequences. Two HSV-1 E promoters, those of the TK and gD genes, have been studied in detail. The TK promoter region, which is positioned immediately upstream from the TK mRNA start site, has been divided into one proximal and two distal regions (dsI and dsII) by means of linker scanning mutations (Fig. 7a; reviewed in McKnight and Tjian, 1986). The proximal region contains the TATA box which has been shown to be especially important for TK transcription when present on the viral genome (Coen et al., 1986). Each of the distal regions contains an Spl binding site (see Page 19) and in addition, dsI contains an inverted CCAAT homologue which binds the CBP/CTF transcription factor (Jones et al., 1985). The gD promoter region has been extensively studied by Everett (1983 and 1984a). Deletion analysis indicated that the sequence requirements for gD-regulated expression reside within an 83bp region located upstream from the mRNA start site (Fig. 7b; Everett, 1983). Further analysis of this region identified two G-rich regions, termed G1 and G2, which are quite similar to an Spl binding site in the human immunodeficiency virus long terminal repeat (Jones, K.A., et al., 1986), and a functional TATA box together with a cap site region (Everett, 1984a). A CCAAT homologue was not identified.

iii) Control of HSV E gene expression. At least two IE gene products, Vmw175 and Vmw110, appear to be important for regulation of E gene expression (see Fig. 6). Transient expression assays demonstrated that Vmw175 and Vmw110 can independently stimulate expression of E promoters whereas, in combination, their effect is synergistic (Everett, 1984b and 1986; O'Hare and Hayward, 1985b; Quinlan and Knipe, 1985; Gelman and Silverstein, 1986; see Results and Discussion, Section E).

The requirement for a functional Vmw175 for E gene expression has also been observed during virus infection; at the NPT, HSV-1 ts-Vmw175 mutants fail to induce E gene expression and fail to repress IE gene expression (Marsden et al., 1976; Preston, 1979; Dixon and Schaffer, 1980; Watson and Clements, 1978 and 1980). In an attempt to


a)
Sp1
TK mRNA
GC
CCAAT
GC
TATA
dsII
dsI
ps
CTF/CBP
-120
-100
-80
-60
-40
-20
+1

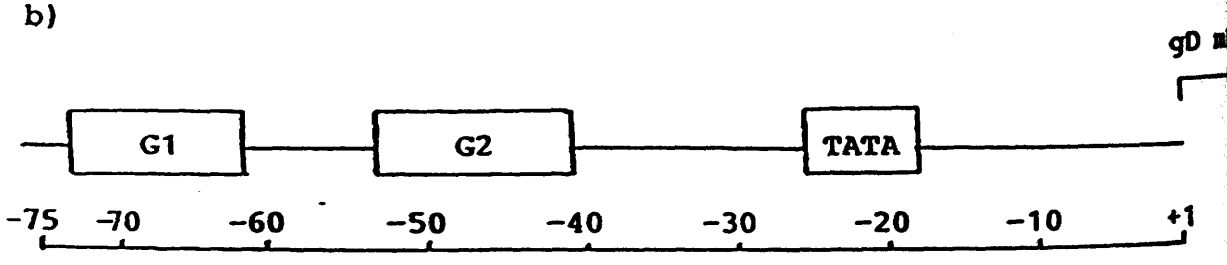

b)
G1
G2
TATA
-75
-70
-60
-50
-40
-30
-20
-10
+1

Figure 7. Cis-acting transcription elements within E gene promoters.

a) Elements within the TK promoter (reviewed in McKnight and Tjian, 1986). The GC boxes represent the binding sites of the Spl transcription factor and arrows above them indicate the relative orientation of the sequences. The CCAAT box indicates the binding site of the CTF/CBP transcription factor. Both the Spl and CTF/CBP transcription factors are depicted to interact with the promoter sequences by curved arrows and the stronger affinity of the Spl site in dsII is depicted by two arrows. A binding factor is thought to interact with the TATA box. The TK promoter sequences are numbered from -120 to beyond the first TK mRNA cap site at +1.

b) Elements within the gD promoter (Everett 1984a). The open boxes labelled G1 and G2 represent the two G-rich regions thought to be binding sites of the Spl transcription factor. A binding factor is thought to interact with the TATA box. The gD promoter sequences are numbered from -75 to the gD mRNA start site at +1.

identify regions important for Vmw175 function, Paterson and Everett (1988) constructed insertion and deletion mutants in a plasmid-borne gene encoding Vmw175. These authors identified a region, between amino acids (aa) 275 and 490, which is important for both E gene trans-activation and IE gene repression, and a carboxy terminal (C-terminal) region which is important primarily for E gene trans-activation.

The fact that HSV-1 ts-Vmw175 mutants fail to activate E gene expression is an interesting finding in view of the ability of Vmw110 to independently trans-activate E promoters in transient expression assays. The reason for this phenomenon appears to be the transdominance of the mutant Vmw175 protein at the NPT. This is evidenced by the fact that certain ts-Vmw175 mutant polypeptides, which fail to accumulate in the nucleus, impair nuclear localisation of Vmw110 (Knipe and Smith, 1986). Further, a different ts-Vmw175 mutant, which localises in the nucleus and does not interfere with nuclear localisation of Vmw110, inhibits Vmw110-mediated trans-activation of E promoters at the NPT (Gelman and Silverstein, 1986). However, the transdominance of mutant ts-Vmw175 proteins may not be the only reason for the inability of Vmw110 to induce E gene expression. It is quite possible that the trans-activating function of Vmw110 is not strong enough and is therefore inhibited in the presence of negative regulators of IE gene expression such as MDBP (see Page 21; Godowski and Knipe, 1986).

The role of Vmw110 in productive infection has been studied in two HSV-1 recombinants with deletions in both copies of IE gene 1 (Stow and Stow, 1986; Sacks and Schaffer, 1987). Although the deleted genes had no detectable stimulatory effect on E gene promoters in transient expression assays (Perry et al., 1986; Sacks and Schaffer, 1987), mutant viruses were able to grow in tissue culture (Stow and Stow, 1986; Sacks and Schaffer, 1987). Growth defects were apparent at low m.o.i., where the plaquing efficiency of mutants was reduced, whereas at high m.o.i. the amount of gene expression and DNA synthesis was indistinguisable from wild-type infection (Stow and Stow, 1986; Sacks and Schaffer, 1987). Thus, it was suggested that although Vmw110 is not absolutely essential for virus

growth in tissue culture, it is required for normal virus growth at low multiplicity infections. Everett (1987a and 1988) constructed insertion and deletion mutants in a plasmid-borne gene encoding Vmw110, recombined them into the viral genome, and studied their effects during recombinant virus infection. The results showed that Vmw110 is required at early stages of low multiplicity infections where its absence results in insufficient viral gene expression to allow productive infection.

Vmw68 and Vmw63 have no stimulatory function on E gene transcription either independently or in combination with other IE polypeptides (O'Hare and Hayward, 1985b; Everett, 1986). By contrast, although Vmw12 does not stimulate E gene expression independently, it appears to augment the synergistic effect of Vmw175 and Vmw110 (O'Hare and Hayward, 1985b).

iv) The mechanism of E gene trans-activation. The mechanism by which Vmw175 and Vmw110 trans-activate E gene promoters is unknown. It appears that, at least in the case of Vmw175, this process requires the formation of a complex between Vwm175 and cellular protein(s); a partially purified Vmw175 preparation failed to bind to DNA in the absence of added cellular proteins, but did so in their presence (Freeman and Powell, 1982). This complex subsequently interacts with the promoter regions of E genes, via the cellular proteins, and positively regulates initiation of transcription. A partially purified nuclear Vmw175 preparation, where Vmw175 represented 5% of the total protein mass, was able to bind to a region flanking the gD mRNA start site and stimulated initiation of transcription from this promoter in vitro (Beard et al., 1986). When this Vmw175 preparation was incubated with an antibody specific for Vmw175, initiation of transcription was significantly impaired (Beard et al., 1986). Interestingly the same preparation stimulated transcritpion from the L gC and VP5 promoters whereas it inhibited transcription from the IE gene 4 promoter (Pizer et al., 1986).

Faber and Wilcox (1986) have identified two regions in the pBR322 plasmid vector sequences and one in the

gD promoter (between positions -111 to -81), which are preferentially bound by a partially purified Vmw175 nuclear preparation. Alignment of the protected sequences revealed a consensus of 5'-ATCGTCNNNNYCGRC-3' (where N=any nucleotide, Y=pyrimidine and R=purine) which was suggested to be the Vmw175/cellular factor(s) binding site (Faber and Wilcox, 1986). The relationship between this consensus with E gene trans-activation or IE gene repression is unclear. First, this consensus is not included in the 83bp promoter region which is required for fully regulated transcription of the gD gene (see Page 22; Everett, 1983 and 1984a). Second, this consensus is not protected by the Vmw175/cellular protein(s) putative complex in the upstream region of IE gene 2 while, the complete consensus or part of it are protected in the promoter regions of IE genes 1 and 3 (Kristie and Roizman, 1986a and b; Muller, 1987). Therefore, it appears that binding sites other than that proposed by Faber and Wilcox (1986) might be involved in the interaction between DNA and the putative Vmw175/cellular protein(s) complex.

c) Late gene expression.

L or γ gene expression requires the presence of functional IE genes and, in addition, viral DNA replication for maximal expression. L transcripts are first detectable at 2h to 3h p.i. and their accumulation peaks at 10h to 16h p.i. (Harris-Hamilton and Bachenheimer, 1985). Two groups of late genes have been described, the leaky-late or $\gamma 1$ and the true-late or $\gamma 2$ and these are distinguisable by the fact that the former are readily detectable before the onset of viral DNA replication whereas the latter are barely detectable (Roizman and Batterson, 1985; reviewed in Wagner, 1985).

Johnson and Everett (1986a) demonstrated that a fusion gene, consisting of the true-late US11 gene promoter linked to a rabbit β-globin gene, was expressed, in the presence of a functional ori_S, with kinetics similar to its viral counterpart after infection with HSV-1. Further, the appearance of both the fusion and viral genes coincided with the onset of viral replication. Expression of this fusion

gene from plasmids lacking ori_S was detectable but severely curtailed. These findings indicate that DNA replication, although not an absolute requirement for activation of a true-late promoter, is essential for abundant expression. It is possible that L gene promoters are relatively weak and therefore high copy numbers are required for abundant expression. Alternatively, it is possible that DNA replication somehow increases L gene expression by alteration of the state of the template; however, such an interpretation would not explain why only L gene products become more abundant after DNA replication and not E gene products.

It appears that a functional TATA box and a cap site are sufficient promoter elements for maximal US11 expression in the presence of ori_S (Johnson and Everett, 1986b). Similar sequence requirements have been described for the true-late gC gene promoter (Homa et al., 1986).

Activation of L promoters requires IE gene products Vmw175 and Vmw110 (see Fig. 6). HSV-1 ts-Vmw175 mutant viruses fail to induce late gene expression at the NPT (Marsden et al., 1976; Preston, 1979; Dixon and Schaffer, 1980; Watson and Clements, 1978 and 1980). Similarly, two different ts-Vmw175 mutants, which express IE and E genes and induce viral DNA synthesis, do not express L gene products at the NPT (DeLuca et al., 1984). In transient expression assays both Vmw175 and Vmw110 have been shown to activate L promoters (Everett, 1986; Mavromara-Nazos et al., 1986). However, Everett (1986) observed that, although a combination of Vmw175 and Vmw110 gave slight activation of the true-late VP5 gene promoter expression, addition of Vmw63 further increased activation.

SECTION D.

HSV POLYPEPTIDE SYNTHESIS.

7. Number and Post-Translational Modifications of HSV-Encoded Polypeptides.

Following infection of cells with HSV-1, about 50 novel polypeptides are induced as identified by one-dimensional SDS-PAGE analysis (Honess and Roizman, 1973; Marsden et al., 1976). However, two-dimensional polyacrylamide gel analysis revealed the existence of some 230 virus induced polypeptide species a number of which most probably are related forms of the same polypeptide (Haar and Marsden, 1981). Virus-specific polypeptide synthesis can be divided into IE (or α), E (or β) and L (or γ) temporal groups. The E polypeptides have been further subdivided into groups $\beta 1$ and $\beta 2$ and in contrast to $\beta 1$, $\beta 2$ synthesis requires fully-functional IE gene products (Pereira et al., 1977). The L polypeptides have been subdivided into groups $\gamma 1$ and $\gamma 2$ which are analogous to the early-late and true-late mRNAs respectively (see Page 25; Powell and Courtney, 1975). Although HSV infection is predominantly regulated at the transcriptional level, there is evidence that translational regulation also occurs. This is shown by the persistence at early and late times p.i. of a relatively large amount of HSV-1 IE mRNA 3 (Harris-Hamilton and Bachenheimer, 1985) while at the same time Vmw175 synthesis is greatly reduced (Dixon and Schaffer, 1980). The observed lack of correlation between mRNA levels and the rate of protein synthesis could be due to translational regulation although this possibility needs to be further examined (Harris-Hamilton and Bachenheimer, 1985).

The failure of some in vitro synthesised HSV polypeptides to comigrate with their in vivo counterparts on SDS-polyacrylamide gels indicates that they undergo certain modifications (Preston, 1977). Four main types of modification occur: phosphorylation, glycosylation, sulphation and cleavage (Marsden et al., 1982).

i) Phosphorylation. This is a reaction by which phosphate groups are esterified to a serine or a threonine residue in the primary structure of the protein, and is catalysed by the enzyme protein kinase (see Page 35). Approximately 16 HSV-1 and 18 HSV-2 polypeptides are phosphorylated (Pereira et al., 1977; Marsden et al., 1978). In several of these phosphoproteins the phosphate can cycle on and off (Wilcox et al., 1980). Vmw175 is present as three species with different electrophoretic mobilities termed a, b, and c; species a and c rapidly cycle between the phosphorylated and the non-phosphorylated forms while species b remains stably phosphorylated. Other phosphoproteins in which the phosphate cycles on and off include Vmw68, Vmw63 and Vmw136 (Wilcox et al., 1980). Phosphorylation has also been shown to change the affinity of certain polypeptides for double-stranded DNA; for example, the affinity of Vmw136 is decreased after phosphorylation (Wilcox et al., 1980).

ii) Glycosylation. Glycosylation is a process by which oligosaccharides are covalently attached to the polypeptide chain. At least seven HSV-1-induced polypeptides are glycosylated (see Page 30). Studies with the antibiotic tunicamycin, which blocks the synthesis of glycoproteins, demonstrated that in HSV the mechanism of oligosaccharide moiety addition to the polypeptide backbone is analogous to that of the glycosylation of the VSV glycoprotein G (Pizer et al., 1980; Hope and Marsden, 1983). In VSV, oligosaccharide-peptide linkages are predominantly N-glycosidic where the oligosaccharide core, which contains N-acetylglucosamine and mannose, is linked to an asparagine residue. However, O-glycosidic linkages between the core and serine or threonine residues have also been reported (Olofsson et al., 1981). Glycosylation appears to occur in discrete steps (Haar and Marsden, 1981; Palfreyman et al., 1983).

iii) Sulphation. At late times of infection, inorganic sulphate is added to the major glycoprotein

species (Hope et al., 1982; Hope and Marsden, 1983). In most cases this is achieved by attachment of the sulphate to N-linked oligosaccharide moieties (Hope and Marsden, 1983).

iv) Proteolytic cleavage. Proteolytic cleavage is another form of HSV polypeptide modification although the mechanism by which it occurs has been poorly investigated. Vmw136 and the IE polypeptides Vmw175, Vmw110 and Vmw63 induced by the HSV-1 mutant tsK at the NPT are degraded to lower mol. wt. products (McDonald, 1980). This degradation appears to be specific as it does not occur in the presence of protease inhibitors. The Vmw136 polypeptide has been shown, by tryptic peptide mapping, to give rise to products of 100,000 mol. wt. and 90,000 mol. wt. which are found in the nucleus of infected cells while the polypeptide is itself predominantly cytoplasmic (McDonald, 1980). Likewise, Ingemarson and Lankinnen (1987) reported that Vmw136 is proteolytically digested to products with mol. wts. of 110,000, 93,000 and 81,000. The maturation of HSV glycoproteins also involves proteolytic cleavage. Eisenberg et al. (1984) demonstrated that 25 amino acids from the translation product of gD were missing from the mature glycoprotein isolated from HSV-infected cells; the missing stretch of amino acids, which includes the entire amino terminal (N-terminal) hydrophobic domain, is thought to constitute the signal sequence which is found at the N-terminus of many transmembrane and secreted proteins.

8. Structural Polypeptides.

a) Virion polypeptides.

Approximately 33 HSV-1 polypeptides have been designated as structural components of the virion (Spear and Roizman, 1972; Marsden et al., 1976). The nucleocapsid contains seven polypeptides ranging in mol. wt. from 12,000 to 155,000. Immune electron microscopy revealed that the major capsid protein, Vmw155, was distributed over the entire capsid surface whereas polypeptide Vmw50 appeared to be located at the capsid vertices (Vernon et al., 1981).

It is quite possible that these two polypeptides bind within the capsid structure with disulphide bonds. A third virion polypeptide, Vmw40 or p40, also was located over the capsid (Vernon et al., 1981), possibly at positions interior to those of Vmw155, and this protein appears to form disulphide-linked complexes, the constituents of which are not resolved. Gibson and Roizman (1972) have suggested that this protein could be involved in DNA encapsidation and this was strengthened by the observation that p40 covers the surface of DNA-containing capsids (full nucleocapsids) but not of empty ones (Braun et al., 1984). However, recent immune electron microscopy studies, using two different monoclonal antibodies, have localised p40 on the surface of partially-cored rather than full nucleocapsids (Rixon et al., 1988).

Located in the tegument of the herpesvirion, of note are a large polypeptide of 273,000 mol. wt. (Heine et al., 1974), the function of which is unclear, and the Vmw65 protein which is responsible for the trans-induction of IE gene transcription (see Page 19). In addition, the presence of a protein kinase activity in capsid-tegument structures has been described (Lemaster and Roizman, 1980), although, similarities between this activity and the host casein kinase II suggest that this enzyme may not be virus-encoded (Stevely et al., 1985).

b) Envelope polypeptides.

The virus particle has an external lipid envelope (Wildy et al., 1960) which, although is normally acquired by budding of the intranuclear particles through the host cell inner nuclear membrane (Darlington and Moss, 1968), is enriched for virus specific glycoproteins (Spear and Roizman, 1972; Heine et al., 1972; Honess and Roizman, 1975). At least seven HSV-1 glycoproteins have been identified, designated B, C, D, E, G, H, and I (reviewed in Marsden, 1987; Longnecker et al., 1987; McGeoch et al., 1987) all of which are present in virions (Spear, 1976; Para et al., 1980; Buckmaster et al., 1984; Frame et al., 1986a; Richman et al., 1986). Immunoprecipitation studies using type common antibodies

have shown that HSV-2 induces equivalent polypeptides to gB, gC, gD, gE and gG (reviewed in Marsden, 1987).

9. HSV-Induced Enzymes.

HSV induces a number of enzyme activities which are involved in nucleotide metabolism, and either directly or indirectly in DNA replication.

a) Enzymes involved in DNA metabolism.

i) Ribonucleotide reductase. This enzyme catalyses the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates and its properties are described in Section E.

ii) Thymidine kinase. The cellular enzyme catalyses the synthesis of deoxypyrimidine nucleotides from preformed nucleosides, which are the products of cell death or catabolic activity. Kit and Dubbs (1963) first described such an HSV-induced activity with properties different from those of the host cell enzyme. The HSV enzyme, originally thought to phosphorylate only thymidine, was shown in addition to phosphorylate deoxycytidine and was thus characterised as a deoxypyrimidine kinase (Jamieson et al., 1974; Jamieson and Subak-Sharpe, 1974). By contrast, PRV and vaccinia virus (VV) specify only a thymidine phosphorylating activity while equine abortion virus (EAV) does not induce either activity (Jamieson et al., 1974). The two HSV phosphorylating activities appear to be catalysed by the same active site although they are quite distinct in their biochemical behaviour (Jamieson and Subak-Sharpe, 1974). Further, the HSV-induced enzyme appears to be associated with a phosphotransferase activity which can utilize AMP as phosphate donor to convert thymidine to thymidylate (Jamieson et al., 1976).

The active enzyme is a dimer consisting of two identical subunits of 42,000 mol. wt. (Honess and Watson, 1974; Jamieson and Subak-Sharpe, 1978). Jamieson et al. (1974) demonstrated that it was indispensable for virus

growth in serum-starved cells but not in actively growing ones indicating that such an activity is required only when the de novo metabolism of the host cell is low.

iii) Deoxyuridine triphosphatase (dUTPase). After infection, HSV specifies a novel dUTPase activity (Wohlrab and Franke, 1980) which is virus-encoded (Preston and Fisher, 1984). This enzyme catalyses the reduction of dUTP to dUMP and pyrophosphate; in the host cell, the role of this enzyme is to reduce the intracellular concentration of dUTP by reducing it to dUMP. Subsequently, dUMP can be methylated to dTMP by the enzyme thymidylate synthetase. The HSV-1-induced dUTPase has a mol. wt. of 53,000 (Williams, 1984) and has been shown not to be essential for virus growth in cultured cells (Fisher and Preston, 1986). Recently, William and Paris (1987) have reported that HSV-2 induces a dUTPase activity utilising dUTP as a substrate and that this activity differs biochemically from the HSV-1-induced activity. It appears that HSV, as well as, HCMV, PRV and EBV do not encode a thymidylate synthetase, although HVS and VZV do (Davison and Scott, 1986b; Honess et al., 1986; Thompson et al., 1987). This could be due to the fact that both HVS and VZV have A+T-rich genomes which indicates a possible greater requirement for thymidylate synthesis.

iv) Uracil-DNA glycosylase. During replication when a cytosine residue in DNA loses its amino group a uracil:guanosine mispair occurs which can lead to a guanosine:cytosine to adenine:thymine transition mutation. The function of the cellular enzyme is the removal of deaminated cytosine residues from DNA thus reducing transition mutations. In HSV-infected cells, this activity appears to be virally encoded and differs biochemically from the host cell enzyme (Caradonna and Cheng, 1981; Caradonna et al., 1987). Initial mapping data suggested that it is located at the left portion of U_L (Caradonna et al., 1987) and recent evidence strongly suggests that the gene encoding the glycosylase is UL2 (see Fig. 2; J. Mullaney, personal communication).

b) Enzymes involved in DNA replication.

i) DNA polymerase. Keir and Gold (1963) demonstrated that HSV-1 induced a DNA polymerase activity which was stimulated by high salt concentration (Keir et al., 1966). The viral enzyme is distinct from the host cell enzyme not only immunologically (Keir et al., 1966), but also biochemically by virtue of its 3' to 5' exonuclease activity and by its sensitivity to low concentrations of phosphonoacetic acid (PAA) a pyrophosphate analogue (Knopf, 1979; Leinbach et al., 1976). The HSV-1 enzyme consists of a single polypeptide chain of 150,000 mol. wt. (Powell and Purifoy, 1977) and in vitro it is active as a monomer (Knopf et al., 1979). Mutations within this gene have shown that it is essential for viral DNA replication (Purifoy et al., 1977).

Purified preparations of the HSV-2 DNA polymerase often contained a polypeptide of 54,000 mol. wt. designated ICSP 34,35 (Powell and Purifoy, 1977; Knopf, 1979; Vaughan et al., 1985). This polypeptide has been shown to be the HSV-2 counterpart of the HSV-1 $65K_{DBP}$ described on Page 13 (H.S. Marsden, personal communication).

ii) DNA primase. Recently, Holmes et al. (1988) reported a novel HSV-1 primase activity which co-eluted with a portion of the HSV-1 DNA polymerase from single-stranded DNA agarose columns loaded with high-salt infected cell extracts. This activity was biochemically distinct from host HeLa cell DNA primase, did not cross-react with polyclonal antibodies raised against the calf thymus DNA polymerase/primase complex and appeared to be coupled with the HSV-1 DNA polymerase. The approximate mol. wt. of this novel activity is 40,000 (Holmes et al., 1988).

iii) Alkaline exonuclease (AE). A novel alkaline exonuclease activity is induced following HSV-1 (Morrison and Keir, 1968; Strobel-Fidler and Franke, 1980) and HSV-2 infection (Hay et al., 1971; Hoffmann and Cheng, 1979) with properties different from those of cellular nucleases.

This enzyme, in addition to its 5' and 3' exonuclease activities, has been shown to possess an endonuclease activity (Hoffmann and Cheng, 1979; Hoffmann, 1981). Marker rescue experiments, using an HSV-2 ts-AE virus (Moss et al., 1979), and in vitro translation in Xenopus laevis oocytes (Preston and Cordingley, 1982) suggest that this enzyme is virally encoded and corresponds to polypeptide Vmw85 (Marsden et al., 1978). Moss (1986) demonstrated that the AE activity is essential for virus replication as a ts-AE mutant exhibited considerably reduced levels of virus DNA synthesis and growth at the NPT.

iv) Topoisomerase. DNA topoisomerases are enzymes that transiently break the phosphodiester backbone of DNA and then rejoin the free DNA ends. To date, all topoisomerases can be divided into two categories. Type I enzymes catalyse the breaking and rejoining of only one strand of DNA at a time, whereas, type II enzymes appear to introduce transient double-strand breaks (reviewed in Gellert, 1981). Both classes relax supercoiled DNA although the reaction mechanisms are clearly different (Brown and Cozzarelli, 1981) and each enzyme class can be distinguished by reaction cofactor requirements. Biswal et al., (1983) first reported an HSV-induced topoisomerase activity which copurified with the DNA polymerase through several chromatographic steps. By contrast, Muller et al. (1985) suggested that the HSV-1 topoisomerase activity, which these authors classified as type I, was a component of the virion envelope or the tegument. Further studies with antibodies specific for the cellular and putative viral topoisomerase activities will be required to resolve the localisation of the enzyme and its role in replication.

c) Interactions between HSV-1 enzymes involved in DNA replication.

There is both biochemical and genetic evidence that at least four virus-encoded polypeptides form a functional complex which is involved in DNA replication.

The constituents of this putative complex are DNA polymerase, AE, $65K_{DBP}$, and MDBP. The latter is a virus induced protein of 128,000 mol. wt. and its *in vitro* properties include the ability to dissociate double-stranded DNA (Powell *et al.*, 1981), to stimulate the activity of the HSV-encoded DNA polymerase activity and to preferentially bind single-stranded DNA (Ruyechan and Weir, 1984). The MDBP has been shown to be essential for virus replication (Weller *et al.*, 1983). Vaughan *et al.* (1984) using monoclonal antibodies against the MDBP, $65K_{DBP}$, and AE demonstrated the existence of: i) two very strong binding complexes, the first between MDBP and AE and the second between DNA polymerase and $65K_{DBP}$, and ii) an intermediate strength association between MDBP and $65K_{DBP}$.

The first genetic evidence of the existence of such a functional complex *in vivo* involved studies with *ts* virus mutants (Littler *et al.*, 1983). These authors demonstrated that two HSV-2 *ts*-MDBP mutant viruses severely restricted DNA polymerase and AE induction in infected cells. Furthermore, *ts* mutations in MDBP induced secondary mutations within the DNA polymerase gene which altered its sensitivity to PAA (Chiou *et al.*, 1985). These secondary mutations could possibly restore the functional interaction between the DNA polymerase and the altered MDBP.

10. Other HSV-Induced Enzyme Activities.

During lytic infection of cells by HSV, virus-induced protein kinase activities have been detected. DNA sequencing studies revealed that the predicted primary structure of the US3 gene product of both HSV serotypes (McGeoch *et al.*, 1985 and 1987) and the corresponding gene of VZV (Davison, 1983) were clearly homologous to members of the protein kinase family of eukaryotes (McGeoch and Davison, 1986b). Frame *et al.* (1987) demonstrated, by means of rabbit antisera raised against an octapeptide corresponding to the eight C-terminal amino acids of the predicted US3 gene sequence, that the product of this gene is indeed a protein kinase.

The substrate specificity of the HSV-induced enzyme has been shown to be broadly similar to that of the PRV-induced enzyme which phosphorylates serine and threonine residues (Purves et al., 1986). The main conclusions regarding the site specificity of the PRV enzyme, as deduced by phosphorylation studies with oligopeptide substrates, are: i) basic residues, preferably arginines, must be present at the N-terminal side of the target residue and ii) at least two basic N-terminal residues are required, whereas, additional ones have a further positive effect. Additional requirements for maximal phosphorylation include the need for the N-terminal arginines to be displaced from the target serine or threonine residue for optimal effect, hydrophobic residues at the N-terminal side of the target residue may cause a decrease in phosphorylation; finally, proline residues adjacent to the target residue on the N-terminal side increase phosphorylation, whereas, on the C-terminal side they decrease it (Purves et al., 1986).

SECTION E.

RIBONUCLEOTIDE REDUCTASES.

11. General Background.

a) Mechanism of the reaction.

Ribonucleotide reductase (EC 1.17.4.1) catalyses the reduction of all four ribonucleotides to the corresponding deoxyribonucleotides, the hydroxyl group at the 2' position of the ribonucleotide ribose moiety being substituted with a hydrogen, and NADPH being the ultimate hydrogen donor (Fig. 8; Thelander and Reichard, 1979; Reichard and Ehrenberg, 1983). This reaction is the first unique step in the de novo pathway of DNA biosynthesis.

b) Hydrogen donor systems.

Efforts to identify the physiological hydrogen donor in this reaction resulted in the discovery of two novel types of hydrogen carrier systems, the thioredoxin and the glutaredoxin systems.

i) The thioredoxin system. This system was originally discovered in Escherichia coli (E. coli; Laurent et al., 1964); it consists of two small proteins, thioredoxin and thioredoxin reductase, and utilizes NADPH as a hydrogen donor. The E. coli thioredoxin is an enzyme of 11,700 mol. wt. and 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 serves as an efficient hydrogen donor for ribonucleotide reductase while the oxidised form is reduced in the cell by the enzyme thioredoxin reductase at the expense of NADPH. 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). Overall, the function of this system involves a

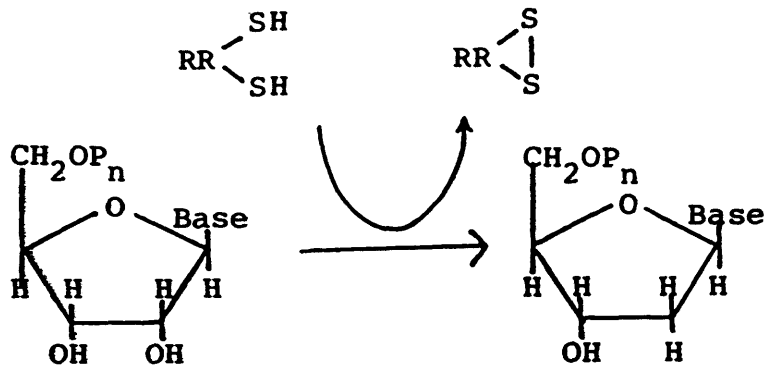


Figure 8. Reduction of a ribonucleotide to a deoxyribonucleotide by ribonucletide reductase (RR). RR-$(SH)_2$ and RR-S_2 represent the reduced and oxidised forms of ribonucleotide reductase respectively. P_n denotes a diphosphoryl or triphosphoryl nucleotide.

shuttle transfer of electrons from NADPH to the substrate via a system of redox-active disulphides as shown in Fig. 9 (reviewed in Thelander and Reichard, 1979)

Mammalian cells specify a thioredoxin system, comprising a thioredoxin reductase and a thioredoxin, similar to that of E. coli. Bacteriophage T4 encodes a thioredoxin but not a thioredoxin reductase; oxidised T4 thioredoxin can be reduced by the host cell thioredoxin reductase (Berglund et al., 1969; Berglund, 1969). Further, T4-induced thioredoxin can also be reduced by the glutathione system (see below) and thus appears to be a functional hybrid between these two hydrogen donor systems (Holmgren, 1978). Although the primary structure of the T4-induced and E. coli thioredoxins are not homologous (Sjoberg and Holmgren, 1972), their three-dimensional structures display large similarities (Sodenberg et al., 1978; Hoog et al., 1983). Attempts to identify the principal hydrogen donor for the HSV-1-induced ribonucleotide reductase resulted in the purification of a protein, present in both infected and mock-infected extracts, with similar physical characteristics to the cellular thioredoxin (Darling, 1988); this result, in conjunction with the lack of DNA sequence-evidence for the existence of a virus-induced thioredoxin, suggests that HSV-1 utilises the cellular enzyme as a hydrogen donor.

ii) The glutaredoxin system. This system was identified in an E. coli mutant which was unable to replicate bacteriophage T7 DNA, failed to express any detectable level of thioredoxin and, at the same time, displayed no decreased capacity to reduce ribonucleoside diphosphates (NDPs) in vitro as compared to the wild-type (Holmgren, 1976). The glutaredoxin system consists of glutathione, glutaredoxin, and glutathione reductase and utilizes NADPH as a hydrogen donor. The E. coli glutaredoxin is an enzyme of 11,600 mol. wt. and contains two redox-active cysteine residues (Holmgren, 1979a). This system functions as shown in Fig. 10 (Holmgren, 1979b).

It is not known which of these two systems is functional in vivo; it is possible that both systems

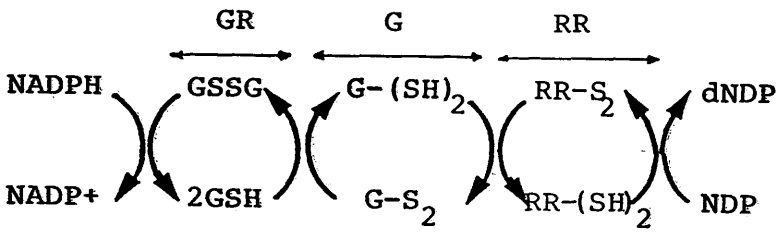


Figure 10. Reduction of a ribonucleoside diphosphate (NDP) to a deoxyribonucleoside diphosphate (dNDP) with the E. coli glutaredoxin system. The diagram shows the involvement of proteins, with oxidation-reduction active sulhydryl groups, in the shuttle electron transfer from NADPH to the NDP. The reactions catalysed by glutaredoxin reductase (GR), glutaredoxin (G) and ribonucleotide reductase (RR) are indicated above the diagram. The reduced forms of GR, G and RR are indicated by GR-$(SH)_2$, G-$(SH)_2$ and RR-$(SH)_2$ while the oxidised forms are indicated by GR-S_2, G-S_2 and RR-S_2 respectively.

substitute for each other under different growth conditions.

c) Classification of ribonucleotide reductases.

Studies on ribonucleotide reductases of prokaryotic, eukaryotic and viral origin have suggested that three different classes of enzyme exist. Class I, which is represented by the enzyme from Lactobacillus leichmannii, is monomeric and is found in several prokaryotes and in a few eukaryotes. Class II is represented by the E. coli enzyme and consists of two non-identical homodimeric subunits. This type is found in some prokaryotes, most eukaryotes and animal viruses. Finally, class III enzymes resemble those of class II in structure but differ from them biochemically. This class is found in a number of gram positive bacteria. The following part of Section E will mainly describe class I and II enzymes which are the best characterised.

12. Monomeric Ribonucleotide Reductases.

As already mentioned, the monomeric ribonucleotide reductases are represented by the Lactobacillus leichmannii enzyme which consists of a single polypeptide chain of 76,000 mol. wt. (Fig. 11; 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 is assumed that the enzyme has a single catalytic site which binds the ribonucleoside triphosphate (NTP) substrates. The existence of the latter has not been shown using direct binding studies with substrates; however, kinetic experiments showed that the best substrate, GTP, binds with a Km as high as 0.24mM (Vitols et al., 1967). Chen et al. (1974) demonstrated that the substrates are also able to bind to the regulatory site although their affinity is 100 to 1,000 lower than that of the effectors.

The activity of this class of enzymes is absolutely dependent on the presence of adenosylcobalamine (AdoCbl, B12-coenzyme). AdoCbl binds directly to the polypeptide chain and is dependent on effector binding to the regulatory

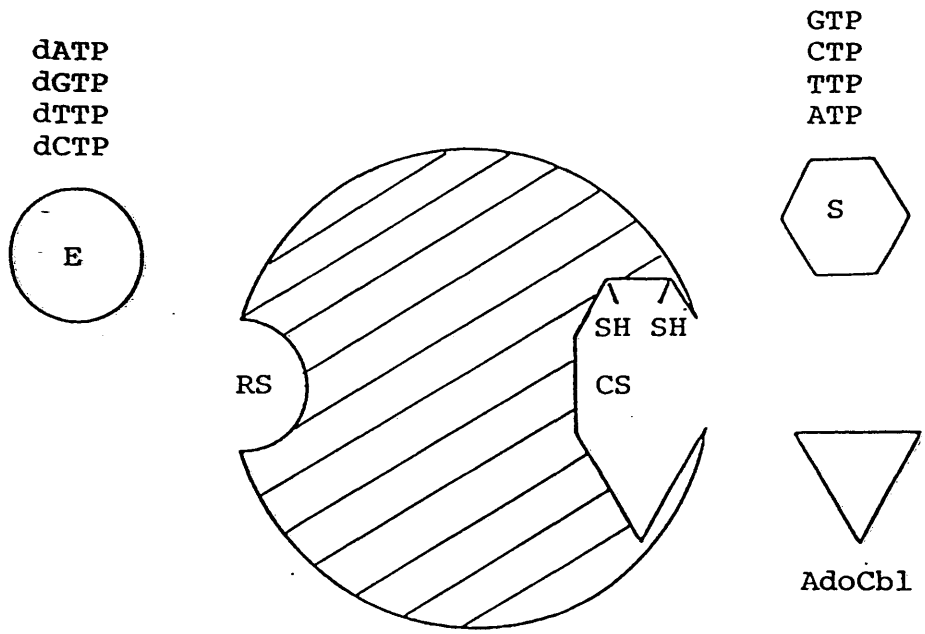


Figure 11. A schematic representation of the proposed structure of the Lactobacillus leichmannii ribonucleotide reductase. The enzyme is depicted as a monomeric protein (hatched region) with a single regulatory site (RS) which binds the allosteric deoxyridonucleoside triphosphate effectors (E) indicated. The enzyme's single catalytic site (CS) contains the active sulphydryl groups (SH) and binds the ribonucleoside triphosphate substrates (S) shown. The CS is also depicted to bind adenosylcobalamine (AdoCbl).

site (Singh et al., 1977). It is thought that AdoCbl functions in a manner similar to that of the class II enzyme subunit which contains the iron centre and the tyrosyl free radical (see below; Ashley et al., 1986).

It is proposed that the enzyme's activity is only positively regulated as no strong 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 reduction of the CTP, UTP, GTP, and ATP substrates respectively (Vitols et al., 1967).

13. Oligomeric Ribonucleotide Reductases.

a) The E. coli enzyme.

The E. coli ribonucleotide reductase is a dimeric enzyme consisting of two non-identical subunits, B1 and B2 (Fig. 12; Brown and Reichard, 1969a). The respective polypeptides are encoded by the nrdA and nrdB genes (Fuchs and Karlstrom, 1976) which are located at adjacent regions on the E. coli chromosome (Bachmann et al., 1976; Yamada et al., 1982). The active enzyme consists of the B1 and B2 subunits bound in a 1:1 stoichiometry in the presence of Mg^{2+} (Brown et al., 1967; Brown and Reichard, 1969a; Thelander, 1973). This binding is rather weak and the two subunits easily dissociate during purification.

i) The B1 subunit. This subunit is a dimer of two identical polypeptides (a_2) and has a mol. wt. of 160,000 (Thelander, 1973). Each B1 subunit contains two catalytic sites which bind the NDP substrates (von Dobeln and Reichard, 1976), and two types of different regulatory sites, each type consisting of two sites, which bind the allosteric effectors (Brown and Reichard, 1969b).

The catalytic sites. Binding studies with separated B1 and B2 subunits showed that only B1 has the capacity to bind substrates, indicating that the catalytic sites are positioned on B1 (von Dobeln and Reichard, 1976). In addition, these authors proposed that all four substrates

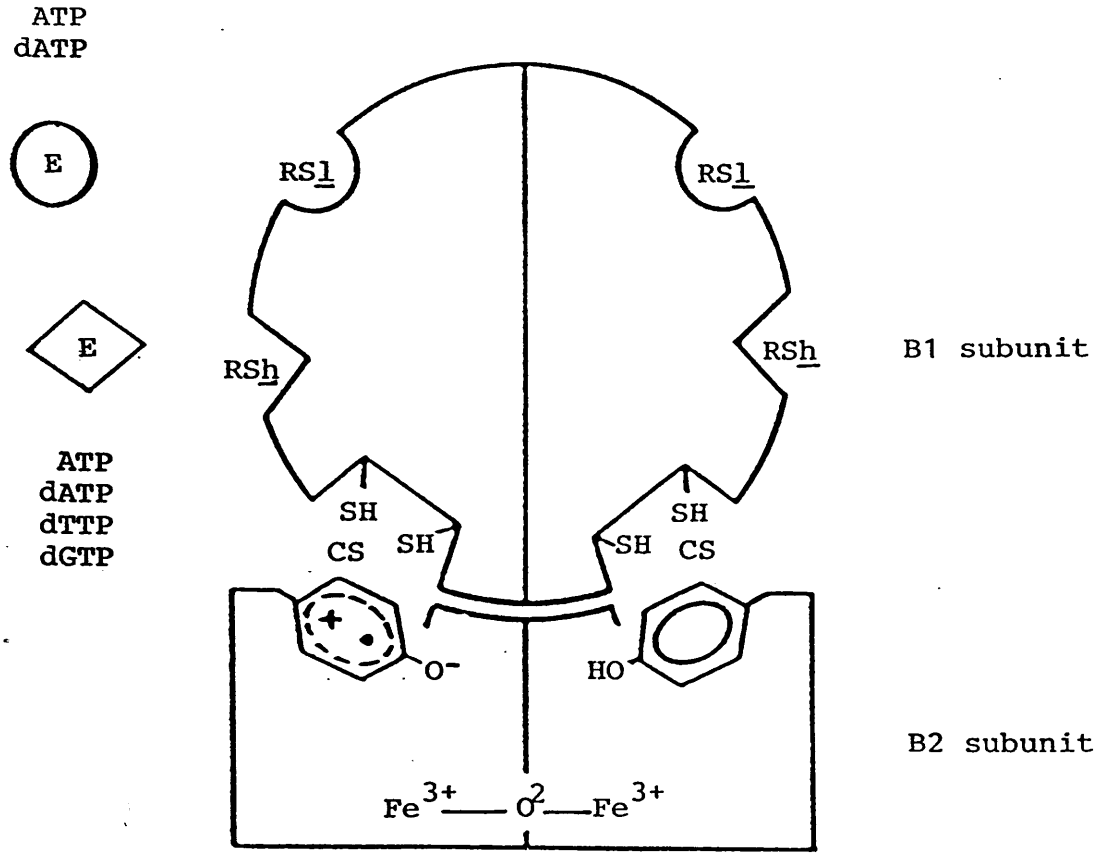


Figure 12. A schematic representation of the proposed structure of the E. coli ribonucleotide reductase. The enzyme is depicted as a dimer of the B1 and B2 subunits, each consisting of two identical polypeptides. The B1 subunit contains two catalytic sites (CS) which contain the active sulphydryl groups (SH), and bind the ribonucleoside diphosphate substrates. The regulatory sites (RS) bind the allosteric effectors (E) shown, and are separated in low (l) and high (h) dATP-affinity sites. The B2 subunit contains the tyrosyl radical (•), which is depicted to conform to half-site reactivity, and the iron centre (Fe^{3+}-O^{2}-Fe^{3+}).

bind to the same site since they are equally competitive for binding. However, as depicted in Fig. 12, the catalytic site, in order to reduce substrates, requires the presence of the active sulphydryl groups provided by two cysteine residues which are located exclusively on B1 (Thelander, 1974). B2 alone cannot reduce the substrates even after incubation with dithiothreitol to increase its sulphydryl active content. Furthermore, reduction of substrates requires the stable free radical which is provided by B2 (see below; Thelander, 1974; Ehrenberg and Reichard, 1972). In conclusion, although substrate binding takes place in B1, the active catalytic site itself is composed by elements from both subunits.

The allosteric sites. Brown and Reichard (1969b) proposed that the allosteric sites can be separated into two types on the basis of their affinity for dATP-effector binding; the h sites with high affinity (K_d=0.03uM) and the l sites with low affinity for this effector (K_d=0.1-0.5uM). Competition experiments demonstrated that the h sites also bind ATP, dTTP, and dGTP, while, the l sites bind only ATP in addition to dATP (Brown and Reichard, 1969b).

ii) The B2 subunit. This subunit is a dimer of two identical polypeptides (b_2) and has a mol. wt. of 78,000 (Thelander, 1973). The B2 subunit has two important elements namely, the iron binuclear centre and the stable free radical.

The iron centre. The E. coli B2 subunit contains two non-heme iron atoms which can be removed after prolonged dialysis against 8-hydroxyquinoline (Brown et al., 1969). These are in an antiferromagnetically coupled binuclear complex and most possibly are liganded with an μ-oxo-group (Atkin et al., 1973; Petersson et al., 1980; Sjoberg et al., 1980). Sjoberg et al., (1985) proposed that the E. coli iron centre is of the same type as in h emerythrin, where the two iron atoms are liganded by one glutamic acid, one aspartic acid and five histidine residues (Stenkamp et al., 1981).

The stable free radical. The E. coli B2 subunit contains a stable free radical as part of its structure

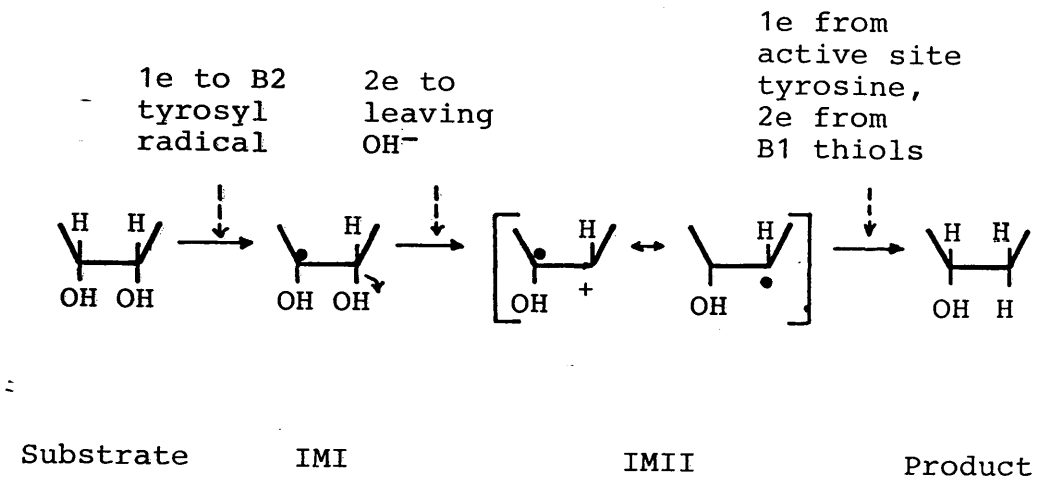


Figure 13. The proposed involvement of the E. coli ribonucleotide reductase radical in the reduction of a ribonucleotide to the corresponding deoxyribonucleotide (see text). The diagram depicts the substitution of the 2' OH group of the substrate ribose moiety with a hydrogen in the product. The balance of electrons (e) is shown and the radical is depicted by (•). IMI and IMII indicate the intermediate states I and II.

(Ehrenberg and Reichard, 1972). The radical can be destroyed after treatment with hydroxyurea or hydroxylamine which are powerful radical scavengers; they most probably act by donating an electron to the radical (Ehrenberg and Reichard, 1972).

Isotope substitution experiments located the radical to a tyrosine (Sjoberg et al., 1977) with its spin density delocalised over the aromatic ring of the residue (Sjoberg et al., 1978). This tyrosine was identified by site directed mutagenesis (Larsson and Sjoberg, 1986); substitution of it with a phenylalanine produced a mutant B2 protein which showed an electron paramagnetic resonance (EPR) spectrum identical to that of the host cell, and was less than 2% active as compared to the wild-type B2. Sjoberg et al., (1987) found a truncated form of B2 dimers (b'_2), lacking the 29 C-terminal amino acids, which resulted from limited proteolysis of the normal B2(b_2) dimers. Although the B2(b'_2) subunit had normal tyrosyl radical content and a normal iron centre, it had no enzymatic activity as it failed to bind the B1 subunit (Sjoberg et al., 1987). Further, by mixing B2(b_2) and B2(b'_2) populations, these authors isolated a heterodimeric form of B2 with a bb' structure which could weakly bind B1 and the resulting B1(a_2)B2(bb') enzyme had low activity. Using this mutant enzyme, they proposed that the tyrosyl radical is randomly generated in either one or the other of the two polypeptides in B2(bb'), thus conforming to half-site reactivity.

The main involvement of the radical in the reaction is the transient transfer of the unpaired electron from the enzyme to the substrate. Sjoberg et al. (1983) proposed a scheme for this reaction (Fig. 13). First, the radical abstracts the hydrogen atom at the 3' position of the substrate ribose moiety. The 3' electron is captured by the radical and this results in the conversion of the radical-containing tyrosine residue to a normal tyrosine residue and in the formation of substrate intermediate state I. Second, the presence of the radical at the 3' position destabilises the ribose moiety which rapidly rearranges by releasing the hydroxyl group from 2' position thus resulting

in intermediate state II. Third, donation of two electrons from the B1 subunit active sulphydryls and one electron from the tyrosine results in formation of a deoxyribose moiety and concomitant regeneration of the radical in the B2 tyrosine residue.

It is unusual to find a stable free radical as an integral part of an enzyme. Atkin et al. (1973) proposed that the iron centre appears to generate and stabilise the radical since when it is chelated out of the protein the radical is also lost. Recovery of the iron centre, by addition of ferrous iron in the presence of oxygen, regenerates the radical as well (Atkin et al., 1973). In contrast, destruction of the tyrosyl radical with hydroxyurea does not destroy the iron centre, and reconstitution of the former requires chelation and subsequent reconstitution of the iron centre. In conclusion, the enzymatic activity of B2 depends upon the presence of the radical which in turn depends upon the presence of the iron centre.

iii) Allosteric regulation. Allosteric control of the E. coli ribonucleotide reductase has been extensively studied. The overall enzyme activity and substrate specificity are regulated by the allosteric effectors (Brown and Reichard, 1969b). As described above, there are two types of binding sites the l and h. The l sites regulate the level of overall activity of the enzyme since binding of ATP activates the enzyme, whereas, binding of dATP leads to inactivation which can be reversed by the addition of ATP. dATP inhibition is accompanied by the formation of complexes (dimers) which contain equimolar amounts of the B1 and B2 subunits and have a sedimentation coefficient of approximately 15.5S while the active enzyme has a coefficient of 9.7S (Brown and Reichard, 1969a). The h sites regulate the substrate specificity of the enzyme; binding of any effector to these sites results in a conformational change of the catalytic site which leads to preferential binding of the appropriate substrate (Brown and Reichard, 1969b).

In vitro binding studies, performed by Larsson and

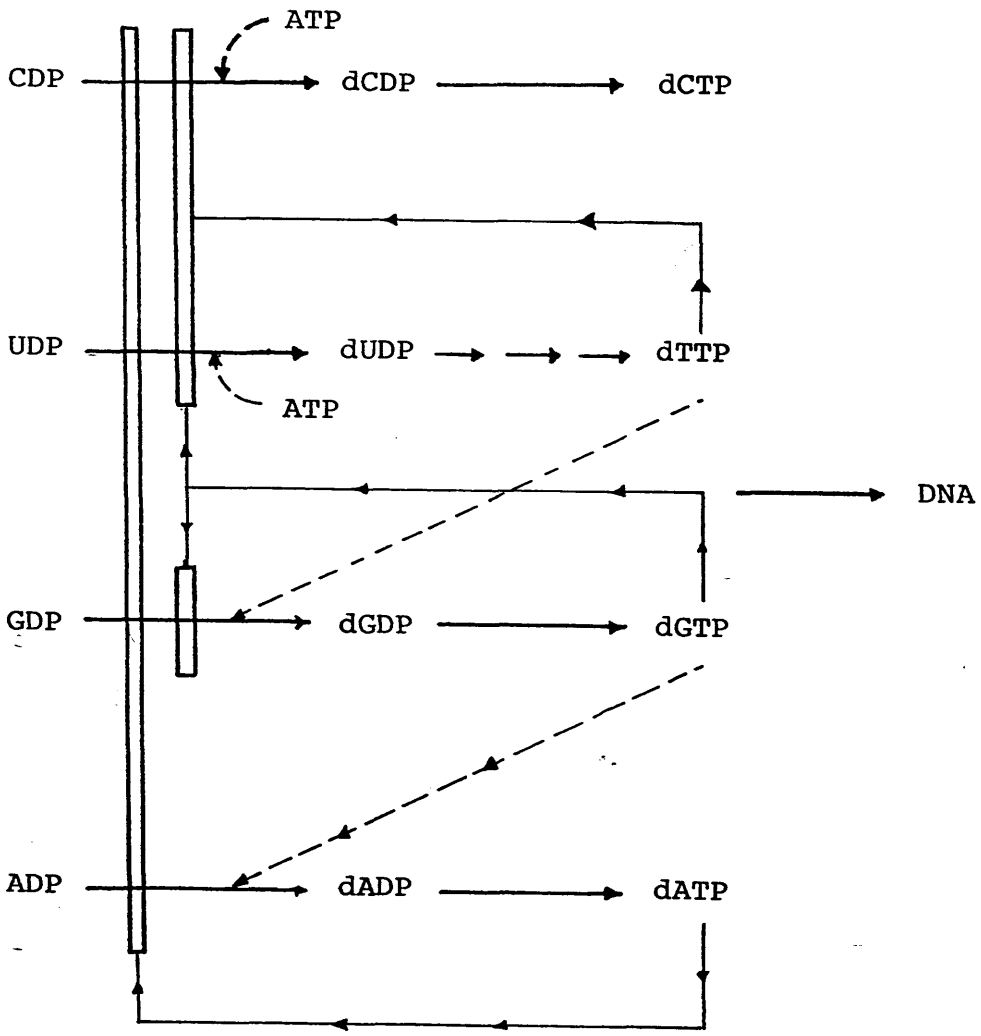


Figure 14. Allosteric control of the E. coli ribonucleotide reductase. Substrates and reaction products are shown, with the direction of reaction pathways indicated by thick-arrowed lines. Reactions inhibited by the negative effectors are shown by open bars; dashed lines indicate reactions stimulated by positive effectors.

Reichard (1966a,b), demonstrated that in the absence of effectors the enzyme activity is low whereas addition of low concentrations of dTTP stimulates reduction of all four substrates. Addition of ATP or low concentrations of dATP selectively stimulates reduction of CDP and UDP, whereas, the presence of dGTP stimulates reduction of ADP and, to a lesser extent, GDP. These conditions refer to the situation where the enzyme has only its h sites occupied; however, in vivo, in all probability, the l sites are at the same time occupied with either ATP or dATP and the ratio of their concentrations determines the activity of the enzyme. Therefore, it is proposed that in vivo three active and one inactive enzyme states exist (reviewed in Thelander, 1969). When ATP is bound to the l sites, binding of either ATP, or dTTP, or dGTP to the h sites will respectively reduce CDP or UDP, GDP (and ADP) and finally ADP (and GDP). When dATP is bound to the l site, the enzyme is inactive irrespective of effector binding to the h site.

These findings can be summarised into the following scheme that links ribonucleotide reduction to DNA synthesis (Fig. 14; reviewed in Reichard, 1978). Deoxyribonucleotide synthesis begins with the reduction of pyrimidine substrates (CDP, UDP) by an ATP-activated enzyme. Subsequently, a dTTP-activated enzyme reduces GDP and a dGTP-activated enzyme reduces ADP. Accumulation of dTTP shuts off pyrimidine substrate reduction whereas accumulation of dGTP shuts off both pyrimidine and GDP substrate reduction. Finally, dATP shuts off reduction of all four substrates.

b) Bacteriophage-induced enzymes.

Infection of E. coli with bacteriophages T2, T4, T5, and T6 results in the appearance of a new ribonucleotide reductase activity (Cohen and Barner, 1962; Biswas et al., 1965; Berglund et al., 1969; Eriksson and Berglund, 1974). In contrast T7 and λ do not synthesise a novel enzyme during infection (Eriksson and Berglund, 1974). The T4-induced enzyme, which is the most well studied, has a mol. wt. of 225,000 and is composed of two subunits called T4B1 and T4B2 (Fig. 15; Berglund, 1972a). In contrast to the E. coli enzyme, the T4 subunits form a very tight

complex (Berglund, 1972a).

i) The T4B1 subunit. This subunit is a dimer of two identical polypeptides (α_2) each of which has a mol. wt. of 85,000 (Berglund 1972a and 1975). This subunit contains, possibly, a single catalytic site which binds the NDP substrates and, possibly, two types of allosteric sites, one with low and one with high affinity for dATP; the latter binds in addition ATP, dTTP, and dGTP (Berglund, 1972b). Cook and Greenberg (1983) demonstrated that a T4B1 amber mutant (nrdA67) lacking the C-terminal amino acid sequences, encoded a truncated polypeptide of 50,000 mol. wt. which failed to bind to dATP affinity columns. Therefore, these authors suggested that either the C-terminus contains the allosteric site or that it is essential for the tertiary structure of that site which is located elsewhere along the polypeptide backbone.

ii) The T4B2 subunit. This subunit is also a dimer and consists of two identical polypeptides (β_2) each of which has a mol. wt. of 34,500 (Berglund, 1972a and 1975; Cook and Greenberg, 1983). Sjoberg et al. (1986) sequenced the region between 136.1kb and 137.8kb positions on the T4 genetic map and identified a putative open reading frame (ORF) which exhibited 47% amino acid homology to the E. coli nrdB gene product. On the basis of this homology, these authors suggested that this ORF encoded the T4B2 subunit and called it the T4nrdB gene. Further, they suggested that an approximately 625 base intron divided the gene in two parts. The proposed exon-intron boundary at the 5' splice donor site ends with a TAA stop codon and the intron-exon boundary of the 3' splice acceptor site has an ATG codon. These intron boundaries were proposed on the basis of homology with those of the well characterised intron of the adjacent T4 thymidylate synthetase gene (td gene; Chu et al., 1984).

The T4B2 subunit contains two non-heme iron atoms per molecule whose optical and EPR spectra are very similar to those of the E. coli B2 iron centre (Berglund, 1972a and 1975). The iron atoms are essential for activity since their removal results in loss of activity. This subunit

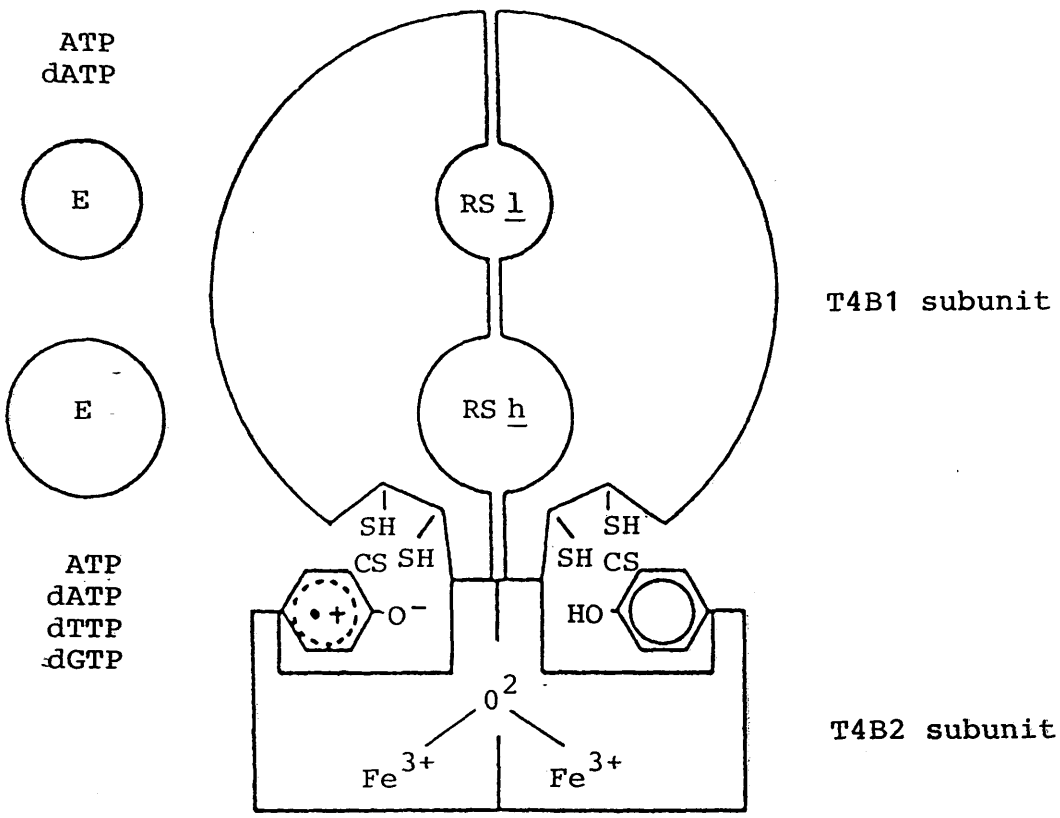


Figure 15. A schematic representation of the proposed structure of the bacteriophage T4 ribonucleotide reductase. The enzyme is depicted as a dimer of the T4Bl and T4B2 subunits. The T4Bl subunit contains two catalytic sites (CS) which contain the active sulphydryl groups (SH) and bind the ribonucleoside diphosphate (NDP) substrates. The regulatory sites (RS) bind the allosteric effectors (E) shown and are separated in low (l) and high (h) dATP-affinity sites. The T4B2 subunit contains the tyrosyl radical (•), which is depicted to conform to half-site reactivity, and the iron centre (Fe^{3+}-O^{2}-Fe^{3+}).

also contains a free radical which is located on a tyrosine residue with its spin density delocalised over the aromatic ring (Berglund, 1972a; Sahlin et al., 1982). The EPR spectra profiles of the bacterial and bacteriophage radicals are overall similar although small differences occur. These have been attributed to the different geometry of the radicals due to a different angle of the β-methylene group relative to the plane of the aromatic ring. Again, in analogy to the E. coli radical, the T4 radical is destroyed by hydroxyurea although it is ten times more sensitive to the drug. Kjoller-Larsen et al. (1982) ascribed the different suseptibility to the drug, to the different topology of the active sites in the bacterial and phage enzymes with the latter site being more exposed.

iii) Allosteric regulation. The allosteric properties of the T4-induced ribonucleotide reductase are in many ways similar to those of the E. coli enzyme (Berglund, 1972b). ATP affects the reduction of CDP and UDP, dTTP affects the reduction of GDP and dGTP affects the reduction of ADP. In contrast to the bacterial enzyme where high concentrations of dATP ($>10^{-5}$ M) inhibit enzyme activity, the same dATP concentrations positively regulate reduction of the pyrimidine substrates by the T4 enzyme (Berglund, 1972b). Further, 5'-hydroxymethyl cytosine, which is essentialy inert with the bacterial enzyme, stimulates pyrimidine reduction by the T4-induced enzyme. The lack of any negative effectors of the T4-induced enzymatic activity may be related to the fact that T4 DNA synthesis is not turned off during infection but continues until the T4-infected cell lyses.

c) 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 (Hopper, 1972 and 1978) and human tissue culture cell lines (Chang and Cheng, 1979). Hopper (1972) demonstrated that the mammalian enzyme's properties resemble those of

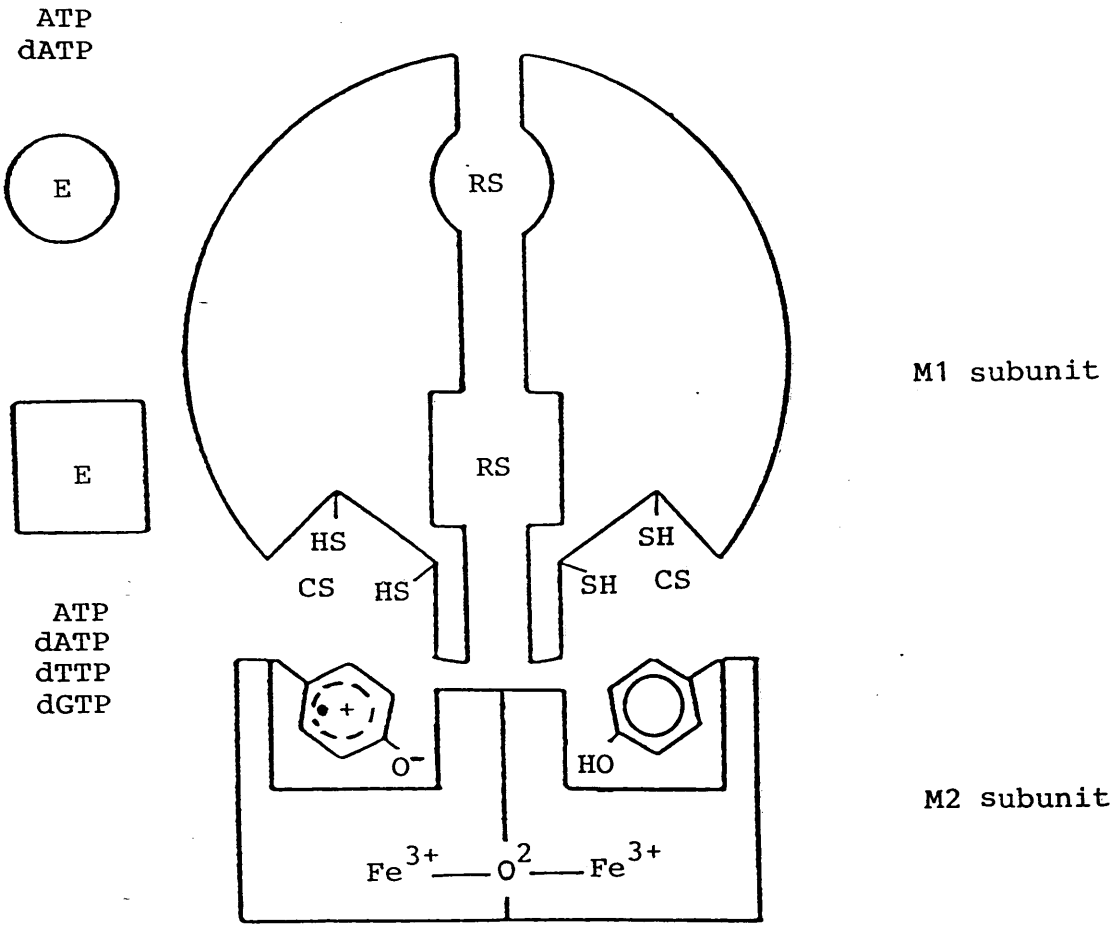


Figure 16. A schematic representation of the proposed structure of the mammalian ribonucleotide reductase. The enzyme is depicted as a dimer of the M1 and M2 subunits, each consisting of two identical polypeptides. The M1 subunit is shown to contain two regulatory sites (RS) which bind the allosteric effectors (E) indicated. The catalytic sites (CS) are shown to contain the active sulphydryl groups (SH). The M2 subunit contains the tyrosyl radical (•), and the iron centre (Fe^{3+}-O^{2}-Fe^{3+}).

E. coli rather than the Lactobacillus enzyme as it contains easily separable subunits, it is inhibited by hydroxyurea, does not require AdoCbl for activity, and contains iron. The mammalian enzyme consists of two non-identical subunits called M1 and M2 (Fig. 16; Thelander et al., 1980).

i) The M1 subunit. This subunit is composed of two identical polypeptides, and its mol. wt. in calf thymus is 170,000 (Thelander et al., 1980). The M1 dimer contains two catalytic sites which bind the NDP substrates and two different types of allosteric effector binding sites. In analogy to the E. coli enzyme, the first of the allosteric effector binding sites binds ATP and dATP while the second binds dTTP and dGTP in addition (Thelander et al., 1980). However, two major differences appear to exist between the bacterial and mammalian enzymes. First, substrate binding studies indicated that the number of sites in M1 is lower than that in B1 (Thelander et al., 1980). Second, the M1 sites show equal affinity for dATP-binding and therefore they cannot be classified as h or l sites as the case is with the E. coli B1 (Thelander et al., 1980). Both of these suggestions require further investigation.

ii) The M2 subunit. This subunit is a dimer composed of two polypeptides; in mouse the M2 molecule has a mol. wt. of 88,000 (Thelander et al., 1985). M2 contains a non-heme iron centre and a tyrosine free radical. Light adsorption spectra for the M2 and B2 subunits gave iron related bands at almost similar wavelengths indicating that both proteins should have the same type of iron centre (Thelander et al., 1985). Isotope substitution studies demonstrated that the free radical is located on a tyrosine residue and its spin density is delocalised over the aromatic ring of this tyrosine (Graslund et al., 1982). In analogy to the E. coli radical, the M2 radical is inhibited by hydroxyurea (Engstrom et al., 1979). However, the M2 radical can be regenerated by the addition of dithiotreitol in the presence of oxygen (Graslund et al., 1982) and this is in sharp contrast to the bacterial radical where radical regeneration requires chelation and subsequent re-introduction of the

iron centre (see Page 43; Atkin et al., 1973). Comparison of the EPR spectra profiles of the M2 and B2 radicals revealed small differences which, as the case is for the T4 radical, are attributed to the slightly different angles of the β-methylene group relative to the plane of the aromatic ring of the tyrosine (Graslund et al., 1982). Another property of the mammalian radical is that it is 75 times more sensitive to hydroxyurea analogues than the bacterial radical. Kjoller-Larsen et al. (1982) and Thelander et al. (1985) suggested that this is due to different active site topologies with the mammalian site being more exposed.

iii) Allosteric regulation. The mammalian enzyme has been shown to be allosterically regulated in a similar fashion to the E. coli enzyme (Eriksson et al., 1979). In the absence of positive effectors, the enzyme is inactive with any substrate. Reduction of pyrimidine substrates is stimulated by ATP, reduction of GDP by dTTP and reduction of ADP by dGTP. Reduction of purine substrates is further stimulated by ATP but only in combination with dTTP and dGTP respectively. Each of dTTP and dGTP inhibits reduction of the other three substrates. Finally, dATP is a general inhibitor but the dATP-mediated inhibition can be reversed by high concentrations of ATP. Overall, the E. coli allosteric regulation scheme (see Fig. 13) is also applicable to the mammalian enzyme, although, allosteric control of the latter enzyme is much more strict.

d) Viral-induced enzymes.

Several animal viruses including HSV-1 (Cohen, 1972), HSV-2 (Cohen et al., 1974), PRV (Lankinen et al., 1982), EHV serotypes 1 and 3 (Cohen et al., 1977; Allen et al., 1978) EBV (Henry et al., 1978), VV (Slabaugh 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. However, direct evidence that this activity is virus-encoded has been obtained only for HSV-1. This part of Section E is mainly concerned with the properties of the ribonucleotide reductase activity induced by HSV-1, HSV-2, PRV, VZV and VV.

i) Constituents and structure of the HSV-1-induced enzyme. Dutia (1983) demonstrated that the HSV-1 enzyme is virus-encoded since the multiple HSV-1 mutant tsG (Brown et al., 1973) expressed significantly reduced levels of ribonucleotide reductase activity at the permissive temperature (PT) while at the NPT no activity was observed. The enzyme is composed of two non-identical subunits, termed RR1 and RR2 (Frame et al., 1985; Cohen et al., 1985; Bacchetti et al., 1986; Ingemarson and Lankinen, 1987). The gene encoding RR1 has been shown to be UL39 by marker rescue of the mutant HSV-1 virus ts1207 (see Results and Discussion, Section D). The product of UL39, Vmw136 or RR1 polypeptide, has a mol. wt. of 136,000 (Marsden et al., 1978).

Other studies have accumulated evidence that the RR1 polypeptide is not the only one involved in ribonucleotide reductase activity. First, amino acid comparisons of the predicted primary structure of the UL40 gene product, Vmw38 or RR2 polypeptide, with that of the small subunit polypeptide of E. coli exhibited high homology (Standart et al., 1985; Sjoberg et al., 1985; McLauchlan 1986; Nikas et al., 1986; see Results and Discussion, Section C). Second, a monoclonal antibody specific for the RR1 polypeptide precipitated along with RR1 a smaller protein species (Preston, V.G. et al., 1984), which was shown to be RR2 (Frame et al., 1985). Conclusive evidence for the involvement of the RR2 polypeptide in enzymatic activity was obtained from the HSV-1 mutant ts1222 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 studies with these ts mutants further demonstrate that the viral enzyme consists of two non-identical subunits. The sedimentation coefficient of the active HSV-1 enzyme is 17S which approximately corresponds to a mol. wt. of 370,000 (Ingemarson and Lankinen, 1987). Given the predicted sizes of the RR1 and RR2 polypeptides, these authors suggested that the active enzyme is a homodimeric complex of the $\alpha_2\beta_2$ structure. The complex appears to be a very strong one as the two subunits

fail to dissociate in columns containing antibodies specific for each subunit nor do they sediment separately in glycerol gradients (Frame et al., 1985; Ingemarson and Lankinen, 1987). This type of tight binding between the two subunits is similar to that of the T4-induced enzyme subunits (Berglund, 1972a).

ii) Physicochemical and binding properties of the virus-induced enzymes. Unlike the bacterial and mammalian enzymes, Mg^{2+} or ATP inhibit the viral enzyme (Averett et al., 1983; Huszar and Bacchetti, 1981; Ponce de Leon et al., 1977). At least for the HSV-1 and HSV-2 enzymes, the inhibition appears to be qualitatively similar, although slight quantitative differences exist (Averett et al., 1984) However, in the presence of a Mg-ATP complex this inhibition is reduced most probably because the formation of the complex lowers the concentration of free ligands in the reaction mixture. The substrate binding properties of the HSV-1 and HSV-2 enzymes are quite similar (Averett et al., 1983 and 1984). Both enzymes have as substrates NDPs which bind at a common catalytic site; competition binding studies with CDP and ADP substrates indicated that both are mutual competitors for binding (Averett et al., 1983). The products of the reaction, deoxynucleoside diphosphates (dNDPs), are inhibitors which compete with the substrates (Averett et al., 1983). Both HSV enzymes are insensitive to inhibition by dNTPs suggesting that they are not allosterically regulated (Averett et al., 1983 and 1984). The same suggestion can be applied to the PRV- (Lankinen et al., 1983) and VZV-induced activities (Spector et al., 1987). Conversely, the VV-induced activity has been shown to be inhibited by dATP and dTTP and has therefore been proposed to be allosterically regulated (Slabaugh et al., 1984).

PRV-infected cells display an EPR spectrum resembling that of the tyrosyl radical of the M2 subunit (Lankinen et al., 1982). Therefore, it is possible that the viral enzyme also contains a free radical. This suggestion is reinforced by the strong conservation of the tyrosine residue and the flanking amino acid sequences of the polypeptides encoding

the E. coli B2 and HSV-1 RR2 subunits (see Results and Discussion, Section C); further it is reinforced by the observed inhibition of the HSV and PRV enzymes by hydroxyurea (Averett et al., 1983; Lankinen et al., 1982) which, as reported above (see Page 43), acts as a radical scavenger.

14. Ribonucleotide Reductase is Essential for Virus Growth.

HSV-1 ts1207 failed to induce any ribonucleotide reductase activity at the NPT and its yield at that temperature was 100-fold lower than that of wild-type virus (Preston, V.G. et al., 1984). These results suggested that the enzyme is essential for virus replication. In contrast, subsequent reports raised questions as to the validity of this proposal. First, Nutter et al., (1985) reported that concentrations of hydroxyurea, which would otherwise inhibit ribonucleotide reductase activity to undetectable levels, reduced the HSV-2 virus yield in proliferating cells only by six-fold. Second, Wu et al. (1988) demonstrated, by means of the plasmid replication assay developed by Challberg (1986), that a significant amount of DNA replication occured in the absence of the ribonucleotide reductase genes. Finally, third, Goldstein and Weller (1988a) reported that the enzyme is dispensable in dividing cells at 34^{o}C. These authors reached this conclusion by studing the growth and DNA replication of a mutant HSV-1 virus, hr3, which was expressing the N-terminal RR1 434 amino acids fused to the β-galactosidase (lacZ) gene.

However, studies with the HSV-1 mutant virus ts1222 reconciled these apparently conflicting data and showed that the enzyme is essential for virus replication. Preston, V.G. et al. (1988) demonstrated that ts1222 failed to grow in resting cells at the PT thus indicating that the enzyme is essential under these conditions. This is the case with TK as well, although, the requirement for actively dividing cells appears to be more stringent for ribonucleotide reductase negative mutant viruses than in TK^{-} viruses. Preston and co-workers further showed that, in exponentially growing cells and at the PT, ts1222 is dependent on the

cellular enzyme for replication, while, it was proposed that at the NPT the mutant fails to replicate due to inactivation of the cellular enzyme at this temperature. Similar suggestions were reached by Goldstein and Weller (1988b), using the HSV-1 mutant ICP6Δ which was expressing less than 10% of the RR1 DNA coding region; these authors showed that growth and DNA replication of ICP6Δ were severely restricted in non-dividing cells or at 39.5°C.

Conclusive evidence that ribonucleotide reductase is an essential HSV function have been obtained from studies on the pathogenicity of the ts1207 and ts1222 mutants following intracranial or intraperitoneal inoculation of albino Charles River mice (Cameron et al., 1988). The virulence of these mutants was reduced by about a million-fold when compared with that of the parental virus (HSV-1 strain 17^+); further, the pathogenicity of a revertant of ts1222 (ts1222 revl) was restored to about 60- to 200-fold of that of the parent virus. Similar evidence was obtained from experiments involving HSV infection in epithelium cells of hairless mice which were scarified on the flank (H.S. Marsden, D.M. Ryan, personal communication). Mice infected with HSV-1 strain 17^+ gave rise to a zosteriform lesion, as did ts1222 revl, while infection at doses higher than 4×10^2 plaque forming units (p.f.u.) per mouse caused death. In contrast, infection with ts1222 did not give rise to zosteriform lesions but instead to small blisters which healed by 6 days p.i.

15. Ribonucleotide Reductase as a Target for Antiviral Compounds.

The herpesvirus-encoded enzyme has been considered as an attractive antiviral target for a number of reasons. Firstly, as reported above, the enzyme is important for pathogenicity in mice. Secondly, the herpesviral enzyme is biochemically distinct from the mammalian enzyme. Thirdly, the interaction between the RR1 and RR2 subunits may itself be exploitable as a target for antiviral compounds.

Effort is currently being put in the identification of a compound or a class of compounds that would inhibit the

interaction of the two subunits. This approach resulted from reconstitution experiments designed to show that both subunits are necessary for activity (Dutia et al., 1986); in these experiments an IgG from serum raised against a synthetic octapeptide corresponding to the C-terminal seven amino acids of RR2 successfully removed this subunit from a crude extract of HSV-1 infected cells. Subsequently, two different groups reported that the same octapeptide inhibited ribonucleotide reductase activity and postulated that this was achieved by dissociation of the RR1/RR2 subunit complex (Dutia et al., 1986; Cohen et al., 1986a). McClements et al. (1988) demonstrated that indeed the synthetic oligopeptide acted by dissociating the RR1 and RR2 subunits and that this resulted in inhibition of the ribonucleotide reductase activity.

Recently, Huang et al. (1988) and Darling et al. (1988) demonstrated that the enzymatic activity can be restored by reassociation of the two subunits both in vivo and in vitro and that no other HSV proteins are required for the formation and activity of the reconstituted enzyme. Furthermore, Darling and co-workers demonstrated that the two subunits exist in a dynamic equilibrium between the associated and dissociated states of the enzyme (A.J. Darling, personal communication). These observations will prove valuable in the further characterisation of the enzyme and in studies with antiviral compounds.

16. Regulation of Ribonucleotide Reductase Synthesis.

As already mentioned, the prokaryotic and eukaryotic enzymes are allosterically regulated by NTPs and dNTPs. However, additional types of regulation exist at the transcriptional, translational or post-translational level.

The E. coli genes encoding the bacterial enzyme are arranged in one operon and are coordinately regulated (Hanke and Fuchs, 1983a). The enzyme appears to be synthesised only once during the cell cycle at a time that coincides with the onset of DNA replication. It appears that subunit synthesis is primarily controlled at the transcriptional level as inhibition of DNA synthesis by thymine starvation

results in an increased rate of transcription in ribonucleotide reductase genes (Hanke and Fuchs, 1983b). It appears that this increase is mediated by a positive regulatory protein(s) which accumulates during inhibition of DNA replication and acts to induce ribonucleotide reductase transcription (Hanke and Fuchs, 1984). Another type of regulation of the E. coli enzyme appears to be at the post-transcriptional level; as reported above (see Page 42), Sjoberg et al. (1987) encountered a truncated form of B2 which resulted from limited proteolysis of normal B2 by a serine protease and which inactivated the enzyme.

Synthesis of the mouse enzyme is regulated in a cell cycle-dependent fashion. Activity measurements of the two subunits in cells separated in G1, S and G2 phases of the cell-cycle indicated that the M1 catalytic activity was constant throughout these phases whereas the M2 activity was substantially decreased in G1 as compared to S and G2 (Eriksson and Martin, 1981; Engstrom et al., 1985). The reduced M2 activity in G1 could be explained either by the existence of a latent M2 protein pool, which was activated at the start of the S phase, or by the de novo synthesis of M2 protein. Eriksson et al., (1984) demonstrated that the latter is correct, since a 3- to 7-fold increase in M2 radical content is observed when cells are passed from the G1 to the S phase. Moreover, these authors proposed that this type of M2 regulation primarily occurs at the transcriptional level.

In analogy to the mouse enzyme regulation, both sea urchin and surf clam oocyte enzymes are regulated at the level of M2, although, in the latter the control of M2 synthesis appears to be at the translational level. Standart et al. (1985) demonstrated that the small subunit is expressed after fertilisation of the embryo and that the mRNA encoding this subunit is one of the stored maternal mRNAs whose translation is induced by fertilisation. On the other hand, the large subunit is already present as a polypeptide in the embryo (Standart et al., 1986).

MATERIALS AND METHODS

SECTION A.

MATERIALS.

1. Viruses.

HSV-2 strain HG52 (Timbury, 1971) and HSV-1 Glasgow strain 17^{+} (Brown et al., 1973) were used during the course of this work.

2. Cells.

Baby hamster kidney cells (BHK-C13; MacPherson and Stoker, 1962) were used for growth and assay of virus. HeLa cells (obtained from Dr J. McLauchlan) were used for transient expression assays of plasmid constructions.

3. Bacterial Strains.

E. coli K12 strain DH1 (recA1, F^{-}, endA1, gyrA96, thi-1, supE44, recA1; Hanahan, 1983) was used as host in all recombinant plasmid experiments. E. coli K12 strain JM101 (Δ(lac;pro), supE, thi, F'traD36, proAB, $lacI^{q}$, Z Δ M15; Messing et al., 1981), which harbours an F factor essential for the transmittance of the male specific phage, was used as host strain of M13 bacteriophage recombinants.

4. Tissue Culture Media.

BHK-C13 cells were grown in Glasgow Modified Eagle's medium (Busby et al., 1964) supplemented with 100ug/ml streptomycin, 100units/ml penicillin, 0.2ug/ml amphotericin, 0.002% (w/v) phenol red. The growth medium used (ETC10) consisted of 80% Eagle's, 10% tryptose phosphate and 10% calf serum.

HeLa cells were grown in Dulbecco's medium (Flow Laboratories), a modified version of Eagle's medium, supplemented with 20mM glutamine, 100units/ml of streptomycin, 100units/ml penicillin and 0.2ug/ml (w/v)

amphotericin. In addition to the above ingredients, growth medium contained 2.5% calf serum and 2.5% foetal calf serum (Gibco Ltd).

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

i) PBS, which contained 170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4 (pH 7.2)

ii) Versene, which consisted of PBS containing 0.6mM EDTA and 0.0015% (w/v) phenol red and,

iii) 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 100units/ml penicillin and 100ug/ml streptomycin.

5. Bacterial Culture Media.

DH1 bacteria were propagated in L-broth which consisted of 177mM NaCl, 10g/l Difco Bactotryptone and 5g/l yeast extract (pH 7.5 prior to sterilisation). Solid medium for the growth of colonies was agar which contained 1.5% (w/v) agar in L-broth. These media were supplemented, where appropriate, with ampicillin or chloramphenicol, both at a final concentration of 100ug/ml. JM101 were grown in 2YT broth which comprised 85mM NaCl, 16g/l Difco Bactotryptone, 10g/l yeast extract (pH 7.5 prior to sterilisation).

6. Frequently Used Buffers and Solutions.

TE	10mM Tris HCl (pH 7.5), 1mM EDTA
TBE	90mM Tris, 90mM boric acid, 1mM EDTA (pH 9.3)
TAE	40mM Tris HAc (pH 8.0), 2mM EDTA
STET	50mM Tris HCl (pH 8.0), 50mM EDTA, 8% (w/v) sucrose, 5% (v/v) Triton X100
5x LB	100mM Tris HCl (pH 7.5), 20mM $MgCl_2$, 20mM DTT
SSC	150mM NaCl, 15mM sodium citrate (pH 7.5)
50x DHB	1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin (BSA) (Denhardt, 1966)
10x AB	100mM Tris HCl (pH 8.5), 100mM $MgCl_2$

TGM	150 ml 40% polyacrylamide (acrylamide/bisacrylamide 20:1), 50ml 10x TBE, 540g urea, made up to 1l with distilled H_2O
BGM	150 ml 40% polyacrylamide (acrylamide/bisacrylamide 20:1), 62.5ml 40x TBE, 540g urea, 50g sucrose, 10ml 1% bromophenol blue, made up to 1l with deionized H_2O
EB	100mM NH_4Ac, 10mM MgAc, 0.1% (w/v) SDS, 1mM EDTA
TE(P)	1mM Tris HCl (pH 7.5), 0.05mM EDTA
HBS	140mM NaCl, 50mM HEPES (pH 7.12), 1.5mM Na_2HPO_4
ILB	150mM NaCl, 1.5mM $MgCl_2$, 10mM Tris HCl (pH 7.8), 0.65% (v/v) nonidet p40.
PEB	7.0M urea, 350mM NaCl, 10mM EDTA, 10mM Tris HCl (pH 7.9), 1% (w/v) SDS
5x SP6	200mM Tris HCl (pH 7.5), 30mM $MgCl_2$, 10mM spermidine
2x PK	100mM Tris HCl (pH 8.0), 20mM EDTA, 20mM NaCl, 0.4% (w/v) SDS
5x M13	3.75M NaCl, 250mM HEPES (pH 6.95), 5mM EDTA
HNE	200mM NaCl, 10mM HEPES (pH 7.9), 1mM EDTA

7. Enzymes.

The majority of restriction enzymes were obtained from New England Biolabs or Bethesda Research Laboratories (BRL). The Klenow fragment of E. coli polymerase was supplied by Boer inger Corporation Ltd or provided by Dr A. Davison, ribonuclease T2 was supplied by Calbiochem Ltd, and lysozyme and proteinase K, were supplied by Sigma Chemical Company. Unless otherwise stated, enzyme digests were performed in 1x core buffer (50mM Tris HCl, pH 8.0, 10mM $MgCl_2$, 50mM NaCl), supplied by BRL.

8. Radiochemicals.

Radioisotopes were supplied by Amersham International PLC at the following specific activities:

5' [γ-^{32}P] adenosine triphosphate, 5000Ci/mmol

5' [α-^{32}P] deoxynucleoside triphosphates, 3000Ci/mmol

5' [α-^{32}P] uridine triphosphate, 800Ci/mmol.

9. Chemicals and Miscellaneous Reagents.

Most chemicals of analytical grade were supplied by BDH Chemicals UK or Sigma Chemical Company. Koch-Light laboratories supplied acrylamide, bisacrylamide, boric acid and caesium chloride; Biorad laboratories supplied ammonium·persulphate (APS), and TEMED (N',N',N',N' -tetramethylethylene diamine). The sequencing primer (5'-GTAAAACGACGGCCAGT-3') was obtained from New England Biolabs. Other primers or oligonucleotide probes used during the course of this work were constructed by Dr J. McLauchlan using a Biosearch 8600 DNA Synthesizer. Dideoxynucleoside triphosphates (ddNTPs) and dNTPs, used in sequencing reactions, were bought from P-L Biochemicals. Ribonucleoside triphosphates, used for the production of RNA probes, were obtained from Promega Biotec. Tissue culture materials were supplied by Sterilin Ltd. and Nunc.

10. Cloning Vectors.

Most of the constructs made during the course of this study used plasmids pUC8 and pUC12 as cloning vectors (Vieira and Messing, 1982).

Bacteriophage M13 was used for subcloning of DNA fragments for sequencing (Messing and Vieira, 1982). The double-stranded DNA replicative form (RF) of this phage can be manipulated as a plasmid for subloning of DNA fragments into the multiple cloning site (MCS). The infectious phage particle contains single-stranded DNA and is secreted, in high titres, from the host cell into the culture medium. The ssDNA can be used as a template for sequencing reactions. Throughout this study the M13mp8 RF bacteriophage was used (obtained from New England Biolabs).

RNA probes used in transient expression experiments were produced with the use of the pGEM-2 Riboprobe Gemini transcription plasmid supplied by Promega Biotec (Fig. 17). This vector comprises part of the pBR322 vector sequences and the bacteriophage SP6 and T7 promoters which are cloned in opposite transcriptional directions and are separated by a short piece of DNA containing an MCS (Melton et al.,

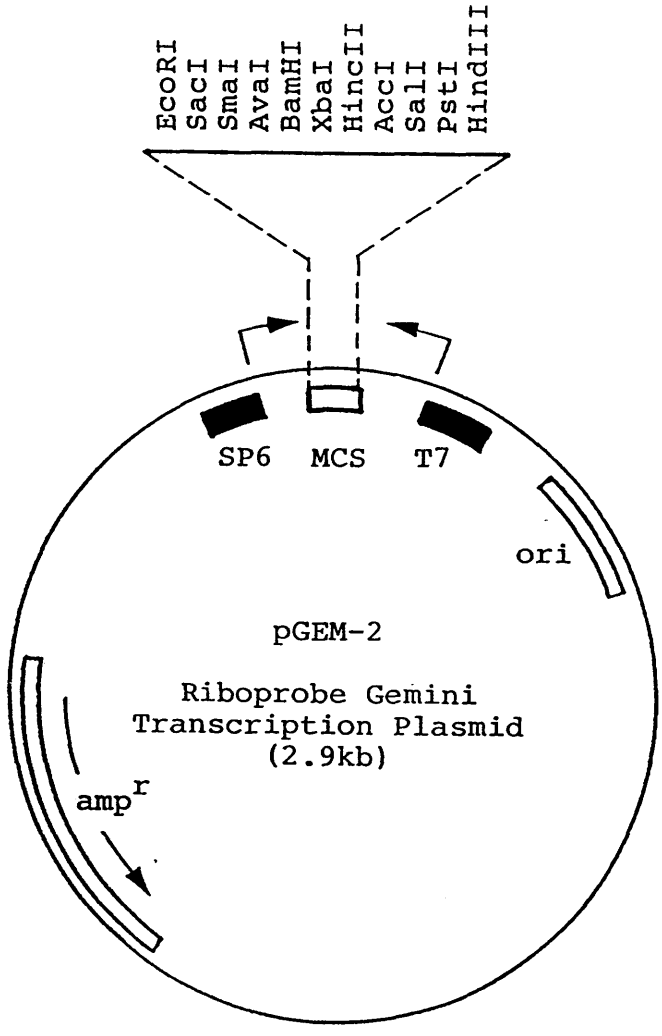


Figure 17. The pGEM-2 Riboprobe Gemini transcription plasmid. Black boxes indicate the SP6 and T7 promoters and arrowed lines above them indicate their respective transcriptional directions. The multiple cloning site (MCS) is expanded above the plasmid to show the restriction endonuclease cleavage sites. The location and orientation of the ampicillin resistance gene (amp^r) and the pBR322 origin of replication (ori) are also indicated.

1984). The pGEM-2 plasmid carries the pBR322 origin of replication and the gene conferring resistance to ampicillin.

11. Recombinant Plasmids.

All recombinant plasmids used during the course of this work were constructed by the author with the exception of: pHindK, pBamE and pBamT, obtained from Dr J. McLauchlan, pBamHIo, obtained from Dr V.G. Preston and pl11 and pl75 obtained from Dr R.D. Everett.

SECTION B.

METHODS.

12. Virus Growth and Assay.

BHK-C13 cells, maintained in 850cm^2 roller bottles, were infected with HSV-2 strain HG52 at a multiplicity of 1 p.f.u. per 350 cells. The infected cells were incubated in 20ml of ETC10 for 3 to 4 days at 31°C until they could be readily harvested by gentle shaking; subsequently, they were pelleted by centrifugation in the cold at 1,500rpm for 10min. The supernatant was removed and centrifuged at 12,000rpm for 2h. The resultant pellet was resuspended in 5ml of ETC10 and stored in -70°C as cell released virus (CRV). The pellet from the initial centrifugation was resuspended in an equal volume to 5ml of ETC10 and sonicated briefly. After centrifugation at 3,000rpm for 15min, the supernatant was recovered and stored at -70°C as cell associated virus (CAV). The CRV and CAV fractions were titrated as described in Brown et al. (1973).

13. Standard Techniques.

a) Restriction enzyme digests.

Restriction digests were performed at 37°C or, in certain cases, at 65°C (for example, BstE II digests). Plasmid DNA and restriction enzymes were incubated for 1h to 4h in 1x core buffer, with the exception of SmaI digests where, instead of NaCl, 20mM KCl was used. For diagnostic purposes, digests were carried out in 15ul of 1x core buffer using 0.5ug of plasmid DNA and 1unit of enzyme for 2h at 37°C, unless the reaction conditions specified by the supplier differed radically. For subcloning of DNA fragments, the enzyme units and the incubation time varied according to the size and amount of the DNA fragment.

b) Ligation of DNA fragments.

DNA fragments, produced by cleavage with restriction enzymes, were ligated to cleaved plasmid vector which contained termini compatible with those of the fragment. The vector and fragment were mixed in a 1:10 ratio in 5x ligation buffer (LB), 0.4mM ATP and 2units of T4 DNA ligase and incubated overnight at 15°C.

DNA fragments excised from low melting point agarose gels (see below) were not removed from gel slices for ligation purposes. The DNA-containing gel slices were mixed, an approximately equal volume of distilled H_2O was added, and the mixture was heated at 65°C until the agarose melted. At that point, the sample was incubated at 37°C and vector DNA, 5x LB and T4 DNA ligase were rapidly added. The ligation mixture was then incubated overnight at 15°C.

In order to prevent reannealing of linearised M13mp8 RF DNA with Sma I, which gives 'blunt-ends', the bacteriophage DNA was treated with 25units of bacterial alkaline phosphatase in 50mM Tris HCl (pH 8.5) at 37°C for 3h.

c) Gel electrophoresis.

i) Non-denaturing agarose gels. These gels were used to analyse DNA digested with restriction enzymes. Horizontal slab gels (260mm x 160mm) comprising 200ml of 0.5% to 1.5% (w/v) agarose in 1x TBE buffer were run at up to 12V/cm submerged in 1x TBE; both the gel and the buffer contained 0.2ug/ml of ethidium bromide. Samples were applied in 1x TBE, 10% (w/v) sucrose, 0.02% (w/v) bromophenol blue and xylene cyanol.

ii) Low melting point agarose gels. These gels were used for the isolation of DNA fragments, of at least 500bp in length, for subcloning purposes; they comprised 200ml of 1% (w/v) agarose in 1x TAE buffer and were electrophoresed in 1x TAE at up to 7.5V/cm.

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 1kbp. Acrylamide stock of 30% (w/v) acrylamide (acrylamide:bisacrylamide 29:1, stored at 4°C) was diluted to the required concentration (4% to 10%) in 75ml of 1x TBE buffer. To this, 0.5ml of 10% (w/v) APS and 50ul TEMED were added to intiate polymerisation. The mixture was poured quickly into the gel mould and allowed to stand for at least 45min prior to electrophoresis. Samples were prepared as in non-denaturing agarose gels (see Page 61) and gels (260mm x160mm x 1mm) were run in vertical kits up to a maximum voltage of 16V/cm. DNA was visualised by ethidium bromide staining or by autoradiography.

iv) Denaturing polyacrylamide gels. This type of gel was used to analyse the products of ribonuclease T2 digestion and were prepared as described by Maxam and Gilbert (1980). A stock of 20% acrylamide (acrylamide:bisacrylamide 19:1, stored at 4°C) was diluted to 8% in 1x TBE buffer containing 7M urea. The solution was filtered, degassed, polymerised and gels (450mm x 230mm x 0.35mm) were pre-run for 30min at 40W. Lyophilised samples were resuspended in 5ul to 10ul of 90% (v/v) formamide, 0.1% (w/v) bromophenol blue and xylene cyanol, denatured at 90°C for 3min and chilled rapidly in ice prior to electrophoresis. Gels were run at 40W for 2h approximately. Radiolabelled RNA was deteced by autoradiography. Where appropriate, the amount of radioactive product was quantified by densitometry of autoradiographs using a Joyce-Loebl scanning densitometer. Then areas under the peaks of the densitometer tracing were measured by a DEC PDP 11/44 computer linked to a digitising tablet.

vi) Gradient polyacrylamide gels (Sequencing gels). This type of gel was used for the analysis of sequencing reaction products. The gradient of potential difference is constructed by using two different ionic strength buffers, the top buffer (TGM), containing a low concentration of TBE,

and the bottom buffer (BGM), containing a high concentration of TBE (Biggin et al., 1983).

Two gel plates, one plain and one notched (430mm x 400mm x 0.35mm), were thoroughly cleansed with ethanol. The inside surface of the plain plate was treated with 1ml of Wackersilicone solution (10ml ethanol, 0.3ml 10% acetic acid, 50ul Wackersilicone) and the inside surface of the notched plate was treated with 1ml of 'Repelcote' (a 2% solution of dimethylchlorosilane in 1,1,1-tricloroethane). The Wackersilicone solution allows the formation of a firm bond between the glass surface and the gel while the 'Repelcote' facilitates plate separation after electrophoresis. The TGM solution contained 0.5x TBE and was polymerised by adding 80ul each of 25% APS and TEMED to 80ml of TGM. The BGM solution contained 2.5x TBE and was polymerised by adding 15ul each of 25% APS and TEMED to 15ml of BGM. 10ml of TGM and 14ml of BGM were mixed in a 50ml syringe and then the mixture was carefully injected into the gel mould. The remaining TGM solution was subsequently injected into the mould, moving the flow from one side to the other in order not to skew the BGM to one side; the gel was allowed to stand for at least 45min prior to electrophoresis and was run for approximately 2h at 40W with 0.5x TBE in both top and bottom reservoirs. Lyophilised DNA samples were taken up in 2ul of formamide dyes (100ml formamide, 0.1g xylene cyanol, 0.1g bromophenol blue and 2ml of 500mM EDTA), heated at 95^{o}C for 1min and quenched in ice prior to electrophoresis.

14. Large Scale Preparation of Plasmid DNA.

A loop of bacteria or a bacterial colony was used to inoculate 10ml of L-broth supplemented with 100ug/ml ampicillin and incubated overnight at 37^{o}C. 5ml of this culture was then diluted in 800ml of L-broth containing ampicillin and the culture was shaken vigorously for 6h at 37^{o}C; then, plasmid DNA was amplified by the addition of chloramphenicol to 50ug/ml and the culture was shaken overnight at 37^{o}C.

Isolation of plasmid DNA was essentially performed

with the method of Holmes and Quigley (1981). Bacteria were recovered by centrifugation at 8,000rpm for 10min and resuspended in 60ml of STET followed by the addition of 8ml of freshly-prepared ice-cold lysozyme (10mg/ml). The suspension was boiled for 40sec, chilled on ice for 2 to 3min and centrifuged for 1h at 15,000rpm. The nucleic acid in the supernatant was precipitated by adding an equal volume of isopropanol and pelleted by centrifugation at 3,000rpm for 20min. In order to isolate plasmid DNA, the pellet was resuspended in 10.5ml of distilled H_2O followed by the addition of 11g of caesium chloride and 0.5ml of ethidium bromide (10mg/ml). DNA was banded at 40,000rpm for 72h. Supercoiled plasmid DNA was visualised under a long wave UV light and recovered by a syringe. The ethidium bromide was removed by multiple extractions with TE-saturated butan-2-ol followed by overnight dialysis against deionised H_2O. The dialysed DNA was then treated with 50ug/ml RNase A for 1h at 65°C and then with 50ug/ml proteinase K and 0.1% (w/v) SDS for 1h at 37°C. The DNA was phenol/chloroform extracted twice and then precipitated with ethanol. Lyophilised DNA was finaly resuspended in TE such that the final concentration was 1ug/ml as estimated by spectrophotometry (OD_{260} 1.0=50ug of DNA/ml).

15. Small Scale Preparation of Plasmid DNA ('Miniprep').

20ml bottles containing 3ml of L-broth supplemented with ampicillin (100ug/ml) were inoculated with single plasmid-transformed bacterial colonies and shaken at 37°C overnight. Half of the bacterial culture was stored at 4°C and the remainder was poured into an 1.5ml reaction vial and centrifuged for 15sec in a MSE microfuge. The bacterial pellet was resuspended in 200ul of STET and 5ul of lysozyme (10mg/ml), heated in a boiling waterbath for 45sec and centrifuged for 10min. An equal volume of isopropanol was added to the supernatant and precipitated nucleic acids were centrifuged for 5min, washed in 70% ethanol and lyophilised. Finally, the pellet was resuspended in 20ul of distilled H_2O and stored at 4°C. When DNA from 'minipreps' was digested with restriction enzymes, 0.2ul of 1mg/ml ribonuclease A

(RNase A) was added to remove RNA and this facilitated visualisation of bands following gel electrophoresis.

16. Transformation and Transfection of E. coli.

a) Preparation of competent bacteria.

100ml of L-Broth or 100ml of 2YT were inoculated with 2ml of an overnight culture of DH1 or JM101 bacteria, respectively, and shaken at 37°C until the OD_{660} reached 0.3. The culture was chilled on ice for 10min and bacteria were pelleted by centrifugation at 8,000rpm for 10min. The pellets were resuspended in a total of 40ml of ice-cold 100mM $CaCl_2$ and incubated on ice for 40min. The cells were then harvested again by centrifugation and resuspended in a total of 2ml of ice-cold 100mM $CaCl_2$. Competent cells were used within 24h.

b) Transformation by plasmid.

10ul of plasmid DNA, usually a ligation mix, were mixed with 100ul to 200ul of competent DH1 bacteria in an 1.5ml reaction vial by gentle shaking and the mixture was then placed on ice for 45min. After incubation at 42°C for 3min, 70ul aliquots were spread on L-broth agar plates containing ampicillin and these were incubated overnight at 37°C. Prior to spreading the transformation mixture, plates were spread with 50ul 5-bromo 4-chloro 3-indonyl β D galactopyranoside (Xgal; 25mg/ml in dimethylformamide), in order to select, on the basis of colour, for insert-containing plasmid vectors.

In the case of ligations performed in low melting point agarose, $CaCl_2$ was added to the ligation mixture to a final concentration of 50mM prior to mixing with competent bacteria.

c) Transformation by M13mp8 RF.

10ul of M13mp8 RF/insert ligation mix (see Page 67) was added to 200ul of competent JM101 and incubated on ice for 40min. After this, the transformation mixture was incubated at 42°C for 2min and then added to a 5ml glass

bottle, maintained at 42°C, which contained: 3ml of molten top agar, 25ul each of isopropyl-D-thiogalactoside (IPTG; 25mg/ml in distilled H2O) and Xgal (25mg/ml in dimethylformamide) and 200ul of a JM101 overnight culture. The final mixture was gently shaken and spread on L-broth agar plates which were pre-warmed at room temperature. The top agar was allowed to set for 5min at room temperature and plates were incubated at 37°C overnight.

17. DNA Sequencing.

The dideoxy/chain termination method was used for the nucleotide sequence determination of the coding region of RR1 and the identification of the nucleotide changes in an RR1 coding region of the HSV-1 ts1207 mutant (see Results and Discussion, Sections A and D respectively). This method was combined in the former study with a 'shotgun' cloning strategy while, in the latter study, a different approach was followed which is described on Page 108.

The principle behind the 'shotgun' cloning strategy is the generation of a bank of single-stranded M13 clones containing random fragments of the DNA region to be sequenced. These can then be sequenced by the dideoxy/chain termination method. A universal synthetic oligonucleotide primer is annealed to the M13 recombinant clones and is then used as a substrate for elongation by the Klenow fragment of the E. coli DNA polymerase. Four separate reactions are set up each containing a combination of four ddNTPs together with dNTPs, one of which is radiolabelled (α-[^{32}P]dATP). Incorporation of a ddNTP in the elongating chain results in termination of elongation; thus, the four reactions result in a set of radiolabelled chains which extend from the primer and randomly terminate at A, T, C and G residues. These chains are subsequently electrophoresed side by side on gradient polyacrylamide gels (see Page 62) and the sequence can then be directly read from the autoradiograph. This procedure generates a series of overlapping sequences which can then be collated computer analysis (see Materials and Methods, Section C) thus producing a contiguous sequence

that spans the DNA region of interest in both the prototype and complementary orientations.

a) DNA cloning into M13mp8 RF by the 'shotgun' method.

20ug of plasmid containing the DNA fragment to be sequenced were dissolved in TE and sonicated as described in Deininger (1983) to give rise to random subfragments. These were subsequently end-repaired using 8units of T4 DNA polymerase, in a reaction 2mM for all four dNTPs in 1x T4 DNA polymerase buffer (100mM Tris HCl (pH 7.9), 100mM $MgCl_2$, 100mM DTT). The reaction was incubated for 1h at 37°C and DNA was phenol/chloroform extracted and ethanol precipitated.

The end-repaired products of sonication were size-fractionated prior to cloning. The DNA was run on a 1.5% agarose gel alongside Hinf I digested pAT153 vector DNA as marker (fragment sizes in bp: 1651, 517, 396, 298, 221, 220, 154, 145 and 75). A trough was cut in the sample track just ahead of the 221bp/220bp bands and was filled with buffer. Electrophoresis was continued and the buffer in the trough was collected and replaced at 1min intervals until the majority of 250bp to 500bp fragments were isolated. DNA was subsequently phenol extracted and ethanol precipitated. Finally, the pellet was taken up in TE buffer to a final concentration of 50 to 500ng/ul.

10ng of SmaI linearised M13mp8 RF was ligated to 10 to 500ng of sonicated, end-repaired and size-fractionated DNA fragments with 2units of T4 DNA ligase in 1x LB buffer for 15h at 15°C (Sanger et al., 1980). Competent JM101 bacteria were transfected with the ligation mixture as described on Page 65.

b) Preparation of single-stranded templates for sequencing.

1ml of an overnight JM101 culture was added to 100ml of 2YT broth and 1.5ml aliquots were placed in 20ml glass bottles. A white M13 plaque was transferred into each bottle and shaken for about 6h at 37°C. The phage cultures were decanted into 1.5ml vials and bacteria were pelleted by centrifugation for 2min in a MSE microfuge. The supernatant

was transfered into a new vial and mixed thoroughly with 150ul of 2.5M NaCl, 20% (v/v) polyethylene glycol 6000. The mixture was left to stand for 30min at room temperature, the bacteriophage were pelleted for 5min in an MSE microfuge and the supernatant was carefully removed. The pellet was taken up in 100ul of 10mM Tris HCl (pH 8.0), 0.1mM EDTA, phenol extracted and ethanol precipitated. Phage DNA was pelleted, lyophilised and dissolved in 30ul TE.

c) Selection of insert-containing recombinant M13 phages.

To select for recombinant phages containing insert HSV DNA fragments, Southern hybridisations were performed. 1ul of single-stranded M13 recombinant DNA was blotted onto duplicate Schleicher and Schuell nitrocellulose filters and these were baked in a vacuum oven for 2h. The filters were then shaken at 70^{o}C with a hybridisation mix, comprising 4ml of 1M Tris HCl (pH 7.5), 60ml of 20x SSC, 40ml of 50x Denhardt's buffer (DHB), 0.8ml of 250mM EDTA, 5ml of 20% (w/v) SDS and 1ml of 10mg/ml salmon sperm single-stranded DNA. After 2h the hybridisation mix was replaced with fresh mix which in addition contained a specific probe for each filter. The first filter, which represented the positive control, was probed with the DNA fragment to be sequenced. The second filter, which represented the negative control, was probed with vector plasmid DNA digested at a unique site. Both probes were end-labelled with [α-^{32}P]dNTPs as described on Page 67, dissolved in 0.7ml of deionised formamide and heated at 95^{o}C for 5min prior to adding to the hybridisation mix. The filters were then incubated at 70^{o}C overnight, washed three times with 300ml of a mixture containing 10ml NaH_2PO_4 (pH 7.5), 100ml of 20x SSC and 10ml of 20% (w/v) SDS and autoradiographed. Clones containing HSV DNA were retained for sequencing.

d) DNA sequencing reactions.

Typically 16 to 18 clones were sequenced at the same time. 1ul of template DNA was added to a vial containing 1ul of oligonucleotide primer (2.5ng), 1ul of 10x AB and 7ul of distillied H_2O and the reaction was incubated for 30min at 37^{o}C. At the end of the incubation 1ul of Klenow

TABLE I

NUCLEOTIDE CONCENTRATIONS USED IN DNA SEQUENCING REACTIONS

dNTP Mixes

	dG mix	dA mix	dt mix	dC mix
0.5mM dT	200	200	10	200
0.5mM dC	200	200	200	10
0.5mM dG	10	200	200	200
dNTP-mix buffer	50	50	50	50

dNTP-mix buffer = 0.05m Tris-HCl (pH8.0), 1mm EDTA

ddNTP Mixes

	0.2mM ddGTP	70uM ddATP	0.3mM ddATP	70uM ddCTP
5mM ddG	40	-	-	-
5mM ddA	-	14	-	-
5mM ddT	-	-	60	-
5mM ddC	-	-	-	14
H_2O	960	986	940	986

dNTP:ddNTP Mix

'Sequencing Mix'	dNTP	ddNTP	H_2O
G	400	50	350
A	400	100	300
T	400	200	200
C	400	50	350

polymerase (1unit/ul) was added to each annealing reaction and mixed thoroughly; 2ul of that mixture was transfered to each of four 0.75ml reaction vials, labelled A, T, C and G.

Meanwhile, four vials labelled A, T, C, and G were set up each containing 36ul of A, T, C or G 'sequencing mix' respectively (see Table 1). To each of these, 5ul of a mix containing 3ul of [α-^{32}P]dATP and 18ul of 12mM dATP was added. 2ul of each of these mixtures were added to the corresponding 0.75ml vials mentioned above. The elongation reactions were allowed to stand for 20min at room temperature and then were incubated with 2ul of 'chase mix', which contained 0.25mM of each dNTP, for a further 30min at room temperature. Finally, samples were electrophoresed as described on Page 62.

18. Transient Expression Experiments.

a) Transfection of DNA into tissue culture.

DNA transfections into HeLa cells were performed with the calcium-phosphate transfection procedure as described by Wigler et al. (1978) and Corsalo and Pearson (1981).

HeLa cells were seeded at 30% to 40% confluence on 90mm petri dishes 12h to 24h prior to transfection. 10ug to 30ug of plasmid DNA was dissolved in TE(P) buffer to a final volume of 420ul. Then, 60ul of 2M $CaCl_2$ was added dropwise to the DNA/TE(P) solution with constant agitation. The DNA/$CaCl_2$ solution was added dropwise with constant agitation to 480ul of 2x HBS and the precipitate was allowed to form at room temperature for 30min. This solution was then applied dropwise to the cell monolayers and swirled into the medium (15ml per 90mm petri dish). After 24h the medium was replaced with fresh medium and cells were incubated at 37^{o}C for a further 24h. When cells were infected with virus the medium was removed after the first 24h incubation and virus added in 5ml of fresh medium. Following adsorption at 37^{o}C for 1h, a further 10ml of fresh medium was added and cells were harvested at 16h p.i.

b) Preparation of cytoplasmic RNA.

HeLa cell cytoplasmic RNA was prepared from tissue culture cells grown on 90mm petri dishes. All solutions and vessels used up to the phenol/chloroform extraction steps were cooled on ice. Following removal of the medium, cells were washed with PBS and then harvested in 4ml of PBS. Cells were centrifuged in the cold at 2,000rpm for 1min, washed in PBS and re-pelleted. Following resuspension in 0.3ml of ILB, cells were left on ice for 3min and the cytoplasmic fraction was separated from nuclei by centrifugation at 3,000rpm for 5min. The supernatant (cytoplasmic fraction) was added to an equal volume of phenol extraction buffer (PEB) and extracted three times with phenol/chloroform and once with chloroform. RNA was precipitated twice with ethanol, washed with 70% ethanol, lyophilised and resuspended in 20ul of distilled H_2O. Concentration of cytoplasmic RNA was determined by spectrophotometry (OD_{260} 1.0=40ug RNA/ml).

c) Production of RNA probes.

Detection of cytoplasmic RNAs was achieved using probes produced by the pGEM-2 transcription vector (see Page 58). The principle behind this system is that in vitro transcription of a DNA fragment, spanning the 5' end of the RNA-specifying DNA region, will produce a transcript which can then be used as a probe for the detection of this RNA. The DNA fragment is inserted into the MCS of pGEM-2 in such an orientation so that the produced transcript is complementary to the RNA of interest. As mentioned on Page 58, pGEM-2 contains two promoters one of which was used for the production of probes (SP6 in this study); in order to obtain run-off transcripts of appropriate length, the pGEM2/insert plasmid is linearised at a restriction enzyme site located doownstream form the insert. The linearised construct is then incubated in a cell-free transcription system which comprised: an SP6 RNA polymerase, a mixture of NTPs and ^{32}P-UTP and a capped dinucleotide which is added to the 5' end of the transcript and is required for its stabilisation. After the completion of the reaction, the mixture is incubated in the presence of an RNase-free DNase

which degrades the template, and RNA transcripts are electrophoresed on polyacrylamide gels and then isolated.

The main advantage of pGEM-2-produced RNA probes as compared to single-stranded DNA probes, usually used for RNA mapping, is the high counts per minute (c.p.m.)/ug ratio which allows the detection of very low amounts of RNA. Further, the RNA probes are specific for the RNA of interest, whereas, after separation of double-stranded DNA on denaturing polyacrylamide gels, single-stranded DNA probes have to be tested for complementarity to the RNA of interest.

1ul of linearised pGEM2/insert plasmid DNA (1ug/ul) was added to 1ul of 2.5mg/ml BSA, 5ul of 5x SP6 buffer, 1.25ul of 0.2M DTT, 2.5ul of 10x NTPs (1mM each of A, U, C and G), 7ul of [α-^{32}P]UTP, 1.25ul of 10mM G(5')ppp(5')G_{OH}, 1ul of RNasin, 1ul of SP6 DNA polymerase (10units/ul) and 4ul of distilled H_2O. The reaction was incubated at 37^{o}C for 1h and then for a further 10min in the presence of 2.5ul of RNase-free DNase (1unit/ul). The reaction was stopped with 175ul of 2x PK buffer and the mixture was phenol/chloroform and chloroform extracted. RNA transcripts were precipitated with 20ul of 6M NH_4Ac, 600ul of ethanol and 2ul of tRNA (20ug/ul) and pelleted by centrifugation for 10min in a MSE microfuge. The pellet was resuspended in 50ul of TE and the suspension was electrophoresed on an 8% denaturing polyacrylamide gel (see Page 62) in order to isolate full-length transcripts. A gel slice containing the transcript was excised from the gel, and RNA was eluted by incubating with 1ml of elution buffer (EB) at 37^{o}C overnight. Following ethanol precipitation the RNA was taken up in 50ul of distilled H_2O.

d) Hybridisations and ribonuclease T2 digestions.

Typically, 0.5ug or 4ug of cytoplasmic RNA was added to approximately 200ng of pGEM2-produced probe (approximate specific activity 2x 10^8 c.p.m./ug), the mixture was lyophilised, and the pellet was resuspended in 15ul of 1x M13 buffer. The solution was heated at 95^{o}C for 10min and then incubated at 50^{o}C overnight.

Following hybridisation, samples were treated with

200ul of HNE buffer and 5units of ribonuclease T2 and incubated at 30^{o}C for 30min. The hybrids were then mixed with 50ug of proteinase K and 40ug of tRNA, incubated as above, phenol/chloroform and chloroform extracted, precipitated and lyophilised. The pellets were taken up in 5ul of formamide dyes, heated for 5min at 95^{o}C, quenched on ice and then loaded on an 8% denaturing polyacrylamide gel. pAT153 plasmid DNA digested with Hpa II was used as DNA size markers (fragment sizes in bp: 622, 492, 404, 242, 238, 217, 201, 190, 160, 160, 147, 122, 110, 90, 76, 67, 34, 26, 26, 15, 9, 9).

SECTION C.

COMPUTER ANALYSIS.

DNA sequencing data were handled and analysed using the Institue of Virology Digital Research computer operating under the RSX-11M operating system. In this Section computer programs utilised throughout this work will be briefly described.

19. Accumulation and Processing of Gel Readings.

Gel autoradiographs were read using the DPAD program of Dr P. Taylor. This program allows gels to be read on a digitising tablet from which the data are entered and stored directly into the computer in a form which can be read by the Staden programs (see below).

The compilation of the individual gel readings entered into the database was achieved using a group of programs designated BATCH, based on the DBAUTO system of Staden (1982). This program compares a 'file of files', which comprises several individual gel readings, with sequences held in the existing database. Gel readings showing homology to the consensus of the database are entered into the database in the appropriate region. Where necessary the program will insert padding characters into either the newly entered gel or in the consensus sequence thus enabling the sequences to be correctly aligned. Gel readings exhibiting no homology to any sequence in the database are held as separate contigs. Initially the number of contigs approximates the number of gel readings, particularly in a large project; however, as the gaps between gel readings are filled by new overlapping sequences, the number of contigs is reduced. Finally, one contig is achieved within which all gel readings overlap. Joining of the individual contigs can be achieved with the DBUTIL program of Staden (1980). This program can further monitor the state of the database by providing lists of all the entered gel readings, details

on their location, length and orientation.

20. Interpretation of Data in the Database.

Analysis of the completed sequence was achieved with a number of programs most of which were designed and/or modified by Dr P. Taylor.

BASES, estimates the frequency of occurence of each base in a nucleotide sequence at a specified position (for example every third base) or in a whole sequence and outputs a table of values.

CINTHOM, compares two nucleotide or amino acid sequences and displays the results in a matrix plot (Pustell and Kafatos, 1982). This program was designed to score for identical amino acids, however, it has been modified by Dr P. Taylor to score in addition for conservative amino acid changes as evaluated by Dayhoff (1983). The sequences are aligned on the X-axis and on the Y-axis. Areas of conserved sequences are represented by a diagonal on the plot while insertions or deletions are represented by diagonals which, when extrapolated, cross the X and Y axis at different points. Homologous sequences are displayed as uppercase or lowercase letters of the alphabet, with A representing the the highest homology (100%) and z the lowest. The parameters of this program are set by the operator.

CHOUFAS predicts the secondary structure of a protein (Chou and Fasman, 1978).

CHOP deletes strings of nucleotide or amino acid sequences from a file.

DENS measures the areas under the peaks of densitometer tracing.

DSPLAY searches a nucleotide sequence for ORFs which can either be displayed or filed to be printed; the minimum

length of the ORFs is defined by the operator.

FRMSCAN (Staden and McLachlan, 1982) identifies potential protein coding regions by evaluating the codon usage of all three reading frames in a DNA sequence using as reference the codon usage of a 'standard' gene; in the case of HSV-1 the codon usage of the TK gene was used as reference.

HOMOL (Taylor, 1984) optimally aligns two nucleotide or amino acid sequences by scoring for identical residues. The parameters are set by the operator.

MWCALC calculates the molecular weight and amino acid composition of an amino acid sequence.

PROFIL produces a graphic display of the hydropathicity profile (Kyte and Doolittle, 1982) of an amino acid sequence.

PTRANS (Taylor, P., 1986) predicts the amino acid sequence from a nucleotide sequence. In addition this program estimates the mol. wt. of the translated sequence and outputs an amino acid content and codon usage table. The positions where translation starts or ends are set by the operator.

SEARCH searches for specified strings of characters in a nucleotide or amino acid sequence.

WORDSEARCH (Dereveux et al., 1984) searches the National Biomedical Research Foundation (NBRF) protein sequence database for homologous sequences. This program was implemented on a VAX computer at the Edinburgh Regional Computing Centre.

21. Structural Analysis of Amino Acid Sequences.

Amino acid sequences of the polypeptides encoding the large and small subunit polypeptides of ribonucleotide reductase from eukaryotic, prokaryotic and viral origin were

aligned with the program of Dr W.R. Taylor (see Results and Discussion, Section C). This program can align a number of polypeptide sequences on the basis of identical amino acids and, in addition, on the basis of amino acids sharing similar physico-chemical properties. For the latter reason amino acids have been classified in the form of a Venn diagram.

a) The Venn diagram of amino acid classification.

This diagram shows the classification in groups (sets) of the twenty naturally occuring amino acids to a selection of physico-chemical properties which are important for the determination of the secondary structure of a protein (Fig. 18; Taylor, W.R., 1986a). The two most prominent sets are the 'polar' and 'hydrophobic' ones; the former comprises amino acids which have a polar group in their side-chain and the latter, which is less rigorously defined, comprises amino acids which are hydrophobic. These two sets overlap, and the amino acids found in the region between them are considered as ambivalent to H_2O. A third major set of amino acids, 'small', contains amino acids with side-chain volume less than 60 Å. This set contains a subset of amino acids with side-chain volume less than 35 Å and this is designated as 'tiny'. The location of cysteine in this diagram is not well defined as the reduced form (C_H) of this residue contains a polarisable sulphur which suggests similarity with the serine residue, while the oxidised form (C_{SS}) lacks this property and appears to be more similar to valine. The remaining sets are defined on the basis of obvious properties such as 'aromatic', with ring-containing side-chains, 'aliphatic', with a branched aliphatic side-chain, and 'charged', which are normally fully ionised. Proline is generally associated with turns or loops on the protein surface due to the structure of its side-chain and therefore, despite its very hydrophobic nature, is not included in the 'hydrophobic' set of amino acids.

b) Protein homology by consensus template alignment.

The consensus template alignment (Fig. 19; Taylor, W.R., 1986b) is a program which can align a number of amino

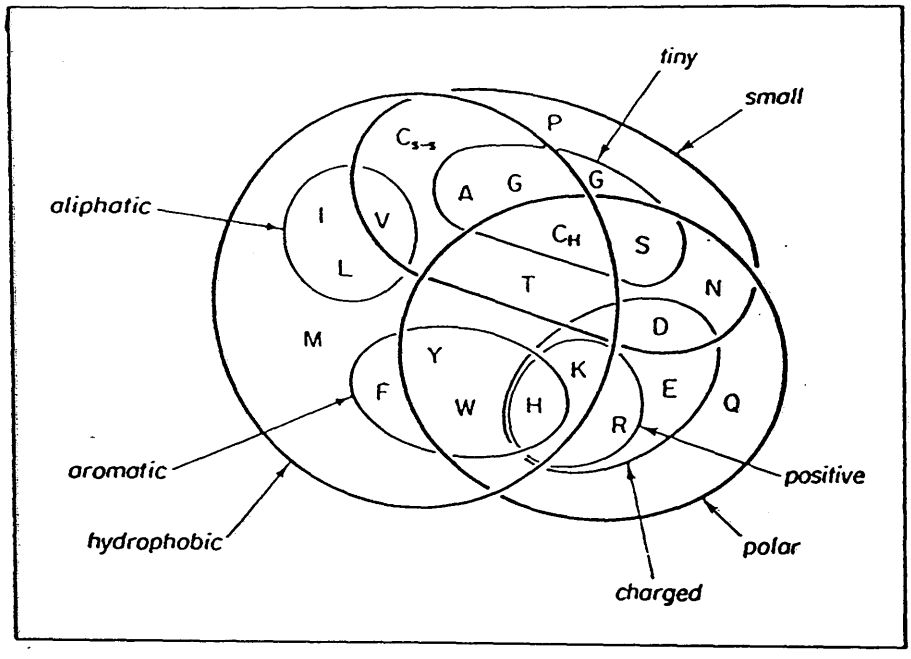


Figure 18. The Venn diagram of amino acid classification showing the relationship of the 20 naturally occuring amino acids to a selection of physicochemical properties (reprinted from Taylor, W.R., 1986a).

acid sequences. The alignment is obtained with the SETEM and FITEM programs and is based on the use of consensus templates. The templates are essentially the core amino acid sequences from conserved regions of related proteins with known three-dimensional structure, which have been aligned to related sequences of unknown structure in order to become more representative (alignment templates).

The program begins with the tentative alignment of sections from a few of the amino acid sequences to be aligned with the use of the alignment templates. Then the SETEM program will process the aligned sections in order to make the alignment templates more representative. Depending on the aligned amino acids at a given template position, the SETEM will assign a property on the basis of the Venn diagram. For example, if at a given template position three polypeptides sequences have R, K, and H residues respectively, then the program will assign the 'POSITIVE' property. Each of the properties is allocated a score ranging from 1 to 20. A score of 1 is given when the same amino acid occurs at a given template position in all sequences, and 20 when the amino acids at that position are classified in totally different sets in the Venn diagram. The SETEM-produced alignment templates are then fitted by FITEM to a non-aligned sequence. If the complete set of templates fits the new sequence then it joins the alignment. The SETEM-FITEM alternating cycles continue until the existing alignment cannot be further altered.

Once the alignment is completed, then the program will predict the secondary structure for each aligned amino acid with the GARNIER algorithm (Garnier et al., 1978), and from the combination of the individual predictions it will derive a consensus.

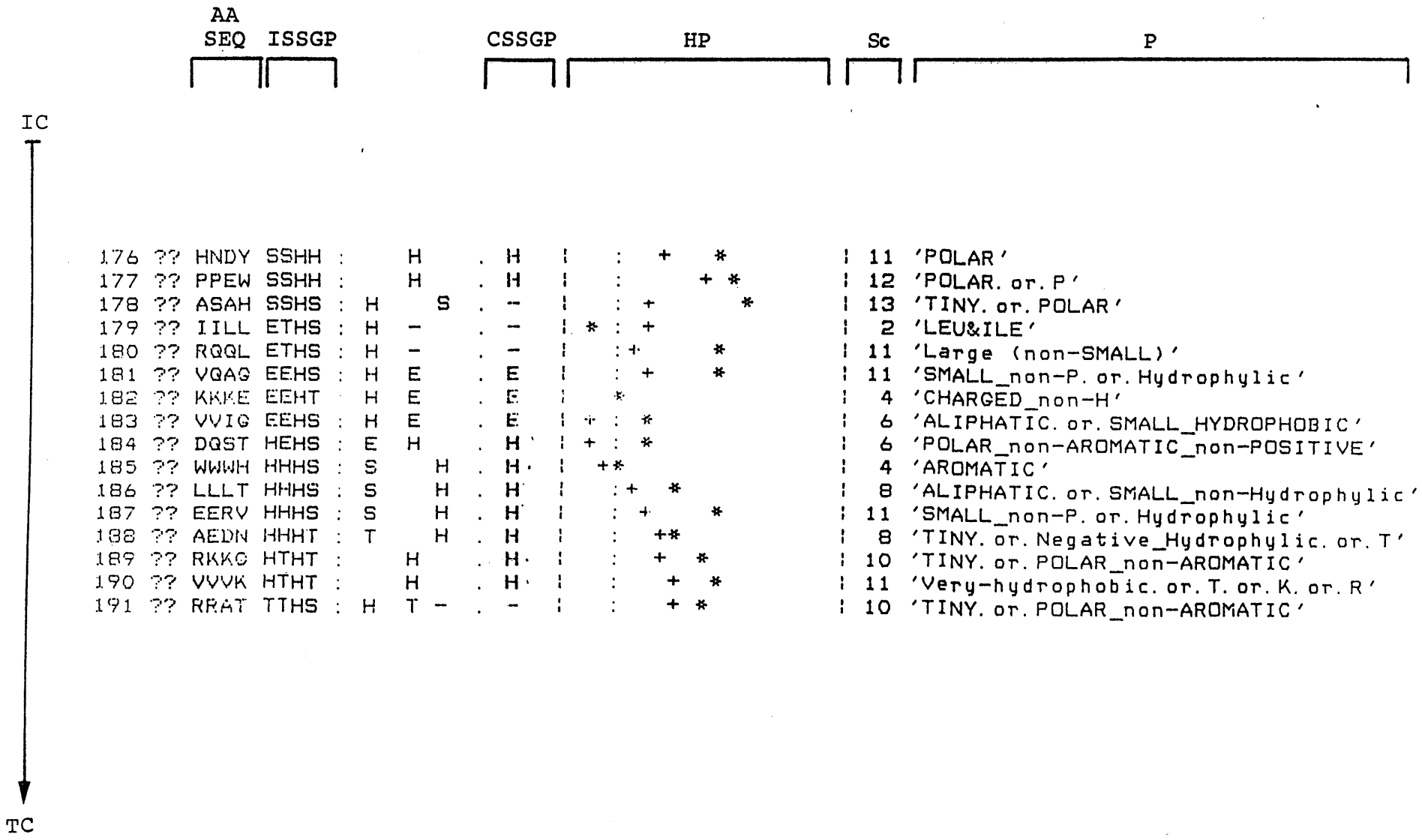

AA
SEQ
ISSGP
CSSGP
HP
Sc
P
IC
TC
176 ?? HNDY SSHH : H . H : 11 'POLAR'
177 ?? PPEW SSHH : H . H : 12 'POLAR. or. P'
178 ?? ASAH SSHS : H S . - : 13 'TINY. or. POLAR'
179 ?? IILL ETHS : H - . - : 2 'LEU&ILE'
180 ?? RQQL ETHS : H - . - : 11 'Large (non-SMALL)'
181 ?? VQAG EEHS : H E . E : 11 'SMALL_non-P. or. Hydrophylic'
182 ?? KKKE EEHT : H E . E : 4 'CHARGED_non-H'
183 ?? VVIG EEHS : H E . E : 6 'ALIPHATIC. or. SMALL_HYDROPHOBIC'
184 ?? DQST HEHS : E H . H : 6 'POLAR_non-AROMATIC_non-POSITIVE'
185 ?? WWWH HHHS : S H . H : 4 'AROMATIC'
186 ?? LLLT HHHS : S H . H : 8 'ALIPHATIC. or. SMALL_non-Hydrophylic'
187 ?? EERV HHHS : S H . H : 11 'SMALL_non-P. or. Hydrophylic'
188 ?? AEDN HHHT : T H . H : 8 'TINY. or. Negative_Hydrophylic. or. T'
189 ?? RKKG HTHT : H . H : 10 'TINY. or. POLAR_non-AROMATIC'
190 ?? VVVK HTHT : H . H : 11 'Very-hydrophobic. or. T. or. K. or. R'
191 ?? RRAT TTHS : H T - . - : 10 'TINY. or. POLAR_non-AROMATIC'

Figure 19. A printout of the consensus template alignment program of Taylor, W.R. (1986b). The aligned polypeptide amino acid sequences (AA SEQ) are shown to run vertically, starting at the initiation codons (IC) and stoping at the termination codons (TC). The secondary structure GARNIER predictions for each polypeptide (ISSGP) are indicated. The consensus secondary GARNIER prediction for each aligned amino acid position (CSSGP) is shown. β-strand conformation is indicated by (E), α-helix conformation by (H), unstructured or turn conformation by (T) and (-) and (S) indicate that the consensus prediction for the given position is unknown. The hydropathicity values (HP) are indicated by asterisks (*). The printout also shows the scores (Sc) assigned by the SETEM program for each aligned position, and the property (P) of the aligned amino acids as deduced from the Venn diagram of amino acid classification (see Fig. 18).

RESULTS AND DISCUSSION

SECTION A.

1. DNA Sequencing Studies Within the Hind III k Fragment of HSV-1.

The HSV-1 strain 17^+ ribonucleotide reductase locus between 0.54 and 0.60 map units (Fig. 20) specifies a family of four nested mRNAs which are comprised of two L and two E transcripts (Anderson et al., 1980; McLauchlan and Clements, 1982 and 1983a; McLauchlan, 1986). The L mRNAs, which are presumed to be 5' coterminal, have sizes of 7.0kb and 1.9kb and encode the same 54,000 mol. wt. polypeptide (Vmw54; McLauchlan, 1986). The E mRNAs, which are encoded by genes UL39 and UL40 are unspliced and have sizes of 5.0kb and 1.2kb, respectively. The 5.0kb mRNA specifies the RR1 polypeptide and the 1.2kb mRNA specifies the RR2 polypeptide. The 5' end of the 5.0kb mRNA is located 194nuc upstream from a Xho I site at 0.561 map units and the 5' end of the 1.2kb mRNA lies 290nuc upstream from a Hind III site at 0.587 map units (McLauchlan and Clements, 1983a). The E mRNAs and the 7.0kb mRNA share a common 3' terminus positioned 770nuc to the right of the Hind III site at 0.587 map units (McLauchlan and Clements, 1982).

Previous partial sequencing studies predicted that the entire coding region of RR1 and the N-terminal portion of RR2 lie within the Hind III k fragment (McLauchlan and Clements, 1982 and 1983a; McLauchlan, 1986). A possible ATG start codon for RR1 was located 34nuc downstream from the Xho I site at 0.561 map units (see Fig. 20). Within the 5' portion of the 1.2kb mRNA two ATG codons were identified, but an in-frame TGA stop codon was positioned 12nuc downstream from the first ATG (McLauchlan and Clements, 1983a). It was proposed that the TGA codon was the C-terminus of RR1 while the second ATG codon represented the initiation methionine at the N-terminus of RR2. The prediction of the latter was based on two reasons (McLauchlan and Clements, 1983a and b; McLauchlan, 1986): first, the first ATG codon was absent from the leader of the equivalent HSV-2 1.2kb mRNA and, second, the nucleotides

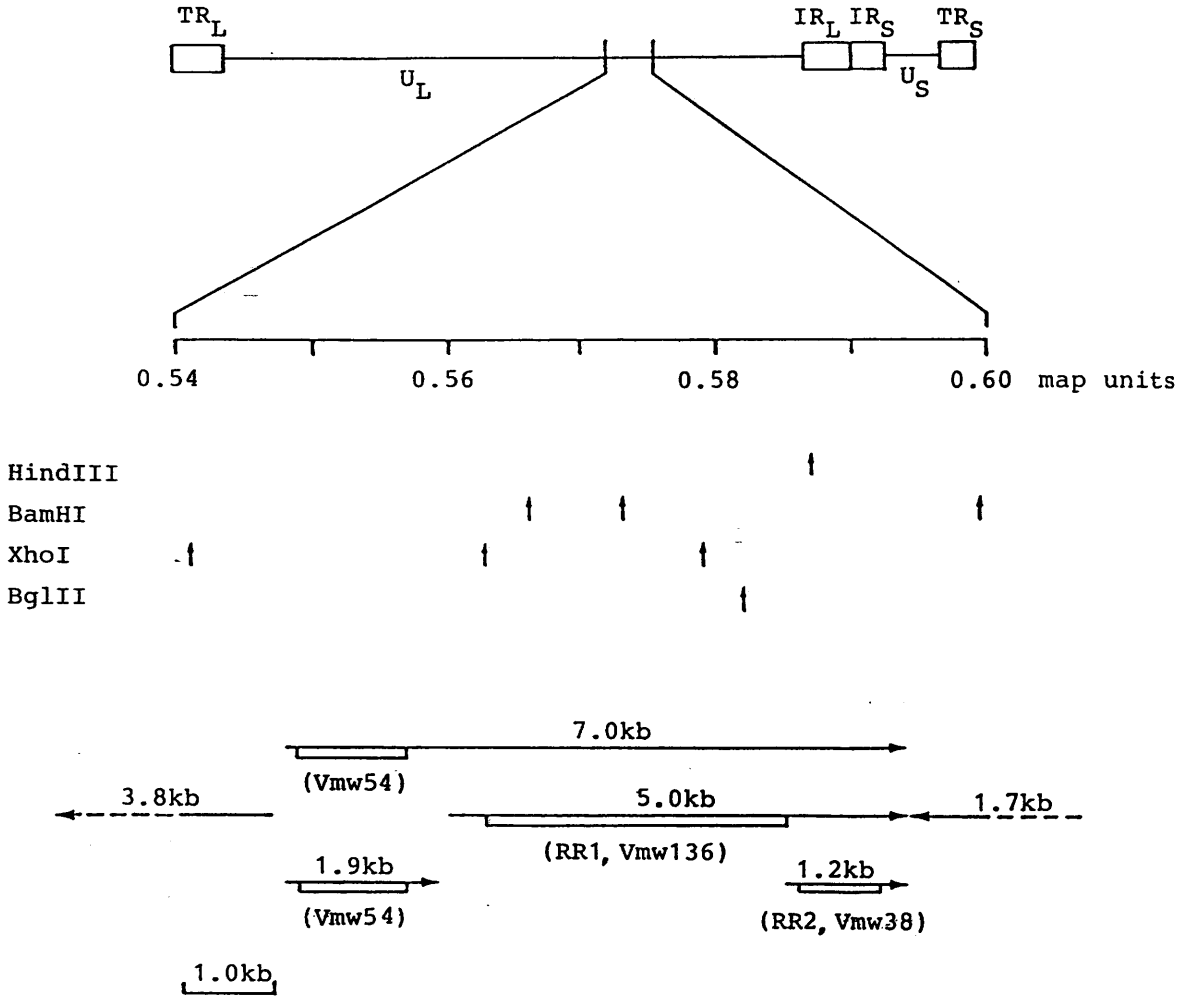


Figure 20. The HSV-1 strain 17^+ ribonucleotide reductase locus. This diagram shows the location of restriction endonuclease cleavage sites and the arrangement of mRNAs between 0.54 and 0.60 map units on the viral genome. The transcripts specified by this locus are shown as arrowed lines and their sizes in kb are indicated above each line. Open boxes denote the polypeptide coding regions of the transcripts and their designation is given in parentheses below each box.

flanking the second ATG of the HSV-1 1.2kb mRNA were identical to those flanking the ATG of the HSV-2 1.2kb mRNA and, furthermore, they resemble those at other functional initiation codons (Kozak, 1981). In conclusion, although the coding regions of the RR1 and RR2 polypeptides do not overlap, the upstream transcription initiation signals and part of the untranslated leader of the 1.2kb mRNA map entirely within the genome region specifying the C-terminus of the RR1 polypeptide.

To determine the nucleotide sequence of the RR1 coding region, a Xho I/Hind III fragment (0.561 to 0.587 map units) was isolated from a cloned Hind III k fragment (obtained from Dr J. McLauchlan), and was subcloned into a Sal I/Hind III digested pUC8 vector (Fig. 21). Therefore, the resultant plasmid, pYN1, contained the entire proposed coding region of RR1 and part of the RR2 coding region. Sequencing data was obtained using the M13 dideoxy chain termination method in combination with a 'shotgun' cloning approach and was subsequently collated by computer programs as described in Materials and Methods (see Page 73).

Manipulation of the sequencing data resulted in the formation of the HINDKK database which consisted of 186 independent gel readings (Fig. 22). There were 34,286 characters, which produced a final contiguous length of 3564bp (Fig. 23). For each contig character an average of 9.62 characters were read from gels.

2. Computer Assisted Analysis of the HINDKK Database.

Analysis of the obtained sequence for potential ORFs and potential coding regions was performed with the DSPLAY and FRMSCAN programs, respectively (see Materials and Methods, Pages 74 and 75). The DSPLAY program identified in the left-to-right stand a single ORF (frame 1 in Fig. 24) starting with the ATG triplet at position 34 and terminating with the TGA stop codon at position 3447. The other two reading frames on this strand (frames 2 and 3 in Fig. 24) are punctuated by stop codons and the longest potential ORF is approximately 600 nucleotides long. Analysis of the complementary strand sequence identified a frame containing

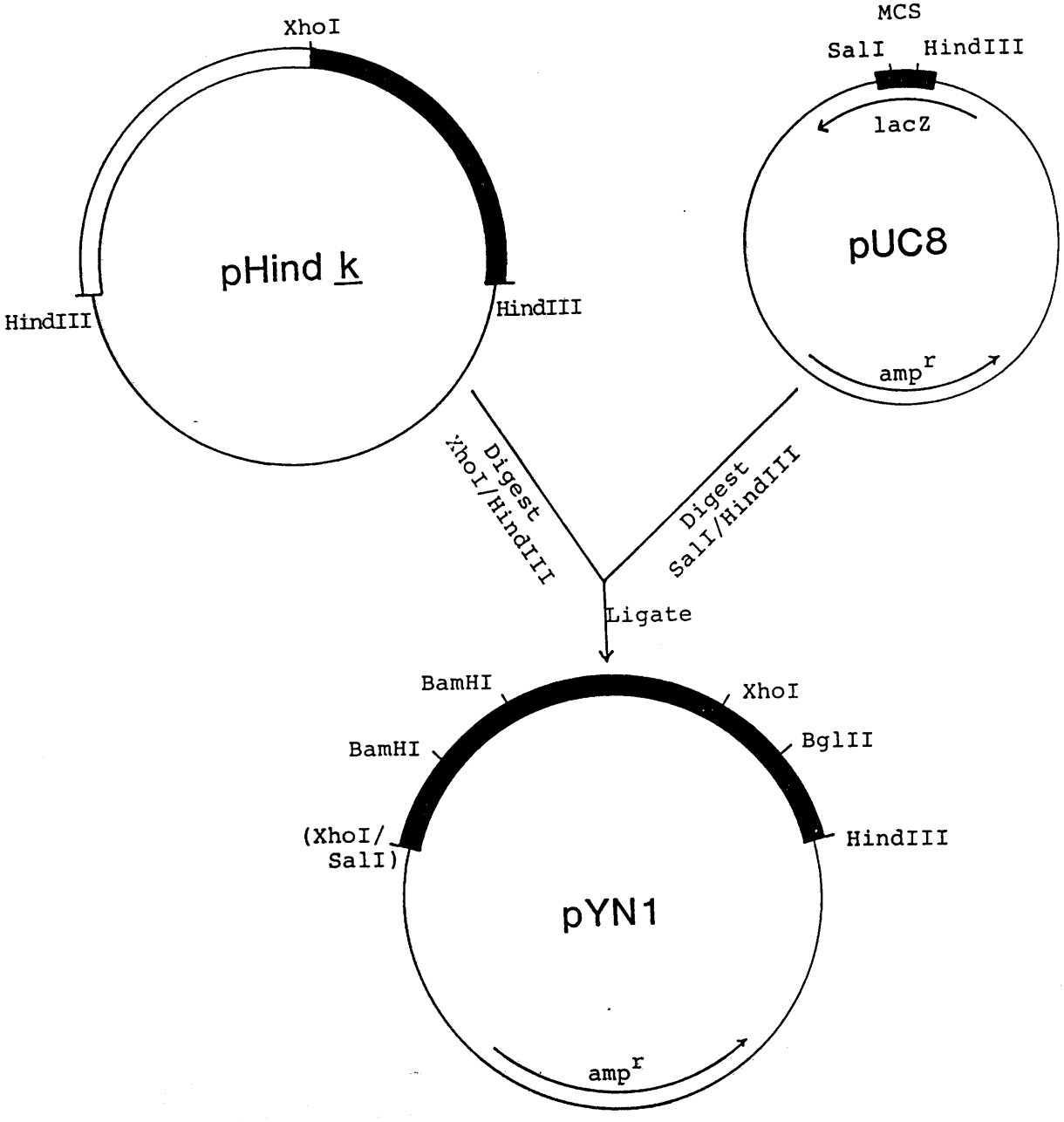

XhoI
pHind k
HindIII
HindIII
MCS
SalI
HindIII
lacZ
pUC8
amp r
Digest XhoI/HindIII
Digest SalI/HindIII
Ligate
BamHI
BamHI
(XhoI/ SalI)
XhoI
BglII
HindIII
pYN1
amp r

Figure 21. Construction of plasmid pYN1. The pHind k plasmid (obtained from Dr J. McLauchlan) was digested with Xho I/Hind III. The fragment which contains the DNA region encoding the RR1 polypeptide and the N-terminus of the RR2 polypeptide (dark area) was ligated into the multiple cloning site (MCS) of a Sal I/Hind III digested pUC8 vector. The location and orientation of the ampicillin resistance (amp^r) and β-galactosidase (lacZ) genes is indicated.

Figure 22. A sorted list (CONTIG LINES) of the gel readings obtained after sequencing the recombinant M13mp8 clones which contained subfragments of the RR1 polypeptide coding region. The column termed GEL LINES represents the name of the clone given by the author. The columns headed A to E are assigned by the DBUTIL program (see Materials and Methods, Page 73) and represent:

A Gel reading database number.

B Position of the left-most base of the gel reading in the contig.

C Length of the gel reading. (-) indicates that the gel reading overlaps on the opposite strand.

D Number of the gel reading overlapping to the left.

E Number of the gel reading overlapping to the right.

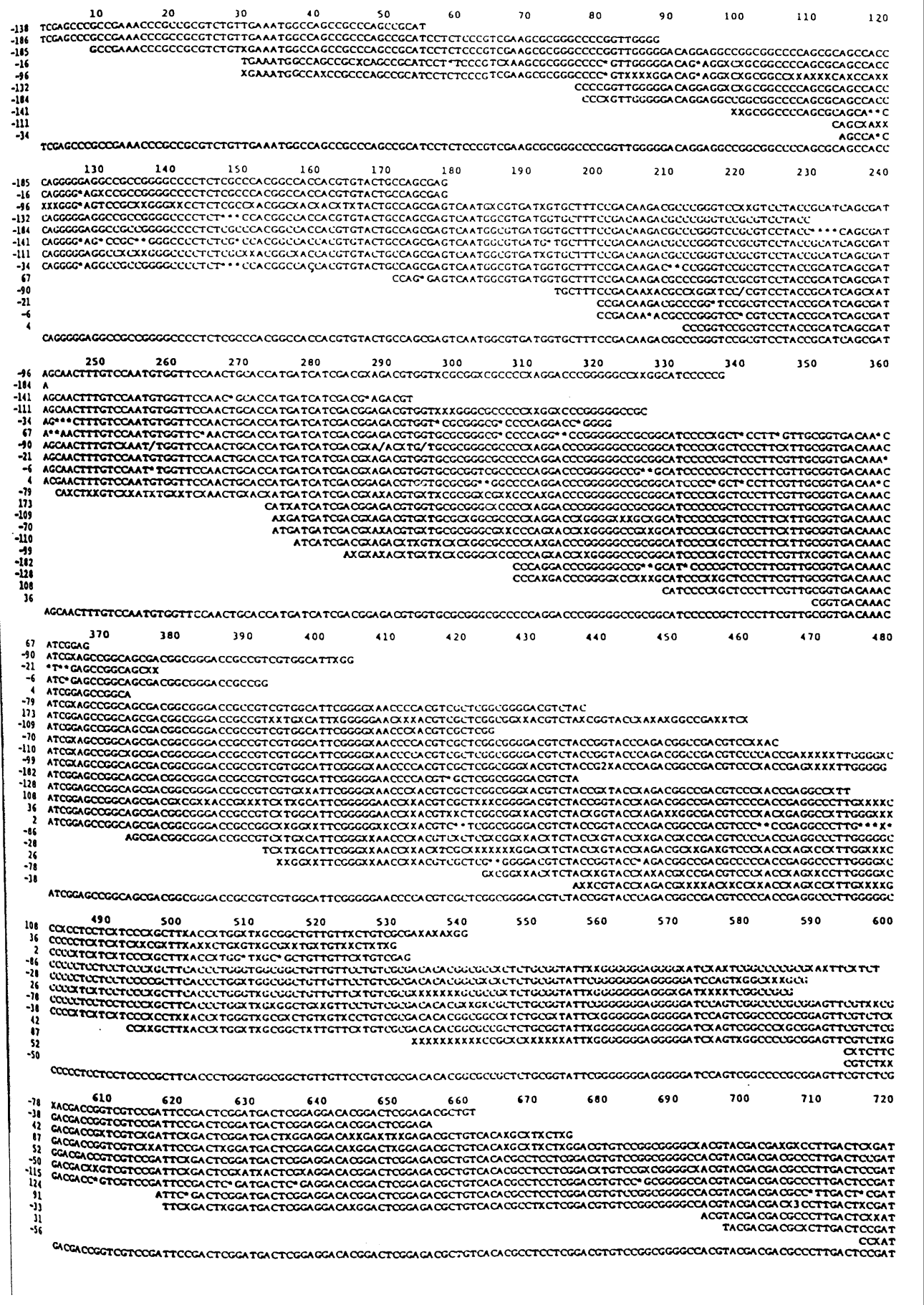
CONTIG LINES

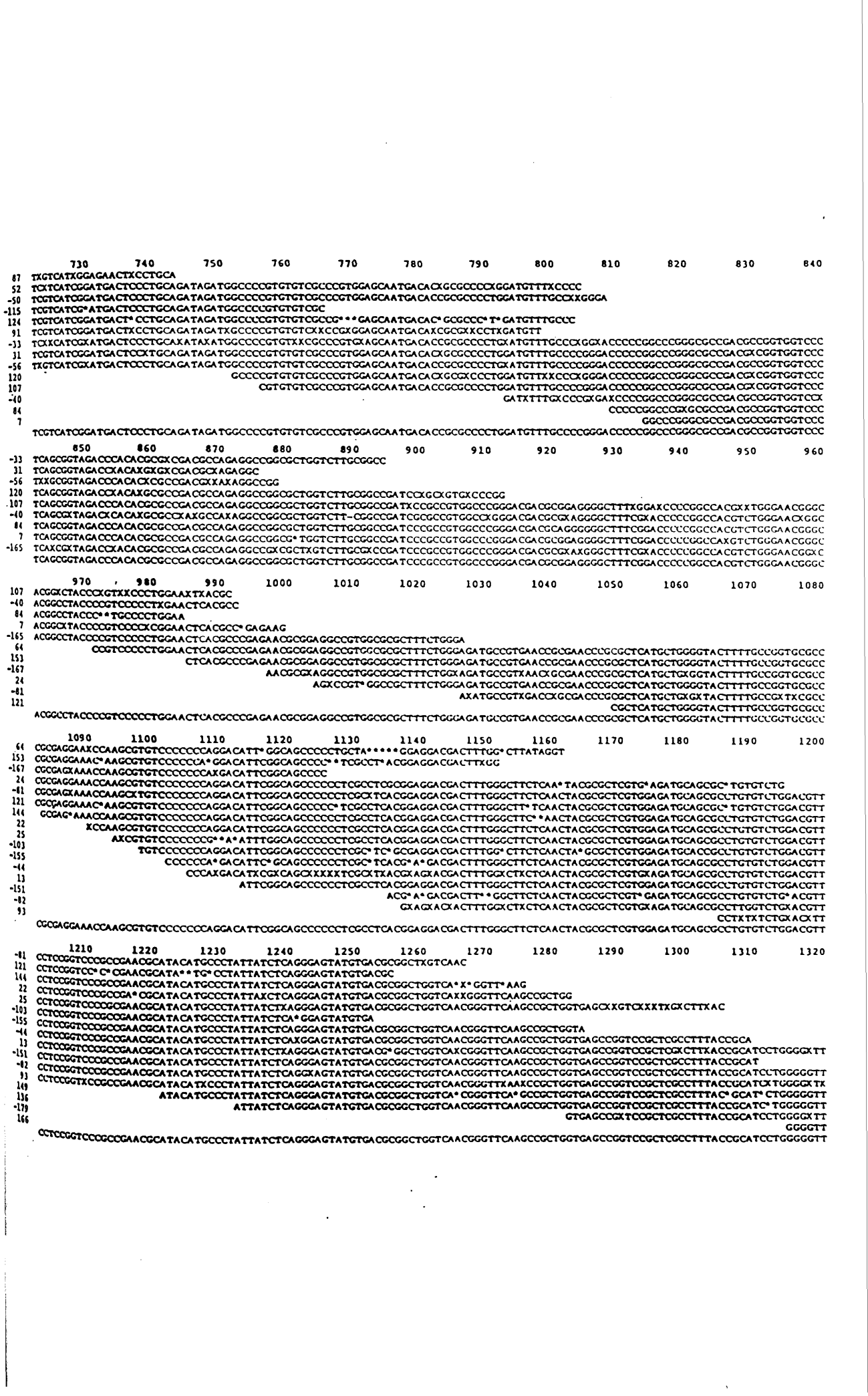
	499	3564	0	138	46
GEL LINES	A	B	C	D	E
112.G4	1	2869	176	117	71
146.G4	2	361	172	36	86
149.G4	3	2574	-179	77	57
150.G4	4	212	180	6	79
154.G4	5	2908	-159	176	23
155.G4	6	200	-193	21	4
163.G4	7	814	186	84	165
144.G4	8	3388	177	137	46
164.G4	9	3201	-162	27	30
356.G5	10	1609	142	168	60
358.G5	11	3098	-246	112	131
361.G5	12	1836	213	152	65
362.G5	13	1113	246	44	151
365.G5	14	2402	206	178	104
412.G6	15	2986	221	183	53
384.G7	16	29	-150	185	96
322.G8	17	2456	-239	140	45
338.G9	18	2164	-143	130	175
343.G9	19	2733	-179	180	117
345.G9	20	2038	160	51	139
303.G10	21	200	-178	90	6
295.G11	22	1090	193	144	25
296.G11	23	2924	-188	5	61
300.G11	24	1004	191	167	81
255.G12	25	1094	212	22	103
256.G12	26	394	193	28	78
260.G12	27	3197	-141	41	9
264.G12	28	392	-197	86	26
266.G12	29	2960	-171	147	183
274.G12	30	3350	-156	9	137
276.G12	31	697	179	33	56
238.G13	32	2267	205	134	118
254.G13	33	694	-201	91	31
233.G14	34	114	-208	111	67
218.G14	35	1952	-201	47	123
363.G5	36	350	181	108	2
372.G5	37	2372	-165	80	122
423.G6	38	436	-220	78	42
382.G7	39	2888	-170	74	176
323.G8	40	793	-200	107	84
329.G8	41	3186	197	146	27
330.G8	42	493	183	38	87
336.G9	43	2431	180	104	127
335.G9	44	1105	-205	155	13
344.G9	45	2467	178	17	94
352.G9	46	3447	118	8	0
298.G11	47	1952	199	95	35
244.G13	48	2878	189	72	74
251.G13	49	3139	-203	131	146
227.G14	50	594	-215	52	115
228.G14	51	2002	-197	69	20
231.G14	52	594	211	87	50
235.G14	53	2989	227	15	97
214.G14	54	1845	214	65	113
393.G6	55	2619	-216	62	85
212.G14	56	716	-163	31	120
223.G14	57	2600	-195	3	83
249.G13	58	3044	178	97	150
243.G13	59	2501	202	161	77
316.G10	60	1611	188	10	116
334.G15	61	2932	203	23	147
108.G15	62	2616	184	83	55
111.G15	63	1771	187	145	133
114.G15	64	971	191	165	153
127.G15	65	1844	-208	12	54
538.G16	66	1386	196	126	163
167.G15	67	171	197	34	90

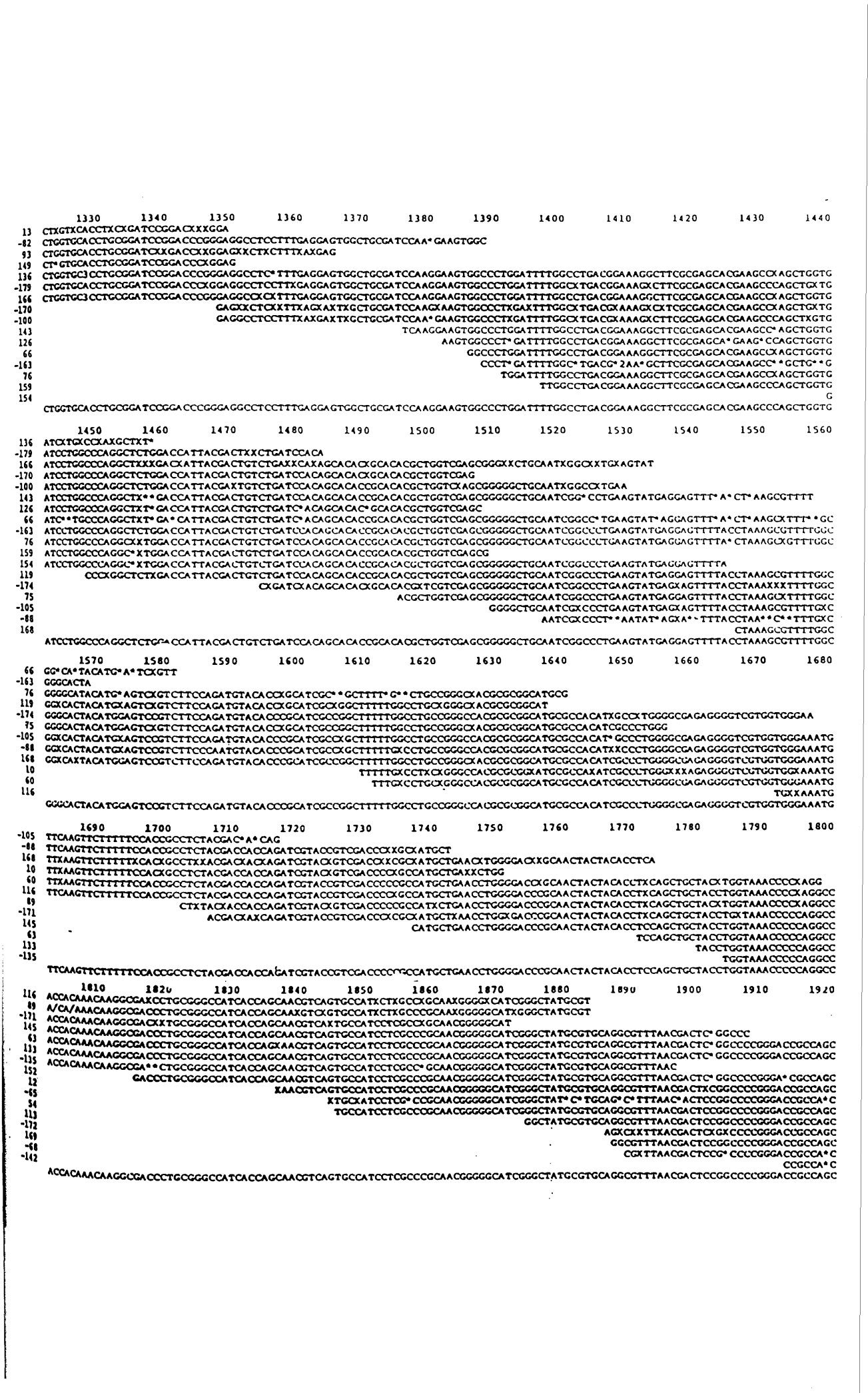
207.G15	68	1889	-198	169	142
539.G16	69	1996	-155	92	51
115.G15	70	274	-191	109	110
118.G15	71	2870	-191	1	72
134.G15	72	2871	-192	71	48
540.G16	73	2651	-143	160	181
552.G16	74	2882	-183	48	39
559.G16	75	1495	161	174	105
563.G18	76	1391	250	163	159
567.G18	77	2531	-224	59	3
561.G18	78	423	-239	26	38
553.G16	79	243	-195	4	173
566.G18	80	2332	242	129	37
569.G18	81	1026	-241	24	121
473.G18	82	1137	-252	151	93
465.G18	83	2609	-266	57	62
113.G02	84	809	176	40	7
116.G02	85	2643	201	55	160
480.G18	86	373	-226	2	28
463.G18	87	533	211	42	52
468.G18	88	1517	-226	105	168
184.G15	89	1702	182	116	171
482.G18	90	194	-211	67	21
105.G15	91	618	181	124	33
558.G16	92	1991	209	125	69
550.G16	93	1185	181	82	149
557.G16	94	2474	-180	45	161
237.G13	95	1931	209	106	47
110.G01	96	29	-309	16	132
122.G02	97	3041	-175	53	58
125.G02	98	2308	-204	118	129
129.G02	99	284	-196	110	182
141.G02	100	1348	-182	170	143
HI61	101	1	121	0	0
HI63	102	1	25	0	0
HA4	103	1098	-155	25	155
169.G19	104	2410	200	14	43
210.G19	105	1509	-208	75	88
539.G19	106	1925	-226	142	95
140.G19	107	756	236	120	40
173.G19	108	329	212	128	36
222.G19	109	274	-151	173	70
129.G19	110	277	-204	70	99
556.G19	111	113	-214	141	34
560.G19	112	3048	230	150	11
130.G20	113	1874	187	54	172
177.G20	114	1	25	0	0
181.G20	115	601	-165	50	124
117.G20	116	1672	212	60	89
119.G20	117	2836	-198	19	1
125.G20	118	2271	-241	32	98
178.G20	119	1448	190	154	174
192.G20	120	752	161	56	107
200.G20	121	1049	207	81	144
195.G20	122	2378	159	37	178
291.G21	123	1953	219	35	125
301.G21	124	617	187	115	91
442.G21	125	1989	-176	123	92
601.G22	126	1382	126	143	66
608.G22	127	2431	-185	43	140
281.G21	128	308	-167	182	108
284.G21	129	2320	-172	98	80
G20.180	130	2155	200	157	18
741.G23	131	3112	-195	11	49
745.G23	132	77	-153	96	184
753.G23	133	1780	183	63	135
621.G24	134	2235	154	175	32
622.G24	135	1784	-113	133	152
623.G24	136	1232	227	149	179
624.G24	137	3383	-178	30	8
629.G24	138	1	-55	0	186
630.G24	139	2092	-188	20	162
631.G24	140	2435	-102	127	17
642.G24	141	99	-195	184	111
658.G25	142	1913	-207	68	106

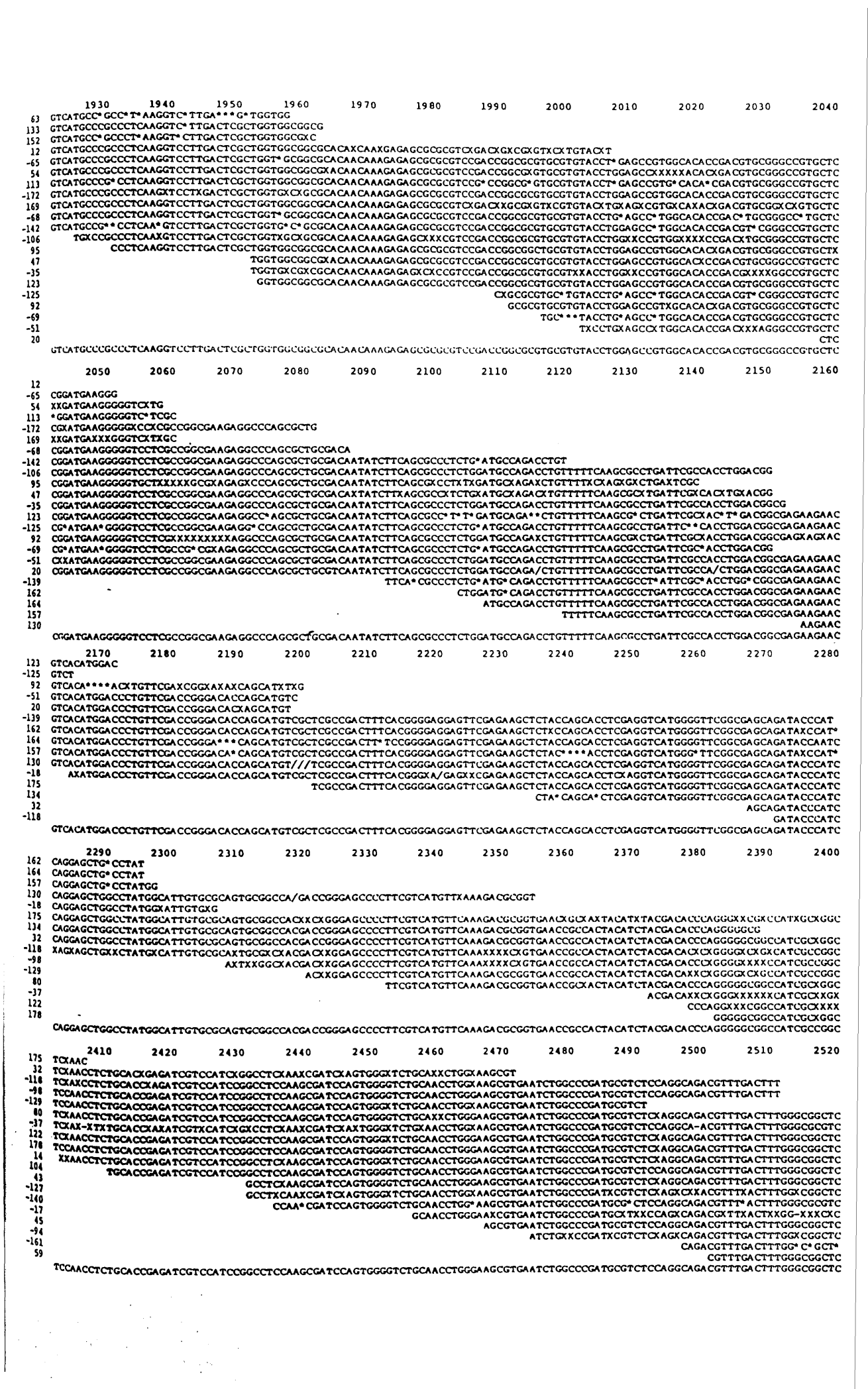
664.G25	143	1376	182	100	126
666.G25	144	1082	194	121	22
667.G25	145	1737	171	171	63
668.G25	146	3180	-190	49	41
671.G25	147	2947	-118	61	29
673.G25	148	1	155	0	0
682.G26	149	1221	130	93	136
686.G26	150	3045	173	58	112
689.G26	151	1135	-176	13	82
695.G26	152	1815	147	135	12
696.G26	153	985	167	64	167
698.G26	154	1440	105	159	119
706.G27	155	1102	-183	103	44
707.G27	156	1	182	0	158
722.G27	157	2119	179	164	130
724.G27	158	22	125	156	0
729.G27	159	1397	112	76	154
730.G27	160	2648	153	85	73
733.G27	161	2497	-172	94	59
735.G27	162	2103	193	139	164
737.G27	163	1388	-181	66	76
738.G27	164	2107	189	162	157
634.G24	165	841	-186	7	64
638.G24	166	1315	219	179	170
639.G24	167	997	-129	153	24
657.G25	168	1546	228	88	10
683.G26	169	1887	174	172	68
684.G26	170	1348	-159	166	100
692.G26	171	1706	-166	89	145
694.G26	172	1886	-196	113	169
704.G27	173	273	188	79	109
711.G27	174	1474	-204	119	75
721.G27	175	2201	206	18	134
448.G21	176	2899	-221	39	5
486.G21	177	2714	241	181	180
728.G27	178	2382	191	122	14
434.G21	179	1282	-202	136	166
678.G25	180	2716	193	177	19
604.G22	181	2657	143	73	177
610.G22	182	308	-129	99	128
613.G22	183	2985	-118	29	15
751.G23	184	78	-164	132	141
G7.138	185	9	-170	186	16
602.G22	186	1	-88	138	185

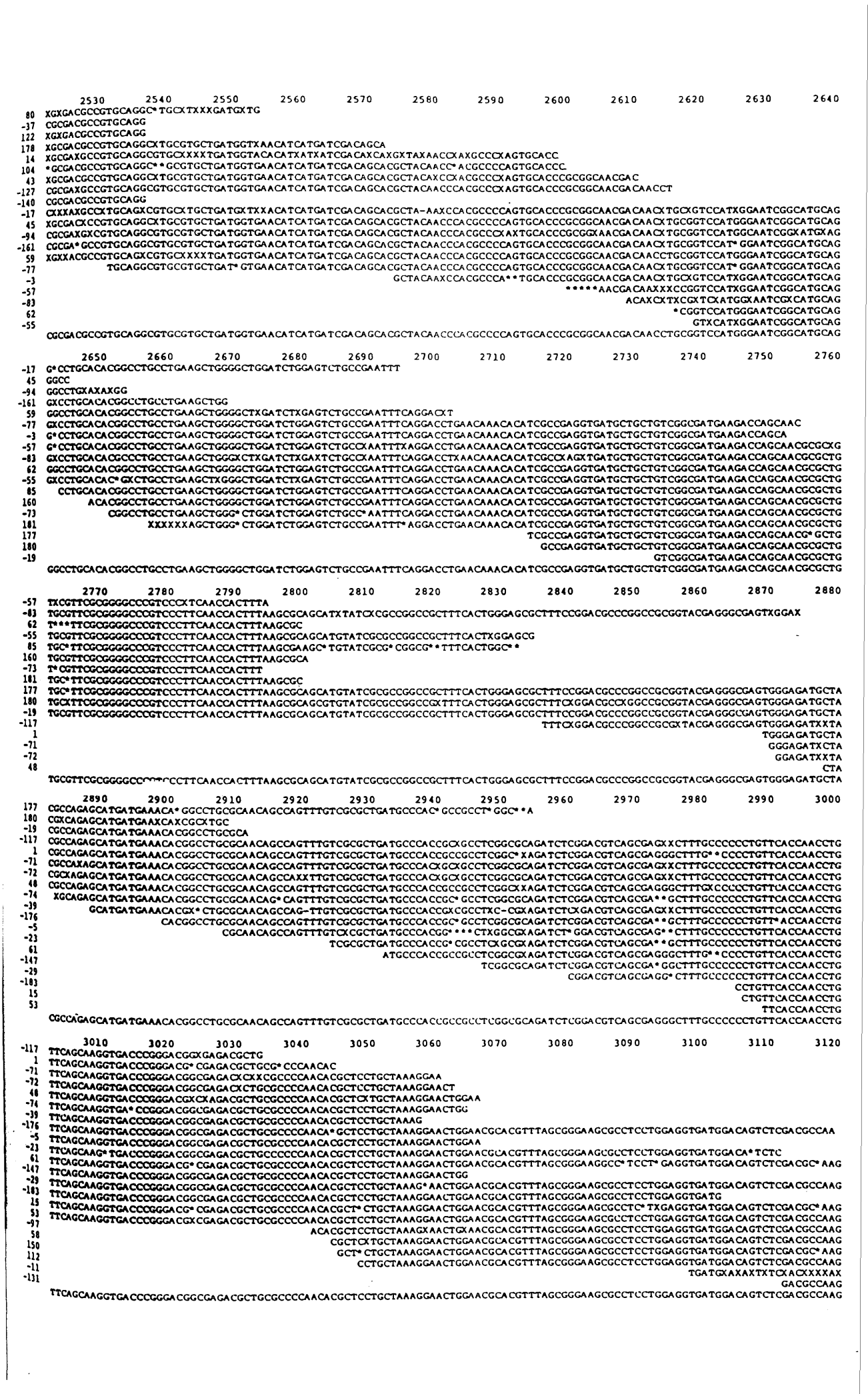
Figure 23. The HINDKK database. This printout shows all the gel readings in the HINDKK database which are numbered (down the left side of the sequences) as in column A of Fig. 22. Computer inserted characters into the gel readings entering the contig database are indicated by (*) and those inserted by the author are indicated by (X), (/) and (-).

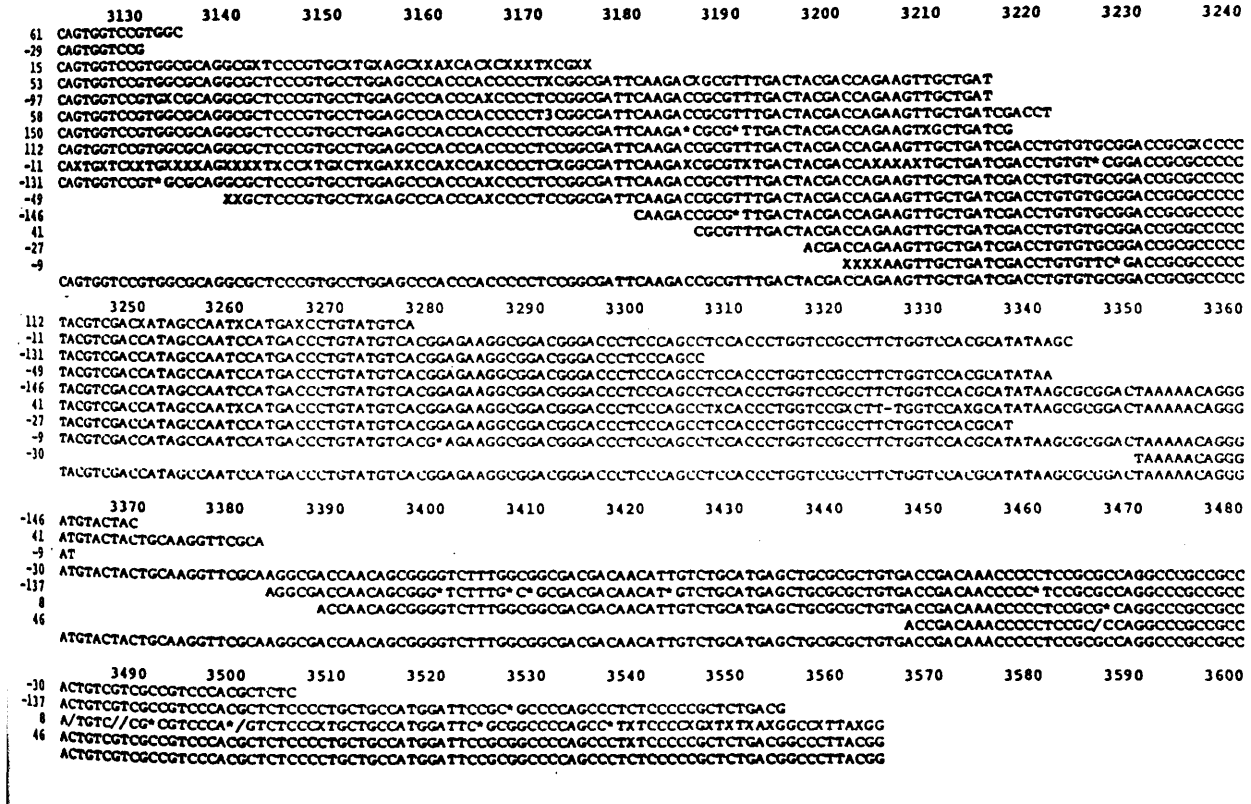












OPEN READING FRAMES IN HINDKK

LENGTH OF SHORTEST POLYPEPTIDE DISPLAYED IS 20 AMINO ACIDS

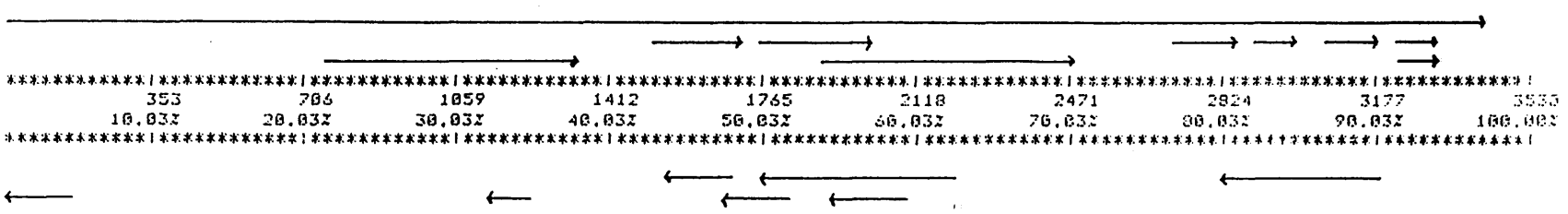


Figure 24. Analysis of the HINDKK database for open reading frames (ORFs) with the DSPLAY program. ORFs are indicated by lines, and in-frame stop codons by arrowheads. The reading frames are numbered on the left; 1, 2 and 3 for the left-to-right DNA strand, and, 4, 5 and 6 for the complementary strand.

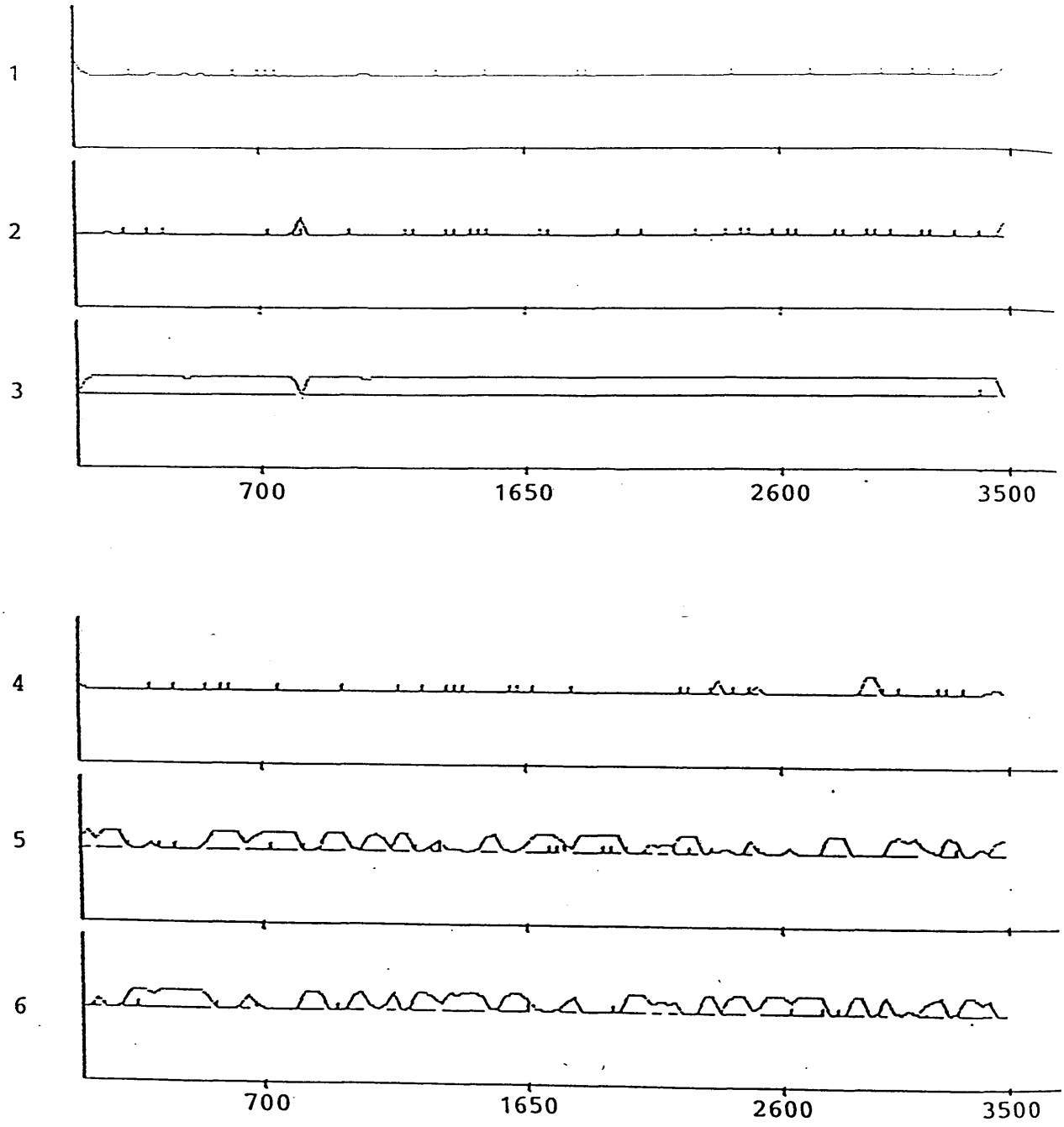


Figure 25. Codon usage evaluation of the HINDKK database with the FRMSCAN program. The top panel (reading frames 1, 2 and 3) represents the codon usage evaluation for the left-to-right strand while the lower panel (reading frames 4, 5 and 6) represents the codon usage evaluation of the complementary strand. The y axis represents the log probability function using an arbitrary scale (Staden and McLachlan, 1982). The x axis shows nucleotide positions in the HINDKK database; on the line parallel to the x axis the in-frame stop codons (ı) are displayed.

no putative ORFs (frame 4 in Fig. 24), while the remaining frames are blocked by stop codons (frames 5 and 6 in Fig. 24); the longest potential ORF is approximately 430 nucleotides.

A representation of the FRMSCAN output is shown in Fig. 25 for both the left-to-right and complementary strands. This program identified in the former strand a single potential coding region spanning the entire sequence (frame 3 in Fig. 25).

These data predict that the coding region of the RR1 polypeptide is an ORF of 3414 nucleotides located between positions 34 and 3447 of the HINDKK contig. Translation of the obtained sequence with the PTRANS program (see Materials and Methods, Page 75) predicted that the encoded polypeptide is 1137 amino acids in length (Fig. 26).

DISCUSSION.

3. The HSV-1 RR1 Polypeptide.

The 1137 amino acid polypeptide predicted from the DNA sequence has a mol. wt. of 124,017 (Table II); this is lower than the estimate of 136,000 obtained from gel electrophoretic mobility of the protein (Marsden et al., 1978). However, RR1 is phosphorylated (Pereira et al., 1977; Marsden et al., 1978, Wilcox et al., 1980), and this may partly account for the observed size discrepancy.

a) Analysis of the RR1 amino acid content.

The overall G+C content across the coding region is 66% (Table II), although, at the third base position of amino acid codons it is markedly higher (83.2%). This is consistent with the codon usage of other HSV proteins which show that, in most cases, the third base position of amino acid codons has a higher G+C content as compared with first and second positions (McGeoch, 1984). The most pronounced triplet periodicity is observed in the alanine residues where the G+C content at the third base position is 91.2%.

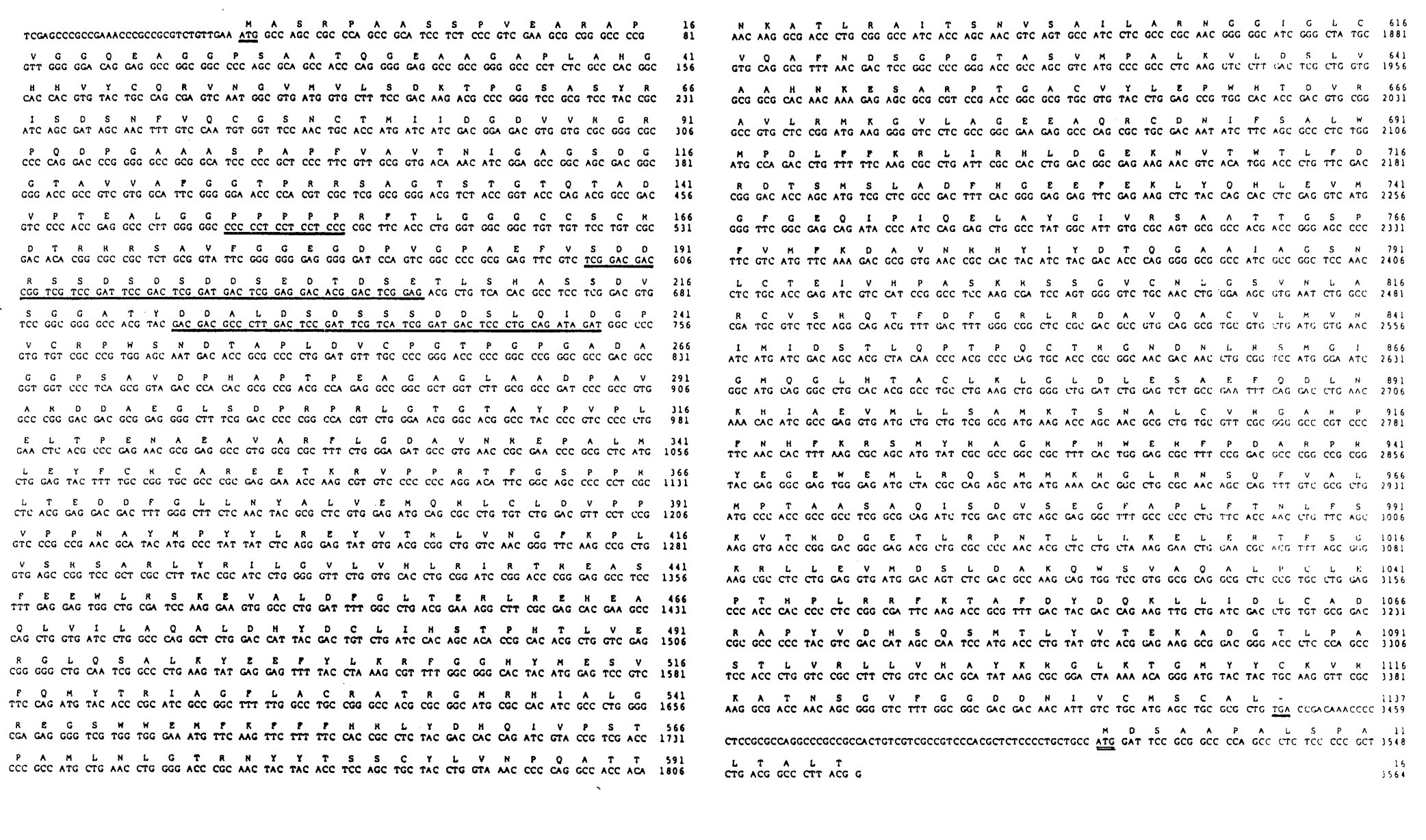


Figure 26. The DNA sequence and the predicted amino acid sequence of the RR1 polypeptide coding region and the N-terminal portion of the RR2 polypeptide. The methionine initiation codon at the N-terminus of RR1 is underlined as is the stop codon at the C-terminus of RR1. The methionine at the RR2 N-terminus is indicated by a double line. Underlined amino acids indicate the run of prolines (P) and the two discrete aspartic acid (D) and serine (S) rich stretches.

TABLE II

AMINO ACID COMPOSITION OF THE HSV-1 RR1 POLYPEPTIDE

Ala	114	10.0%	Val	75	6.6%	Leu	107	9.4%	Ile	30	2.6%
Gly	94	8.3%	Pro	71	6.2%	Cys	29	2.6%	Met	32	2.8%
His	29	2.6%	Phe	46	4.0%	Tyr	33	2.9%	Trp	10	0.9%
Asn	35	3.1%	Gln	33	2.9%	Ser	84	7.4%	Thr	69	6.1%
Lys	32	2.8%	Arg	84	7.4%	Asp	71	6.2%	Glu	59	5.2%

Approximate Molecular Weight = 124016.63

CODON USAGE OF THE HSV-1 RR1 POLYPEPTIDE DNA CODING REGION

TTT Phe	24	2.1%	TCT Ser	4	0.4%	TAT Tyr	8	0.7%	TGT Cys	8	0.7%
TTC Phe	22	1.9%	TCC Ser	27	2.4%	TAC Tyr	25	2.2%	TGC Cys	21	1.8%
TTA Leu	0	0.0%	TCA Ser	3	0.3%	TAA ---	0	0.0%	TGA ---	1	0.1%
TTG Leu	2	0.2%	TCG Ser	18	1.6%	TAG ---	0	0.0%	TGG Trp	10	0.9%
CTT Leu	10	0.9%	CCT Pro	6	0.5%	CAT His	3	0.3%	CGT Arg	6	0.5%
CTC Leu	24	2.1%	CCC Pro	40	3.5%	CAC His	26	2.3%	CGC Arg	45	4.0%
CTA Leu	6	0.5%	CCA Pro	8	0.7%	CAA Gln	4	0.4%	CGA Arg	6	0.5%
CTG Leu	65	5.7%	CCG Pro	17	1.5%	CAG Gln	29	2.5%	CGG Arg	23	2.0%
ATT Ile	3	0.3%	ACT Thr	0	0.0%	AAT Asn	4	0.4%	AGT Ser	4	0.4%
ATC Ile	25	2.2%	ACC Thr	38	3.3%	AAC Asn	31	2.7%	AGC Ser	28	2.5%
ATA Ile	2	0.2%	ACA Thr	7	0.6%	AAA Lys	5	0.4%	AGA Arg	0	0.0%
ATG Met	32	2.8%	ACG Thr	24	2.1%	AAG Lys	27	2.4%	AGG Arg	4	0.4%
GTT Val	7	0.6%	GCT Ala	4	0.4%	GAT Asp	12	1.1%	GGT Gly	6	0.5%
GTC Val	31	2.7%	GCC Ala	67	5.9%	GAC Asp	59	5.2%	GGC Gly	39	3.4%
GTA Val	4	0.4%	GCA Ala	6	0.5%	GAA Glu	12	1.1%	GGA Gly	9	0.8%
GTG Val	33	2.9%	GCG Ala	37	3.3%	GAG Glu	47	4.1%	GGG Gly	40	3.5%

BASE COMPOSITION OF THE HSV-1 RR1 POLYPEPTIDE DNA CODING REGION

	Y	T	C	A	G	R	ALL
NUMBER	1841	595	1246	617	1106	1723	3564
PERCENT	51.7	16.7	35.0	17.3	31.0	48.3	100.0

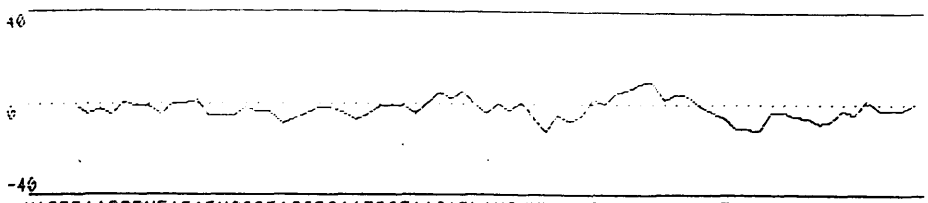
The most common amino acids in RR1 are alanine, glycine and leucine, comprising 27.7% of all residues. Examination of the amino acid distribution throughout the length of RR1 revealed a region at the N-terminus (aa 150 to 280, see Fig. 26) which is extremely rich in aspartic acid, serine, proline and glycine residues. At the start of this region there is a run of five prolines and further on there are two discrete stretches rich in aspartic acid and serine residues (underlined regions in Fig. 26). Aspartic acid and serine residues together comprise 32% of all amino acids in this region, whereas, their overall percentage of occurence in RR1 is 13.6%.

Analysis of other HSV-1 polypeptide N-termini revealed that stretches of prolines occur in the AE polypeptide (Draper et al., 1986), in the IE trans-inducing factor Vmw65 (Dalrymple et al., 1985) and in Vmw175. The latter contains two stretches of prolines the first of which is located at the N-terminus while the second is located approximately in the middle of the polypeptide (McGeogh et al., 1986a). However, it should be stated that in at least 10 other HSV-1 polypeptides proline-rich stretches were identified in the C-terminus. Examination of HSV-1 sequences for stretches of aspartic acid and serine residues revealed, notably, that these were present in the N-terminal region of all the IE polypeptides with the exception of Vmw12 (Perry et al., 1986; McGeoch et al., 1985, 1986a and 1988) and in the protein kinase (McGeoch et al., 1985). Further, a stretch rich in aspartic acid and glutamic acid was identified approximately in the middle of the DNA polymerase (Quinn and McGeoch, 1985).

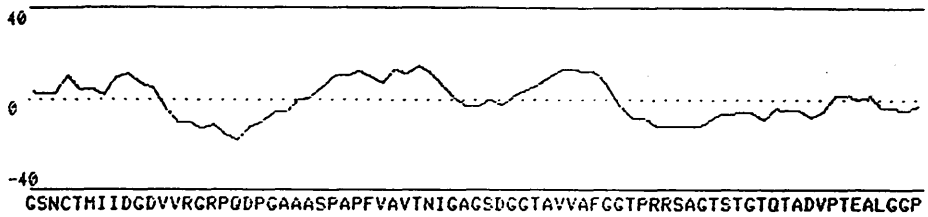
b) The RR1 hydrophathicity profile.

The RR1 hydropathicity plot (Fig. 27) was obtained with the PROFILE program (see Materials and Methods, Page 75). The program predicted two major hydrophilic regions; the first one corresponding to the first stretch of aspartic acid and serine residues and is the most hydrophilic region, while the second, from aa 914 to 961, is the longest predicted region. The most hydrophobic regions are located between aa 833 to 847 and at the

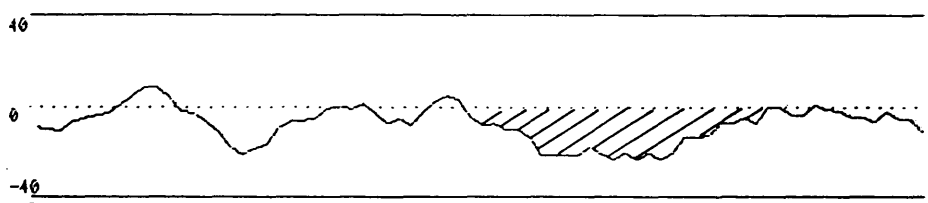
Figure 27. Hydropathicity of the RR1 polypeptide. Hydrophobic regions lie between 0 and 40, and hydrophilic regions between 0 and -40. The RR1 amino acid sequence is shown below the plot and is numbered as in Fig. 26. Hatched regions represent the most hydrophilic regions while dotted regions represent the most hydrophobic regions. Program settings were: group length = 9, shift = 1, spacing of plotted points = 10. The underlined sequence represents a block of highly conserved amino acids between HSV-1 RR1 and other homologue herpesviral polypeptides for which a putative function has been postulated (see General Discussion, Page 130).



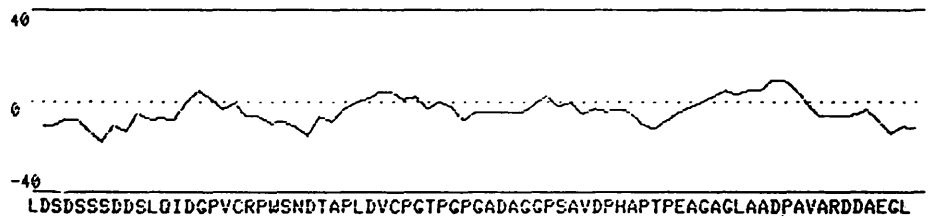
MASRPAASSPVEARAPVGGDEAGGPSAATGGEAAGAPLAHGHHVYCGRVNGVHVLSDKTPGSASYRISDSNFVGC 75



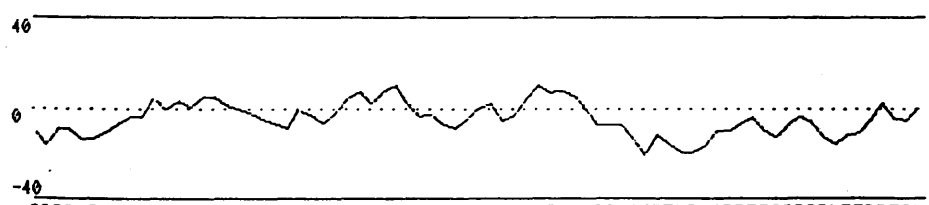
GSNCTMIIDGDVVRGRPGDPGAAASPAPFVAVTNIGAGSDGGTAVVAFGGTPRRSAGTSTGTQTADVPTEALGGP 150



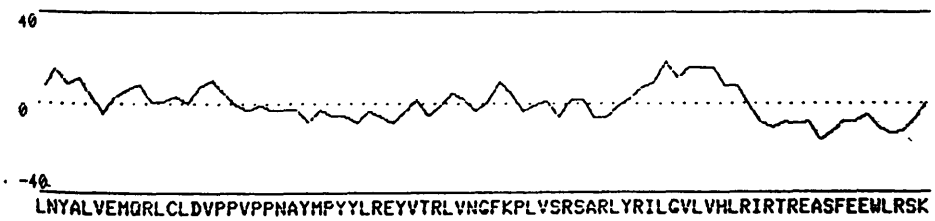
PPPPRFTLGGGCCSCRDTRRRSAVFGGEGDPVGPAEFVSDDRSSDSDSDDSEDTDSETLSHASSDVSGGATYDDA 225



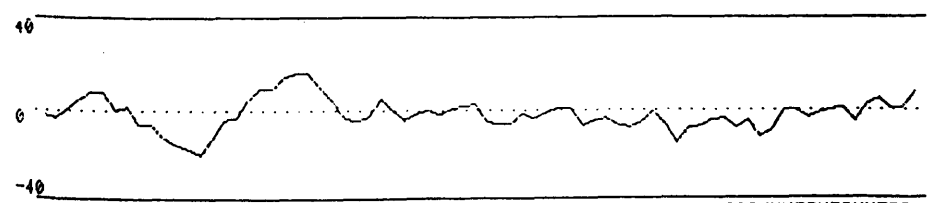
LDSDSSSDDSLGIDGPVCRPWSNDTAPLDVCPGTPGPGADAGGPSAVDPHAPTPEAGAGLAADPAVARDDAEGL 299



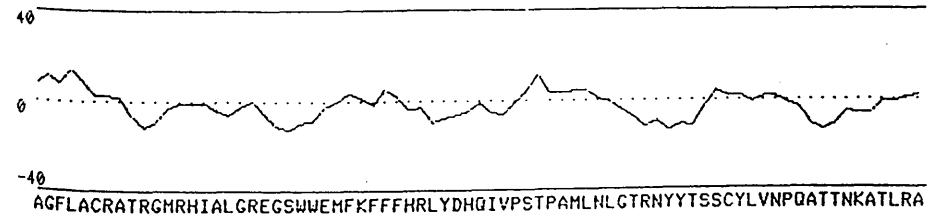
SDPRPRLGTGTAYPVPLELTPENAEAVARFLGDAVNREPALMLEYFCRCAREETKRVPPRTFGSPPRLTEDDFGL 374



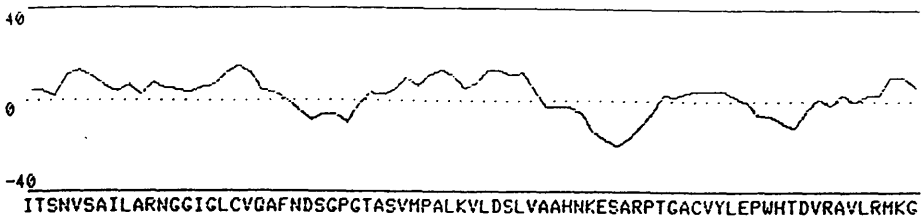
LNYALVEMGRLCLDVPPVPPNAYMPYYLREYVTRLVNGFKPLVSRSARLYRILGVLVHLRIRTREASFEEWLRSK 449



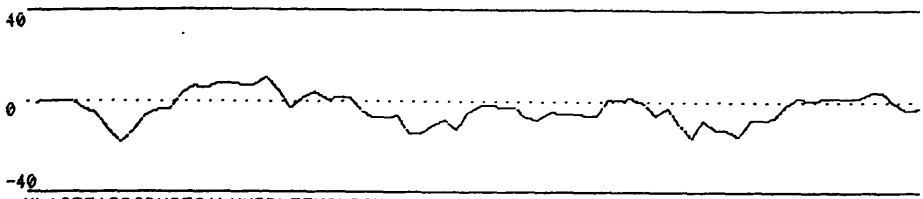
EVALDFGLTERLREHEAQLVILAQALDHYDCLIHSTPHTLVERGLQSALKYEEFYLKRFGGHYMESVFQMYTRI 523



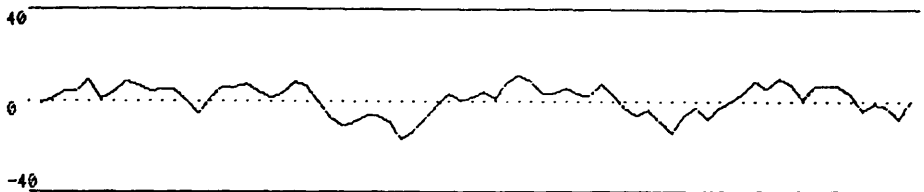
AGFLACRATRGMRHIALGREGSWWEMFKFFFHRLYDHQIVPSTPAMLNLGTRNYYTSSCYLVNPQATTNKATLRA 598



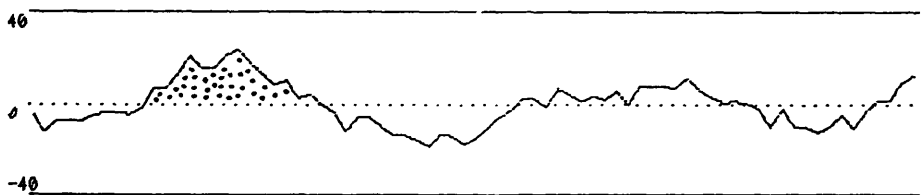
ITSNVSAILARNGGIGLCVGAFNDSGPGTASVMPALKVLDSLVAAHNKESARPTGACVYLEPWHTDVRAVLRMKG 673



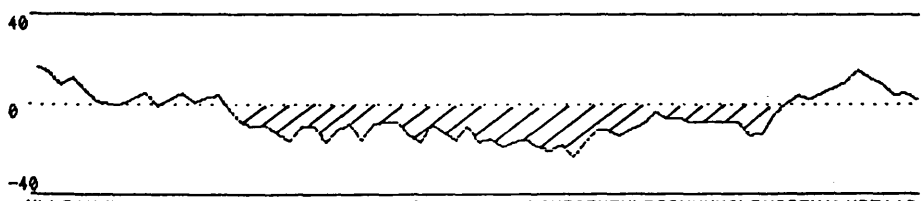
VLAGEEAQRCDNIFSALWMPDLFFKRLIRHLDGEKNVTWTLFDRDTSMSLADFHGEEFEKLYQHLEVMGFGEQIP 748



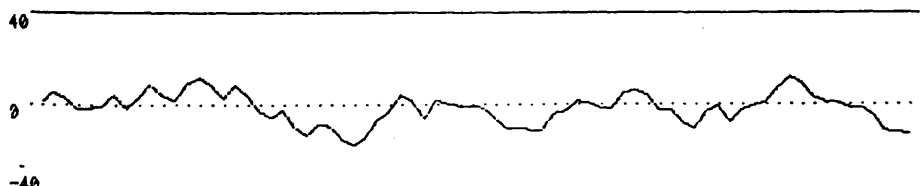
IQELAYGIVRSAATTGSPFVMFKDAVNRHYIYDTQGAAIAGSNLCTEIVHPASKRSSGVCNLGSVNLARCVSRG 822



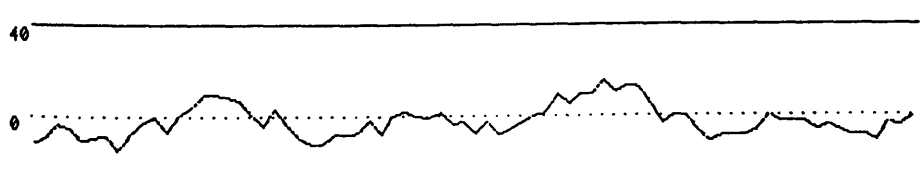
TFDFGRLRDAVQACVLMVNIMIDSTLQPTPQCTRGNDNLRSMGIGMQGLHTACLKLGLDLESAEFQDLNKHIAEV 897



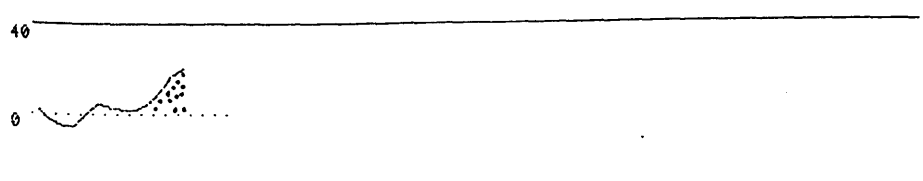
MLLSAMKTSNALCVRGARPFNHFKRSMYRAGRFHWERFPDARPRYEGEWEMLRQSMMKHGLRNSQFVALMPTAAS 927



AQISDVSEGFAPLFTNLFSKVTRDGETLRPNTLLLKELERTFSGKRLLEVMDSLDAKQWSVAQALPCLEPTHPL 1046



RRFKTAFDYDQKLLIDLCADRAPYVDHSQSMTLYVTEKADGTLPASTLVRLLVHAYKRGLKTGMYYCKVRKATNS 1121



GVFGGDDNIVCMSCAL 1137

C-terminal five residues of the polypeptide.

c) Repetitions within the RR1 N-terminus.

Examination of the RR1 amino acid sequence for possible repetitions was performed with the CINTHOM program (see Materials and Methods, Page 74; Fig. 28). As can be seen, a number of small letter homology diagonals were identified indicating that amino acid sequences positioned in different parts of the RR1 N-terminus share homology. These sequences were then aligned with the HOMOL program (see Materials and Methods, Page 75), and the results are shown in a graphical form in Fig. 29. Seven tandemly repeated stretches of amino acids were identified which mostly contained identical and/or conserved residues as determined by the Venn diagram of amino acid classification (Taylor, W.R., 1986a). Interestingly, the stretch designated 7 comprises two blocks of residues from which the first one is inverted in 7' and this is indicated by arrows in Fig. 29.

Examination of other HSV-1 polypeptide N-termini with the analysis described above demonstrated the existence of five tandemly repeated stretches of amino acids in the Vmw175 polypeptide and these are shown in Fig. 30.

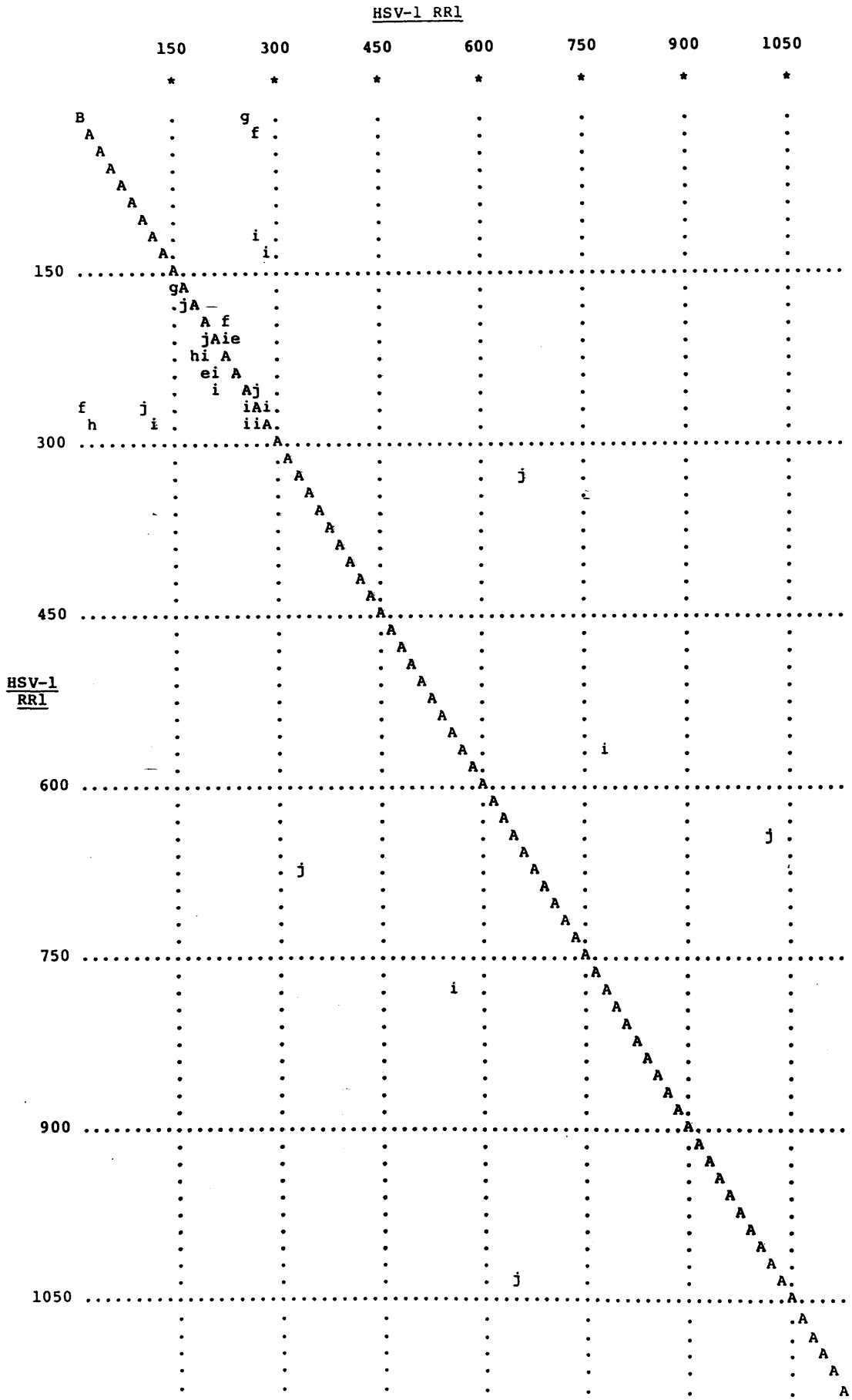

HSV-1 RRl
150
300
450
600
750
900
1050
HSV-1
RRl

Figure 28. Analysis of the HSV-1 RR1 polypeptide amino acid sequence for repetitions with the CINTHOM program. The small letter homology diagonals correspond to RR1 regions containing amino acid repetitions. Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x15.

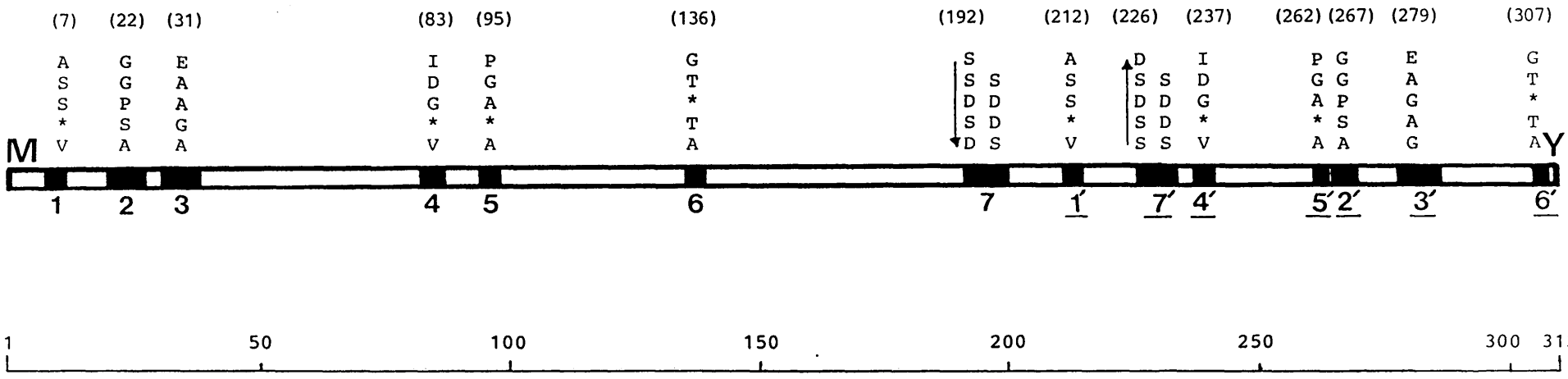

(7) (22) (31) (83) (95) (136) (192) (212) (226) (237) (262) (267) (279) (307)
A G E I P G S A D I P G E G
S G A D G T S S S D D G G A T
S P A G A * D D S D D G A P G *
* S G * * T S D S S D G * * S A T
V A A V A A D S V S S V A A G A Y
M
1 2 3 4 5 6 7 1′ 7′ 4′ 5′ 2′ 3′ 6′
1 50 100 150 200 250 300 312

Figure 29. Repeated amino acid sequences within the HSV-1 RR1 N-terminus. The diagram depicts the region in a linear form starting at the N-terminal methionine (M) and terminating at a tyrosine residue (Y) at amino acid position 312. Black boxes indicate the position of each repetition; the first set of boxes are numbered 1 to 7 and their corresponding boxes are numbered 1' to 7'. The amino acid sequence of each repetition is given above each box and the first amino acid is numbered as in Fig. 26. Asterisks within the sequences indicate any amino acid and the arrows indicate the stretch of residues in block 7 which is inverted in block 7'.

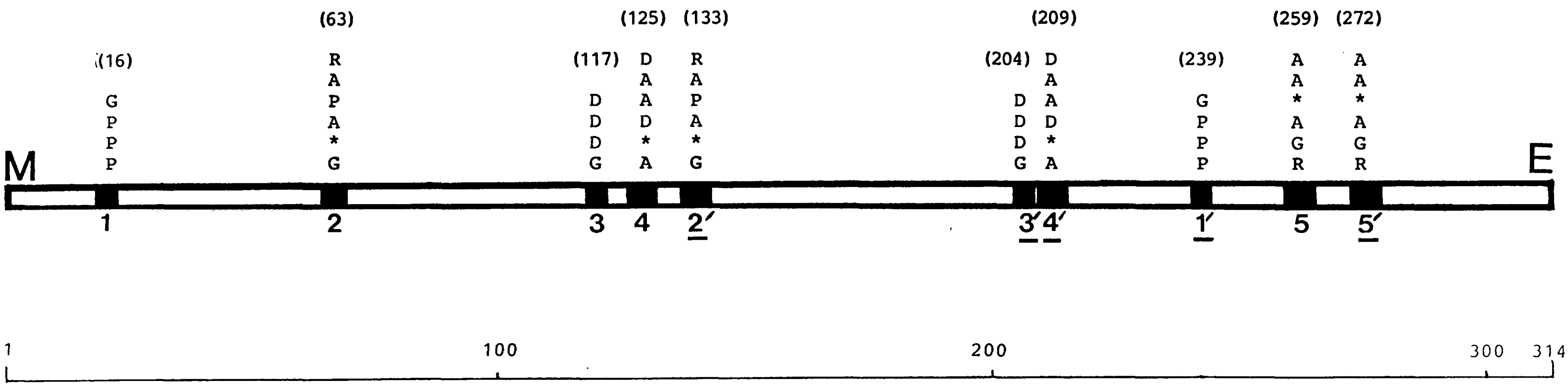

M
E
(16) GPPP 1
(63) RAPA*G 2
(117) DDDG 3
(125) DAAD*A 4
(133) RAPA*G 2′
(204) DDDG 3′
(209) DAAD*A 4′
(239) GPPP 1′
(259) AA*AGR 5
(272) AA*AGR 5′
1
100
200
300
314

Figure 30. Repeated amino acid sequences within the HSV-1 Vmw175 N-terminal region. The diagram depicts the region in a linear form starting at the N-terminal methionine (M) at amino acid position 1 and terminating at a glutamic acid (E) residue at amino acid position 314. Black boxes indicate the position of each repetition; the first set of boxes are numbered 1 to 5 and their corresponding boxes are numbered 1' to 5'. The amino acid sequence of each repetition is given above each box and the first amino acid is numbered as in McGeoch et al. (1986a).

SECTION B.

4. Amino Acid Sequence Conservation of HSV-1 RR1 with Other Viral, Eukaryotic and Prokaryotic Equivalent Polypeptides.

Comparisons of the N- and C-terminal portions of HSV-1 RR1 with herpesviral polypeptides and the polypeptides encoding the mouse M1 and E. coli B1 subunits demonstrated the existence of homology (McLauchlan, 1986; Swain and Galloway, 1986). In this part of Section B the complete sequence of RR1 is compared, using the CINTHOM and HOMOL programs, with these polypeptides and, in addition, with a VV polypeptide and a recently obtained putative ORF of HCMV. The purpose of this analysis was to determine whether RR1 shares any homology with the equivalent viral, eukaryotic and prokaryotic polypeptides and if so, to identify conserved features between these polypeptides.

a) Amino acid sequence conservation of HSV-1 RR1 with other viral polypeptides.

i) HSV-1 RR1 shares homology with HSV-2 RR1.

Sequencing studies in HSV-2 strain 333 identified a polypeptide, Vmw138 or RR1, which was homologous to the N- and C-terminal portions of HSV-1 RR1 (McLauchlan, 1986; Swain and Galloway, 1986). A CINTHOM comparison between these polypeptides revealed that they are essentially colinear (Fig. 31). The lettering in the diagonal indicates that the N-terminal 315 amino acids of both polypeptides are relatively poorly conserved whereas the remainder show extremely high homology. Optimal alignment of the polypeptides with the HOMOL program predicted that the highly conserved regions extend from the tyrosine residues at HSV-1 RR1 aa 312 and HSV-2 RR1 aa 319 to the respective C-termini (Fig. 32). The percentage similarity for these regions is 93% with 57 changes out of 827 aligned residues. However, most of these changes are conservative ones according to the Venn diagram of amino acid conservation (Taylor, W.R., 1986a) and are predominantly between amino

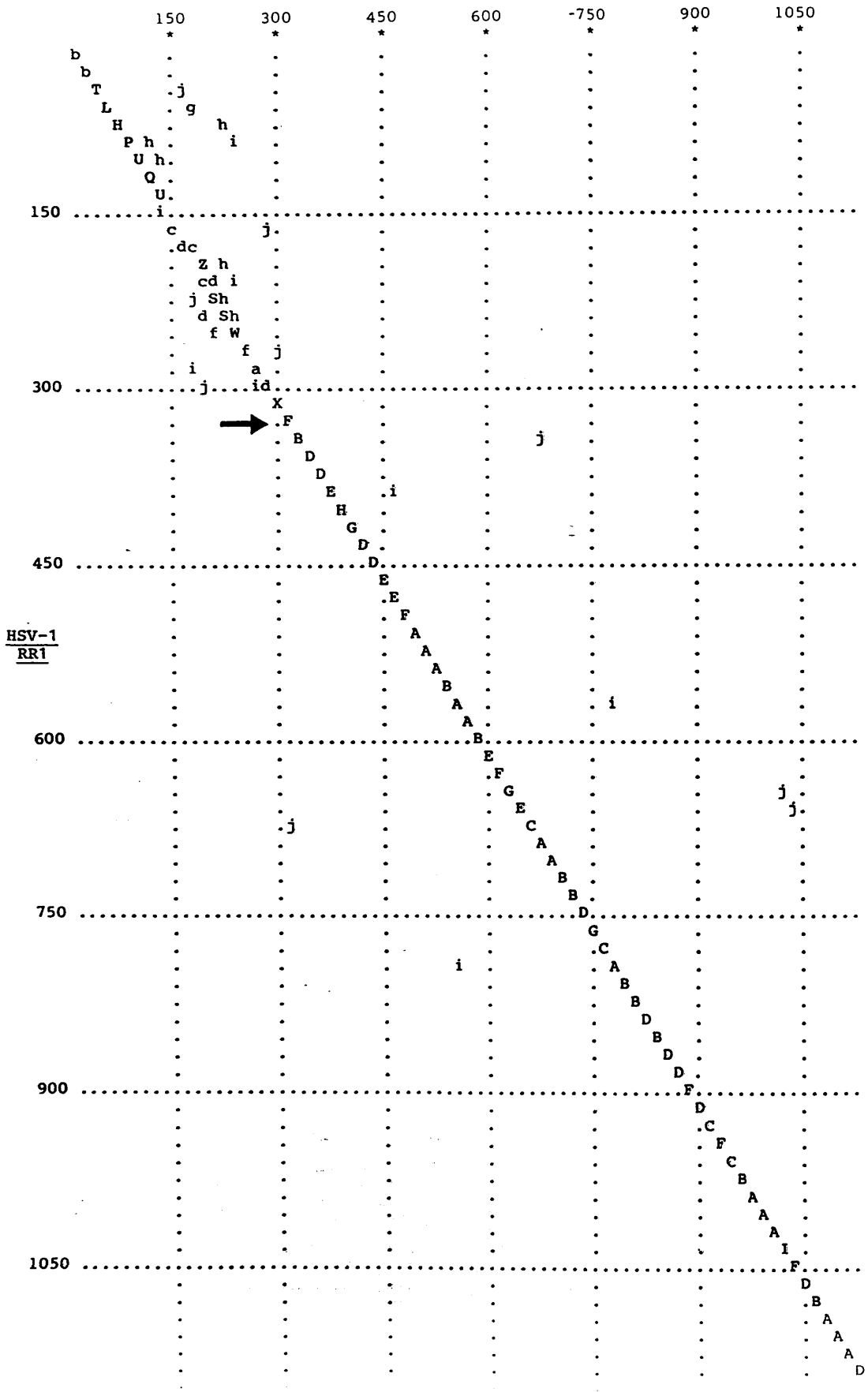

HSV-2 RR1
150
300
450
600
-750
900
1050
HSV-1
RR1

Figure 31. A CINTHOM plot comparing the amino acid sequence of HSV-1 RR1 with that of HSV-2 RR1. The position of the homology diagonal after which the two amino acid sequences share higher homology is indicated by an arrow. Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x15.

```
First  sequence:  HSV-1 RR1
Second sequence:  HSV-2 RR1

   1 MASRPAASSPVEARAPVGGQEAGGPSAATQGEAAGAPLAHGHHVYCQRVNGVMVLSDKTPGSASYRISDSNFVQ
     ** *****    ** *   **   *  *  *          * ** *  * ******   ** * ******* ***
   1 MANRPAASALAGARSPSERQEPREPEVAPPG          GDHVFCRKVSGVMVLSSDPPGPAAYRISDSSFVQ

  75 CGSNCTMIIDGDVVRGRPQDPGAAASPAPFVAVTNIGAGSDGGTAVVAFGGTPRRSAGTSTGTQTADVPTEALG
     ***** ******* **   *   * *   *** *  ** ** ***** ***   ** ** ****     * *
  66 CGSNCSMIIDGDVARGHLRDLEGATSTGAFVAISNVAAGGDGRTAVVALGGTSGPSATTSVGTQT   SGEFLH

 149 G               PPPPPRFTLGGGCCSCRDTRRRSAVFGGEGDPVGPAEFVSDDRSSDS   DSDDS EDT
     *               ***** *  *  **  ** *       **    ** **  **  ****   *** * ***
 137 GNPRTPEPQGPQAVPPPPPPPFPWGHECCARRDAR       GGAEKDVGAAESWSDGPSSDSETEDSDSSDEDT

 205   DSETLSHASSDVSGGATYDDALDSDSSSDDSLQIDGPVCRPWSNDTAP          LDVCPGTPGPGADAGG
        *****  **     *** **  **** **** * *  * * **   **            ** ** **  *
 205 GSGSETLSRSSSIWAAGATDDDDSDSDSRSDDSVQPDVVVRRRWSDGPAPVAFPKPRRPGDSPGNPGLGAGTGP

 269 PSAVDPHAPTPEAGAGLAADPAVARDDAEGLSDPRPRLGTGTAYPVPLELTPENAEAVARFLGDAVNREPALML
     ** ** *      *  **  *   * *  * * *  **  ************************ *******
 279 GSATDPRASADSDSAAHAA  APQADVAPVL DSQPTVGTDPGYPVPLELTPENAEAVARFLGDAVDREPALML

 343 EYFCRCAREETKRVPPRTFGSPPRLTEDDFGLLNYALVEMQRLCLDVPPVPPNAYMPYYLREYVTRLVNGFKPL
     ********** ********** ********** ** ***** ******** ** **** **********
 350 EYFCRCAREESKRVPPRTFGSAPRLTEDDFGLLNYALAEMRRLCLDLPPVPPNAYTPYHLREYATRLVNGFKPL

 417 VSRSARLYRILGVLVHLRIRTREASFEEWLRSKEVALDFGLTERLREHEAQLVILAQALDHYDCLIHSTPHTLV
     * ********** ********** ***** ********** ******  ********* ***
 424 VRRSARLYRILGILVHLRIRTREASFEEWMRSKEVDLDFGLTERLREHEAQLMILAQALNPYDCLIHSTPNTLV

 491 ERGLQSALKYEEFYLKRFGGHYMESVFQMYTRIAGFLACRATRGMRHIALGREGSWWEMFKFFFHRLYDHQIVP
     ********************************************************* ********************
 498 ERGLQSALKYEEFYLKRFGGHYMESVFQMYTRIAGFLACRATRGMRHIALGRQGSWWEMFKFFFHRLYDHQIVP

 565 STPAMLNLGTRNYYTSSCYLVNPQATTNKATLRAITSNVSAILARNGGIGLCVQAFNDSGPGTASVMPALKVLD
     ******************************* ******* ************** ***** ***** ********
 572 STPAMLNLGTRNYYTSSCYLVNPQATTNQATLRAITGNVSAILARNGGIGLCMQAFNDASPGTASIMPALKVLD

 639 SLVAAHNKESARPTGACVYLEPWHTDVRAVLRMKGVLAGEEAQRCDNIFSALWMPDLFFKRLIRHLDGEKNVTW
     ******** * ************ ************************************************
 646 SLVAAHNKQSTRPTGACVYLEPWHSDVRAVLRMKGVLAGEEAQRCDNIFSALWMPDLFFKRLIRHLDGEKNVTW

 713 TLFDRDTSMSLADFHGEEFEKLYQHLEVMGFGEQIPIQELAYGIVRSAATTGSPFVMFKDAVNRHYIYDTQGAA
     ********************** *** ***** **** *** ********** ** ******************
 720 SLFDRDTSMSLADFHGEEFEKLYEHLEAMGFGETIPIQDLAYAIVRSAATTGSPFIMFKDAVNRHYIYDTQGAA

 787 IAGSNLCTEIVHPASKRSSGVCNLGSVNLARCVSRQTFDFGRLRDAVQACVLMVNIMIDSTLQPTPQCTRGNDN
     ************** ********************** ***** ************************** ** **
 794 IAGSNLCTEIVHPSSKRSSGVCNLGSVNLARCVSRRTFDFGMLRDAVQACVLMVNIMIDSTLQPTPQCARGHDN

 861 LRSMGIGMQGLHTACLKLGLDLESAEFQDLNKHIAEVMLLSAMKTSNALCVRGARPFNHFKRSMYRAGRFHWER
     ********************* ********* *** ******** ********************* ***********
 868 LRSMGIGMQGLHTACLKMGLDLESAEFRDLNTHIAEVMLLAAMKTSNALCVRGARPFSHFKRSMYRAGRFHWER

 935 FPDARPRYEGEWEMLRQSMMKHGLRNSQFVALMPTAASAQISDVSEGFAPLFTNLFSKVTRDGETLRPNTLLLK
     *  * ************************** ************************************************
 942 FSNASPRYEGEWEMLRQSMMKHGLRNSQFIALMPTAASAQISDVSEGFAPLFTNLFSKVTRDGETLRPNTLLLK

1009 ELERTFSGKRLLEVMDSLDAKQWSVAQALPCLEPTHPLRRFKTAFDYDQKLLIDLCADRAPYVDHSQSMTLYVT
     ****** *****  ** * ************** * ************** ************************
1016 ELERTFGGKRLLDAMDGLEAKQWSVAQALPCLDPAHPLRRFKTAFDYDQELLIDLCADRAPYVDHSQSMTLYVT

1083 EKADGTLPASTLVRLLVHAYKRGLKTGMYYCKVRKATNSGVFGGDDNIVCMSCAL
     ********************************************* ******* ****
1090 EKADGTLPASTLVRLLVHAYKRGLKTGMYYCKVRKATNSGVFAGDDNIVCTSCAL
```

Figure 32. A HOMOL plot comparing the amino acid sequence of HSV-1 RR1 with that of HSV-2 RR1. Arrowheads denote the amino acid positions where the sequences become highly homologous and asterisks denote conserved residues. The program parameters were: mutation weight = 5, gap weight = 3k+7, k up to 2000.

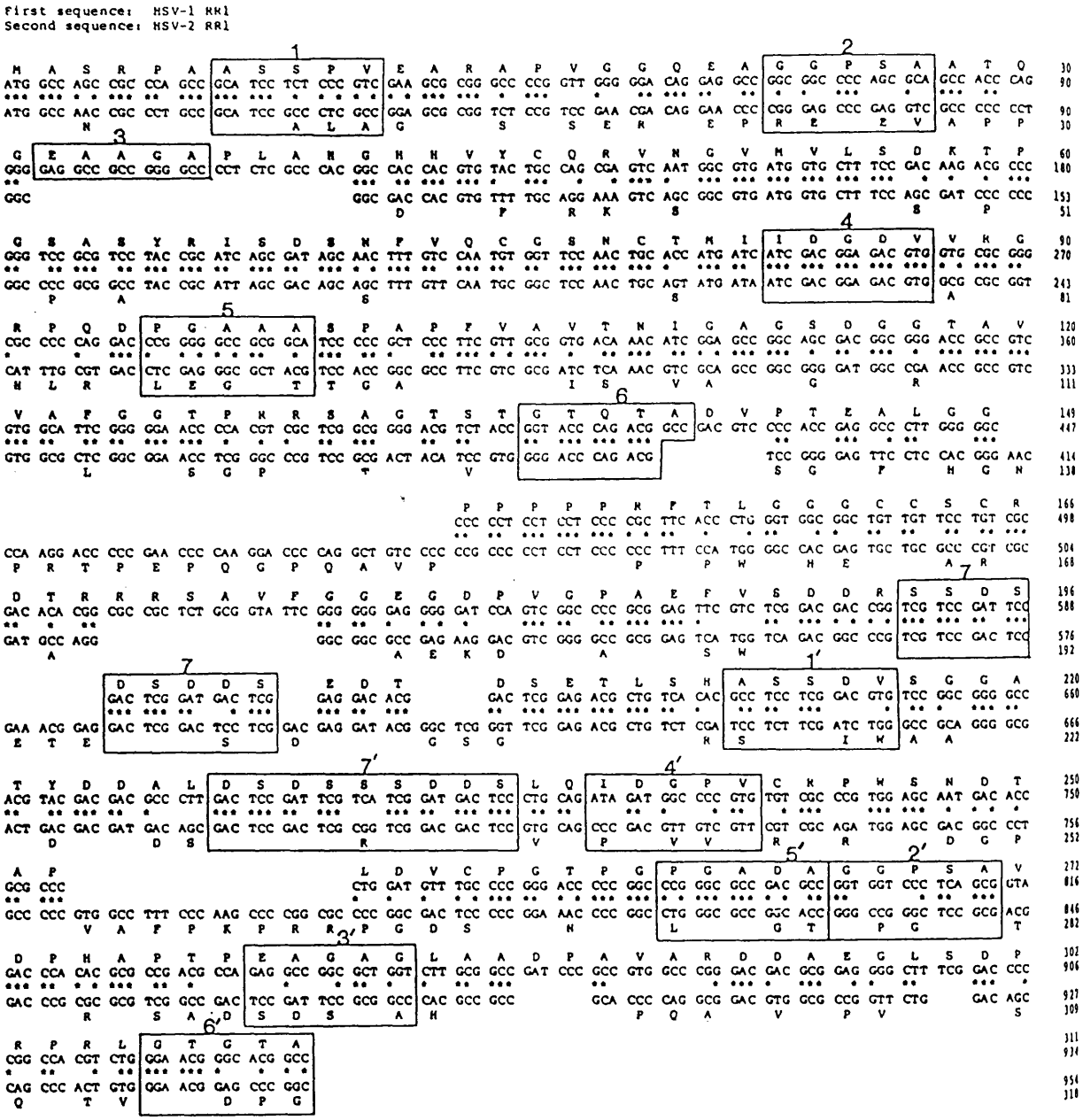


Figure 33. Alignment of the DNA sequences and predicted amino acid residues at the N-termini of HSV-1 RR1 and HSV-2 RR1. Conserved bases are indicated by asterisks. The predicted amino acid sequence of the HSV-1 RR1 polypeptide is shown above the HSV-1 DNA sequence and amino acid residues which are not conserved in HSV-2 RR1 are indicated below the HSV-2 DNA sequence. The HSV-1 RR1 repeated amino acid sequences, shown in Fig. 29, are boxed as are the corresponding HSV-2 RR1 amino acid sequences.

acids with small or tiny side-chains, such as glycine, alanine, serine and threonine (15 changes) or between hydrophobic residues such as methionine, isoleucine, leucine and valine (10 changes).

In contrast, the N-terminal HSV-1 312 and HSV-2 319 amino acids of both polypeptides, designated as the N-terminal regions, show considerably lower homology (38%), and contain a number of predicted insertions and deletions, the longest being 14 amino acids (Fig. 33, HSV-1 RR1 aa 149). Interestingly, the prolines and the stretches rich in aspartic acid and serine residues are well conserved. Further, the only HSV-1 RR1 repetitions which are well conserved in the HSV-2 sequence are , 4, 6, 7, 1' and 7'; the remainder share either limited homology or are not conserved at all (Fig. 33). The low conservation of the N-terminal regions can also be observed at the DNA level. Nucleotide comparison of shared amino acid sequences indicates that the strongest homology is 73% (Fig. 33, HSV-1 RR1 aa 41 to 139). This percentage homology is lower than that obtained from nucleotide sequence comparisons of the colinear parts of both polypeptides (90%, data not shown).

ii) HSV-1 RR1 shares homology with VZV Vmw87. The entire sequence of the VZV genome has been determined by Davison and Scott (1986b). Analysis of the sequence predicted that the genome contained 71 potential ORFs distributed almost equally between the two strands. One of these encodes a polypeptide with a predicted size of 87,000 mol. wt. (Vmw87) which is homologous to HSV-1 RR1 (Davison and Scott, 1986b; Nikas et al., 1986). Comparison of RR1 and Vmw87 with the CINTHOM program (Fig. 34) revealed that the N-terminal 400 amino acids of RR1 are not present in the VZV polypeptide, whereas, the remainder of both sequences are quite homologous and no major insertions or deletions occur. The colinear parts of the sequences display 43% similarity and are separated into three highly conserved regions (marked A, B and C in Fig. 34) by stretches of low homology. Optimal alignment of the colinear sequences with the HOMOL program (Fig. 35) indicated that the homology percentage for the A, B, and C regions is 54%, 61%, and 56% respectively.

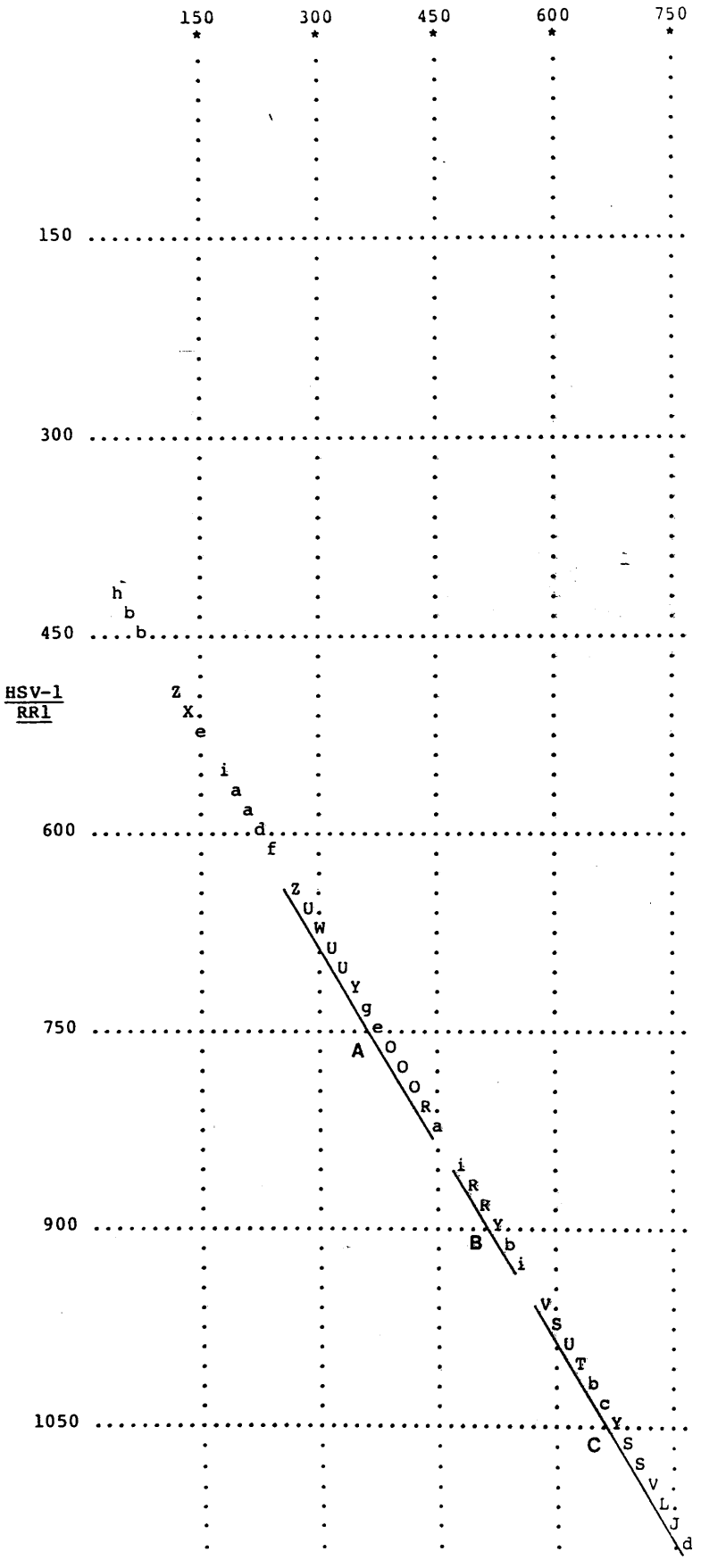

VZV Vmw87
150
300
450
600
750
150
300
450
600
750
900
1050
HSV-1
RR1
A
B
C

Figure 34. A CINTHOM plot comparing the amino acid sequence of HSV-1 RR1 with that of VZV Vmw87. Underlined parts of the homology diagonal denote the A, B and C polypeptide regions of higher homology. Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x15.

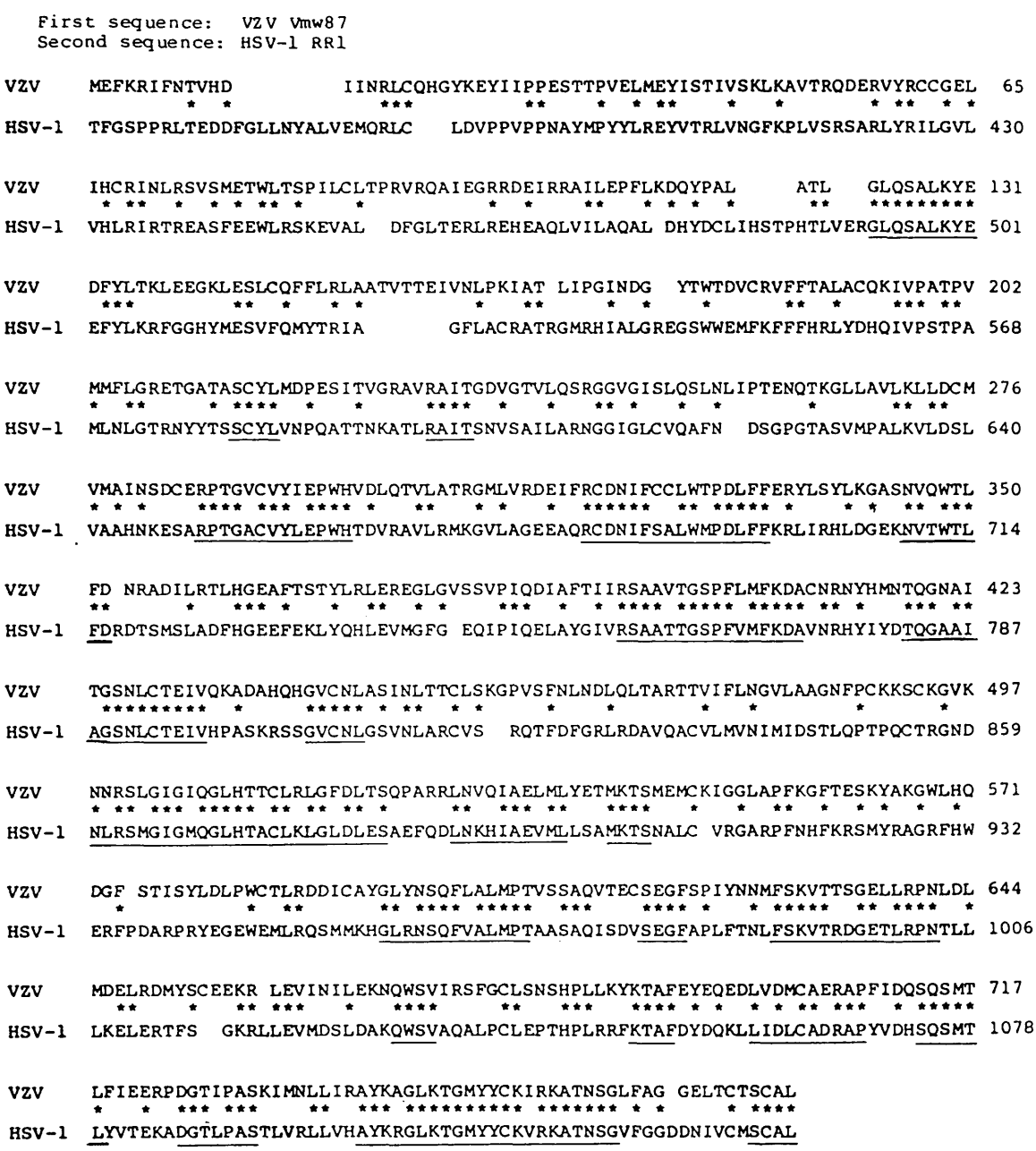


Figure 35. Optimal alignment of the amino acid sequences of HSV-1 RR1 with those of VZV Vmw87. Regions of clustered homology are underlined. Asterisks denote conserved residues. The program parameters were: mutation weight = 5, gap weight = 3k+7, k up to 2000.

Interestingly, most of the identical residues in the colinear parts are clustered rather than distributed evenly throughout the sequences. The longest of these clusters is located close to the C-termini (Fig. 35, HSV-1 RR1 aa 1101 to 1123) and displays 90% homology.

iii) HSV-1 RR1 shares homology with EBV Vmw93. The sequence of the entire EBV strain B 95-8 genome has been obtained (Baer et al., 1984), and mapping data exist for a number of genes. One of these encodes a polypeptide with a predicted size of 93,000 mol. wt. (Vmw93) which is homologous to HSV-1 RR1 (Gibson et al., 1984; Nikas et al., 1986). Comparison of RR1 and Vmw93 with the CINTHOM program indicated that the RR1 N-terminal 400 residues are not present in the EBV polypeptide (Fig. 36). Beyond this region, homology extends to the C-terminus of the HSV-1 polypeptide and the remaining 80 C-terminal residues of Vmw93 are absent in RR1. The major interruption in the homology diagonal is due to an insertion of 35 amino acids in the EBV sequence (Fig. 37, EBV aa 420 to 454). It is of interest to note that the insertion corresponds by position to the non-homologous stretch of amino acids separating the highly conserved A and B regions in the HSV-1 RR1/VZV Vmw87 comparison (see Fig. 34). As indicated in Fig. 37, identical residues are clustered and the overall homology of the colinear parts is 33%. This percentage homology, which is lower than that obtained in the HSV-1 RR1/VZV Vmw87 comparison, is an interesting result considering that the G+C DNA content of HSV (66%) is closer to that of EBV (54%) than to that of VZV (40%) in these coding regions.

iv) HSV-1 RR1 shares homology with VV Vmw86. Recently, Tengelsen et al., (1988) identified and sequenced a VV strain WR gene (I-3) encoding a polypeptide of 86,000 mol. wt. (Vmw86). As the I-3 deduced amino acid sequence was found to be highly homologous to the mouse M1 polypeptide, these authors suggested that I-3 encodes the VV-induced large ribonucleotide reductase subunit polypeptide. CINTHOM comparison of HSV-1 RR1 and VV Vmw86 displayed significant homology extending approximately

EBV Vmw93

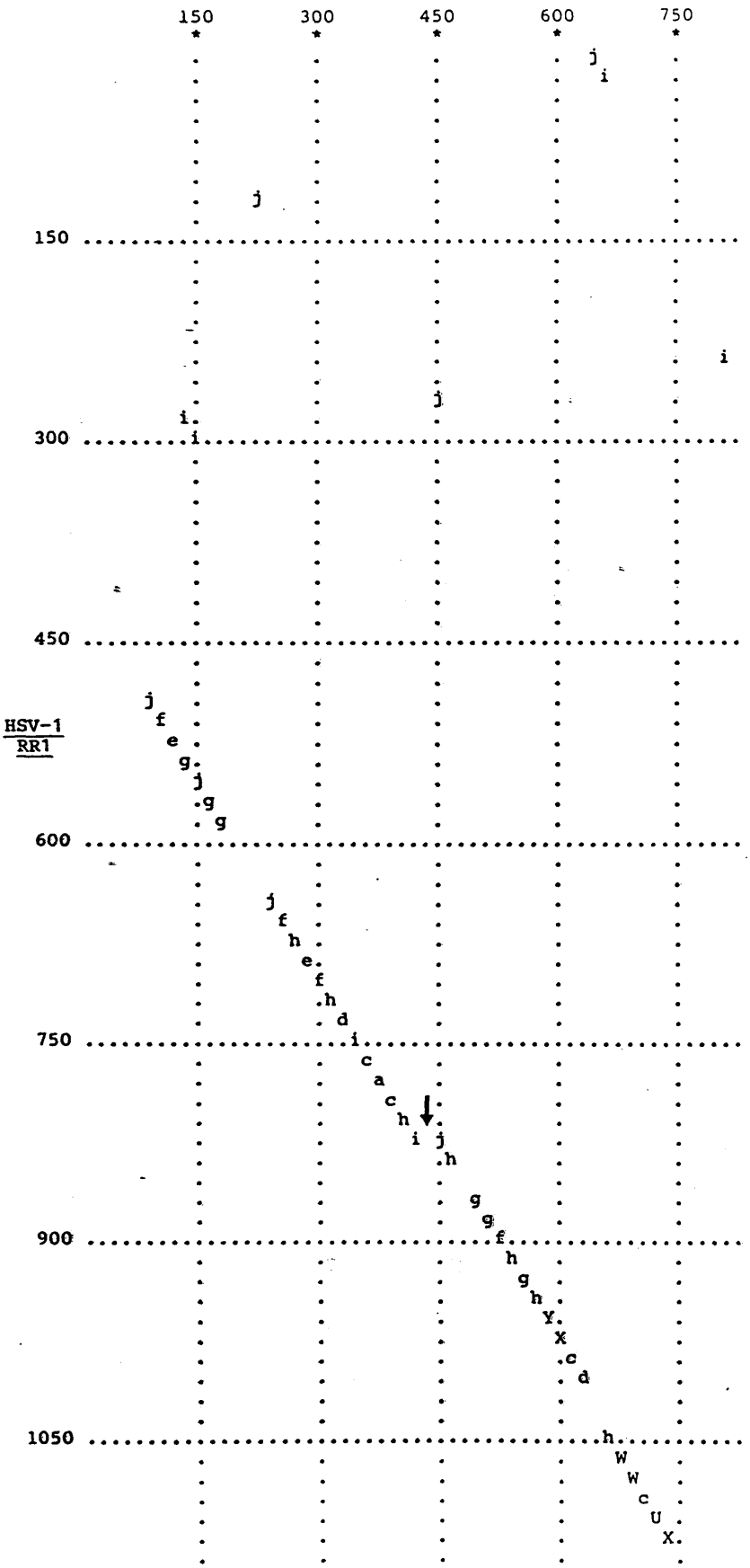


Figure 36. A CINTHOM plot comparing the amino acid sequence of HSV-1 RR1 with that of EBV Vmw93. A major insertion in the EBV sequence is indicated by an arrow. Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x15.

```
First sequence:   EBV Vmw93
Second sequence:  HSV-1 RR1

  90            VSSGYLSALRYYDTYLYVGRSGKQESVQHFYMRLAGFCASTTCLYAGLRAALQRARPEIESDME
                *  *  *** *   **        ***   * * *** *        *     *    * *
 480  CLIHSTPHTLVERGLQSALKYEEFYLKRFGGHYMESVFQMYTRIAGFLA      CRATRGMRHIALGREGSWWE

 154  VFDYYFEHLTSQTVCCSTPFMRFAGVENSTLASCILTTPDLSSEWDVTQALYRHLGRYLFQRAGVGVGVTGAGQ
       *   *  *       *** *   *  *    ** *  *          *        *    * *  *
 549  MFKFFFHRLYDHQIVPSTPAMLNLGTRNYYTSSCYLVNPQATTNKATLRAITSNVSAILARNGGIGLCVQAFND

 228  DGKHISLLM   RMINSHVEYHNYGCKRPVSVAAYMEPWHSQIFKFLETK   LPENHERCPGIFTGLFVPELF
       *      *       * *  **    **     * ****      * *    *   **  **  *  * **
 623  SGPGTASVMPALKVLDSLVAAHNKESARPTGACVYLEPWHTDVRAVLRMKGVLAGEEAQRCDNIFSALWMPDLF

 296  FK LFR   DTPWSDWYLFDPKDAGDLERLYGEEFEREYYRLVTAGKFCGRVSIKSLMFSIVNCAVKAGSPFIL
      ** * *       * ***      *    *****  *  *   * *     *  *   **  *   ****
 697  FKRLIRHLDGEKNVTWTLFDRDTSMSLADFHGEEFEKLYQHLEVMG FGEQIPIQELAYGIVRSAATTGSPFVM

 366  LKEACNAHFWRDLQGEAMNAANLCAE VLQPSRKSVATCNLANICLPRCLVNAPLAVRAQRADTQGDELLLALP
       * * * *  * ** *   *** * *  *  *   ***    * **
 770  FKDAVNRHYIYDTQGAAIAGSNLCTEIVHPASKRSSGVCNLGSVNLARCVRSQ <---------- 35aa

 439  RLSVTLPGEGAVGDGFSLARLRDATQCATFVV   ACSILQGSP  TYDSRDMASMGLGVQGLADVFADLGWQY
                    *  ***** *     *     * **  *  *       *** * ***      **
 823  ----------> TFDFGRLRDAVQACVLMVNIMIDSTLQPTPQCTRGNDNLRSMGIGMQGLHTACLKLGLDL

 508  TDPPSRSLNKEIFEHMYFTALCTSSLIGLHTRKIFPGFKQSKYAGGWFHWHDWAGTDLSIPREIWSRLSERIVR
           *** * * *   * **          *  ** * *  * ***           * *  *
 883  ESAEFQDLNKHIAEVMLLSAMKTSNALCVRGARPFNHFKRSMYRAGRFHWERFPDARPRYEGE WEMLRQSMMK

 582  DGLFNSQFIALMPTSGCAQVTGCSDAFYPFYANASTKVTNKEEALRPN        RSFWRHVRLDDREALNLV
       ** **** *****   **    *  * *  *   ***   * ****        * *     *    *
 956  HGLRNSQFVALMPTAASAQISDVSEGFAPLFTNLFSKVTRDGETLRPNTLLLKELERTFSGKRLLEVMDSLDAK

 648  GGRV SCLPEALRQRYL RFQTAFHYNQEDLIQMSRDRAPFVDQSQSHSLFLRE  EDAARASTLANLLVRSYE
        *   **       * ** *** * *  **    **** ** ***  *   *       **** *** *
1030  QWSVAQALPCLEPTHPLRRFKTAFDYDQKLLIDLCADRAPYVDHSQSMTLYVTEKADGTLPASTLVRLLVHAYK

 718  LGLKTIMYYCRIEKAADLGV    MEC
       **** ****   **   **
1104  RGLKTGMYYCKVRKATNSGVFGGDDNI
```

Figure 37. Optimal alignment of the amino acid sequences of HSV-1 RR1 with those of EBV Vmw93. Regions of clustered homology are underlined, and the major EBV insertion identified in Fig. 36 is indicated. Asterisks denote conserved residues. The program parameters were: mutation weight = 5, gap weight = 3k+7, k up to 2000.

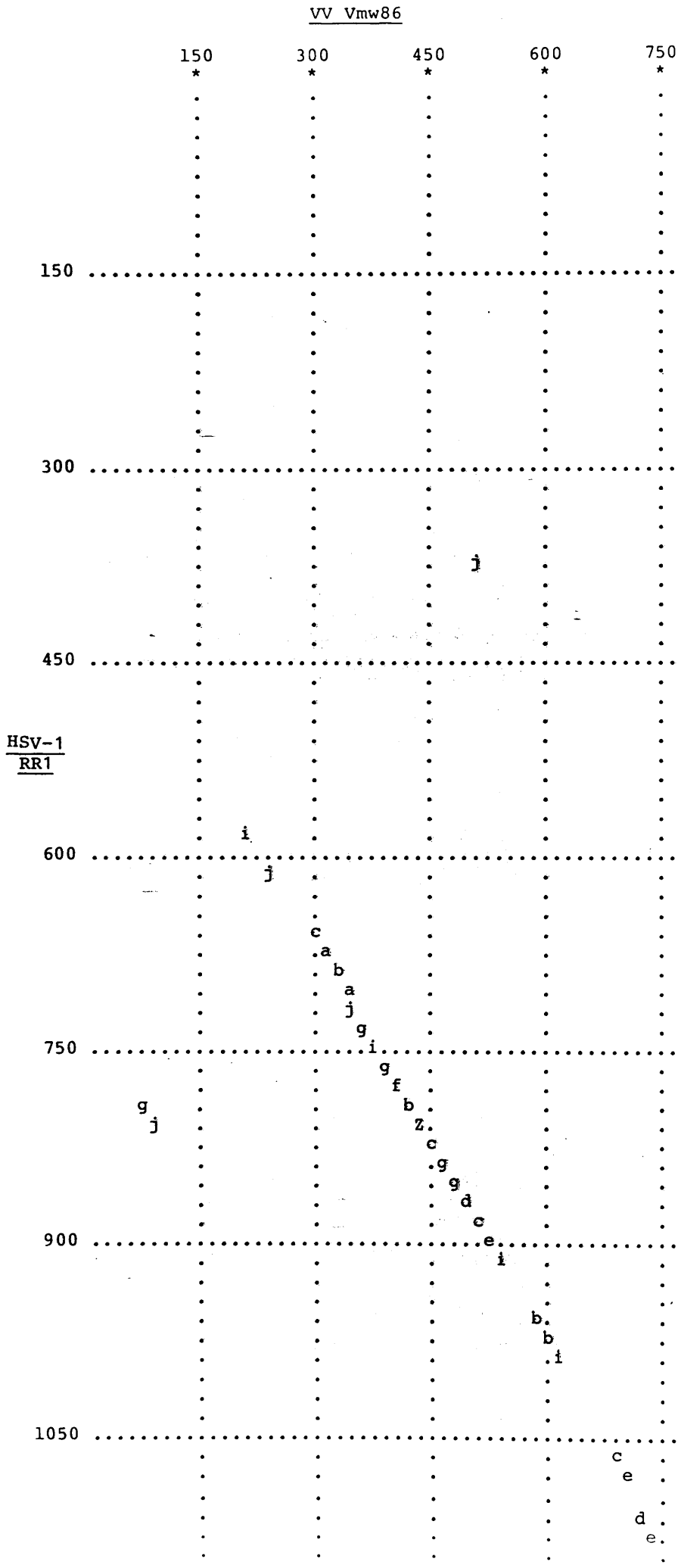

VV Vmw86
150
300
450
600
750
150
300
450
HSV-1
RR1
600
750
900
1050

Figure 38. A CINTHOM plot comparing the amino acid sequence of HSV-1 RR1 with that of VV Vmw86. Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x15.

```
First sequence:    VV Vmw86
Second sequence:   HSV-1 RR1

 150  TLEKSYLLKINNKIVERPQHMLMRVAVGIHQWDIDSAIETYNLLSEKWFTHASPTLFNAGTSRHQMSSCFLLN
                *       *       *                       *          *   * **      *** * *
 540           LGREGSWWEMFKFFFHR                        LYDHQIVPSTPAMLNLGTRNYYTSSCYLVN

 223   MIDDSIEGIYDTLKRCALISKMAGGIGLSISNIRASGSYISGTNGISNGIIPMLRVYNNTARYIDQGGNKRP
                    *    * *    *****          **     **          * * *                **
 588  QATTNKATLRAITSNVSA ILARNGGIGLCVQAFNDSG    PGT     ASVMPALKV LDSLVAAHNKESARP

 296  VMAIYLEPWHSDIMAFLDLK KNTGNEEHRTRDLFIALWIPDLFMKR   VKDDGE     WSLMCPDECPGLDN
          ****** *  * *  *     * *  *     * *** **** **      ***     * *    *     *
 653  GACVYLEPWHTDVRAVLRMKGVLAGEEAQRCDNIFSALWMPDLFFKRLIRHLDGEKNVTWTLFDRDTSMSLAD

 363  WGDEFERLYTLYERERRYKSIIKARVVWKAIIESQIETGTPFILYKDACNKKSNQQNLG TIKCSNLCTEIIQ
       * *** **   *         *         * *   ** **   *** *          *  *  *******
 727  HGEEFEKLYQHLE VMGFGEQIPIQELAYGIVRSAATTGSPFVMFKDAVNRHYIYDTQGAAIAGSNLCTEIVH

 436  ADANEVAVCNLASVALNMFVIDGRFDFLKLKVVVKVIVRNLNKIIDINYYPIPEAEISNKRHRPIGIGVQGLA
      *      **** ** *   *     *** *   *   *   *   **     * *      *   *  *** ***
 800  ASKRSSGVCNLGSVNLARCVSRQTFDFGRLRDAVQACVLMVNIMIDSTLQPTPQCTRGNDNLRSMGIGMQGLR

 510  AFILLNYPFDSLEAQDINKKIFETIYYGALEASCEL AEKEGPYDTYVGS YASNGILQYDLWNVVPSDLWNK
      *   *     * * ** ** * *     *   *  *       *      * *                 *    *
 874  ACLKLGLDLESAEFQDLNKHIAEVMLLSAMKTSNALCVRGARPFNHFKRSMYRAGRFHWERFPDARPRYEGEW

 582  PLKDKIRTYGLRNSLLVAPLPLHQHAQILGNNESVEPYTSNIYTRRVLSGEFQVVNPHLLRVL  TERKLWND
       *       *****  **  *    ***    *   *   *         **    *  **  *  *
 948  MLRQSMMKHGLRNSQFVALMPTAASAQISDVSEGFAPLFTNLFSKVTRDGETLRPNTLLLKELERTFSGKRLL

 654  IKNRIMADGGS   IQNTNLPEDIKRVYKTIWEIPQKTIIKMAADRGAFIDQSQSM   NIHIAD  PSYSKL
          *   *          *    *  **     **  *   ***    * ****       **     * *
1022  VMDSLDAKQWSVAQALPCLEPTHPLRRFKTAFDYDQKLLIDLCADRAPYVDHSQSMTLYVTEKADGTLPASTL

 771  SMHFYGWSLGLKTGMYYLRTKPASAPIQFTLDKDKIKPPVVCDSEICTSCSG
           ********     *    *  *          *   * **
1096  RLLVHAYKRGLKTGMYYCKVRKATNSGVFGGD          DNIVCMSCAL
```

Figure 39. Optimal alignment of the amino acid sequences of HSV-1 RR1 with those of VV Vmw86. Regions of clustered homology are underlined. Asterisks denote conserved residues. The program parameters were: mutation weight = 5, gap weight = 3k+7, k up to 2000.

between RR1 aa 645 and Vmw86 aa 300 to the respective C-termini (Fig. 38). Comparison of the colinear parts of these polypeptides with the HOMOL program demonstrated that they share 29% homology which, as the case is with the other viral polypeptide comparisons, is primarily retained in stretches of identical amino acids (Fig. 39). The overal percentage homology between these polypeptides is 21% (data not shown).

v) HSV-1 RR1 shares limited homology with a putative HCMV ORF. Sequencing studies by Barrell and co-workers in HCMV strain AD169 revealed the existence of a putative ORF, termed M4.PRO, which exhibits homology to RR1 (B.G. Barrell, personal communication). Amino acid comparison of RR1 and M4.PRO with the CINTHOM program displayed limited but detectable homology extending approximately between RR1 aa 855 to 975 and M4.PRO aa 650 to 750 (Fig. 40). Comparison of the colinear parts of RR1 and M4.PRO with the HOMOL program demonstrated that they share 22% homology and that two regions of more than 60% clustered homology exist (underlined regions in Fig. 41). The overall percentage homology is 18% (data not shown).

b) Amino acid sequence comparison of HSV-1 RR1 and mouse M1 polypeptides.

Comparison of HSV-1 RR1 with the mouse M1 polypeptide (Caras et al., 1985) is represented as a CINTHOM plot in Fig. 42. As is the case with the viral polypeptide comparisons, the RR1 N-terminal region is not present in the M1 polypeptide whereas the remainder of the sequences are colinear; the homology diagonal extends approximately between RR1 aa 720 and M1 aa 330 to the respective C-termini and contains a major shift at aa 335 of the mouse sequence (indicated by arrow in Fig. 42). Comparison of the polypeptides with the HOMOL program demonstrated that the shift is due to a deletion of 13 amino acids in the mouse sequence (Fig. 43). The plot also demonstrated a major insertion of 18 amino acids at the C-terminus of the mouse polypeptide. It is of interest to note, that 15 out of 18 residues comprising this insertion are charged ones (lysine,

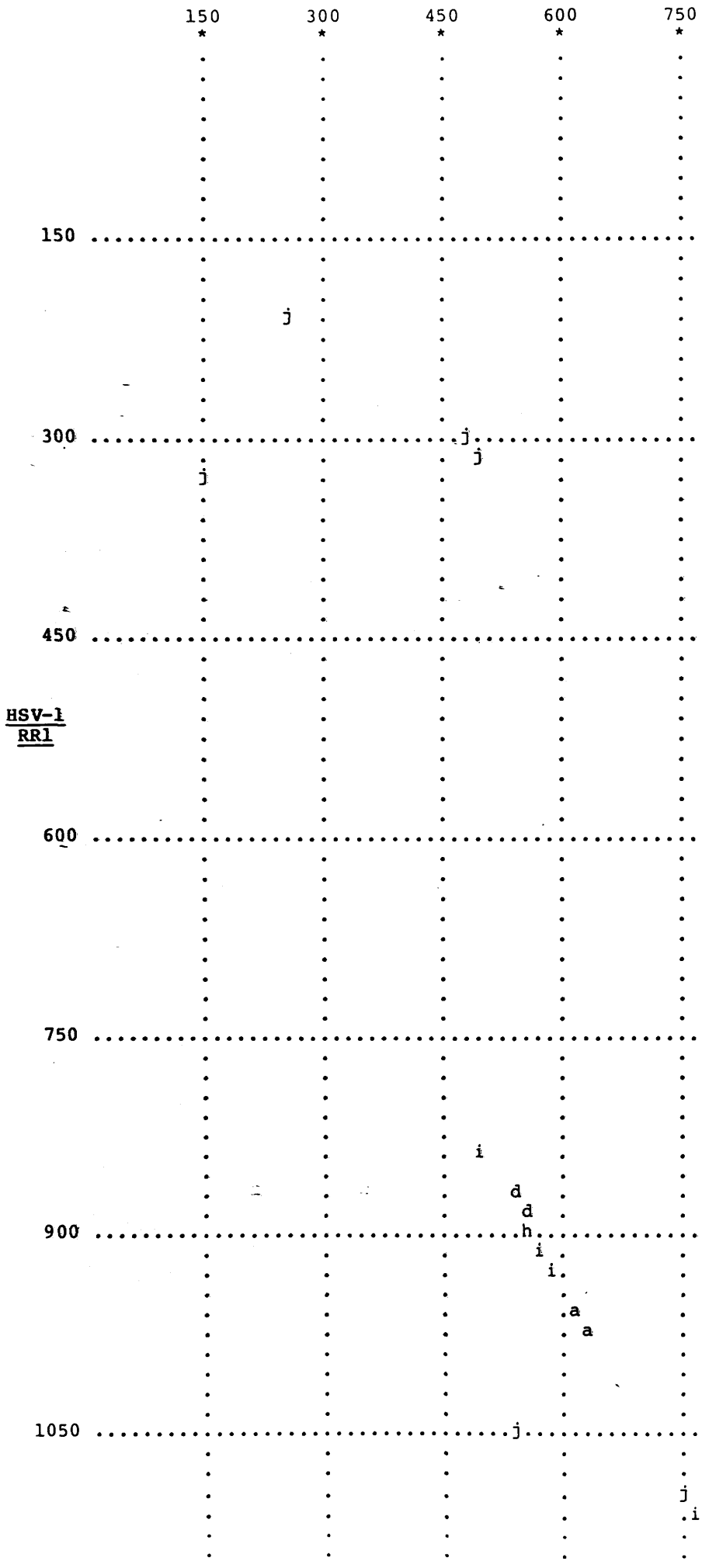

HCMV M4.PRO
150
300
450
600
750
150
300
450
HSV-1
RR1
600
750
900
1050

Figure 40. A CINTHOM plot comparing the amino acid sequence of HSV-1 RR1 with that of a putative HCMV ORF termed M4.PRO (B. Barrell, personal communication). Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x15.

```
First  sequence:  HCMV M4.PRO
Second sequence:  HSV-1 RR1

  600 CDMPVQRLTVNVARCVFARSDEQKLHLPDVVLGNTRRYFDLSVLRELVTEAVVWGNARLDALMSASEWWVESAL
          **   *  **          *                          *    **
  825    DFGRLRDAVQACVLMVNIMIDSTL                       QPTPQCTRGN

  674 EKLRPLHIGVAGLHTALMRLG  FTYFASWDLIERIFEHMYFAAVRASVDLCKSGLPRCEWFERTIYQEGKFIF
        **   **  *****   **          **   * * *   *   * **  *       * *  *  * *
  859 DNLRSMGIGMQGLHTACLKLGLDLESAEFQDLNKHIAEVMLLSAMKTSNALC VRGARPFNHFKRSMYRAGRFHW

  746 ELYRLPRLSIASARWEALRADMLEFGLRNCQFLAVGPDDEVAHLWGVTPSVWASRGTVF      EEETVWSLC P
    *     *        ** **  *   **** ** *  *    *    *           *       **
  933 ERFPDAR PRYEGEWEMLRQSMMKHGLRNSQFVALMPTAASAQISDVSEGFAPLFTNLFSKVTRDGETLRPNTL

  815 PNRECYFPTVVRRPLRVPVVNYA   WLEQHQEEGKATQCLFQAAPAIQND      VEMAAVNLSVFVDQCVAL
        *        * * *    *       *       * *   *  *         *     *  *  *
 1006 LLKELERTFSGKRLLEVMDSLDAKQWSVAQALPCLEPTHPLRRFKTAFDYDQKLLIDLCADRAPYVDHSQSMTL

  881  VFYYDSGMTPDVLLARMLKWPYHWRFKVG                    VYKYCAS
       *     * *  * * *   *    * *                      *   **
 1080 YVTEKADGTLPASTLVRLLVHAYKRGLKTGMYYCKVRKATNSGVFGGDDNIVCMSCAL
```

Figure 41. Optimal alignment of the amino acid sequences of HSV-1 RR1 with those of a putative HCMV ORF termed M4.PRO (B. Barrell, personal communication). Polypeptide regions of higher homology are underlined. Asterisks denote conserved residues. The program parameters were: mutation weight = 5, gap weight = 3k+7, k up to 2000.

MOUSE M1

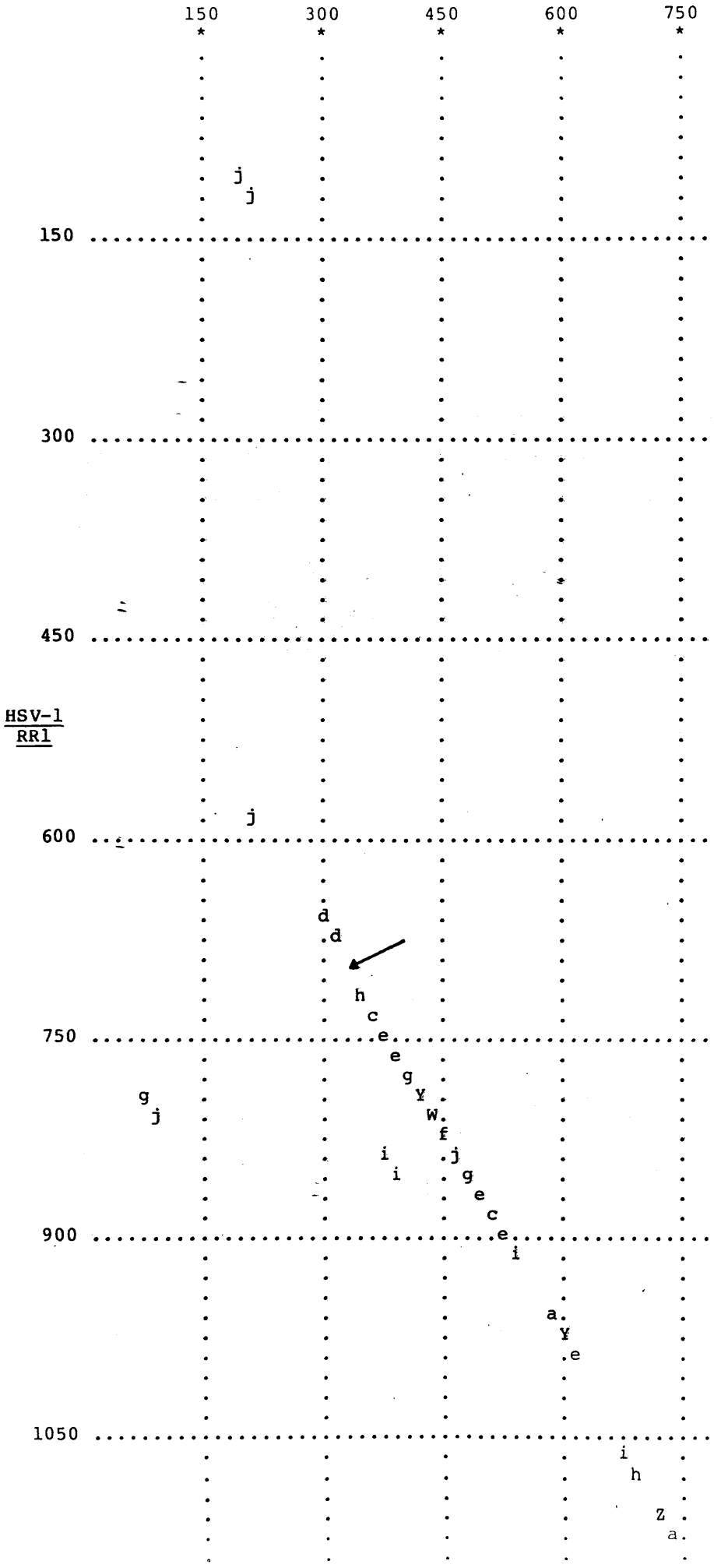


Figure 42. A CINTHOM plot comparing the amino acid sequences of HSV-1 RR1 and mouse M1. The location of a major deletion in the mouse sequence is indicated by an arrow. Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x15.

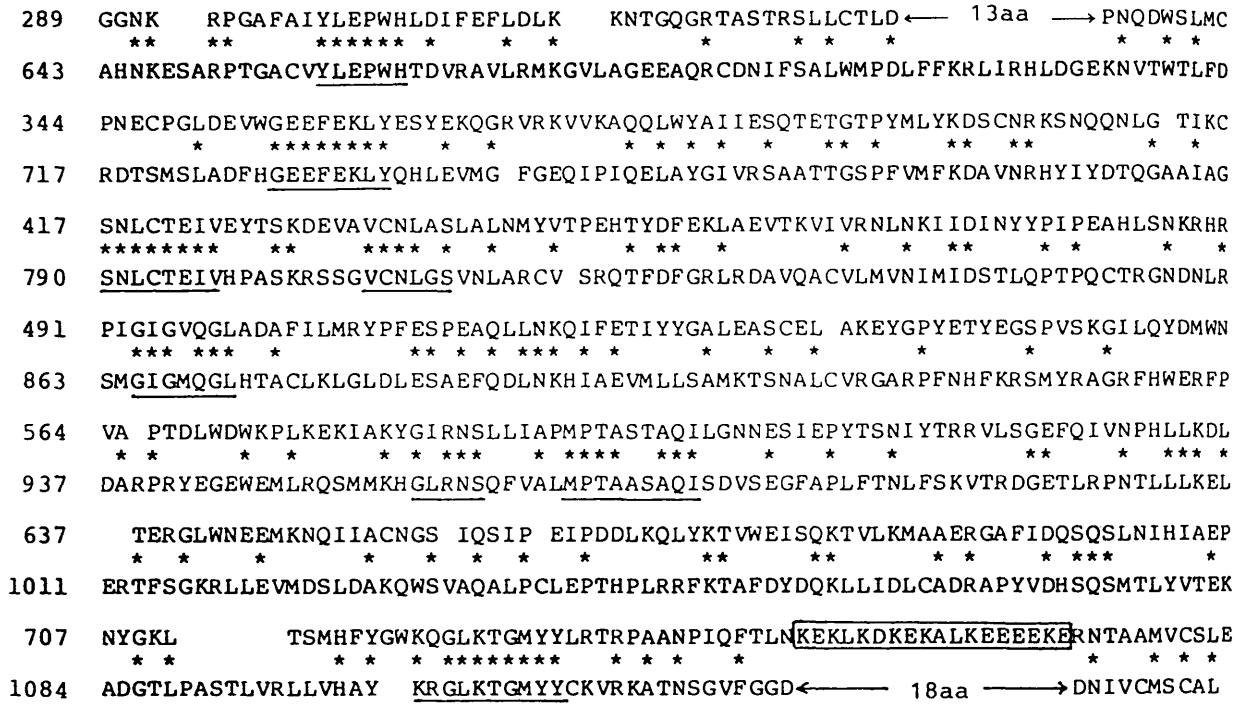


Figure 43. Optimal alignment of the amino acid sequences of HSV-1 RR1 with those of mouse M1. Regions of clustered homology are underlined. The major insertion in the M1 sequence identified in Fig. 42 is indicated and the region of high percentage of occurence of charged residues in M1 is boxed. Asterisks denote conserved residues. The program parameters were: mutation weight = 5, gap weight = 3k+7, k up to 2000.

aspartic acid, glutamic acid), although, the immediate flanking sequences do not contain such an extreme amino acid composition. The HOMOL plot also indicated the existence of clustered homology; the overall percentage homology of the colinear sequences is 31%.

c) Amino acid sequence comparison of HSV-1 RR1 and E. coli B1 polypeptides.

Carlson et al., (1984) reported the amino acid sequence of the E. coli B1 polypeptide, which subsequently was amended (J. Fuchs, personal communication; Nilsson et al., 1988a). Comparisons of RR1 with the amended B1 sequence are represented as CINTHOM and HOMOL plots in Figs. 44 and 45, respectively. The CINTHOM plot displayed no homology at the N-terminus of RR1 for 720 amino acids and for 360 amino acids at the N-terminus of B1 (Fig. 44). After these non-homologous regions, both sequences exhibit low but detectable amino acid conservation. The HOMOL alignment predicted a deletion of 20 residues close to the C-terminus of the E. coli polypeptide (Fig. 45, at E. coli B1 aa 700), and demonstrated that the colinear parts share 21% homology.

5. Amino Acid Sequence Conservation of HSV-1 RR2 with Other Viral Polypeptides.

The amino acid sequences of the polypeptides encoding the small ribonucleotide reductase subunit have been determined for HSV-2 strain HG52 (RR2; McLauchlan and Clements, 1983), E. coli (B2; Carlson et al., 1984), surf clam Spisula solidisima (M2; Standart et al., 1985) and mouse (M2; Thelander and Berg, 1986), and comparisons of these polypeptides have been performed by these authors. The sequence of the HSV-1 RR2 polypeptide has also been reported for HSV-1 strain KOS (Draper et al., 1982) and was subsequently amended (K. Draper, personal communication), and for HSV-1 strain 17^+ (McLauchlan, 1983a; McGeoch et al., 1988). Further, the potential small ribonucleotide reductase subunit sequences have been reported for EBV (Vmw34; Baer et al., 1984), VZV (Vmw35; Davison and Scott,

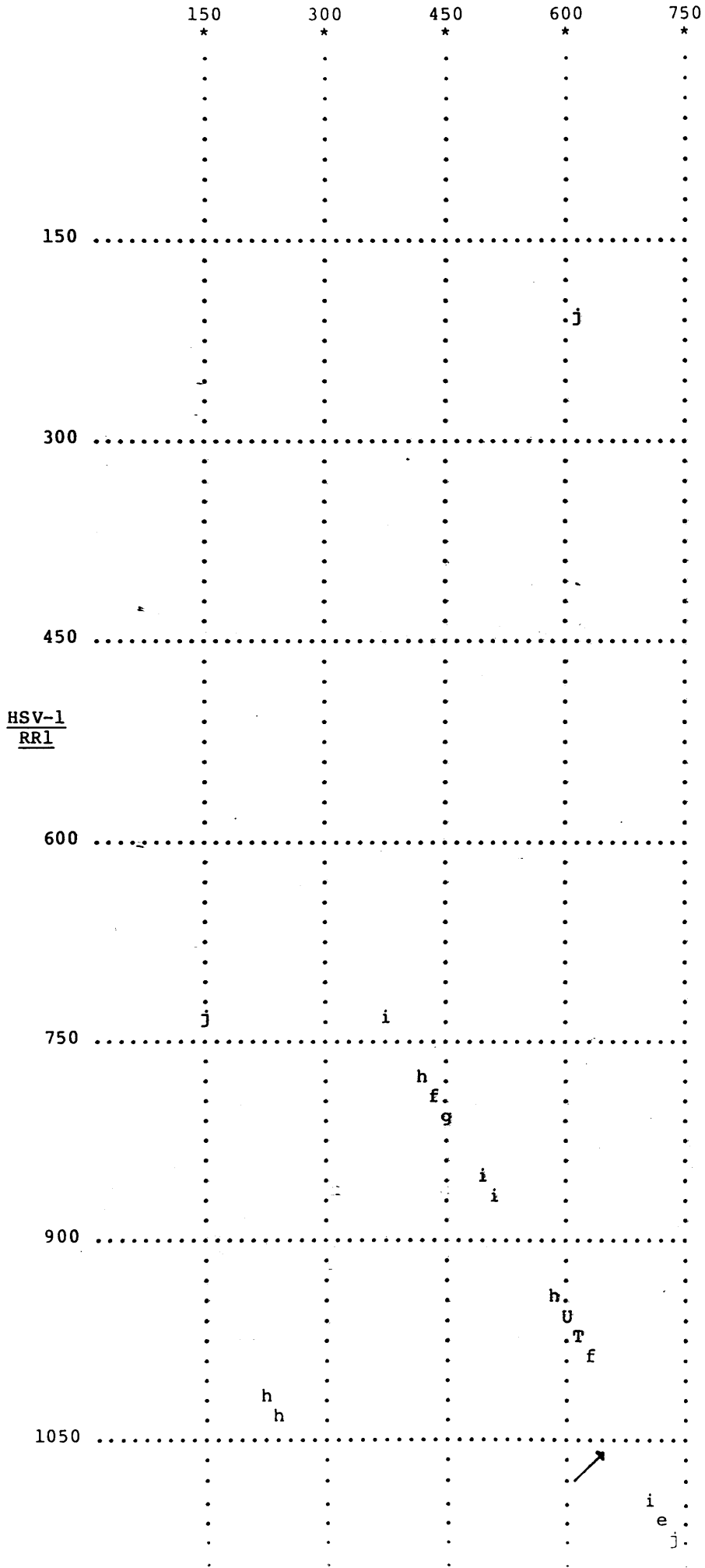

E. coli B1
150
300
450
600
750
150
300
450
HSV-1
RR1
600
750
900
1050

Figure 44. A CINTHOM plot comparing the amino acid sequence of HSV-1 RR1 with the amended *E. coli* B1 polypeptide sequence (Nilsson *et al.*, 1988a). A major deletion in the B1 sequence is indicated by an arrow. Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x15.

First sequence: E. coli B1
Second sequence: HSV-1 RR1

```
 345  TRLLKGEDITLFSPSDVPGLYDAFFADQEEFERLYTKYEKDDSIRKQRVKAVELFSLMMQERASTGRIYIQNVD
        *                      * *   **** **   *              **        * **      *
 720  SMSL                    ADF HGEEFEKLYQHLE  VMGFGEQIPIQELAYGIVRSAATTGSPFVMFKD

 419  HCNTHSPFDPAIAPVRQSNLCLEIALPTKPLNDVNDENGEIALCTLSAFNLGAINNLDELEELAILAVRALDAL
       * *   *   *    **** **  *          *   *              *        * *
 773  AVNRHYIYDTQGAAIAGSNLCTEIVHPASKRSSGVCNLGSVNLARCVSRQTFDFGRLRDAVQACVLMV   NIM

 493  LDYQDYPIPAAKRGAMGRRTLGIGVINFAYYLANDGKRYSDGSANNLTHKTFEAIQYYLLKASNELAKEQGACP
       *   * *   **    *  ***           *          *     *       * ** *    ** *
 844  IDSTLQPTPQCTRGNDNLRSMGIGMQGLHTACLKLGLDLESAEFQDLNKHIAEVMLLSAMKTSNAL CVRGARP

 567  WFNETTYAKGILPIDTYKKDLDTIANEPLHYDWEALRESIKTHGLRNSTLSALMPSETSSQISNATNGIEPPRG
       **                  *  *       ** ** *   ******   ****   * ***    *  *
 917   FNHFKRSMYRAGRFHWERFPD  ARPRYEGEWEMLRQSMMKHGLRNSQFVALMPTAASAQISDVSEGFAPLFT

 641  YVSIKASKDG ILR   QVVPDYEHLHDAYELLWEMPGNDGYLQLV<------ 20aa ------>GIMQK FI
        *   **  **         *       **  *   *     *                    **  *
 988  NLFSKVTRDGETLRPNTLLLKELERTFSGKRLLEVMDSLDAKQWSVAQALPCLEPTHPLRRFKTAFDYDQKLLI

 690  DQSISANTNYDPS         RFPSGKVPMQQLLKDLLTAYKFGVKT LYYQNTRDGAEDAQDDLVPSIQDDGC
       *        * *            *  *   *   *  *** * **  **   *      *    *    *
1062  DLCADRAPYVDHSQSMTLYVTEKADGTLPASTLVRLLVHAYKRGLKTGMYYCKVR    KATNSGVFGGDDNIV

 755  ESGACKI

1132  CMSCAL
```

Figure 45. Optimal alignment of HSV-1 RR1 with the amended amino acid sequence of E. coli B1 (Nilsson et al., 1988a). The major deletion in the B1 sequence predicted in Fig. 44 is indicated. Regions of clustered homology are underlined and asterisks denote conserved residues. The program parameters were: mutation weight = 5, gap weight = 3k+7, k up to 2000.

1986b; Nikas et al., 1986), VV (Vmw37; Slabauch et al., 1988), bacteriophage T4 (T4B2; Sjoberg et al., 1986) and Saccharomyces cerevisiae (Elledge and Davis, 1987; Hurd et al., 1987); likewise, each of these sequences has been compared with other proposed or identified small subunit polypeptide sequences by the respective authors. In this part of Section B the polypeptide sequences of HSV-2 RR2, VZV Vmw35, VV Vmw37 and the putative HCMV ORF M3.PRO are compared with HSV-1 RR2.

i) HSV-1 RR2 is highly homologous to HSV-2 RR2. Comparisons of the RR2 polypeptides of HSV-1 strain 17^+ and HSV-2 strain HG52 are shown as CINTHOM and HOMOL plots in Figs. 46 and 47. The lettering of the CINTHOM diagonal indicates that the N-terminal 40 amino acids of both polypeptides exhibit relatively low homology while the remainder are essentialy colinear (Fig. 46). Analysis of homology with the HOMOL program indicated that the colinear parts extend from HSV-1 aa 34 and HSV-2 aa 31 to the respective C-termini (Fig. 47). The percentage homology for these regions is 93% and from 308 aligned residues 19 changes were observed; five of these are conservative ones according to the Venn diagram of amino acid conservation (Taylor, W.R., 1986a). The percentage homology for the N-terminal 40 amino acids is 44% and no major insertions or deletions are observed which is in contrast to the case with the equivalent regions of HSV-1 RR1 and HSV-2 RR1 (see Page 84).

Larsson and Sjoberg (1986) reported the localisation of the ribonucleotide reductase radical at a tyrosine residue at E. coli B2 aa 122. Comparison of the E. coli B2 amino acid sequence with that of HSV-2 RR2 demonstrated that the tyrosine and adjacent amino acids are well conserved in the RR2 polypeptide; it was therefore proposed that the HSV-2 radical is localised on this tyrosine (McLauchlan, 1986). As can be seen in Fig. 47 the tyrosine residue (indicated by •) is conserved in the HSV-1 RR2 polypeptide as well, and therefore, by analogy with HSV-2 RR2, it is proposed that the HSV-1 RR2 radical is localised on this particular tyrosine.

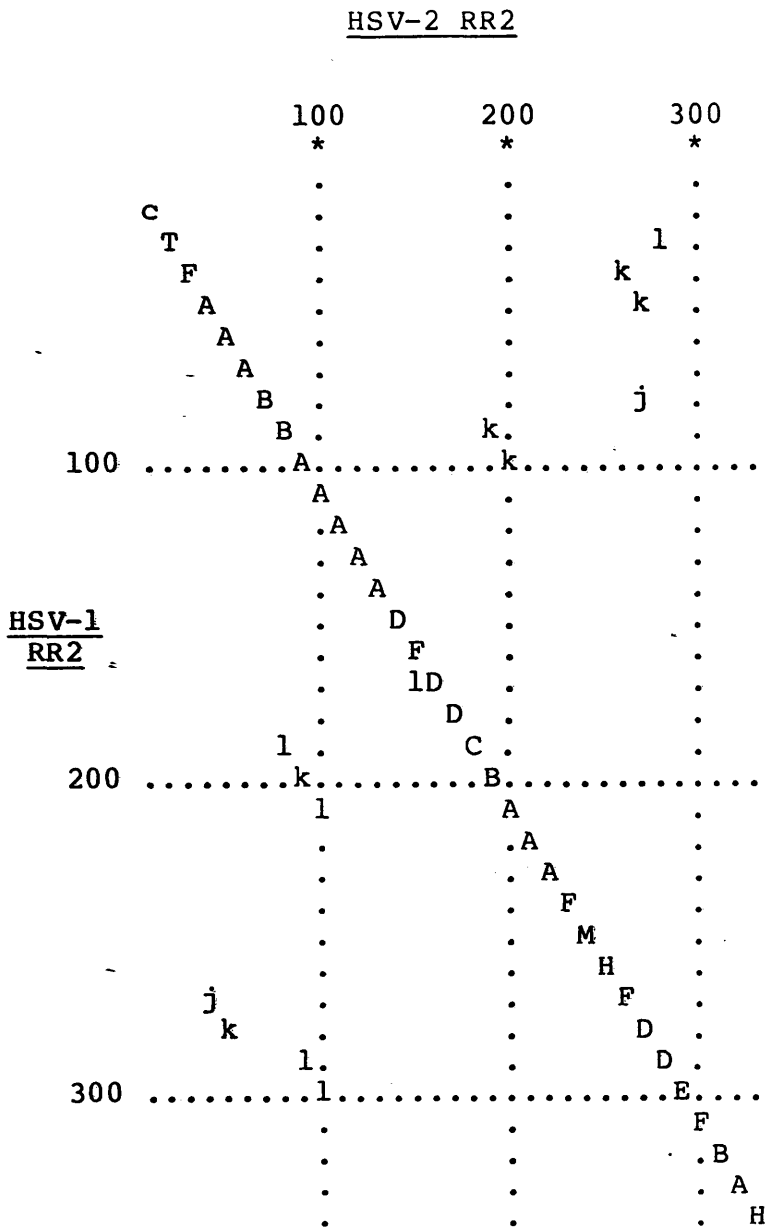


Figure 46. A CINTHOM plot comparing the amino acid sequence of HSV-1 RR2 with that of HSV-2 RR2. Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x10.

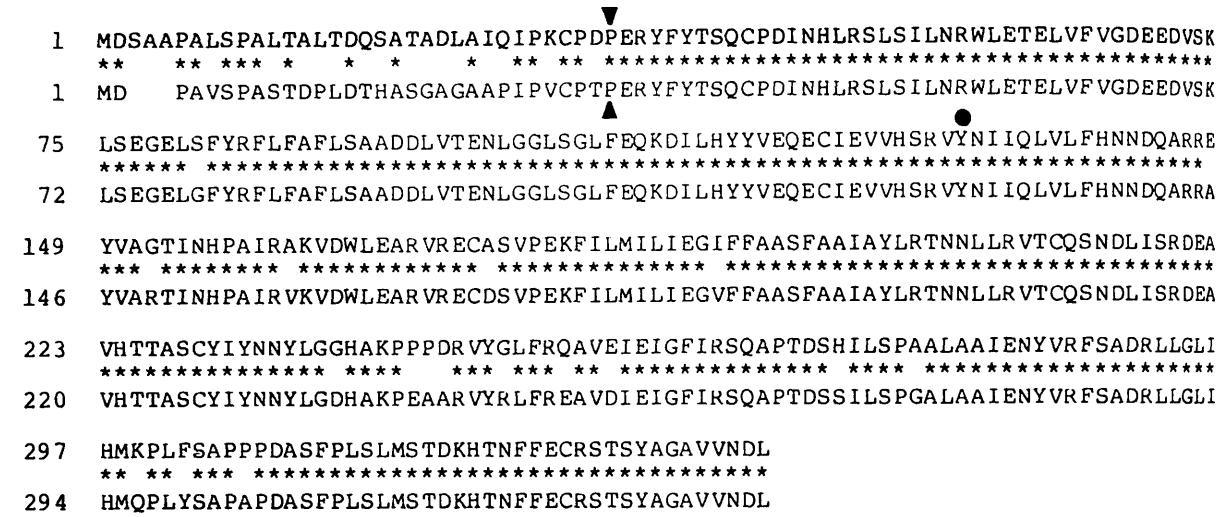


Figure 47. Optimal alignment of the amino acid sequences of HSV-1 RR2 with those of HSV-2 RR2. Arrowheads indicate the amino acid positions where the two sequences become highly homologous. The proposed location of the tyrosine radical (●) is indicated and asterisks denote conserved residues. The program parameters were: mutation weight = 5, gap weight = 3k+7, k up to 2000.

ii) HSV-1 RR2 shares homology with VZV Vmw35. A VZV potential ORF encoding a polypeptide of 35,000 mol. wt., Vmw35, was shown to share homology with HSV-1 RR2 (Davison and Scott, 1986b; Nikas et al., 1986). A CINTHOM comparison of VZV Vmw35 and HSV-1 RR2 revealed that both sequences were colinear, although the position of the homology diagonal indicates the existence of additional amino acids at the N-terminus of RR2 (Fig. 48). Alignment of the two sequences with the HOMOL program indicated that this region consisted of 28 amino acids (Fig. 49); the remainder of both sequences share 54% homology. In common with the HSV-1 RR1/VZV Vmw87 comparison (see Fig. 35), clusters of conserved amino acids exist and these are underlined in Fig. 49. The longest cluster, positioned between HSV-1 aa 111 to 139 and VZV aa 83 to 112, contains a tyrosine residue (indicated by •) which corresponds by position to the RR2 tyrosine on which the radical could be localised. In addition, this cluster contains an RR2 sequence of KDILHYYVE from which six residues are identical in VZV and one residue is replaced by a conserved one (isoleucine to valine, see Fig. 49). Interestingly, McLauchlan (1986) reported that the HSV-2 equivalent block is retained at the C-terminal portion of the homologous E. coli B2 polypeptide.

iii) HSV-1 RR2 shares homology with VV Vmw37. Recently, Slabaugh et al. (1988) identified and sequenced a gene which encodes a polypeptide of 37,000 mol. wt. (Vmw37) as deduced by translation of the nucleotide sequence. This polypeptide was found to be highly homologous to the mouse M2 polypeptide and was therefore suggested to encode the small subunit of the VV-induced ribonucleotide reductase. Amino acid comparison of HSV-1 RR2 with VV Vmw37 with the CINTHOM program revealed the existence of homology (Fig. 50). Alignment of the two polypeptides with the HOMOL program identified three clusters of identical amino acids and the sequences displayed 27% overall homology (Fig. 51). Further, one of these clusters contain a tyrosine residue corresponding by position to the RR2 tyrosine on which the radical could be localised. Analysis of the amino acid

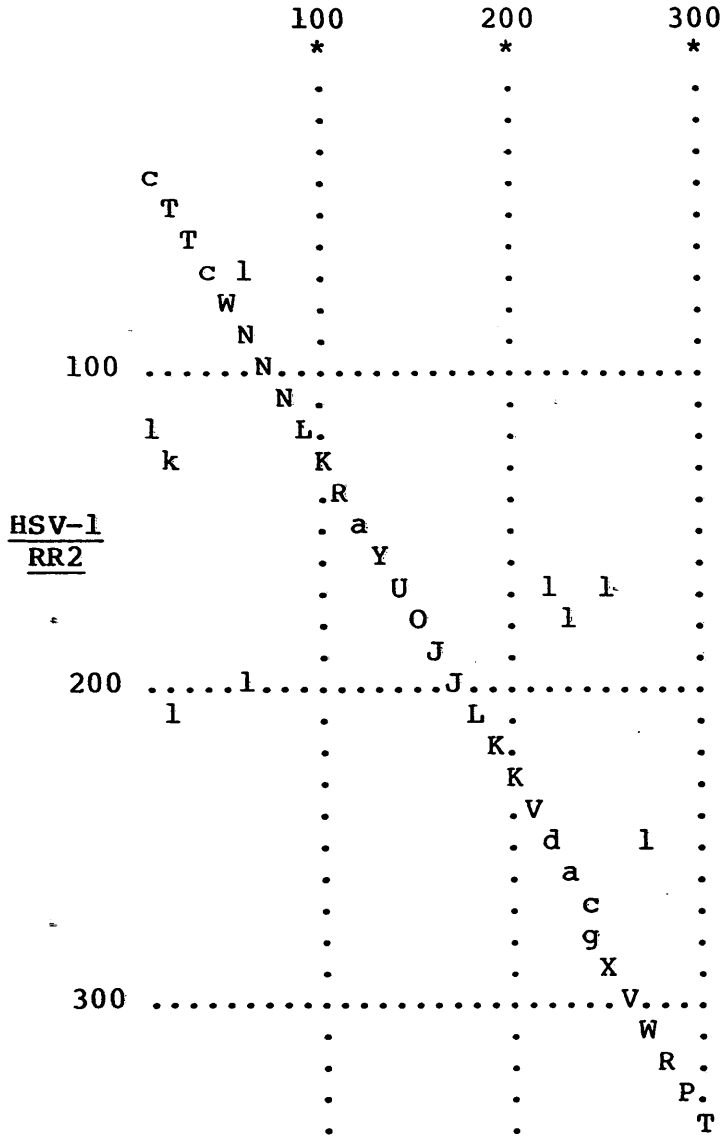


Figure 48. A CINTHOM plot comparing the amino acid sequence of HSV-1 RR2 with that of VZV Vmw35. Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x10.

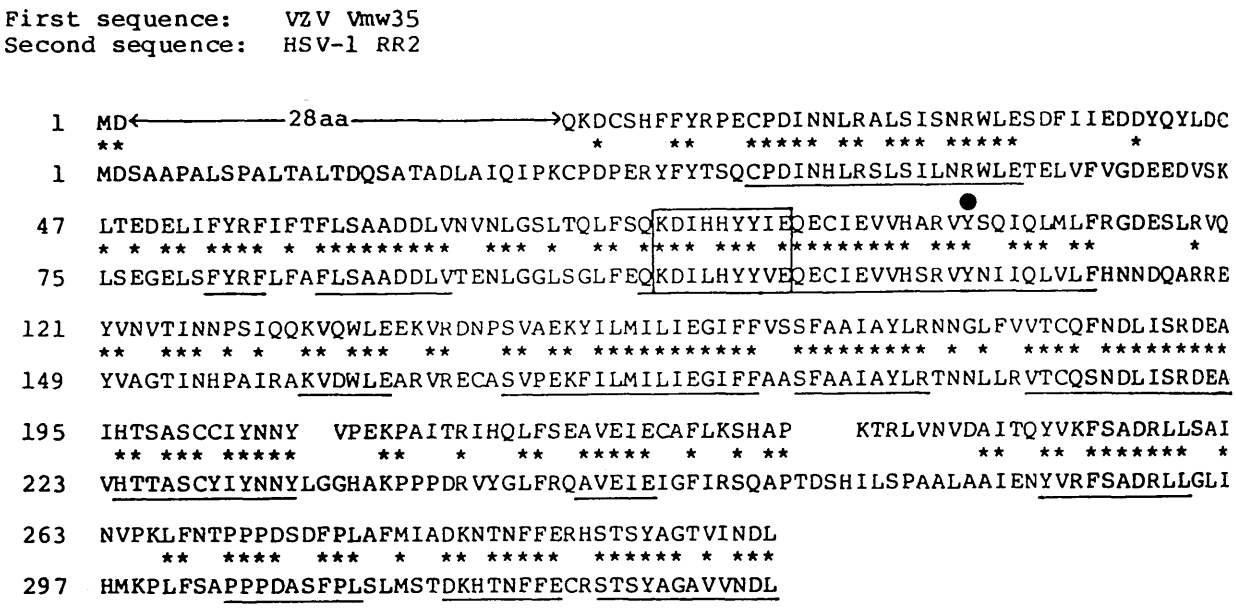


Figure 49. Optimal alignment of the amino acid sequences of HSV-1 RR2 with those of VZV Vmw35. The proposed location of the tyrosine radical (●) is indicated and regions of clustered homology are underlined. Boxed amino acids indicate N-terminal sequences which are conserved at the C-terminus of the E. coli B2 polypeptide (McLauchlan, 1986). Asterisks denote conserved residues. The program parameters were: mutation weight = 5, gap weight = 3k+7, k up to 2000.

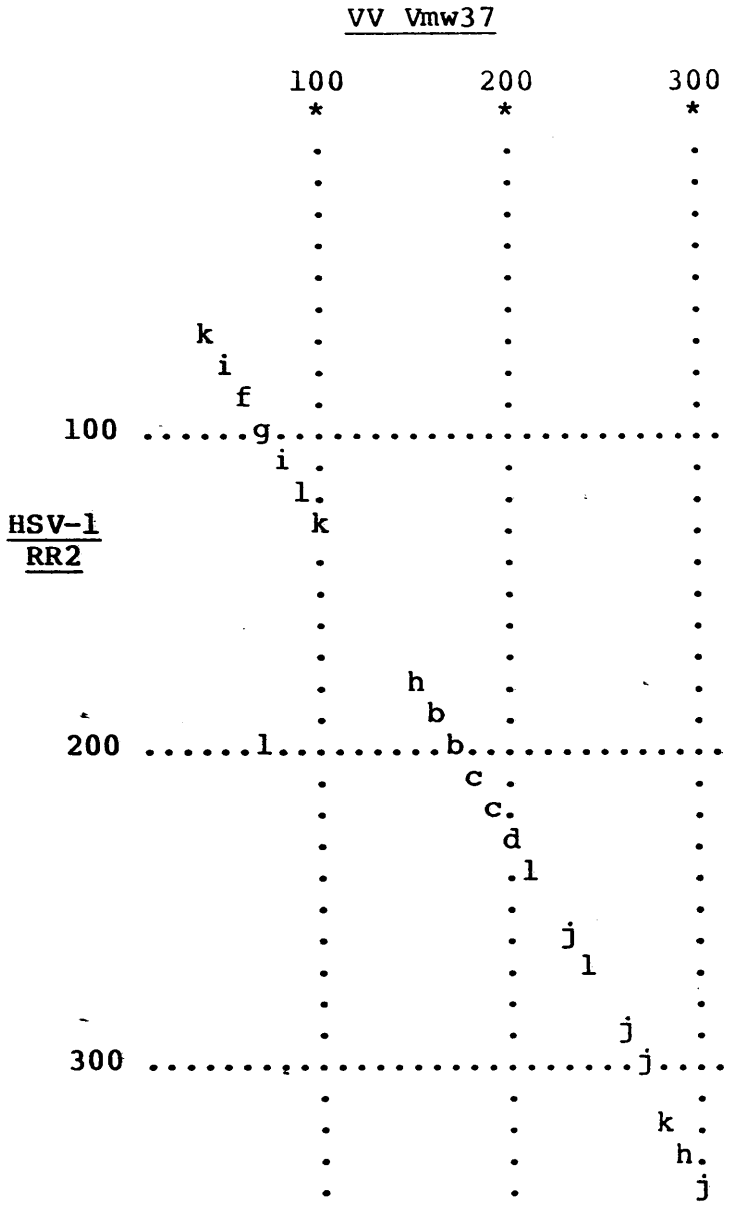


Figure 50. A CINTHOM plot comparing the amino acid sequence of HSV-1 RR2 with that of VV Vmw37. Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x10.

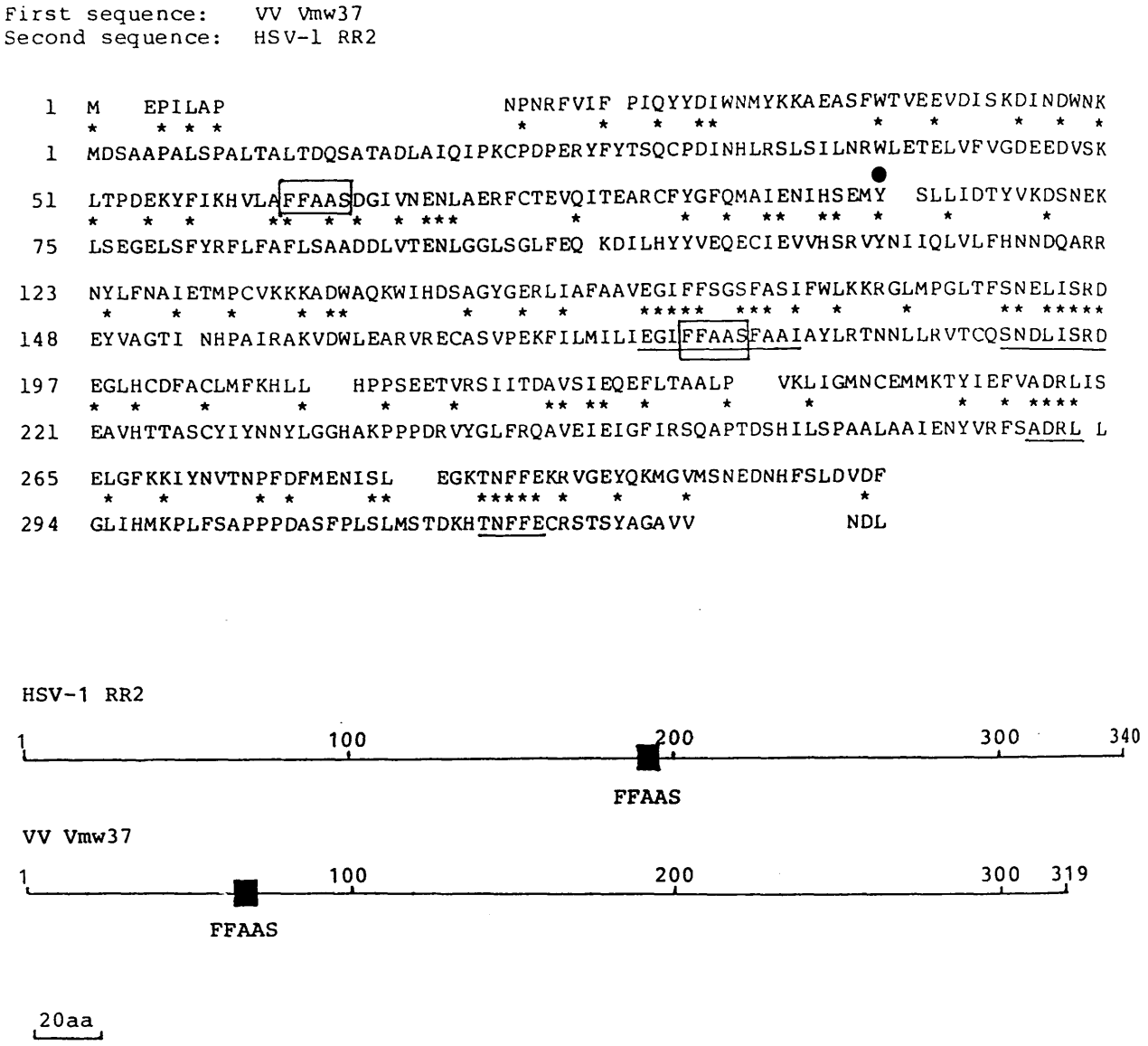


Figure 51. Optimal alignment of the amino acid sequences of HSV-1 RR2 with those of VV Vmw37. Regions of clustered homology are underlined and the proposed location of the tyrosine radical (●) is indicated. Boxed residues indicate a N-terminal RR2 amino acid sequence which is conserved at the C-terminus of the VV polypeptide. Asterisks denote conserved residues. The program parameters were: mutation weight = 5, gap weight = 3k+7, k up to 2000. Below the alignment, the two polypeptides are depicted in a linear form showing the respective locations of the conserved amino acid sequence.

sequences demonstrated the existence of one stretch of residues (boxed residues in Fig. 51) which is present in different positions along each polypeptide backbone.

iv) HSV-1 RR2 is not homologous to HCMV M3.PRO. The sequencing studies of Barrell and co-workers in HCMV strain AD169 identified a putative ORF, termed M3.PRO, which corresponds by position to HSV-1 RR2 (B.G. Barrell, personal communication). This ORF has an extreme C-terminal composition of three interrupted stretches of 7, 21 and 12 glycine residues. CINTHOM comparison of HSV-1 RR2 with M3.PRO revealed low conservation (Fig. 52). This was clearly demonstrated with the HOMOL alignment of the two sequences; the homology percentage was 17%, and only one small stretch of identical amino acids exists (underlined in Fig. 53). Further, the RR2 tyrosine residue (indicated by •) on which the radical could be localised is not conserved in the HCMV sequence. Interestingly, the amino acid sequence LGGLSG which is located close to the N-terminus of RR2 (Fig. 53, HSV-1 aa 101 to 106), is retained close to the C-terminus of M3.PRO (Fig. 53, M3.PRO aa 326 to 331) with only one conservative change from a serine to a glycine.

DISCUSSION.

In the first part of Section B the HSV-1 RR1 polypeptide was compared with homologous herpesvirus sequences and with the equivalent polypeptides of the eukaryotic and prokaryotic enzymes. In the second part, the HSV-1 RR2 polypeptide was compared with recently obtained viral polypeptide sequences. The main feature that emerges from this analysis is thatsimilaritybetween colinear polypeptide regions is primarily retained in stretches of identical or conserved amino acids. Other features are: i) the RR1 N-terminal region is conserved only in HSV-2 RR1, whereas it is absent from other viral, eukaryotic and prokaryotic polypeptides, ii) EBV Vmw93 contains an

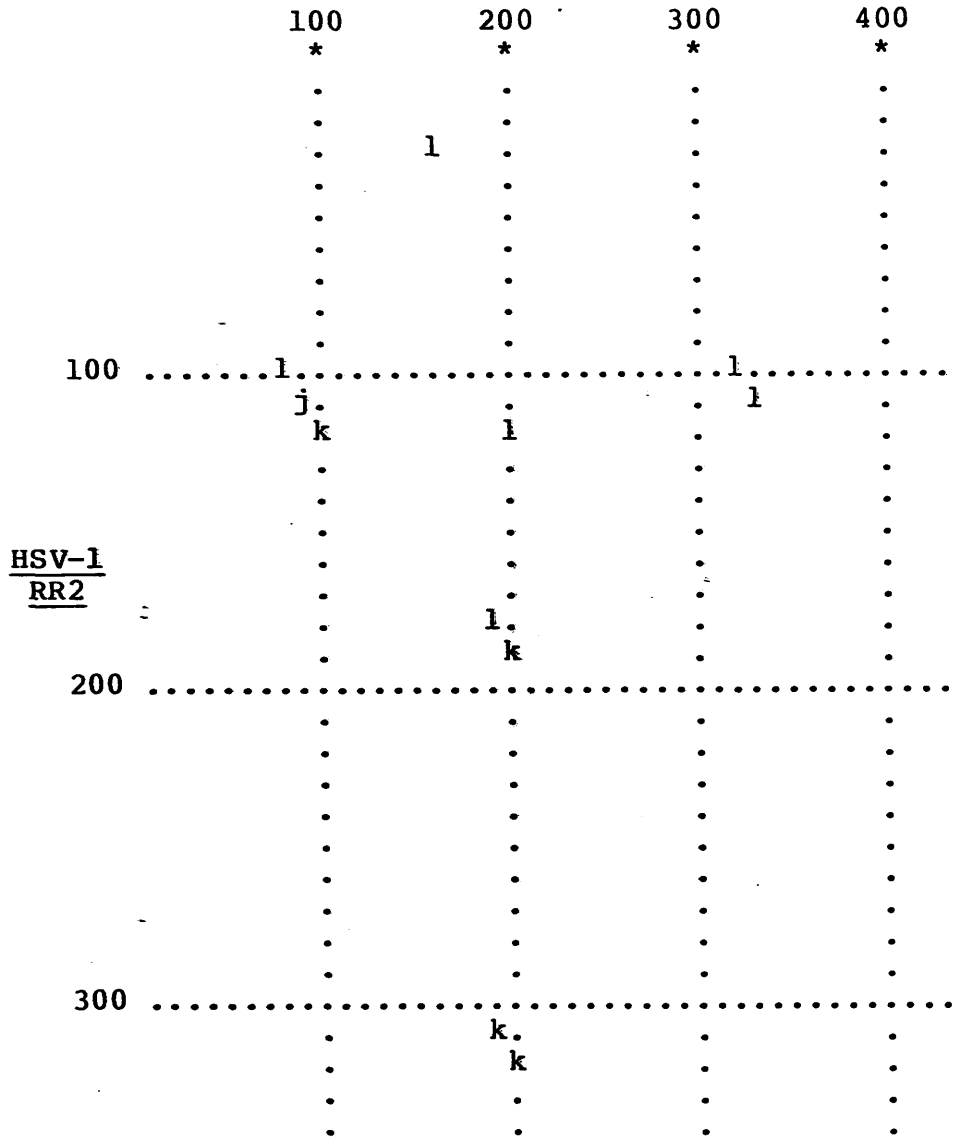


Figure 52. A CINTHOM plot comparing the amino acid sequence of HSV-1 RR2 with that of putative HCMV ORF termed M3.PRO (B. Barrell, personal communication). Program settings were: range = 15; scale factor = 0.95; minimum value plotted = 30%; compressed x10.

```
First sequence:   HCMV M3.PRO
Second sequence:  HSV-1 RR2

  1  MDRKTRLSEPPTLALRLKPYKTAIQQLRSVIRALKENTTVTFLPTPSLILQTVRSHCVSKITFNSSCLYITDKS
     **           * * *  **                       * * *             * * *
  1  MD          SAAPALSPALTA                  LTDQSATADLAIQIPKCPDPERYFYTSQCPDI

 75  FQPKTINNSTPLLGNFMYLTSSKDLTKFYVQDISDLSAKISMCAPDFNMEFSSACVHGQDIVRESENSAVHVDL
               *     *          * *  * * * *                      * * *
 47            NHLRSLSILNRWLETELVFVGDEEDVS KLS                    EGELSFYRFLF

149  DFGVVADLLKWIGPHTRVKRNVKKAPCPTGTVQILVHAGPPAIKFILTNGSELEFTANNRVSFHGVKNMRINVQ
      *   ** *                    *         *    * **   * *      *   * *   *
 88  AFLSAADDL                   VTENLGGLSGLFEQKDILHYYVEQE   CIEVVHSRVYNIIQLVL

223  LKNFYQTLLNCAVTKLPCTLRIVTEHDTL    LYVASRNGLFAVENFLTEEPFQRGDPFDKNYVGNSGKSRGG
      * *     *       *    * *      **    *    * * *       *       *
139  FHNNDQ ARREYVAGTINHPAIRAKVDWLEARVRECASVPEKF ILMILIEGIFFAASFAAIAYLRTNNLLR V

293  GGGGGSLSSLANAGGLHDDGPGLDNDLMNEPMGLGGLGGGGGGGGKKHDRGGGGGSGTRKMSSGGGGGDHDHGL
         * *   *          * *          **      **  *         *
210  TCQSNDLISRDEAVHTTASCYIYNNYL            GGHAKPPPDRVYGLFRQAVEIEIGFIRSQAPTDS

367  SSKEKYEQHKITSYLTSKGGSGGGGGGGGGGLDRNSGNYFNDAKEES DSEDSVTNFEFVPNT    KKQKCG
              *  *      *    *        *          *  * *  ***     *
271  HILSPAALAAIENY   VRFSADRLLGLIHMKPLFSAPPPDASFPLSLMSTDKHTNFFECKSTSYAGAVVNDL
```

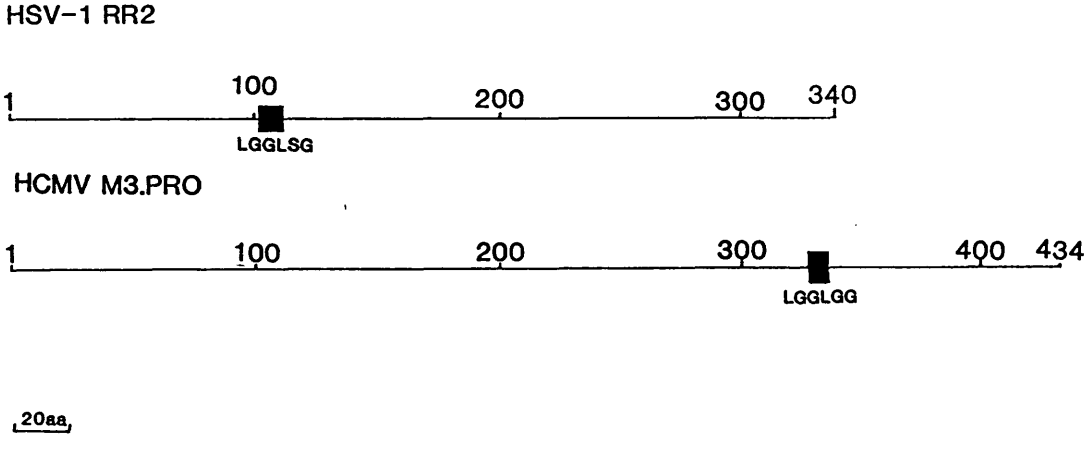


Figure 53. Optimal alignment of the amino acid sequences of HSV-1 RR2 with those of a putative HCMV ORF termed M3.PRO (B. Barrell, personal communication). The single region of clustered homology is underlined and the proposed location of the tyrosine radical in RR2 (●) is indicated. Boxed residues indicate a N-terminal RR2 amino acid sequence which is conserved at the C-terminus of the M3.PRO. Asterisks denote conserved residues. The program parameters were: mutation weight = 5, gap weight = 3k+7, k up to 2000. Below the alignment, the two polypeptide sequences are depicted in a linear form showing the respective positions of the conserved amino acid sequence.

insertion which separates highly conserved regions in the HSV-1, HSV-2 and VZV homologue polypeptides, and iii) the first of two putative HCMV ORFs, which corresponds by position to RR1 exhibits low but detectable homology; the second ORF, which corresponds by position to RR2 exhibits extremely low conservation.

6. Relationship Between HSV-1 and Other Herpesviral and Non-herpesviral Polypeptides.

a) Polypeptide conservation between HSV-1 and HSV-2.

The comparisons presented in this section clearly demonstrate that the highest percentage homology is observed between the HSV-1 and HSV-2 polypeptides. The DNA sequences for a number of genes such as TK (McKnight, 1980; Wagner et al., 1981), gC (Swain et al., 1985), AE (Draper et al., 1986) and for most of the polypeptides encoded by the U_S segment (McGeoch et al., 1985 and 1987), have been obtained for both HSV serotypes. Comparisons between equivalent polypeptides indicate relatively low homology for the N-termini, as compared with the remainder of the coding regions which are quite well conserved (McGeoch et al., 1987); the only exception reported so far is the HSV-1 IE Vmw63 which differs in one out of twenty N-terminal amino acids from its HSV-2 counterpart (Whitton et al., 1983). In some instances, most of the N-terminal amino acid changes are conservative and, therefore, their predicted secondary structures are quite similar as is the case for the AE (Draper et al., 1986) and RR1 (Nikas et al., 1986; see Results and Discussion, Section C). Only two cases of major differences within the N-termini of homologous HSV-1 and HSV-2 polypeptides have been reported so far; the first one is for HSV-2 gG, which contains an insertion of approximately 480 residues (McGeoch et al., 1987), and the second for HSV-1 gC, which contains an additional 28 amino acids (Swain et al., 1985).

b) Polypeptide conservation between HSV-1 and VZV.

The genomes of HSV-1 and VZV are quite similar in that they both have U_L, R_S and U_S segments. VZV does have an R_L segment but this is only 88bp (Davison, 1984). Comparisons of equivalent HSV-1 and VZV polypeptide coding regions demonstrated a wide variation of homology, ranging from similar polypeptides, such as the HSV-1 Vmw175 and VZV Vmw140 (McGeoch et al., 1986) and the DNA polymerase (McGeoch et al., 1988), to the very limited relationship of the polypeptides encoded by the HSV-1 US10 and VZV RS3 genes (Davison and McGeoch, 1986). In most comparisons reported so far, there is a significant dissimilarity in length and amino acid composition at the C- and, particularly, the N-termini, the latter being the least conserved regions (Davison and McGeoch, 1986). In that respect, these authors reported that HSV-1 Vmw68, US3, US10 and VZV Vmw140, US1, US4, have more residues in their N-terminal portions than their counterparts; in contrast, HSV-1 Vmw65, the IE trans-inducing factor, has 80 C-terminal amino acids which are not present in the VZV Vmw54 homologue (Dalrymple et al., 1985). However, an N-terminal difference of approximately 360 residues, as the one observed in the HSV-1 RR1/VZV Vmw87 comparison (see Page 84), has not been reported as yet (D.J. McGeoch, personal communication).

c) Polypeptide conservation between HSV-1 and EBV.

Although the genome sequences of both HSV-1 and EBV have been obtained, comparisons have been performed for only a limited number of polypeptides such as the DNA polymerase (Quinn and McGeoch, 1985) and gB (Pellet et al., 1985) where homology is approximately 40%; a systematic analysis such as the one performed for HSV-1 and VZV has not been carried out as yet. However, Davison and Taylor (1987) have compared all the VZV and EBV ORFs and found three major regions of approximate colinearity in the U_L segments (Fig. 54, regions A, B and C) while no relationship was observed for the repeats or the U_S segments. Within the A, B and C regions, certain genes are strongly conserved while the majority have barely detectable homology; their identification

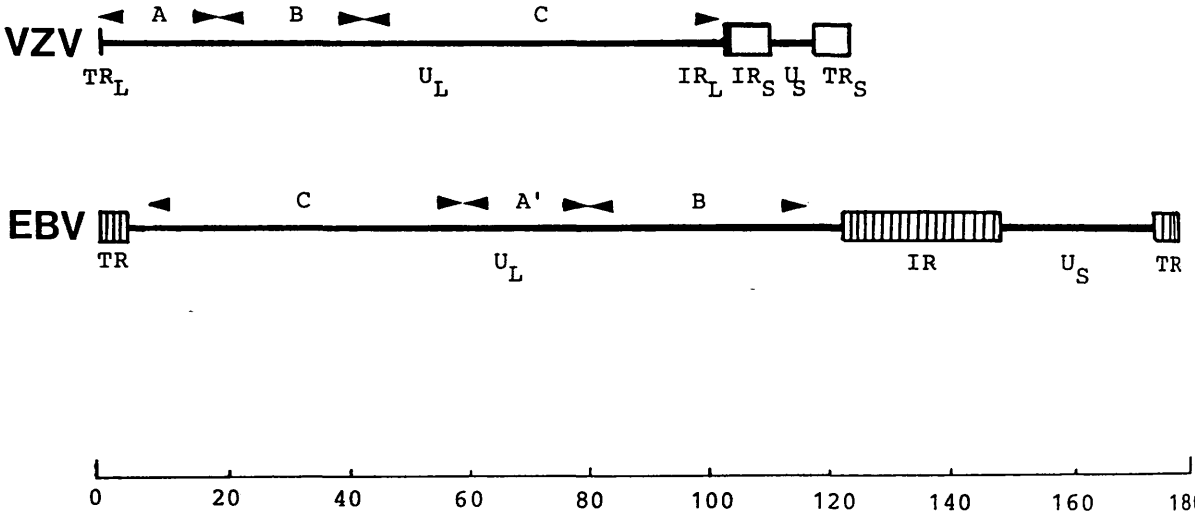


Figure 54. This diagram shows the relationship between the VZV and EBV genomes. The VZV genome comprises two unique components (U_L and U_S) each flanked by inverted repeats (TR_L, IR_L, IR_S and TR_S). The EBV genome also consists of two unique components (U_L and U_S) joined by a variable number of copies of a direct repeat (IR). The VZV U_L component is represented as three major regions (A, B and C) which correspond on the basis of homology to regions C, A' and B in the EBV U_L component. A' denotes the A region in reverse orientation.

as homologues was based on characteristics such as hydropathicity profiles or glycoprotein-like sequences. Further, the VZV genes 9 through to 17 which map between the A and B regions have no obvious homologue in the EBV genome. The HSV-1 counterparts of these VZV genes are UL41 through to UL49 and these include the genes for the IE trans-inducing factor Vmw65, gC and the $65K_{DBP}$ protein.

d) Polypeptide conservation between HSV-1 and HCMV.

As reported above, the lowest percentage homology was observed in the RR1 and RR2 comparisons with the respective putative HCMV ORFs. To date, the only HCMV available sequencing data are for the U_S, R_S, a portion of the TR_L segment (Weston and Barrell, 1986), and a 20kbp fragment in U_L (Kouzarides et al., 1987). Comparisons of the HCMV U_S ORFs with HSV-1, VZV and EBV U_S sequences revealed no homology at all (Weston and Barrell, 1986). However, from the eight ORFs in the U_L fragment one exhibits 31% homology to the HSV-1 DNA polymerase and another to HSV-1 gB (Kouzarides et al., 1987). These homologous pairs of sequences exhibit extremely low conservation at the N-termini while close to the C-termini a substantial degree of conservation can be observed (Kouzarides et al., 1987).

e) Polypeptide conservation between HSV-1 and non-herpesviral polypeptides.

Apart from the ribonucleotide reductase genes, which, as already mentioned, share homology with non-herpesviral polypeptides only two other HSV-1 genes have been shown to share homology. Earl et al. (1986) reported that the herpesvirus DNA polymerase shares homology with the corresponding adenovirus gene, and McGeoch and Davison (1986b) demonstrated that a non-characterised HSV-1 ORF exhibited homology, spanning an 80 amino acid region at the middle of the ORF, with a eukaryotic protein kinase. This ORF was subsequently shown to encode a protein kinase (see Introduction, Page 35) and is the first HSV-1 gene for which a function has been assigned by this approach.

7. Constituents of the Viral Ribonucleotide Reductases.

As already mentioned (see Introduction, Page 49), the HSV-1-specified enzyme consists of the RR1 and RR2 polypeptides. On the basis of the strong homology observed in the HSV-1 RR1/VZV Vmw87 and HSV-1 RR2/VZV Vmw35 comparisons it appears that these VZV ORFs code for the large and small subunits of the VZV-induced enzyme, respectively. This is further supported by the similarity of the properties of the HSV- and VZV-induced enzymes (Spector et al., 1987). Dutia et al. (1986) attempted to immunoprecipitate the VZV activity by means of an oligopeptide antiserum directed against the 7 C-terminal amino acids of HSV-2 RR2. This approach, based on the observation that the C-terminal portions of the HSV-2 and VZV small subunit polypeptides are highly conserved (McLauchlan, 1986; Nikas et al., 1986), resulted in the precipitation of the Vmw35 protein species. However, the antiserum did not precipitate the Vmw87 polypeptide, most probably because different immunoprecipitation conditions are required to achieve this.

On the basis of the strong homology observed in the HSV-1 RR1/EBV Vmw93 (see Page 85) and in conjunction with the observed similarity of HSV-1 RR2 with EBV Vmw34 (McLauchlan, 1986) it appears likely that these polypeptides encode the large and small subunits of the EBV-specified activity in infected cells. Equally, the observed percentage homologies of the VV Vmw86 and Vmw37 polypeptides with HSV-1 RR1 and RR2 would indicate that they code for the large and small subunits of the VV-induced enzyme.

The same suggestions cannot be applied with certainty to the HCMV putative ORFs. The larger of these, M4.PRO, exhibited some homology to HSV-1 RR1 and as can be seen in Fig. 41 two regions were conserved. The first region contains a sequence that resembles the proposed nucleotide binding site identified in the ribonucleotide reductase large subunit polypeptides (see Page 103). The second region corresponds to a region in the large subunit polypeptides which appears to be important for structure of the enzyme (see Page 112). These similarities would suggest that M4.PRO resembles a remnant of a protein which contains

a nucleotide binding site; this could possibly be the large subunit of ribonucleotide reductase although for the time being there are no reports describing such an HCMV-specified activity.

On the other hand, M3.PRO exhibits the lowest similarity to HSV-1 RR2 observed so far. Further, the RR2 tyrosine residue on which the radical is thought to be localised and a block of residues which are highly conserved in all the small subunit polypeptides (see Page 100) are not conserved in M3.PRO. The observed lack of similarity would suggest that this ORF does not code for the ribonucleotide reductase small subunit, although, it should be stated that lack of homology of the primary structure of two proteins is not adequate evidence for assigning different functions to these proteins. The latter has been clearly shown in the EBV/VZV comparisons (see Page 92) and in the case of the HSV-1 protein kinase which shares limited degree of conservation with the eukaryotic equivalent polypeptide (McGeoch and Davison, 1986b). In conclusion, although the available data are insufficient to propose whether the HCMV ORFs code for a ribonucleotide reductase, it is suggested that at least in the corresponding HSV-1 genomic position this virus does not code for such an activity.

8. Putative Common Epitopes in the HSV-1 RR1 and RR2 Subunits.

A number of immunoprecipitation studies using monoclonal antibodies or anti-oligopeptide sera specific for either the RR1 or RR2 polypeptides resulted in the co-precipitation of both subunits (Frame *et al.*, 1985; Bacchetti *et al.*, 1986; Cohen *et al.*, 1986b; Dutia *et al.*, 1986; Langelier *et al.*, 1986). It was therefore suggested that this is due either to the tight binding of the RR1 and RR2 subunits or that these subunits share common epitopes. Cohen *et al.* (1986b) investigated the latter suggestion by raising a polyclonal antiserum(P9) against a nonapeptide representing the C-terminus of RR2 and demonstrated that P9 could precipitate RR2 and in addition, albeit less efficiently, RR1. The P9 antiserumwas reacted with RR2

TABLE III

CONSERVED AMINO ACID STRETCHES IN THE HSV-1 RR1 AND RR2 POLYPEPTIDES

Sequence	RR1 AA Position*	RR2 AA Position*
TAD	139	21
LGG	147	236
TDS	204	268
AVA	119	335
AGA	281	333
PAL	338	10
REYV	403	147
HLR	432	48
NLG	571	100
AAIA	785	196
LGL	878	293
PDA	936	307
DVS	977	71
RLL	1018	287

(*) The numbers indicate the position of the first amino acid (AA) in the RR1 and RR2 sequences shown.

which was bound to nitrocellulose paper and RR2-specific antibodies were eluted; these were then reacted with a partially purified HSV-1-infected cell extract which was separated by SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose paper. The RR2-specific antibodies reacted with both the RR2 and RR1 polypeptides thus indicating that the two subunits share common antigenic determinants.

Comparison of the nonapeptide amino acid sequence with the RR1 amino acid sequence showed that the peptides AVA and AVV were present in RR1 (Cohen et al., 1986b). These authors suggested that, although the homologous sequences were not particularly stri king, they could be considered as common epitopes. It is generally believed that protein epitopes comprise five to seven amino acids which can either be consecutive or reside in different parts of the primary structure of the protein (conformational epitopes). However, shorter epitopes of three consecutive amino acids have been reported (Wehland et al., 1984), and on that basis the suggestion of Cohen et al. (1986b) is plausible. Further to these RR1 and RR2 homologous stretches, a number of others were identified which could represent common epitopes (Table III). However, it is equally possible that these may simply reflect similarities of the codon usage in the RR1 and RR2 polypeptide DNA coding regions. Further studies are required in order to distinguish between these possibilities.

SECTION C.

9. Alignment and Secondary Structure Predictions of the Identified or Potential Large and Small Subunit Ribonucleotide Reductase Polypeptides.

The results presented in Section B and other reports (see Page 87) indicate that there is a high degree of clustered conservation between the identified or potential large (RR_L) and small (RR_S) ribonucleotide reductase subunits of viral, prokaryotic and eukaryotic origin. Thus, it became of interest to identify conserved structural or functional elements and to determine, if possible, the secondary structure of the enzyme's subunits. The program used for this alignment was the consensus template alignment program (Taylor, W.R., 1986b; see Materials and Methods, Page 77). In contrast to the HOMOL program, which can align only two sequences and scores only for identical amino acids, the consensus template alignment program can align more than two sequences and scores for both identical residues and residues with similar physico-chemical properties. Due to the difference in scoring between these programs the sequence alignments are slightly different and some of the insertions or deletions predicted with the HOMOL program map within aligned polypeptide regions of the consensus template alignment output. The latter program can further predict the secondary structure for each amino acid sequence with the GARNIER algorithm (Garnier et al., 1978), and from the combination of the individual predictions a consensus one can be derived. Below the aligned sequences there are two consensus lines; the first line indicates conserved amino acids and the second line contains the consensus secondary structure prediction. As individual predictions did not give identical answers, the most frequent prediction was taken for each position. Gaps introduced into the sequences for alignment were designated as unstructured areas and boxed regions represent blocks within which the majority of amino acids are conserved.

a) Alignment of the RR_L polypeptides.

The RR_L polypeptide alignment contained the HSV-1 RR1, HSV-2 RR1, VZV Vmw87, EBV Vmw93, the amended E. coli B1 and the mouse M1 polypeptide sequences. On the basis of the secondary structure predictions, the RR_L sequences can be divided into four regions.

Region 1 contains the HSV-1 RR1 and HSV-2 RR1 N-terminal regions, spanning HSV-1 positions 1 to 312 (see Page 84) and, in addition, sequences up to HSV-1 position 400 (Fig. 55); the latter were included because they are not present in other RR_L polypeptides (see Page 91). The predicted secondary structures for each Region 1 were homologous and 80% of aligned residues had similar structural predictions (data not shown). The consensus secondary structure emphasised four distinct parts. The first part starts at HSV-1 RR1 position 1 and terminates at the short run of prolines at aa 150 to 155 where there are two deletions comprising a total of 12 amino acids in the HSV-1 sequence. Within this portion of the sequence, 30% of aligned amino acids were predicted to adopt β-strand conformation and 1% to adopt α-helical conformation. Most of the amino acids adopting β-strand conformation were clustered so that the first part of the sequence is punctuated by short β-strand stretches. The intervening sequences were predicted as unstructured regions and contain all the predicted insertions and deletions. Interestingly, 90% of amino acids in the β-strands are either identical or replaced by similar residues and 36% of them are hydrophobic ones, predominantly isoleucine, valine and alanine. The second and third parts extend beyond the stretch of prolines up to HSV-1 RR1 position 255 where there is a deletion of eight amino acids in the HSV-1 sequence; these parts are separated between them by a deletion of six amino acids at HSV-1 RR1 position 205. Parts 2 and 3 are predicted as unstructured, and within them the sequence becomes more hydrophilic with few conserved clusters of hydrophobic residues. The fourth part spans HSV-1 RR1 amino acid positions 256 through to 400. Within this part the sequence becomes increasingly α-helical with a small number of predicted β-strands.


P1
1
2
3
4
5
50
100
HSV-1 RR1 MASRPAASSP VEARAPVGGQ EAGGPSAATQ GEAAGAPLAH GHHVYCQRVN GVMVLSDKTP GSASYRISDS NFVQCGSNCT MIIDGDVVRG RPQDPGAAAS
HSV-2 RR1 MANRPAASAL AGARSPSERQ EPREPEVAPP G..... GDHVFCRKVS GVMVLSSDPP GHAAYRISDS SFVQCGSNCS MIIDGDVARG HLRDLEGATS
C H---HHHHHH -EEEEE---E --------HH H--------- --EEEEE--- EEEEEE---- --EEEEE--- -E-------E EEEEE---H- ---E------
6
7
P1
P2
143
189
HSV-1 RR1 PAFFVAVTNI GAGSDGGTAV VAFGGTPRRS AGTSTGTQTA DV.....P TEALGG.... PPPPPRFTLG GGCCSCRDTR RRSAVFGGEG DPVGPAEFVS
HSV-2 RR1 TGAFVAISNV AAGGDGRTAV VAIGGTSGPS ATTSVGTQTS GEFLHGNPRT PEPQGPQAVP PPPPPPFPWG HECCARRDAR ......GGAE KDVGAAESWS
C -HEEEEEEE- ------EEEE EEE------- -EEEE----- EE-----E-- EE-----EE- ----EEEE-- -----EHH-- --EEEE---H HHHHHH-EEE
P2
P3
P3
P4
233
275
HSV-1 RR1 DDRSSDSDSD DSEDTD.... ..SETLSHAS SDVSGGATYD DALDSDSSSD DSLQIDGPVC RPWSNDTAPL DV..... CPGTPGPGAD AGGPSAVDPH
HSV-2 RR1 DGPSSDSETE DSDSSDEDTG SQSETLSRSS SIWAAGATDD DDSDSDSRSD DSVQPDVVVR RRWSDGPAPV AFPKPRRPGD SPGNPGLGAG TGPGSATDPR
C --------HH ----HH---- --H--HH--- --EE----EE EE--------- EEEEEEEEEE ----------E EE-------- E------EEE E----EEEEE
325
375
HSV-1 RR1 APTPEAGAGL AADPAVARDD AEGLSDPRPR LGTGTAYPVP LELTPENAEA VARFLGDAVN REPALMLEYF CRCAREETKR VPPRTFGSPP RLTEDDFGLL
HSV-2 RR1 ASADSDSAAH AAAP...QAD VAPVLDSQPT VGTDPGYPVP LELTPENAEA VARFLGDAVD REPALMLEYF CRCAREESKR VPPRTFGSAP RLTEDDFGLL
C E-HHHHHHHH HHHHHHHHHH -EEEE--EE- ---EEEEEEE E-EHHHHHHH HHHHHH---H HHHHHHHHHH HHHHHH---- ---------- --EE--HHHH
P4
400
HSV-1 RR1 NYALVEMQRL CLDVPPVPPN AYMPY
HSV-2 RR1 NYALAEMRRL CLDLPPVPPN AYTPY
C HHHHHHHHH- --E------E EEEEE

Figure 55. Alignment of the HSV-1 and HSV-2 RR1 polypeptide Region 1. The consensus line below the alignment (C) shows the consensus GARNIER secondary structure prediction for each aligned position; (E) represents β-strand conformation and (H) represents α-helical conformation. The blocks of prolines are boxed and amino acid sequences in numbered boxes indicate the short β-strand stretches thought to punctuate the RR1 region between positions 1 to 150. The Regions are shown separated in four parts (P) on the basis of the consensus GARNIER prediction and the predicted HSV-1 RR1 deletions. Underlined amino acids indicate the potential nuclear localisation signals (see General Discussion, Page 127). The amino acid positions of the alignment are numbered as in HSV-1 RR1 (see Fig. 26) and asterisks, introduced by the operator, denote conserved amino acids. Dots within the aligned sequences have been introduced by the program to optimise the alignment.

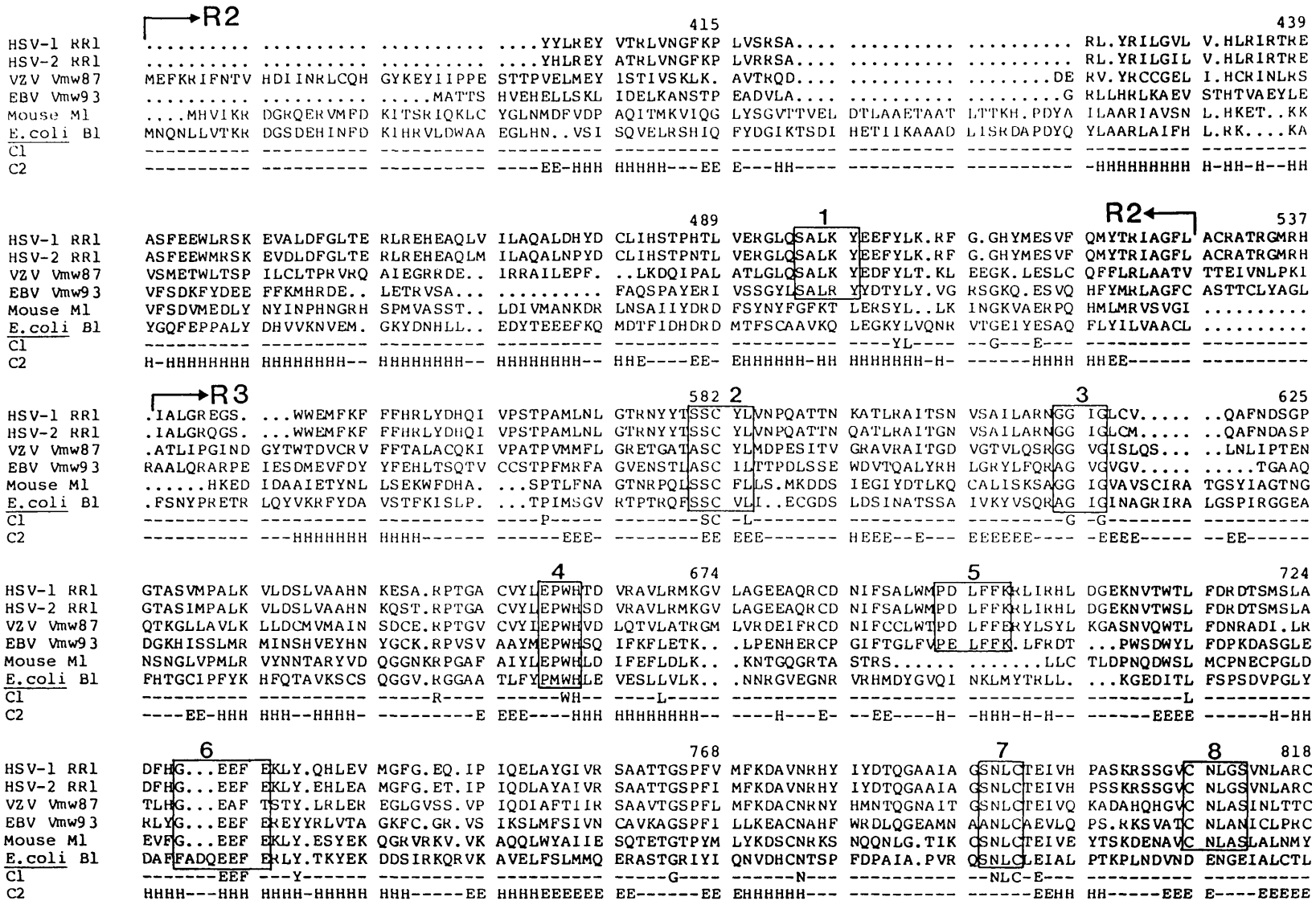
Region 2 extends approximately from HSV-1 aa 400 to 530 where there are two major insertions of 16 and 12 residues in the eukaryotic and prokaryotic polypeptides, respectively (Fig. 56). Within this region, 50% of the amino acids were predicted to adopt α-helical conformations and no β-strands were observed. Region 2 contains 4 conserved residues and one block of identical amino acids, labelled 1 in Fig. 56, which is conserved in the viral RR_L.

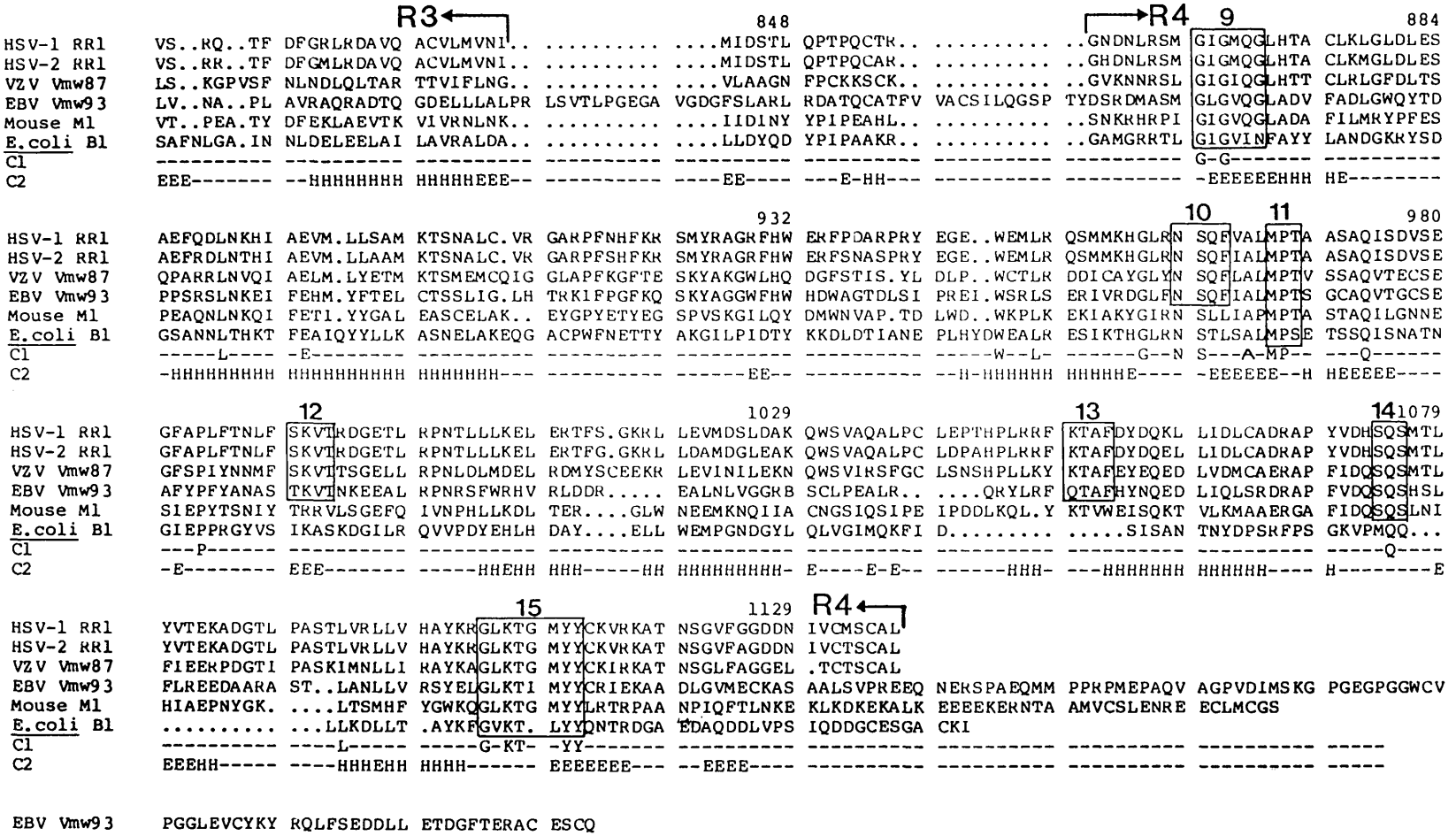
The remainder of the polypeptides have been separated into Regions 3 and 4 by two EBV insertions at HSV-1 RR1 aa 842 and aa 856 (Fig. 56). These insertions, which comprise a total of 30 residues, approximately correspond to the major insertion of 34 amino acids in the HSV RR1/EBV Vmw93 comparison (see Fig. 37); however, as described on Page 97, the length and positions of these insertions differ slightly between different programs. The secondary structure prediction for Regions 3 and 4 identified areas of both β-strand and α-helix, which occur one after the other so that most of the α-helices are separated by β-strands along the sequences and vice versa. Region 3 is the most conserved RR_L region; it contains 21 conserved amino acids and the majority of the blocks are retained in all the polypeptides (Fig. 56, blocks 2, 3, 4, 6 and 7). In Region 4 only blocks 9, 11 and 15 are conserved in all the RR_L and the program identified 20 conserved amino acids.

b) Alignment of the RR_S polypeptides.

The small subunit comparison contained the HSV-1 RR2, HSV-2 RR2, VZV Vmw34, EBV Vmw34 and E. coli B2 polypeptides (Fig. 57). The program identified 33 conserved amino acids and two major insertions of 19 and 23 amino acids in the E. coli B2 polypeptide (Fig. 57, HSV-2 RR2 aa 156 and 315). In common with RR_L Region 2 (see above), 50% of the amino acids were predicted to adopt α-helical conformation although there were a few β-strand structures observed close to the C-termini of the polypeptides. The RR_S alignment further identified six blocks of conserved residues (Fig. 57, blocks 16 through to 21); these were subsequently compared with other RR_S sequences reported on Page 87, which were not previously available. In these additional

Figure 56. Alignment of Regions 2, 3 and 4 of the identified or proposed ribonucleotide reductase large subunit polypeptides of viral eukaryotic and prokaryotic origin (RR_L). Below the alignment, the first consensus line (C1) displays the amino acids conserved in all RR_L and the second line (C2) shows the consensus GARNIER secondary structure prediction for each aligned position. (E) represents β-strand conformation and (H) represents α-helical conformation. The amino acid positions of the alignment are numbered as in HSV-1 RR1 (see Fig. 26), and amino acids sequences of higher homology are boxed. Dots within the aligned sequences denote gaps introduced by the program for optimal alignment.


R2
415
439
HSV-1 RRl
HSV-2 RRl
VZV Vmw87
EBV Vmw93
Mouse Ml
E.coli Bl
Cl
C2
1
R2
489
537
R3
582
2
3
625
4
674
5
724
6
768
7
8
818


R3
848
R4
9
884
HSV-1 RR1 VS..RQ..TF DFGRLKDAVQ ACVLMVNI.. ....MIDSTL QPTPQCTK.. ....GNDNLRSM GIGMQGLHTA CLKLGLDLES
HSV-2 RR1 VS..RR..TF DFGMLKDAVQ ACVLMVNI.. ....MIDSTL QPTPQCAR.. ....GHDNLRSM GIGMQGLHTA CLKMGLDLES
VZV Vmw87 LS..KGPVSF NLNDLQLTAR TTVIFLNG.. ....VLAAGN FPCKKSCK.. ....GVKNNRSL GIGIQGLHTT CLRLGFDLTS
EBV Vmw93 LV..NA..PL AVRAQRADTQ GDELLLALPR LSVTLPGEGA VGDGFSLARL RDATQCATFV VACSILQGSP TYDSRDMASM GLGVQGLADV FADLGWQYTD
Mouse M1 VT..PEA.TY DFEKLAEVTK VIVRNLNK.. ....IIDINY YPIPEAHL.. ....SNKRHRPI GIGVQGLADA FILMKYPFES
E.coli B1 SAFNLGA.IN NLDELEELAI LAVRALDA.. ....LLDYQD YPIPAAKR.. ....GAMGRRTL GIGVINFAYY LANDGKRYSD
C1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- G-G------- ----------
C2 EEE------- --HHHHHHHH HHHHHEEE-- ---------- ----EE---- ---E-HH--- ---------- ---------- -EEEEEHHH HE--------
10
11
932
980
HSV-1 RR1 AEFQDLNKHI AEVM.LLSAM KTSNALC.VR GARPFNHFKR SMYRAGRFHW ERFPDARPRY EGE..WEMLR QSMMKHGLRN SQFVALMPTA ASAQISDVSE
HSV-2 RR1 AEFRDLNTHI AEVM.LLAAM KTSNALC.VR GARPFSHFKR SMYRAGRFHW ERFSNASPRY EGE..WEMLR QSMMKHGLRN SQFIALMPTA ASAQISDVSE
VZV Vmw87 QPARRLNVQI AELM.LYETM KTSMEMCQIG GLAPFKGFTE SKYAKGWLHQ DGFSTIS.YL DLP..WCTLR DDICAYGLYN SQFLALMPTV SSAQVTECSE
EBV Vmw93 PPSRSLNKEI FEHM.YFTEL CTSSLIG.LH TRKIFPGFKQ SKYAGGWFHW HDWAGTDLSI PREI.WSRLS ERIVRDGLFN SQFIALMPTS GCAQVTGCSE
Mouse M1 PEAQNLNKQI FETI.YYGAL EASCELAK.. EYGPYETYEG SPVSKGILQY DMWNVAP.TD LWD..WKPLK EKIAKYGIRN SLLIAPMPTA STAQILGNNE
E.coli B1 GSANNLTHKT FEAIQYYLLK ASNELAKEQG ACPWFNETTY AKGILPIDTY KKDLDTIANE PLHYDWEALR ESIKTHGLRN STLSALMPSE TSSQISNATN
C1 -----L---- -E-------- ---------- ---------- ---------- ---------- -----W--L- ------G--N S---A-MP-- ---Q------
C2 -HHHHHHHHH HHHHHHHHHH HHHHHHH--- ---------- ------EE-- ---------- --H-HHHHHH HHHHHE---- -EEEEEE--H HEEEEE----
12
13
14
1029
1079
HSV-1 RR1 GFAPLFTNLF SKVIRDGETL RPNTLLLKEL ERTFS.GKRL LEVMDSLDAK QWSVAQALPC LEPTHPLRRF KTAFDYDQKL LIDLCADRAP YVDHSQSMTL
HSV-2 RR1 GFAPLFTNLF SKVIRDGETL RPNTLLLKEL ERTFG.GKRL LDAMDGLEAK QWSVAQALPC LDPAHPLRRF KTAFDYDQEL LIDLCADRAP YVDHSQSMTL
VZV Vmw87 GFSPIYNNMF SKVITSGELL RPNLDLMDEL RDMYSCEEKR LEVINILEKN QWSVIRSFGC LSNSHPLLKY KTAFEYEQED LVDMCAERAP FIDQSQSMTL
EBV Vmw93 AFYPFYANAS TKVINKEEAL RPNRSFWRHV RLDDR..... EALNLVGGRB SCLPEALR.. ....QRYLRF QTAFHYNQED LIQLSRDRAP FVDQSQSHSL
Mouse M1 SIEPYTSNIY TRRVLSGEFQ IVNPHLLKDL TER....GLW NEEMKNQIIA CNGSIQSIPE IPDDLKQL.Y KTVWEISQKT VLKMAAERGA FIDQSQSLNI
E.coli B1 GIEPPRGYVS IKASKDGILR QVVPDYEHLH DAY....ELL WEMPGNDGYL QLVGIMQKFI D..... .....SISAN TNYDPSRFPS GKVPMQQ...
C1 ---P------ ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----Q----
C2 -E-------- EEE------- -----HHEHH HHH-----HH HHHHHHHHH- E----E-E-- ------HHH- ---HHHHHHH HHHHHH---- H--------E
15
1129
R4
HSV-1 RR1 YVTEKADGTL PASTLVRLLV HAYKRGLKTG MYYCKVRKAT NSGVFGGDDN IVCMSCAL
HSV-2 RR1 YVTEKADGTL PASTLVRLLV HAYKRGLKTG MYYCKVRKAT NSGVFAGDDN IVCTSCAL
VZV Vmw87 FIEEKPDGTI PASKIMNLLI RAYKAGLKTG MYYCKIRKAT NSGLFAGGEL .TCTSCAL
EBV Vmw93 FLREEDAARA ST..LANLLV RSYELGLKTI MYYCRIEKAA DLGVMECKAS AALSVPREEQ NERSPAEQMM PPKPMEPAQV AGPVDIMSKG PGEGPGGWCV
Mouse M1 HIAEPNYGK. ....LTSMHF YGWKQGLKTG MYYLRTRPAA NPIQFTLNKE KLKDKEKALK EEEEKERNTA AMVCSLENRE ECLMCGS
E.coli B1 ....LLKDLLT .AYKFGVKT. LYYQNTRDGA EDAQDDLVPS IQDDGCESGA CKI
C1 ---------- ----L----- -----G-KT- -YY------- ---------- ---------- ---------- ---------- ---------- -----
C2 EEEHH----- ----HHHEHH HHHH------ EEEEEEE--- --EEEE---- ---------- ---------- ---------- ---------- -----
EBV Vmw93 PGGLEVCYKY RQLFSEDDLL ETDGFTERAC ESCQ

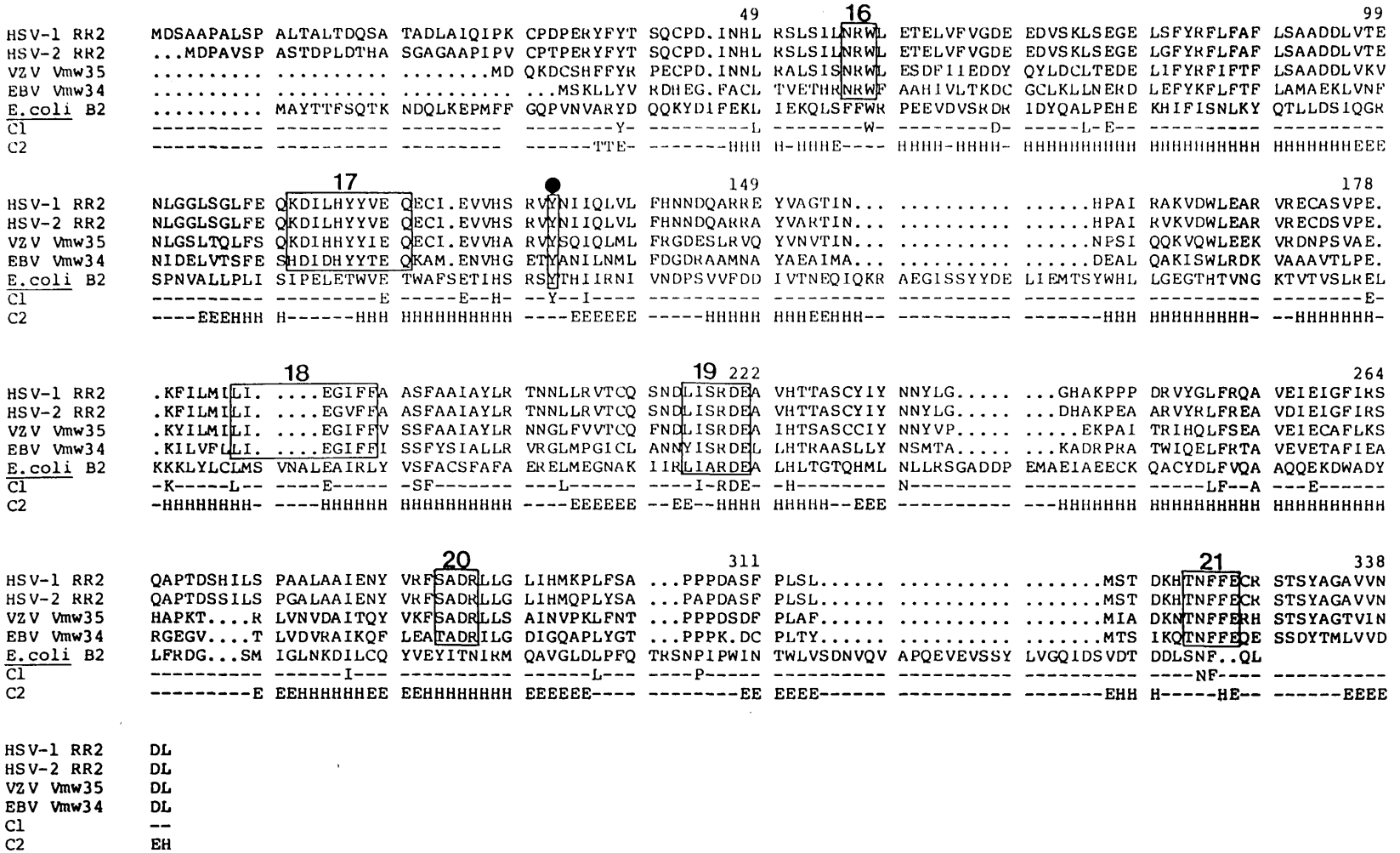

16
17
18
19
20
21
49
99
149
178
222
264
311
338
HSV-1 RR2
HSV-2 RR2
VZV Vmw35
EBV Vmw34
E.coli B2
C1
C2

Figure 57. Alignment of the identified or proposed ribonucleotide reductase small subunit polypeptides of viral eukaryotic and prokaryotic origin (RR_S). The first consensus line (C1) displays the amino acids conserved in all RR_S and the second line (C2) shows the consensus GARNIER secondary structure prediction for each aligned position. (E) represents β-strand conformation and (H) represents α-helical conformation. The proposed location of the tyrosyl radical is indicated (●) and amino acids sequences of higher homology are boxed. The amino acid positions of the alignment are numbered as in HSV-1 RR2 (McGeoch et al., 1988), and dots within the aligned sequences denote gaps introduced for optimal alignment.

comparisons only block 19 was retained in all the RR_S polypeptides.

DISCUSSION.

In the discussion of this Section the available biochemical data are combined with structural features identified from the RR_L and RR_S alignments.

10. Structural and Functional Features of the RR_L Polypeptides.

a) Region 1.

The function of the HSV Region 1 is at present unknown. The absence of this region from other viral RR_L and the amino acid length of the mammalian and E. coli polypeptides would probably suggest that it is not required for enzyme activity. As reported in the Introduction (see Page 29), proteolytic degradation of HSV-1 RR1 gives rise to products of 110,000, 93,000 and 81,000 mol. wt. (Ingemarson and Lankinen, 1987). In vitro enzyme activity assays with these products demonstrated that at least the 93,000 mol. wt. was able to associate with RR2 to form an active complex. The 81,000 mol. wt. may be active as well, however, at that stage of proteolysis the RR2 subunit had dissappeared and therefore a functional complex was not formed. So far there is no indication about the exact polypeptide positions where proteolysis takes place; perhaps it occurs after HSV-1 RR1 aa 146 since an oligopeptide antiserum directed against amino acids 137 through to 146 failed to label the proteolysis products in immunoblots (H. Lankinen, personal communication). Thus, it can be proposed that, at least in vitro, the first part of the N-terminal region is not required for enzymatic activity.

On the other hand, the observed conservation of these regions in both HSV serotypes and the similarity of their predicted secondary structures suggest that there is

evolutionary pressure at the level of protein structure. A possible structure that would incorporate the GARNIER predictions for these regions and a putative function are discussed in the General Discussion.

b) Region 2.

On the basis that conserved regions of enzymes which catalyse the same reaction are important for activity it is possible that Region 2, which is the least conserved RR_L region, is not directly involved in enzymatic activity. However, this region is present in all RR_L polypeptides and is well conserved between the viral RR_L (percentage homology ranging from 24% to 32%) and between the prokaryotic and eukaryotic RR_L (percentage homology 26%); thus it is possible that Region 2 has a structural role in the adopted tertiary structure of the enzyme.

The DNA regions of the HSV-2 genome contained in the Bgl II c and n fragments (HSV-2 map units 0.54 to 0.58 and 0.58 to 0.62 respectively) appear to be involved in morphological transformation of cultured cells (reviewed in Macnab, 1987). Jones, C., et al. (1986) reported that the minimal region of the right-hand 30% of Bgl II c fragment, responsible for transformation of immortalised cell lines, comprises the DNA region coding for the last 60 amino acids of RR1 Region 1 and the first 100 amino acids of Region 2. These authors proposed that transformation may be initiated by insertion sequences and alternating purine- and pyrimidine-rich stretches contained within these DNA coding regions; further, the inability of the respective HSV-1 RR1 DNA coding regions to induce transformation was attributed to the absence of similar structures from the latter regions. However, such sequence elements are frequently present in the HSV-2 DNA and hence their correlation with any function remains to be established (reviewed in Macnab, 1987).

c) Region 3.

This region, which is the most well conserved region, appears to be directly involved in enzymatic activity.

Seventeen amino acids upstream from block 4, Eriksson et al. (1986) identified, using photoaffinity labelling reactions, an E. coli cysteine residue on which the dTTP allosteric effector was localised; this cysteine was part of a tryptic octapeptide with sequence of KSCSQGGV (see Fig. 56, HSV-1 RR1 aa 642 to 649). Similar photoaffinity experiments with the mouse M1 subunit gave several-fold lower dTTP incorporation (Eriksson et al., 1986). This result could be explained by the fact that, although the equivalent M1 sequence aligns well with the B1 sequence, the former does not contain a cysteine residue (see Fig. 56; octapeptide RYVDQGGN). It was therefore proposed that the cysteine is part of the h allosteric site of the E. coli enzyme. However, photoaffinity labelling experiments with a mutant B1 polypeptide where the cysteine was substituted to an alanine showed that, although the mutant B1 was not labelled in the presence of dTTP, it still had 60% activity in the presence of the general effector ATP and 40% in the presence of dTTP (Nilsson et al., 1988b); these results indicate that the mutation did not confer a substantial conformational change of the allosteric site. Clearly, further studies are required to assess whether this cysteine binds the effector or that it provides an active side-chain in the vicinity of the allosteric site.

As already mentioned (see Introduction, Page 50) the VV-specified activity appears to be allosterically regulated. Interestingly, the VV Vmw86 polypeptide which is thought to be the large subunit polypeptide of the VV-specified enzyme, aligns extremely well with the B1 octapeptide. In contrast, the HSV enzyme which is insensitive to allosteric regulation by dNTPs (see Introduction, Page 50) does not contain an equivalent region (see Fig. 56).

Further downstream from the proposed allosteric site, there is a highly conserved sequence of GEEFE (see Fig. 56, block 6). Comparison of this sequence with protein sequences contained in the NBRF data base with the WORDSEARCH program (see Materials and Methods, Page 75) revealed that it was present in adenylate kinase (AK). AK is an enzyme involved in the dephosphorylation of MgATP to

MgADP and binds the substrate in a site similar to the proposed RR_L binding site (see below). The tertiary structure of AK revealed that GEEFE is part of an α-helix located close to the ATP-binding site (von Zabern et al., 1976); further, the side-chain of a leusine which is located 11 residues upstream from GEEFE was proposed to participate in the formation of the hydrophobic pocket that binds the adenine moiety of ATP (Fry et al., 1985). In common with the crystallographic data of AK, the RR_L GEEFE block is shown to adopt an α-helical conformation and, moreover, upstream from this block there are two conserved leucines (see Fig. 56). Although in the RR_L polypeptides the block and the adjacent leucines are positioned 130 amino acids upstream from the RR_L binding site (see below), whereas, in AK they are 90 amino acids downstream from the binding site, it is possible that they participate in the formation of the hydrophobic pocket of the RR_L binding site.

Thirty six amino acids downstream from block 6 there is a conserved glycine (see Fig. 56, at HSV-1 aa 764). Platz et al. (1985) reported that substitution of this residue with serine in the E. coli B1 polypeptide drastically reduced the subunit activity (90 to 95%) for all four substrates in vitro. Thus, it was argued that this glycine participates in the formation of the proposed binding site. Alternatively, the serine may have disrupted the three-dimensional structure of the enzyme in such a way, that the enzymatic activity is abolished although it is notable that the glycine and serine residues are considered as conserved ones according to the Venn Diagram of amino acid classification (Taylor, W.R., 1986a).

Finally, Region 3 contains all the conserved cysteines; two in blocks 2 and 7 and a third one in block 8 although the latter is not present in the E. coli polypeptide. These conserved cysteines are of special interest because two of them could contribute redox-active sulphydryls to the composite catalytic site of the enzyme.

d) Region 4.

The first block within this region (block 9), has a consensus sequence of GxGxxG, although the last glycine

is replaced by a glutamine in the E. coli polypeptide (see Fig. 56). This consensus is identical with nucleotide binding site sequences which are shown to adopt a βαβ secondary structure (βαβ-fold). Wierenga et al. (1985) reported that the most important ('fingerprint') amino acids of these sites are (Fig. 58a):

i) three glycines; the first one forms the C-terminus of the first β-strand and the second is the N-terminus of the adjacent α-helix. The first and third glycines form a sharp turn between the β-strand and the α-helix, whereas, the second interacts with the nucleotide phosphate moiety.

ii) six hydrophobic residues, which are indicated by squares in Fig. 58a. Two of these are located upstream and four downstream from the GxGxxG pattern and form the hydrophobic core of the fold.

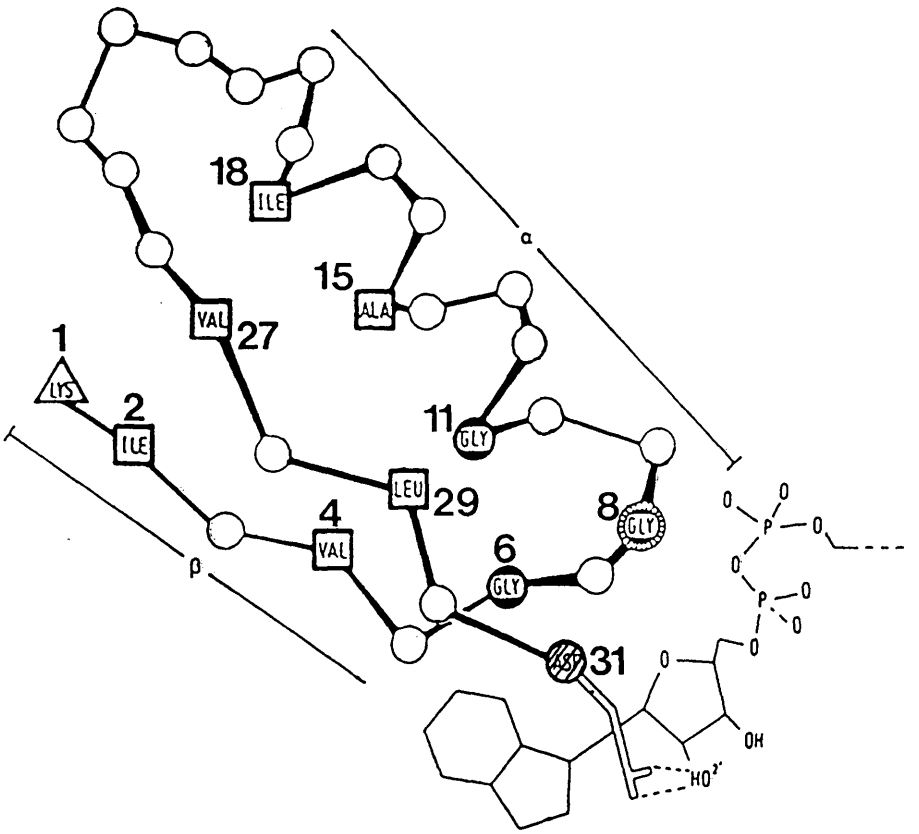
iii) a negatively charged residue (either aspartic acid or glutamic acid) at the end of the second β-strand which hydrogen bonds with the 2'-hydroxyl group of the nucleotide ribose moiety and, finally,

iv) a hydrophilic residue which is indicated by a triangle in Fig. 58a. This is positioned 5 amino acids upstream of the first glycine and its function is not known.

In most of the binding sites identified so far, the glycines and the negatively charged residue at the end of the fold are strongly retained, whereas, variation has been observed in the hydrophobic residues (Sternberg and Taylor, 1984; Wierenga et al., 1986). The length of the binding folds varies from 29 to 31 residues due to the variable length of the loop connecting the α-helix with the second β-strand.

The alignment of the RR_L polypeptides in this region shown in Fig. 58b is different from the one shown in Fig. 56 as spaces (indicated by *) were introduced to optimise the binding fold requirements as reported by Wierenga et al. (1986). All the spaces were introduced between positions 21

(a)



(b)

	1	2	4	6	8	11	15	18				27	29	31
	△	■	■	G	G	G	■	■				■	■	◕
HSV RR1	N	L	S	G	G	G	A	K			*	A	F	D
VZV Vmw87	N	N	S	G	G	G	T	R		*	*	Q	A	R
EBV Vmw93	D	M	S	G	G	G	V	D	*	*	*	T	P	S
Mouse M1	R	H	P	G	G	G	A	L			*	P	A	L
E. coli B1	G	R	T	G	G	N	Y	K			*	G	A	N
HCMV M4.PRO	K	L	P	H	G	G	A	R				W	L	E
VV Vmw86	R	H	P	G	G	G	A	L			*	L	A	D

Figure 58.

a) A representation of the secondary structure of the spiny dogfish M-lactate dehydrogenase ADP-binding fold (reprinted from Wierenga et al., 1986). The 'fingerprint' amino acids of the fold are shown in the three letter amino acid code and are numbered. The triangle represents the hydrophilic residue and the hatched circle the negatively charged residue. Boxes represent the hydrophobic residues and semi-filled circles the glycines. The negatively charged residue (aspartic acid) is depicted to form a hydrogen bond with the 2'-hydroxyl group of the nucleotide ribose moiety.

b) Alignment of RR_L sequences proposed to be nucleotide binding folds. The 'fingerprint' amino acids are designated and numbered as above. Underlined amino acids represent residues not conforming with the 'fingerprint' positions of the fold as reported by Wierenga et al. (1986). Asterisks denote gaps introduced between positions 21 to 24 of the fold for optimal alignment.

through to 24 of the fold which correspond to the variable loop region. Further, homologous sequences of the HCMV M4.PRO ORF and the VV Vmw86 polypeptide, which were not available at the time of the alignment, have been included. As can be seen, the hydrophilic residues at the start of the sequences are well conserved, with the exception of B1 and M4.PRO, while a greater degree of variation is observed in the positions corresponding to hydrophobic residues; this is clearly apparent at position 4 where none of the aligned residues is hydrophobic. The glycines are well conserved in all the polypeptides, except the last one in the E. coli polypeptide and the first one in M4.PRO. Finally, the C-terminal negatively charged residue is present only in the HSV and VV polypeptides and in the M4.PRO. The secondary structure predictions identified the first β-strand and the adjacent α-helix but not the second β-strand (see Fig. 56). It is therefore apparent that the proposed RR_L binding fold differs in structure and in amino acid composition from the folds observed so far. However, as reported in the Introduction (see Section E), studies with the E. coli, HSV and VZV enzymes indicate one substrate binding site for all four substrates, and this could possibly influence the structure of the binding site (Nikas et al., 1986).

Wierenga et al., (1986) reported that the $\beta\alpha\beta$ binding folds always occur near the N-terminus of the nucleotide binding domains studied. In the RR_L alignment this sequence is located just 3 amino acids downstream from the second EBV insertion, and given that insertions are indicative of domain junctions, it can be proposed that Region 4 or part of it participates in the formation of the RR_L binding domain. Another feature of these folds is that, as they are located at the N-terminus of the binding domain and are formed by local interactions, it is possible that during the translation process they form spontaneously and then function as a nucleation centre for the folding of the domain (Wierenga et al., 1986).

11. Structural and Functional Features of the RR_S Polypeptides.

The RR_S comparison highlighted a number of important features, namely: the conservation of the tyrosine residue on which the radical is thought to be localised, conserved potential iron ligands and the primary structure of the RR_S C-termini.

a) The tyrosyl radical and the iron ligands.

As described in the Introduction (see Page 43) the E. coli tyrosine radical is stabilised by the iron centre which is liganded by one aspartic acid, one glutamic acid and five histidine residues. Further, individual alignments performed in the second part of Section B indicated that the E. coli tyrosine at position 122 was conserved in all of the herpesviral polypeptides with the exception of the HCMV M3.PRO ORF. It therefore became of interest to investigate whether the consensus template alignment program would predict that this tyrosine residue is conserved in all the RR_S polypeptides and, further, to identify conserved potential iron ligands.

As can be seen in Fig. 57 the tyrosine residue was predicted to be conserved in all the aligned RR_S polypeptides (at HSV-1 RR2 position 132); moreover, it is retained at the same position in other RR_S sequences which were not available at the time of the alignment with the exception of the HCMV M3.PRO. The program also identified two conserved aspartic acid residues at HSV-1 RR2 positions 68 and 220 which are conserved at identical positions in the non-aligned RR_S sequences. Finally, seven conserved glutamic acid residues were identified from which those at HSV-1 RR2 positions 119, 124 and 221 are conserved in non-aligned RR_S. Of particular interest are the conserved histidine and glutamic acid residues which are located upstream from the tyrosine residue. These residues are part of a predicted α-helix and the distance between them is approximately one helix turn. Thus it is possible that in the tertiary structure of the enzyme they are brought close to each other and ligate the iron atoms which subsequently stabilise the adjacent tyrosyl radical. Conserved aspartic

acid and glutamic acid residues can be found upstream from the second hystidine and these could equally be the iron ligands.

b) The RR_S C-terminal regions.

Another important RR_S region is at the C-termini. As mentioned in the Introduction (see Page 42) Sjoberg et al. (1987) encountered a truncated form of the E. coli B2 protein which lacked thirty C-terminal amino acids and which failed to associate with B1 although it had an almost identical conformation to the normal B2. Therefore, it is likely that the two subunits interact via the C-terminus of RR_S. The same suggestion can be proposed for the viral enzyme although the viral termini exhibit no homology with the respective E. coli region; in fact, only eleven E. coli C-terminal amino acids align with the viral terminal sequences and from these nine are not conserved. The suggestion is indirectly supported by studies with the oligopeptide antiserum directed against the seven C-terminal amino acids of HSV-2 RR2 (Frame et al., 1985). These authors demonstated that the antiserum precipitated only the free form of RR2 while it failed to precipitate its complexed form under non-denaturing conditions, thus indicating that the C-terminus of RR2 is masked by RR1.

Further, the sequncing data of Preston, V.G. et al. (1988) localised the nucleotide change in HSV-1 mutant ts1222 within the HSV-1 RR2 C-terminus. The change was shown to be a deletion of a thymine which resulted in the alteration of all but one amino acids at the RR2 C-terminus and the removal of the termination codon; the latter was positioned 42 residues downstream from the wild-type RR2 C-terminus. In the RR_S alignment, the mutation is localised within block 21 (see Fig. 57), and alters the second phenylalanine residue to a serine.

A summary of the RR_L and RR_S structural and functional features discussed in this Section is shown in Fig. 59.

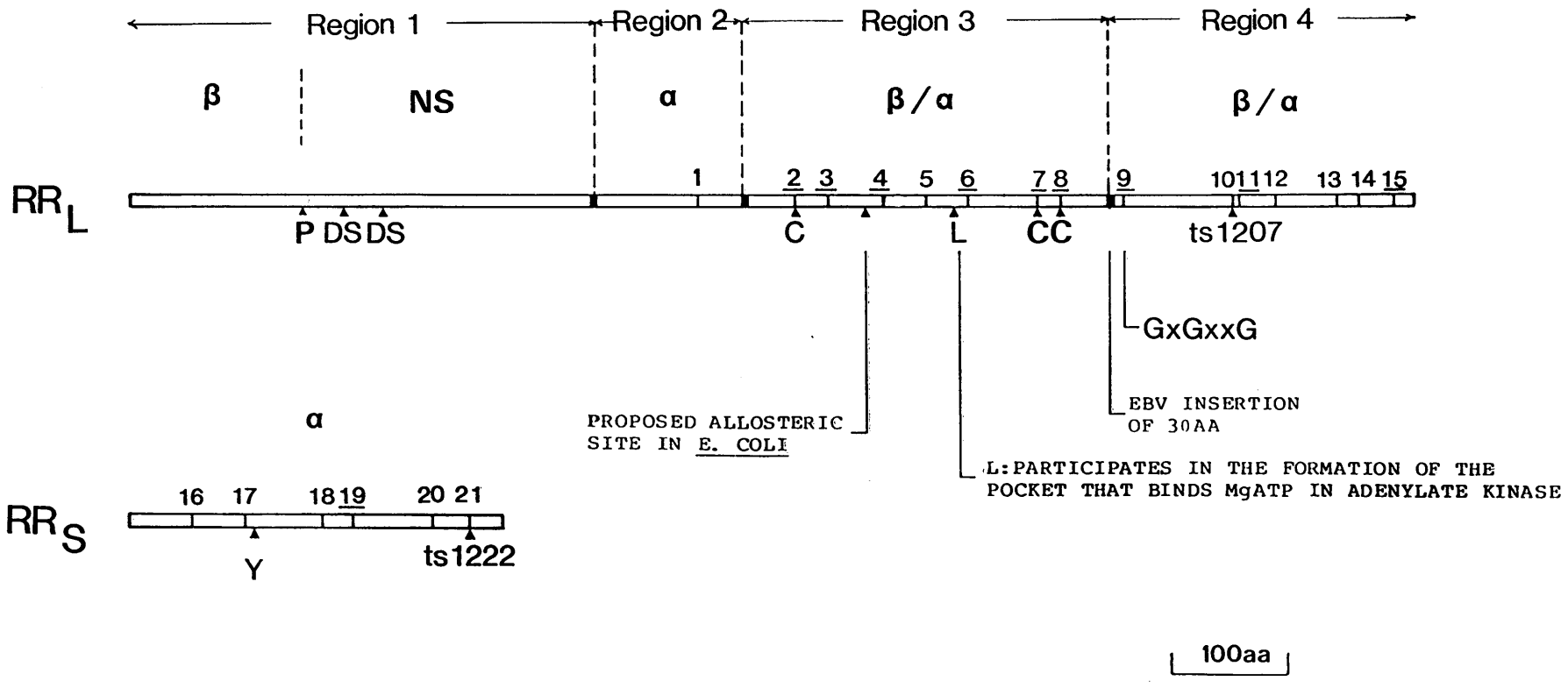

Region 1
Region 2
Region 3
Region 4
β
NS
α
β / α
β / α
RR L
1
2 3
4
5
6
7 8
9
10 11 12
13 14 15
P DS DS
C
L
CC
ts1207
GxGxxG
EBV INSERTION
OF 30AA
PROPOSED ALLOSTERIC
SITE IN E. COLI
L: PARTICIPATES IN THE FORMATION OF THE
POCKET THAT BINDS MgATP IN ADENYLATE KINASE
α
RR S
16 17
18 19
20 21
Y
ts1222
100aa

Figure 59. This diagram shows in a schematic form the combination of structural and biochemical features of the RR_L and RR_S polypeptides. Amino acid blocks of higher homology are indicated by numbers and blocks conserved in all the RR_L and RR_S polypeptides are underlined.

The RR_L polypeptides are divided in four Regions on the basis of the secondary structure predictions shown. (α) represents α-helix conformation, (β) represents β-strand conforamtion and unstructured or loop conformation is depicted by (NS). The locations of the run of prolines (P), the stretches of roughly alternating aspartic acid and serine residues (DS) and conserved cysteine residues (C) are indicated. A leucine residue which could participate in the formation of the RR_L nucleotide binding site (GxGxxG) is depicted by (L). Other RR_L features include the proposed allosteric site in E. coli, the major EBV insertion of 30 amino acids and the location of the amino acid substitution in the RR1 polypeptide encoded by the HSV-1 mutant ts1207 (see Page 111).

The RR_S polypeptides are shown as a single Region on the basis of the secondary structure predictions. The location of the conserved tyrosine residue on which the E. coli radical is localised is depicted by (Y) and the amino acid substitution in the RR2 polypeptide encoded by the HSV-1 mutant ts1222 is indicated.

SECTION D.

12. Sequence Determination of the Mutation(s) in the RR1 Xho I/Bgl II DNA Fragment of the HSV-1 mutant ts1207.

Dutia (1983) reported that the multiple HSV-1 mutant tsG failed to induce detectable ribonucleotide reductase levels at the NPT. As this isolate had multiple mutations, Preston, V.G. et al. (1984) isolated the Bam HI o fragment from tsG DNA, which spans the region between map units 0.574 to 0.600, and recombined it into HSV-1 strain 17^+ DNA giving rise to the HSV-1 ts1207 mutant virus. The mutation(s) was located, with marker rescue experiments, within the Xho I/Bgl II fragment contained in the coding sequences of RR1 (Fig. 60). Thus, it was of interest to identify any nucleotide changes within this fragment and from the deduced amino acid sequence to determine any effect of the mutation(s) on the predicted secondary structure of the enzyme.

For these reasons, the ts1207 Xho I/Bgl II fragment was isolated from a cloned Bam HI o fragment (obtained from Dr V.G. Preston) and sequenced with the M13 dideoxy/chain termination method. However, given the relatively small size of the fragment, it would be inappropriate to follow the 'shotgun' cloning approach followed for the nucleotide sequence determination of the RR1 coding region. Instead, the intact Xho I/Bgl II fragment was cloned into a Sma I digested M13mp8 RF vector. Then, with the use of the New England Biolabs primer (see Materials and Methods, Page 58), approximately 200 bases were read in either orientation. From the sequences at the ends of the obtained gel readings two new pentadecamers were synthesised, shown in Fig. 62, and these were used as primers in sequencing reactions thus advancing the data by approximately 200 bases in either direction. This procedure was repeated and finally the entire sequence was obtained in both orientations.

The sequencing data were collated with computer programs and gave rise to the M1207 data base. This data base contained 26 gel readings (Fig. 61). There were 4865 characters, which produced a final contiguous length of

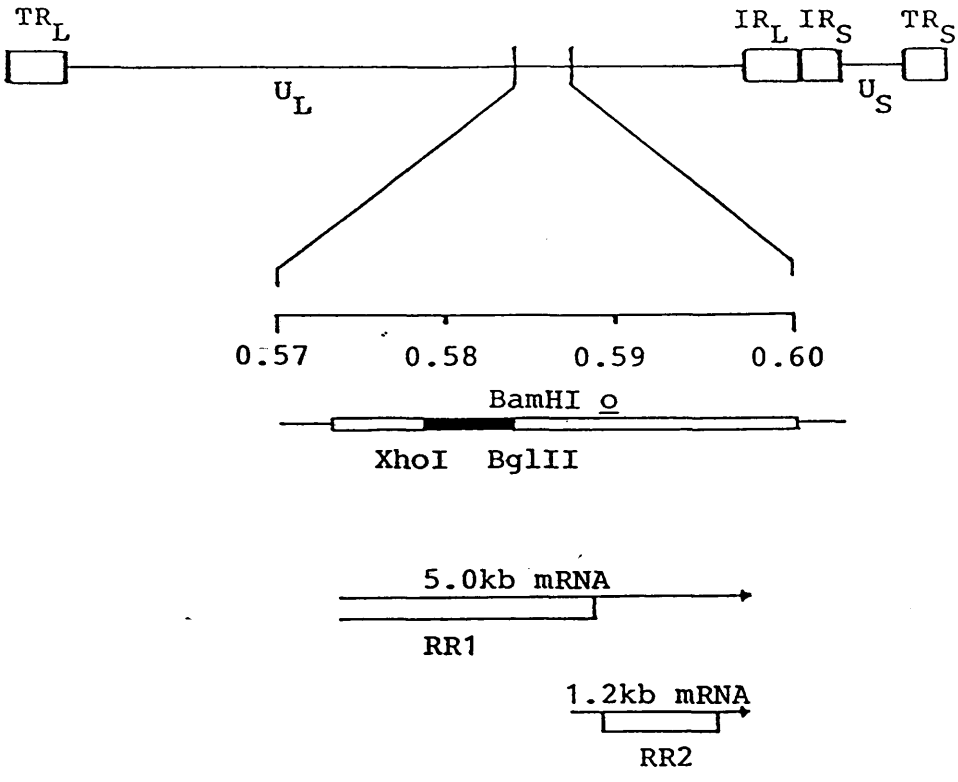


Figure 60. This diagram shows the location of the Xho I/ Bgl II fragment (black box) of the HSV-1 mutant ts1207 with respect to the Bam HI o fragment between 0.57 to 0.60 map units. The mRNAs specifying the RR1 and RR2 polypeptides are indicated as arrowed lines and their lengths are given above each line. The polypeptide coding regions are indicated as open boxes.

CONTIG LINES

	99	715	0	1	23
GEL LINES	A	B	C	D	E
G2.002	1	1	238	0	2
G2.003;1	2	1	268	1	26
G2.005;1	3	467	-223	4	9
G2.007;1	4	452	-238	7	3
G3.029;1	5	1	-47	0	0
G3.076;1	6	1	219	0	0
G3.041;1	7	391	228	18	4
G3.073;2	8	328	-38	19	11
G3.040	9	528	-188	3	24
G2.1P3	10	156	212	25	12
G3.073	11	328	-38	8	16
G4.1P2	12	156	241	10	15
G5.1P2	13	158	229	15	21
G5.1	14	2	228	26	25
G6.1P1	15	158	201	12	13
G6.1P4	16	377	218	11	17
G6.2P4	17	380	217	16	18
G6.3P4	18	381	208	17	7
G8.38	19	264	-180	22	8
G8.D	20	1	-193	0	0
G9.40	21	234	-209	13	22
G9.41	22	264	-174	21	19
G9.44	23	622	94	24	0
G9.45	24	617	99	9	23
G10.62	25	11	-216	14	10
G10.63	26	2	-221	2	14

Figure 61. A sorted list (CONTIG LINES) of the gel readings within the M1207 data base. The column termed GEL LINES represents the name of the clone given by the author. The columns headed A to E are assigned by the DBUTIL program (see Materials and Methods, Page 73) and represent:

A Gel reading database number.

B Position of the left-most base of the gel reading in the contig.

C Length of the gel reading. (-) indicates that the gel reading overlaps on the opposite strand.

D Number of the gel reading overlapping to the left.

E Number of the gel reading overlapping to the right.

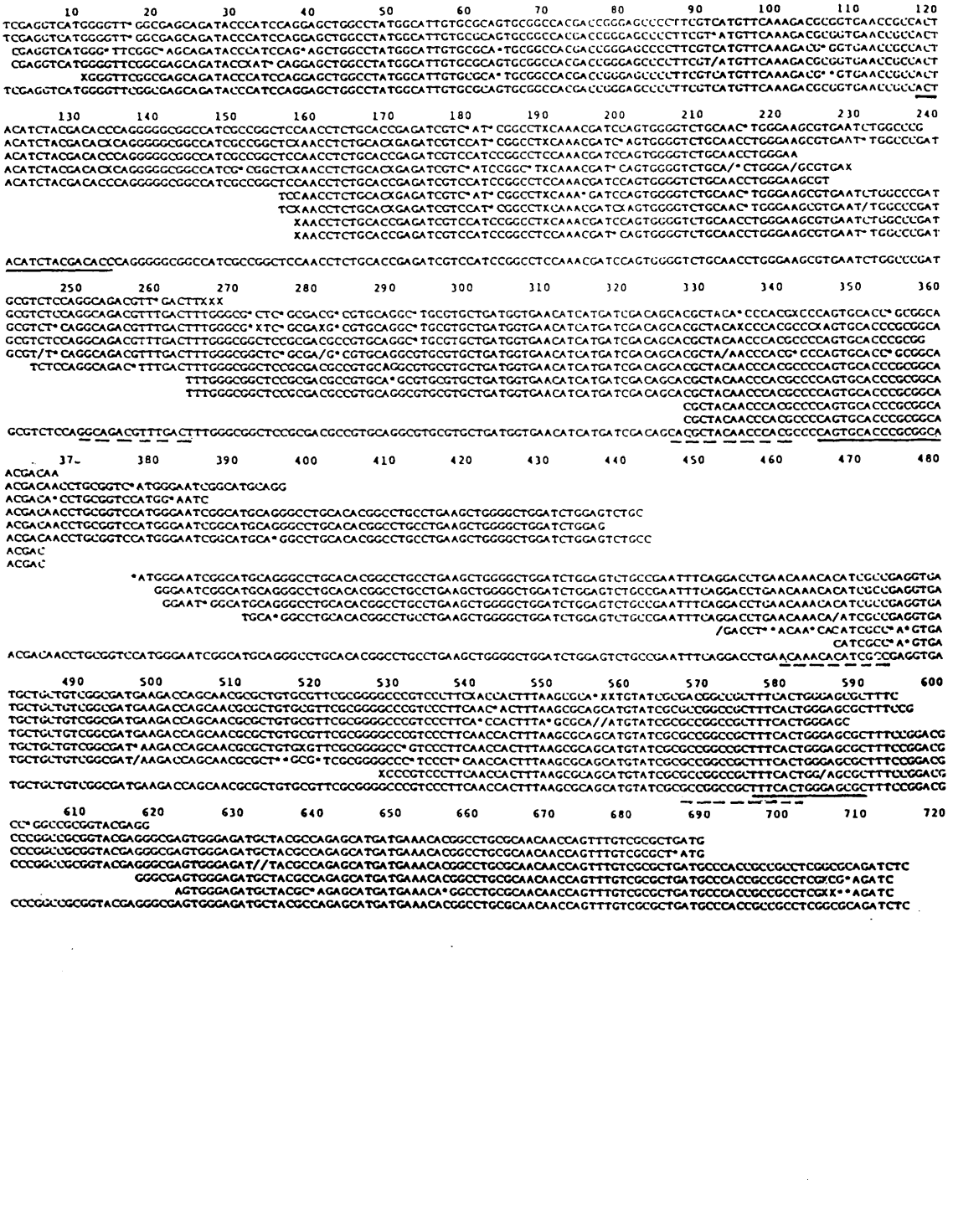


Figure 62. The M1207 database. All the gel readings in the database are shown numbered down the left side of the sequences as in column A of Figure 61. Computer inserted characters into the gel readings entering the contig database are indicated by (*) while those inserted by the author are indicated by (X) or (/). Underlined sequences represent synthetic oligonucleotide primers used in sequencing reactions; primers built in the left-to-right orientation are indicated by solid lines and those built in the complementary orientation are indicated by dashed lines.

715bp (Fig. 62). For each contig character, an average of 6.8 characters were read from gels.

The obtained nucleotide sequence of the mutant Xho I/Bgl II fragment was compared with that of the equivalent wild-type fragment with the HOMOL program (Fig. 63). As can be seen, the program identified two nucleotide changes both from a guanine to an adenine at positions 2439 and 2915 of the HINDKK database consensus sequence (see Fig. 26); the nucleotide changes can also be observed in the sequence autoradiograph comparisons in Fig. 64. Translation of the wild-type and mutant fragments with the PTRANS program indicated that the first nucleotide change did not alter the encoded amino acid whereas the second changed a serine residue to an asparagine (see Fig. 63).

DISCUSSION.

13. The Mode of Action of BUdR as a Mutagen.

Both nucleotide changes in the ts1207 fragment were transitions from a guanine:cytosine pair to an adenine:thymine pair. These changes are compatible with the procedure followed in order to isolate the initial mutant tsG by Brown et al., (1973); these authors treated HSV-1 strain 17 syn infected cells with 5'-bromodeoxyuridine (BUdR). The base of BUdR, 5'-bromouracil (BU), is similar to thymine with the exception that BU has a bromine atom in place of the 5'-methyl group of the thymine ring (reviewed in Freese, 1963). This mutagen can be in the keto and enol states.

In the keto state, the deoxyribonucleoside triphosphate of BU (dBUTP) can base pair to adenine with two hydrogen bonds thus incorporating the deoxyribonucleoside monophosphate of BU (dBUMP) in DNA. This increases the probability of this base pair to mutate because BU undergoes pairing mistakes more frequently than thymine; in the next DNA duplication event, dGTP may pair with dBUMP thus leading to the appearance of a guanine:cytosine pair in the progeny

First sequence: HSV-1 RR1
Second sequence: HSV-1 ts1207 RR1

```
 E   V   M   G   F   G   E   Q   I   P   I   Q   E   L   A   Y   G   I   V   R   S   A   A   T   T   G   S   P     766
GAG GTC ATG GGG TTC GGC GAG CAG ATA CCC ATC CAG GAG CTG GCC TAT GGC ATT GTG CGC AGT GCG GCC ACG ACC GGG AGC CCC   2331
GAG GTC ATG GGG TTC GGC GAG CAG ATA CCC ATC CAG GAG CTG GCC TAT GGC ATT GTG CGC AGT GCG GCC ACG ACC GGG AGC CCC   2331

 F   V   M   F   K   D   A   V   N   R   H   Y   I   Y   D   T   Q   G   A   A   I   A   G   S   N   L   C   T     794
TCC GTC ATG TTC AAA GAC GCG GTG AAC CGC CAC TAC ATC TAC GAC ACC CAG GGG GCG GCC ATC GCC GGC TCC AAC CTC TGC ACC   2415
TCC GTC ATG TTC AAA GAC GCG GTG AAC CGC CAC TAC ATC TAC GAC ACC CAG GGG GCG GCC ATC GCC GGC TCC AAC CTC TGC ACC   2415

 E   I   V   H   P   A   S   K   R   S   S   G   V   C   N   L   G   S   V   N   L   A   R   C   V   S   R   Q     822
GAG ATC GTC CAT CCG GCC TCC AAG CGA TCC AGT GGG GTC TGC AAC CTG GGA AGC GTG AAT CTG GCC CGA TGC GTC TCC AGG CAG   2499
GAG ATC GTC CAT CCG GCC TCC AAA CGA TCC AGT GGG GTC TGC AAC CTG GGA AGC GTG AAT CTG GCC CGA TGC GTC TCC AGG CAG   2499

 T   F   D   F   G   R   L   R   D   A   V   Q   A   C   V   L   M   V   N   I   M   I   D   S   T   L   Q   P     850
ACG TTT GAC TTT GGG CGG CTC CGC GAC GCC GTG CAG GCG TGC GTG CTG ATG GTG AAC ATC ATG ATC GAC AGC ACG CTA CAA CCC   2583
ACG TTT GAC TTT GGG CGG CTC CGC GAC GCC GTG CAG GCG TGC GTG CTG ATG GTG AAC ATC ATG ATC GAC AGC ACG CTA CAA CCC   2583

 T   P   Q   C   T   R   G   N   D   N   L   R   S   M   G   I   G   M   Q   G   L   H   T   A   C   L   K   L     878
ACG CCC CAG TGC ACC CGC GGC AAC GAC AAC CTG CGG TCC ATG GGA ATC GGC ATG CAG GGC CTG CAC ACG GCC TGC CTG AAG CTG   2667
ACG CCC CAG TGC ACC CGC GGC AAC GAC AAC CTG CGG TCC ATG GGA ATC GGC ATG CAG GGC CTG CAC ACG GCC TGC CTG AAG CTG   2667

 G   L   D   L   E   S   A   E   F   Q   D   L   N   K   H   I   A   E   V   M   L   L   S   A   M   K   T   S     906
GGG CTG GAT CTG GAG TCT GCC GAA TTT CAG GAC CTG AAC AAA CAC ATC GCC GAG GTG ATG CTG CTG TCG GCG ATG AAG ACC AGC   2751
GGG CTG GAT CTG GAG TCT GCC GAA TTT CAG GAC CTG AAC AAA CAC ATC GCC GAG GTG ATG CTG CTG TCG GCG ATG AAG ACC AGC   2751

 N   A   L   C   V   R   G   A   R   P   F   N   H   F   K   R   S   M   Y   R   A   G   R   F   H   W   E   R     934
AAC GCG CTG TGC GTT CGC GGG GCC CGT CCC TTC AAC CAC TTT AAG CGC AGC ATG TAT CGC GCC GGC CGC TTT CAC TGG GAG CGC   2835
AAC GCG CTG TGC GTT CGC GGG GCC CGT CCC TTC AAC CAC TTT AAG CGC AGC ATG TAT CGC GCC GGC CGC TTT CAC TGG GAG CGC   2835

 F   P   D   A   R   P   R   Y   E   G   E   W   E   M   L   R   Q   S   M   M   K   H   G   L   R   N   S   Q     962
TTT CCG GAC GCC CGG CCG CGG TAC GAG GGC GAG TGG GAG ATG CTA CGC CAG AGC ATG ATG AAA CAC GGC CTG CGC AAC AGC CAG   2919
TTT CCG GAC GCC CGG CCG CGG TAC GAG GGC GAG TGG GAG ATG CTA CGC CAG AGC ATG ATG AAA CAC GGC CTG CGC AAC AAC CAG   2919
                                                                                                    N

 F   V   A   L   M   P   T   A   A   S   A   Q   I                                                             975
TTT GTC GCG CTG ATG CCC ACC GCC GCC TCG GCG CAG ATC                                                           2958
TTT GTC GCG CTG ATG CCC ACC GCC GCC TCG GCG CAG ATC                                                           2958
```

Figure 63. Alignment of the nucleotide sequences of the Xho I/Bgl II fragment of the HSV-1 wild-type and HSV-1 ts1207 RR1 DNA coding regions. The predicted amino acid sequence of the HSV-1 RR1 polypeptide is shown above the HSV-1 DNA sequence, and the amino acid residue which is altered in HSV-1 ts1207 RR1 is indicated below the ts1207 DNA sequence. Conserved bases are indicated by asterisks and the nucleotide and amino acid positions are numbered as in Fig. 26.

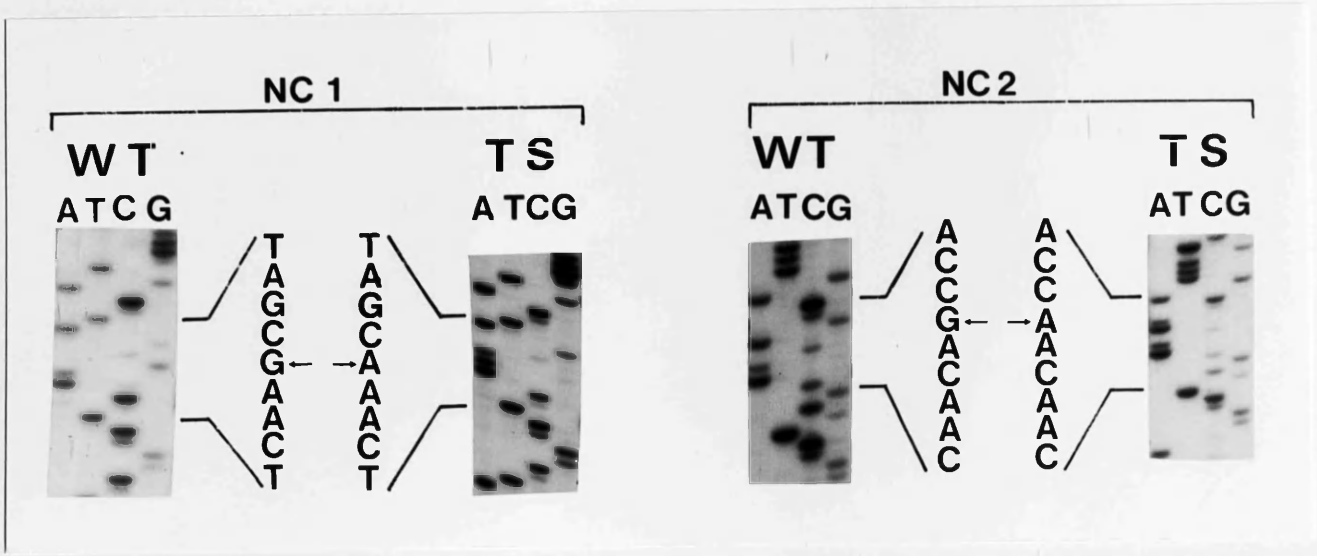

NC 1
WT
ATCG
TS
ATCG
TAGCGAACT
TAGCAAACT
NC 2
WT
ATCG
TS
ATCG
ACCGACAAC
ACCAACAAC

Figure 64. Autoradiographs of sequencing gels showing the nucleotide changes (NC) in HSV-1 ts1207 (TS) compared to the HSV-1 wild-type (WT) sequence. The wild-type and substituted nucleotides are indicated by arrows. The panel designated NC1 shows the first nucleotide change at position 2439 and the panel designated NC2 shows the second nucleotide change at position 2915 (see Fig. 63).

DNA in place of the original adenine:thymine pair.

In the enol state, dBUTP can base pair with a guanine present in DNA with three hydrogen bonds thus incorporating dBUMP in DNA. In the next DNA duplication event, dBUMP can base pair with dATP, adenine being the 'normal' complementary base of BU, and dAMP is incorporated into the new strand. Thus, an adenine:thymine pair appears in some of the progeny DNA in place of the original guanine:cytosine pair.

14. How does the Mutation in HSV-1 ts1207 Inhibit the Association of the RR1 and RR2 Subunits?

As reported in the Introduction (see Page 49), Preston, V.G. et al. (1984) demonstrated that the mutation in the RR1 polypeptide resulted in the inability of ts1207 to express any detectable activity at the NPT. The mechanism by which the mutation inhibits ribonucleotide reductase activity was investigated by means of immunoprecipitation studies using a monoclonal antibody directed against RR1 and an anti-oligopeptide serum directed against the RR2 (Frame et al., 1985). It was shown that, at the PT and in the presence of 0.5% SDS, the antibody and the antiserum precipitated both polypeptides. At the NPT and in the presense of equal SDS concentrations, however, the antibody precipitated only RR1 while the antiserum precipitated only RR2.

These results indicated that, although at the NPT both RR1 and RR2 polypeptides are expressed, the mutation in RR1 prevents the respective subunits from forming a functional complex. On that basis and in the absence of any biochemical data it was proposed that the region where the ts1207 mutation lies could either be a subunit contact region or an important structural RR1 region. These suggestions resulted from a comparison of the predicted secondary structures of the wild-type and mutant RR1 polypeptides with the CHOUFAS program (see Materials and Methods, Page 74). This program predicted that the wild-type RR1 serine residue is located in an unstructured region between an α-helix and a β-strand (Table IV); this

TABLE IV

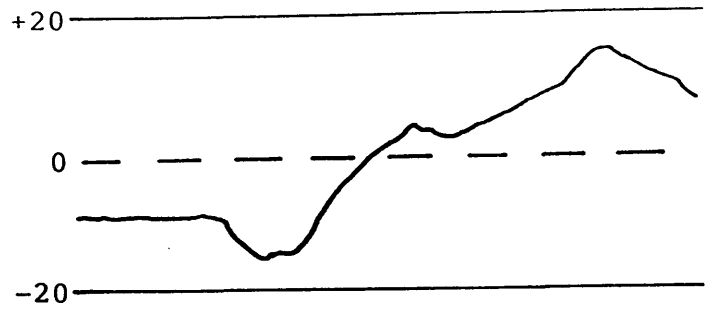
SECONDARY STRUCTURE PREDICTIONS FOR THE HSV-1 RR1 AND HSV-1 ts1207 MUTANT RR1 POLYPEPTIDES

AA Number	HSV-1 RR1 AA	Hy	SS	HSV-1 ts1207 RR1 AA	Hy	SS
950	R	0.300	H	R	0.300	H
951	Q	-0.314	H	Q	-0.314	H
952	S	0.300	H	S	0.300	H
953	M	0.486	H	M	0.486	H
954	M	0.057	H	M	0.057	H
955	K	-0.229	H	K	-0.229	H
956	H	0.157	H	H	0.157	H
957	G	0.371	H	G	0.371	H
958	L	0.600	H	L	0.568	H
959	R	0.200	t	R	0.186	t
960	N	-0.086	t	N	-0.100	t
961	S	-0.300		N	-0.314	t
962	Q	-0.114	B	Q	-0.129	H
963	F	-0.800	B	F	-0.814	H
964	V	-1.014	B	V	-1.029	H
965	A	-1.057	B	A	-1.057	H
966	L	-1.143	B	L	-1.143	H
967	M	-0.857	h	M	-0.857	H
968	P	-0.714	h	P	-0.714	h
969	T	-0.600	h	T	-0.600	h
970	A	-0.414	h	A	-0.414	h

AA = amino acid
Hy = hydropathicity value
SS = secondary structure prediction
H = strong α-helical conformation
h = α-helical conformation
B = β-strand conformation
t = turn

prediction is in good agreement with the individual GARNIER prediction for the HSV-1 RR1 polypeptide obtained with the consensus template alignment program (data not shown). In contrast, the asparagine of the mutant RR1 polypeptide, which substituted the wild-type serine, was predicted to adopt a turn conformation; further, the β-strand following the wild-type serine was altered to an α-helix in ts1207 (ts1207 positions 961 to 968; Table IV). These were the only changes in the secondary structure of the mutant RR1 as compared to that of the wild-type RR1 (data not shown). Hence, if the region where the ts1207 mutation is located is a subunit contact region, alteration of its secondary structure would inhibit subunit association. Equally, if this region is an essential structural RR1 region not directly involved in subunit association, alteration of its secondary structure may alter the structure of a 'true' subunit contact region(s) positioned elsewhere along the polypeptide backbone; as a result, the two subunits unable to associate.

Another reason for proposing that the mutation in ts1207 may lie within a contact region or within a region important for RR1 structure is the primary structure of this region. As can be seen in Fig. 65, the wild-type serine is located within block 10 of the RR_L alignment. This residue is a polar one according to the Venn diagram of amino acid classification (see Fig. 18). Polar amino acids are usually positioned in a hydrophilic region (exposed region) of the protein and are the least conserved amino acids between related proteins (Creighton, 1983). As can be seen in Fig. 65, the serine and the adjacent polar asparagine residue are located close to the end of a hydrophilic HSV-1 RR1 region which, notably, was predicted to be the longest hydrophilic region of the polypeptide (see Page 81). However, both amino acids are highly conserved in the herpesviral, mammalian and E. coli RRL polypeptides and this would suggest that these residues may have a specific function in RR1. On the basis that substitution of the serine in ts1207 inhibits subunit association, this function would most probably be related to subunit interaction or to the structure of RR1 although in the latter case it would not be



RR_L	Sequence	AA Position
HSV-1 RR1	M K H G L R N S Q F V A L M P T A	(954-970)
HSV-2 RR1	M K H G L R N S Q F I A L M P T A	(961-967)
VZV Vmw87	C A Y G L Y N S Q F L A L M P T V	(592-608)
EBV Vmw93	V R D G L F N S Q F I A L M P T S	(580-596)
Mouse M1	A K Y G I R N S L L I A P M P T A	(580-596)
E. coli B1	K T H G L R N S T L S A L M P S A	(607-623)

Block 10 Block 11

Figure 65. Alignment of the proposed or identified large subunit ribonucleotide reductase polypeptides (RR_L) in the region where the HSV-1 *ts*1207 amino acid change is located. The amino acid position of each polypeptide region is given in parentheses. RR_L sequences contained within blocks 10 and 11 of Fig. 56 are shown. The diagram above the alignment shows the hydropathicity plot corresponding to the aligned HSV-1 RR1 region. Hydrophobic regions lie between 0 and 20 and hydrophilic regions between 0 and -20. Program settings were: group length = 9, shift = 1, spacing of plotted points = 10.

directly involved in contact with RR2. In either case, the ts1207 mutation would result in the two subunits being unable to associate.

Experiments designed to distinguish between these possibilities suggest that the region where the mutation lies is not involved in subunit association (A.J. Darling, personal communication). First, high concentrations of a synthetic oligopeptide representing the amino acid sequences flanking the wild-type serine residue (oligopeptide RNSQFVALMPTA, spanning HSV-1 RR1 positions 959 to 969; see Fig. 63), failed to inhibit wild-type enzymatic activity. Second, this oligopeptide failed to inhibit the *in vitro* reconstituted activity from the non-defective ribonucleotide reductase large and small subunits encoded by the HSV-1 ts1222 and ts1207 mutants respectively, as did an antibody raised against this oligopeptide. Third, a second synthetic oligopeptide with the serine residue positioned approximately in the middle (oligopeptide MKHGLRNSQFVALMPT at HSV-1 RR1 positions 954 to 968; see Fig. 63) failed to inhibit the ts1207/ts1222 *in vitro* reconstituted activity. Therefore, it appears more likely that the region where the ts1207 mutation is located is an important structural RR1 region.

Finally, another possibility, which was not previously considered, was that the region where the mutation lies is a contact region between the RR1 polypeptides forming the RR1 subunit as opposed to an RR1/RR2 contact region. If the former was the case, the ts1207 mutation may prevent the formation a functional RR1 subunit in ts1207-infected cells and this may be the reason for the two subunits being unable to associate. However, this possibility was also excluded since the size of the HSV-1 ts1207-encoded RR1 subunit was found to be identical to the size of the wild-type RR1 subunit by means of gel filtration (A.J. Darling, personal communication).

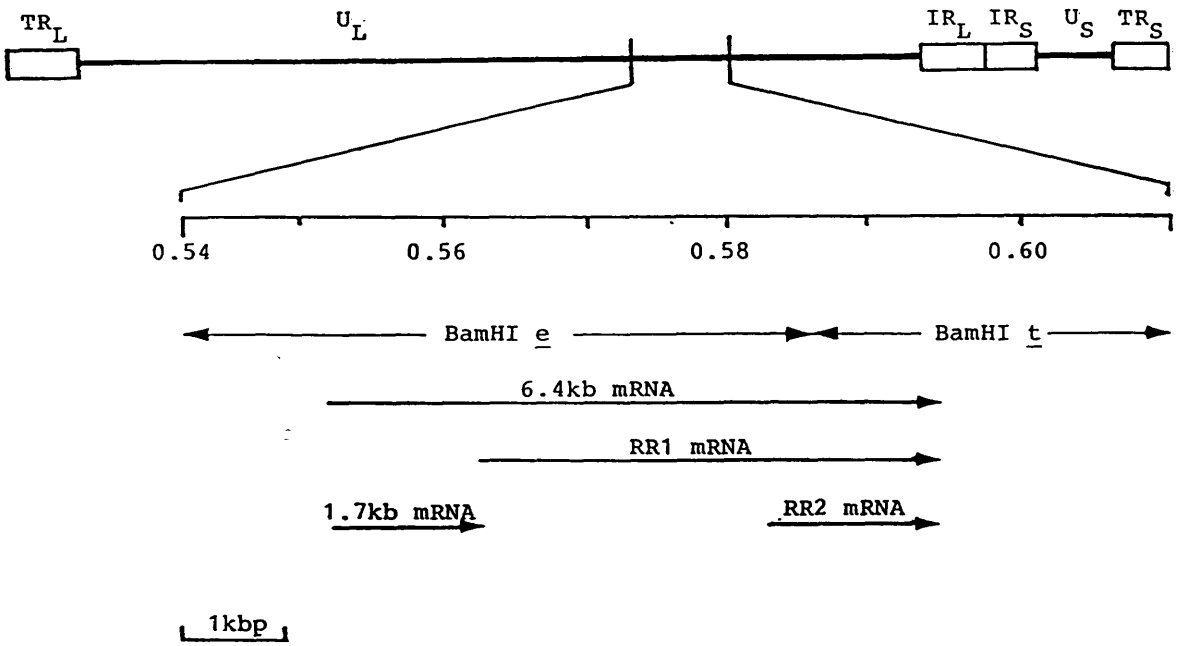


Figure 66. The HSV-2 strain HG52 ribonucleotide reductase locus. This diagram shows the genomic location of the Bam HI e and t fragments and the arrangement of mRNAs between 0.55 and 0.60 map units on the viral genome. The transcripts specified by this locus are shown as arrowed lines. The lengths of the late transcripts are indicated above each line and the mRNAs specifying the ribonucleotide reductase are labelled RR1 and RR2.

SECTION E.

Having identified a number of structural and functional features arising from the enzyme's primary structure, experiments were designed to express the HSV-encoded ribonucleotide reductase in eukaryotic cell lines. These experiments are reported in this Section and, in addition, studies on the transcriptional regulation of the ribonucleotide reductase genes in the presence of HSV trans-activating functions are described.

15. The HSV-2 Ribonucleotide Reductase Locus.

The HSV-2 strain HG52 ribonucleotide reductase locus (Fig. 66) specifies two L and two E transcripts which are similarly sized and arranged as in the equivalent HSV-1 locus (McLauchlan and Clements, 1983b; McLauchlan, 1986; Swain and Galloway, 1986). The L mRNAs are 5' co-terminal, have sizes of 6.4kb and 1.7kb and are presumed to encode the same 54,000 mol. wt. polypeptide. The E mRNAs share a common 3' terminus with the L 6.4kb mRNA and have sizes of 4.5kb (RR1 mRNA) and 1.2kb (RR2 mRNA; Fig. 66). These mRNAs encode the RR1 and RR2 polypeptides which constitute the HSV-2-encoded ribonucleotide reductase (McLauchlan, 1986).

DNA sequencing and RNA mapping studies within this locus positioned the 3' end of the 1.7kb mRNA 404nuc downstream from the Sst I site at 0.558 map units and the 5' end of the RR1 mRNA 582nuc downstream from the same site (McLauchlan, 1986; Fig. 67). The start site of the RR2 mRNA was positioned 139nuc upstream from the Bam HI site which separates the Bam HI e and t fragments (Fig. 67). Finally, the 3' co-terminus of the ribonucleotide reductase mRNAs and the 6.4kb mRNA was located approximately 1000nuc downstream from this Bam HI site (McLauchlan and Clements, 1983b). In common with the HSV-1 equivalent locus, although the HSV-2 ribonucleotide reductase mRNAs overlap, the coding regions of the RR1 and RR2 polypeptides do not (McLauchlan, 1986).

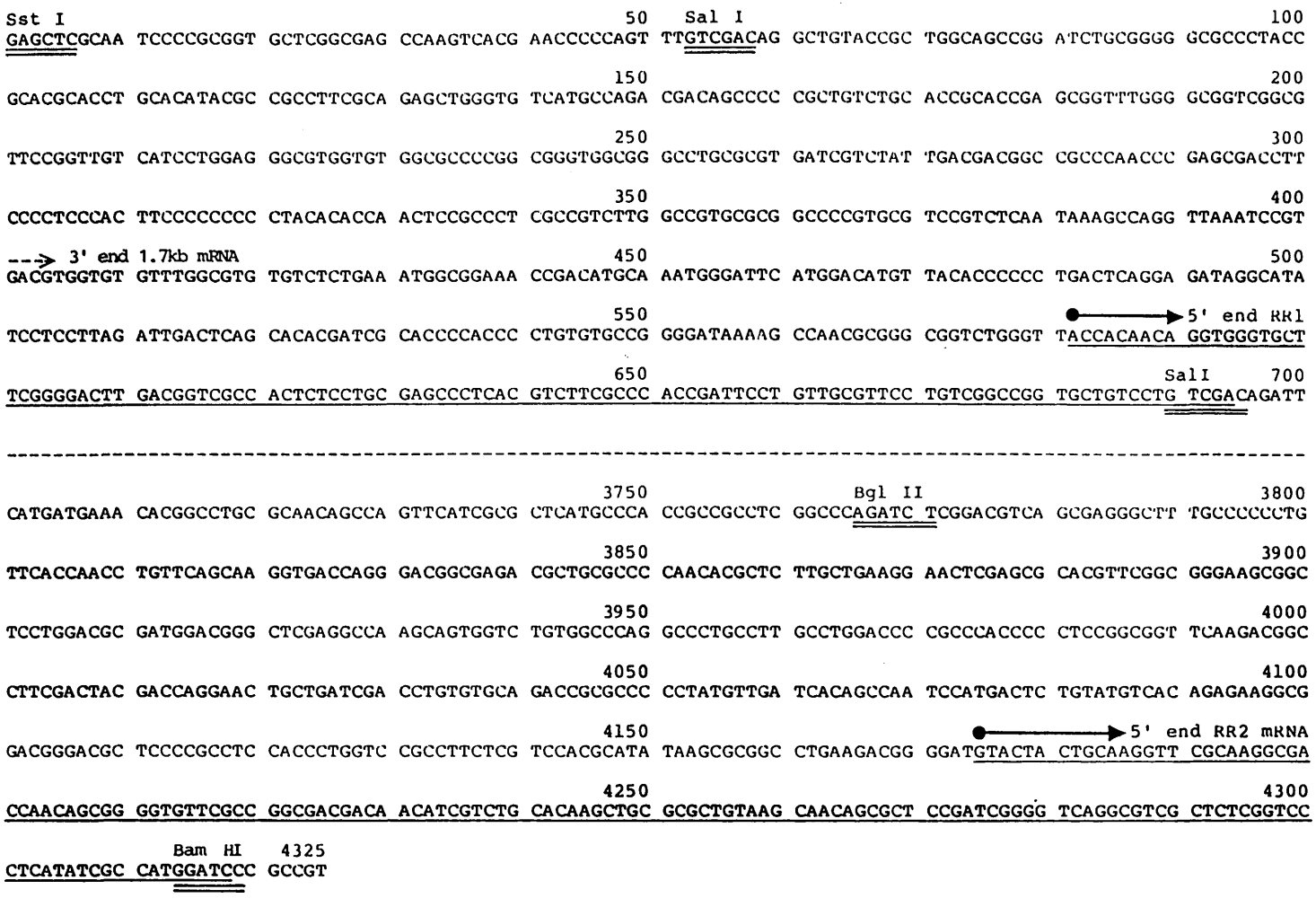

Sst I
50
Sal I
100
GAGCTCGCAA TCCCCGCGGT GCTCGGCGAG CCAAGTCACG AACCCCCAGT TTGTCGACAG GCTGTACCGC TGGCAGCCGG ATCTGCGGGG GCGCCCTACC
150
200
GCACGCACCT GCACATACGC CGCCTTCGCA GAGCTGGGTG TCATGCCAGA CGACAGCCCC CGCTGTCTGC ACCGCACCGA GCGGTTTGGG GCGGTCGGCG
250
300
TTCCGGTTGT CATCCTGGAG GGCGTGGTGT GGCGCCCCGG CGGGTGGCGG GCCTGCGCGT GATCGTCTAT TGACGACGGC CGCCCAACCC GAGCGACCTT
350
400
CCCCTCCCAC TTCCCCCCCC CTACACACCA ACTCCGCCCT CGCCGTCTTG GCCGTGCGCG GCCCCGTGCG TCCGTCTCAA TAAAGCCAGG TTAAATCCGT
--> 3' end 1.7kb mRNA
450
500
GACGTGGTGT GTTTGGCGTG TGTCTCTGAA ATGGCGGAAA CCGACATGCA AATGGGATTC ATGGACATGT TACACCCCCC TGACTCAGGA GATAGGCATA
550
5' end RR1 mRNA
TCCTCCTTAG ATTGACTCAG CACACGATCG CACCCCACCC CTGTGTGCCG GGGATAAAAG CCAACGCGGG CGGTCTGGGT TACCACAACA GGTGGGTGCT
650
SalI
700
TCGGGGACTT GACGGTCGCC ACTCTCCTGC GAGCCCTCAC GTCTTCGCCC ACCGATTCCT GTTGCGTTCC TGTCGGCCGG TGCTGTCCTG TCGACAGATT
3750
Bgl II
3800
CATGATGAAA CACGGCCTGC GCAACAGCCA GTTCATCGCG CTCATGCCCA CCGCCGCCTC GGCCCAGATC TCGGACGTCA GCGAGGGCTT TGCCCCCCTG
3850
3900
TTCACCAACC TGTTCAGCAA GGTGACCAGG GACGGCGAGA CGCTGCGCCC CAACACGCTC TTGCTGAAGG AACTCGAGCG CACGTTCGGC GGGAAGCGGC
3950
4000
TCCTGGACGC GATGGACGGG CTCGAGGCCA AGCAGTGGTC TGTGGCCCAG GCCCTGCCTT GCCTGGACCC CGCCCACCCC CTCCGGCGGT TCAAGACGGC
4050
4100
CTTCGACTAC GACCAGGAAC TGCTGATCGA CCTGTGTGCA GACCGCGCCC CCTATGTTGA TCACAGCCAA TCCATGACTC TGTATGTCAC AGAGAAGGCG
4150
5' end RR2 mRNA
GACGGGACGC TCCCCGCCTC CACCCTGGTC CGCCTTCTCG TCCACGCATA TAAGCGCGGC CTGAAGACGG GGATGTACTA CTGCAAGGTT CGCAAGGCGA
4250
4300
CCAACAGCGG GGTGTTCGCC GGCGACGACA ACATCGTCTG CACAAGCTGC GCGCTGTAAG CAACAGCGCT CCGATCGGGG TCAGGCGTCG CTCTCGGTCC
Bam HI
4325
CTCATATCGC CATGGATCCC GCCGT

Figure 67. The nucleotide sequences flanking the HSV-2 RRl and RR2 mRNA start sites. The positions of the 5' ends of these mRNAs and the 3' end of the late 1.7kb mRNA are shown. Underlined sequences denote the length of the protected RRl and RR2 mRNAs after hybridisation to the specific pGEM-2-produced RNA probes and digestion of hybrids with ribonuclease T2 (see Pages 116 and 117). Restriction endonuclease cleavage sites are indicated by a double line. The sequences are numbered with respect to the Sst I site (+1) at HSV-2 0.558 map units.

16. Construction of Plasmids Used in Transient Expression Assays.

In order to express the RR1 and RR2 mRNAs in eukaryotic cell lines, plasmid pRR was constructed which contains the DNA sequences specifying these mRNAs as they are arranged on the HSV-2 genome (Fig. 68). An Sst I/Bam HI fragment from a cloned Bam HI e fragment (obtained from Dr J. McLauchlan) was subcloned into an Sst I/Bam HI digested pUC12 vector to give plasmid pYNE. Then, a Bam HI/Xho I fragment from a cloned HSV-2 Bam HI t fragment (obtained from Dr J. McLauchlan) was subcloned into a Bam HI/Sal I digested pUC12 vector resulting in plasmid pYNT. Lastly, the Bam HI/Hind III fragment from pYNT was ligated into Bam HI/Hind III digested pYNE to give pRR. Therefore, pRR contains the entire DNA region specifying the RR1 and RR2 mRNAs and, in addition, the region specifying the 3' end of the L 1.7kb mRNA.

RNA probes specific for the detection of the RR1 and RR2 mRNAs were constructed with the use of pGEM-2 Riboprobe Gemini transcription vectors (see Materials and Methods, Page 58). For RR1 mRNA, a 636nuc Sal I fragment spanning the DNA sequences specifying the 5' end of the RR1 mRNA (see Fig. 67), was ligated into Sal I digested pGEM-2. For RR2 mRNA, a 547nuc HSV-2 Bgl II/Bam HI fragment spanning the DNA sequences specifying the 5' end of the RR2 mRNA (see Fig. 67) was ligated to Bam HI linearised pGEM-2 vector. Recombinants were analysed by restriction endonuclease digestion for appropriate insert orientation relative to the SP6 promoter, such that transcripts synthesised from the SP6 promoter would be complementary to RR1 and RR2 mRNAs. This screening procedure resulted in the selection of plasmids pR45 and pR12 (Fig. 69). Subsequently, both plasmids were digested to completion with Hind III and SP6 transcripts labelled with [α-^{32}P]UTP were prepared as described in Materials and Methods (see Page 71).

17. Transient Expression Assays with Plasmid pRR.

Expression of RR1 and RR2 mRNAs in eukaryotic cells was investigated by short-term transfection assays.

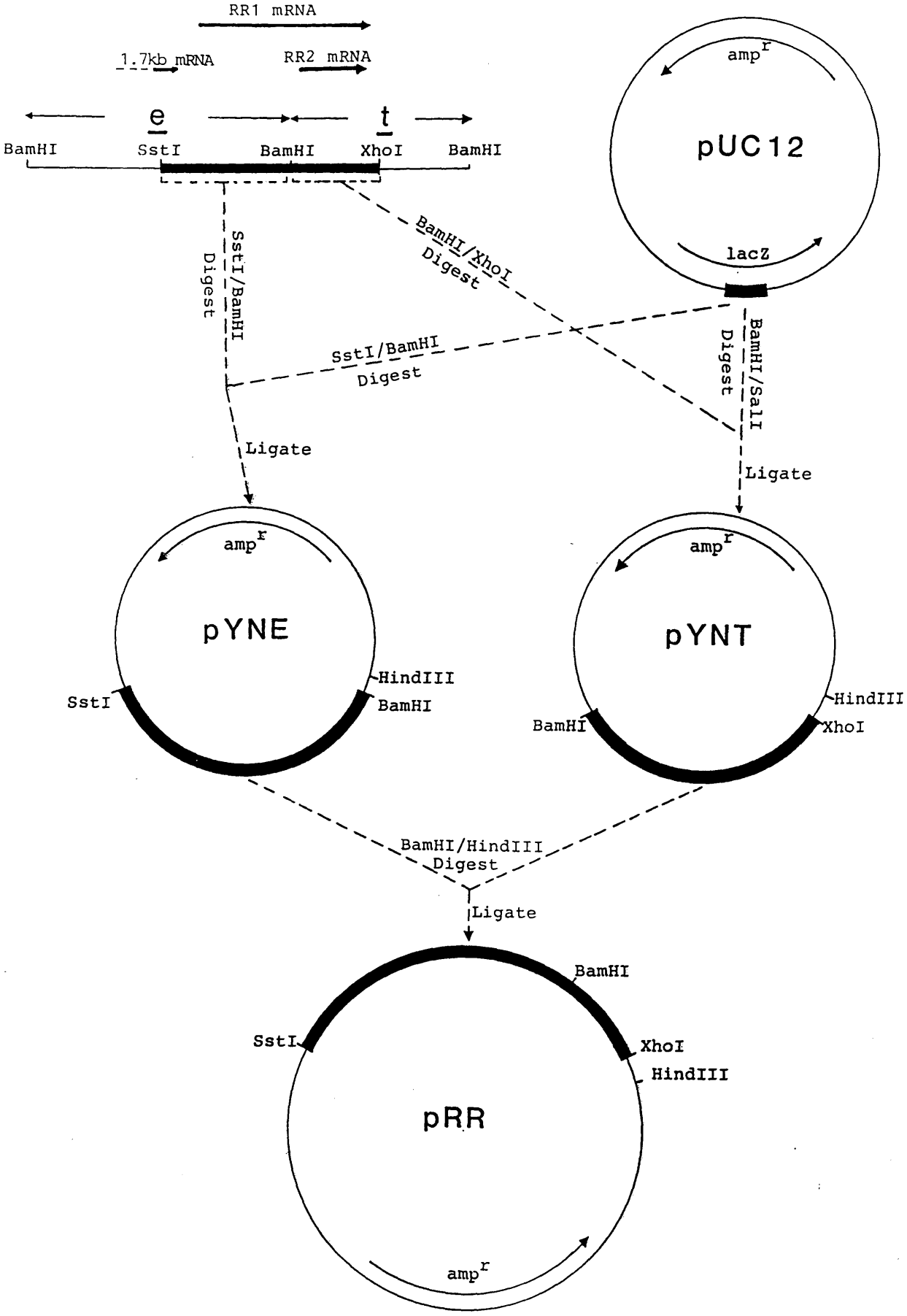
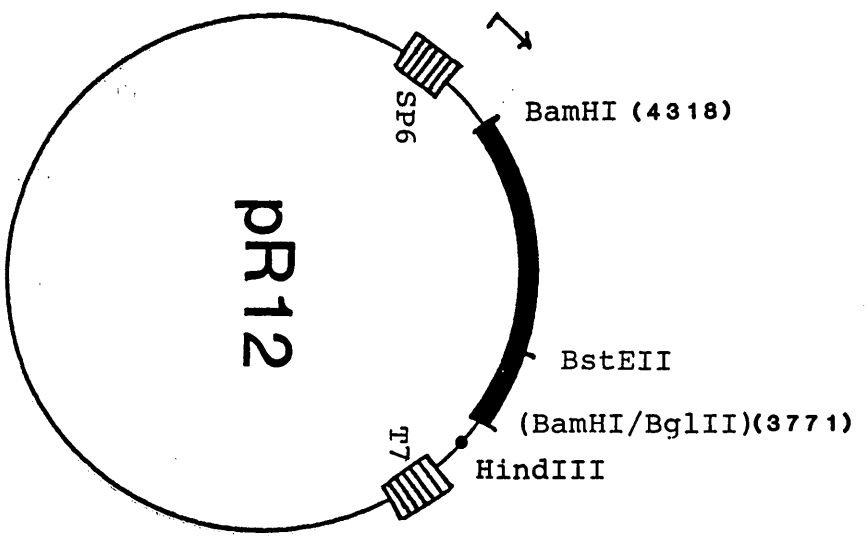

RR1 mRNA
1.7kb mRNA
RR2 mRNA
e
t
BamHI
SstI
BamHI
XhoI
BamHI
SstI/BamHI Digest
BamHI/XhoI Digest
pUC12
amp^r
lacZ
SstI/BamHI Digest
BamHI/SalI Digest
Ligate
Ligate
pYNE
amp^r
HindIII
BamHI
SstI
pYNT
amp^r
HindIII
XhoI
BamHI
BamHI/HindIII Digest
Ligate
pRR
BamHI
XhoI
HindIII
SstI
amp^r

Figure 68. Construction of plasmid pRR. At the top left of the diagram, the HSV-2 Bam HI e and t DNA fragments are depicted in a linear form showing the region (dark area) specifying the RR1 and RR2 mRNAs and the 3' end of the 1.7kb mRNA. The mRNAs are shown as arrowed lines and the part of the 1.7kb mRNA specified outwith this area is indicated by a dashed line. To construct pRR, the Sst I/Bam HI subfragment of Bam HI e was ligated to a Sst I/ Bam HI digested pUC12 vector to give pYNE. The Bam HI/Xho I subragment of Bam HI t was ligated to a Bam HI/Sal I digested pUC12 vector to give pYNT. Finally, the pYNT Bam HI/Hind III fragment was ligated to a Bam HI/Hind III linearised pYNE to give pRR. The ampicillin resistance (amp^r) and β-galactosidase genes (lacZ) of pUC12 are indicated.


pR12
Sp6
T7
BamHI (4318)
BstEII
(BamHI/BglII)(3771)
HindIII

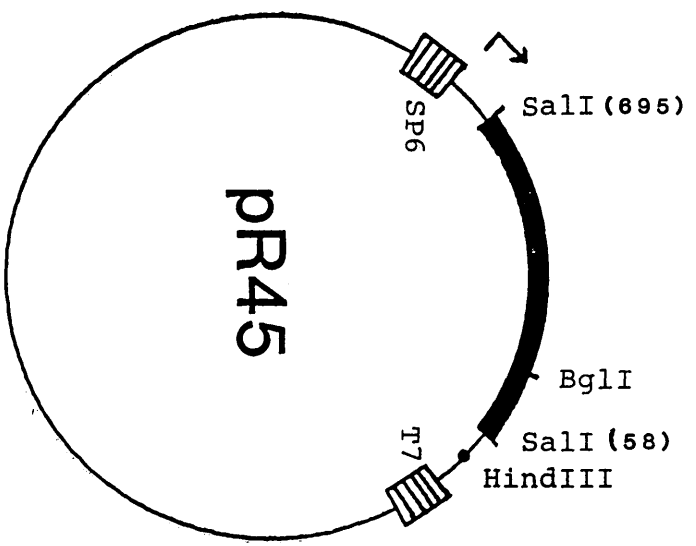

pR45
Sp6
T7
SalI (695)
BglI
SalI (58)
HindIII

Figure 69. The structure of the pR45 and pR12 plasmids used for the production of RNA probes specific for the RR1 and RR2 mRNAs respectively. pR45 plasmid was constructed by inserting a Sal I fragment, spanning positions 58 to 695 relative to the Sst I site at HSV-2 0.558 map units (see Fig. 67), into a Sal I digested pGEM-2 vector. pR12 plasmid was constructed by inserting a Bam HI/Bgl II fragment, spanning positions 3771 to 4318 relative to the above mentioned Sst I site (see Fig. 67), into a Bam HI digested pGEM-2 vector. The lost Bgl II site is shown in brackets. Recombinant plasmids were screened for appropriate insert orientation with the use of the restriction endonuclease site indicated within each insert. The SP6 and T7 promoters are shown as hatched boxes and the transcriptional direction of SP6 is depicted by an arrow. The location of the Hind III site used to linearise the pR45 and pR12 plasmids is depicted by (●).

Sub-confluent monolayers of HeLa cells in 90mm petri dishes were transfected each with 20ug of pRR using the calcium-phosphate transfection procedure (see Materials and Methods, Page 69). However, the RR1 and RR2 genes have been considered as essentially E genes and as such they should require the presence of HSV trans-activating functions for maximal expression (see Introduction, Page 21). Therefore, some dishes of pRR-transfected HeLa cells were infected with HSV-1 strain 17^+ at a multiplicity of 10 p.f.u./cell. This virus was selected, in preference to HSV-2 strain HG52, to allow descrimination between virus-encoded (HSV-1) and plasmid-encoded (HSV-2) ribonucleotide reductase mRNAs. Further, this virus was selected because, although pRR specifies the HSV-2 ribonucleotide reductase mRNAs under the control of their own promoters, HSV-1 IE functions can trans-activate HSV-2 E promoters (O' Hare and Hayward, 1985b). The controls consisted of HeLa cells infected with: HSV-2 strain HG52 at a multiplicity of 10 p.f.u./cell, HSV-1 strain 17^+ at a multiplicity of 10 p.f.u./cell or mock-infected. Cells from all five treatments were harvested at 16h p.i. and cytoplasmic RNA was isolated as described in Materials and Methods (see Page 70). Then, 4ug of cytoplasmic RNA from the pRR transfected dishes and 0.5ug from the control dishes were hybridised to approximately 200ng of RNA probes specific for RR1 and RR2 mRNA. RNA hybrids were digested with ribonuclease T2 and the products were analysed on 8% denaturing polyacrylamide gels. Size standards were Hpa II digested pAT153 DNA fragments which were end-labelled with [α-^{32}P]dCTP. After suitable exposure of the gel, the relative levels of hybridisation were measured by densitometry of autoradiographs as described in Materials and Methods (see Page 62).

a) pRR-borne constitutive and induced levels of RR1 mRNA.

Fig. 70 shows the results obtained after hybridisation of cytoplasmic RNA with the RNA probe produced from plasmid pR45. The RNA samples analysed were isolated from HeLa cells: transfected with pRR (track 1), transfected with pRR and infected with HSV-1 (track 2), infected with HSV-2 (track 3), mock-infected (track 4) and infected with HSV-1

(track 5). Comparison of the protected RNA bands appearing on tracks 1 and 2 with the negative and positive control tracks (tracks 3, 4 and 5), indicates that the bands arising from hybridisation of the probe to RR1 mRNA migrate at approximately 115nuc (indicated by solid arrowheads in Fig. 70). This is in good agreement with the size of the protected RNA (113nuc) as deduced from DNA sequence and RNA mapping data (McLauchlan, 1986; see Fig 67). The size difference observed probably reflects the higher mol. wt. of RNA as compared to the DNA size standards.

Ribonuclease T2 analysis generated a number of additional bands on tracks 2 and 3 of Fig. 70. As shown in Fig. 67, the HSV-2 DNA fragment which was subcloned in pGEM-2 in order to obtain the probe specific for RR1 mRNA, spans the 5' end of the RR1 mRNA and in addition the 3' end of the L 1.7kb mRNA. Comparison of the HSV-2 nucleotide sequence of the probe with the corresponding HSV-1 region revealed the existence of a 209nuc highly conserved region within the 3' end of the 1.7kb mRNA (HSV-2 probe positions 58 to 267 in Fig. 71). Therefore, in HSV-1 infected samples the HSV-2 probe is likely to hybridise to the HSV-1-specified 1.7kb mRNA and this would generate the bands at around 217nuc on track 2 (indicated by ◆ in Fig. 70). These bands are also present in the HSV-1-infected control sample (track 5 in Fig. 70).

Three other obvious bands can be observed on track 2 (indicated by open arrowheads) from which the longest one migrates above the RR1 mRNA main bands and the others have sizes of approximately 95nuc and 80nuc. As already mentioned (see Results and Discussion, Page 78), the HSV-1 ribonucleotide reductase locus specifies a L 7.0kb mRNA which overlaps entirely with the RR1 and RR2 mRNAs. Therefore, these bands of track 2 probably result from hybridisation of the probe to portions of the 7.0kb mRNA spanning the intergenic region between the 1.7kb and RR1 mRNAs of the infecting HSV-1 virus (see Fig. 71). As expected, these bands also appear on the HSV-1-infected control track (track 5 in Fig. 70), although, their intensity is low due to the small amount of RNA analysed on this track (0.5ug) as compared to that analysed on track 2

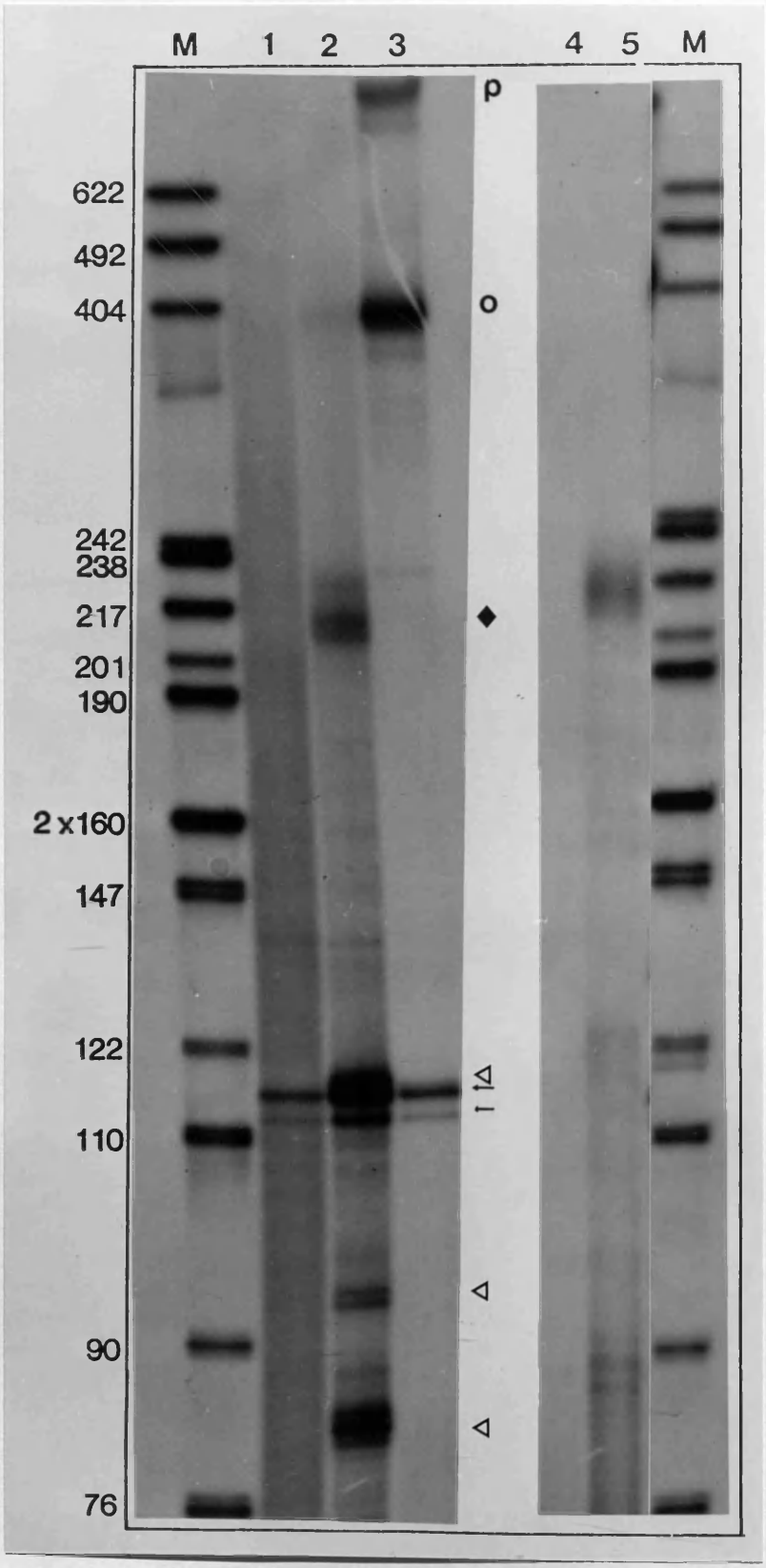

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Figure 70. Ribonuclease T2 analysis of pRR-borne constitutive and HSV-1-induced RR1 mRNA levels with the pR45-produced RNA probe. Cytoplasmic RNA samples were from HeLa cells transfected with plasmid pRR1 which were either mock-infected (track 1) or HSV-1-infected (track 2). The negative and positive control cytoplasmic RNA samples were: HSV-2-infected HeLa RNA (track 3), mock-infected HeLa RNA (track 4) and HSV-1-infected HeLa RNA (track 5). Following hybridisation at 50°C, samples were digested with ribonuclease T2 and the T2-resistant products were analysed on 8% denaturing polyacrylamide gels. The position of the RR1 mRNA main hybrids is indicated by arrowheads; other obvious bands are indicated by symbols and have been accounted for in the text (see Pages 117 and 118). The DNA size standards (M) were pAT153 DNA digested with Hpa II.

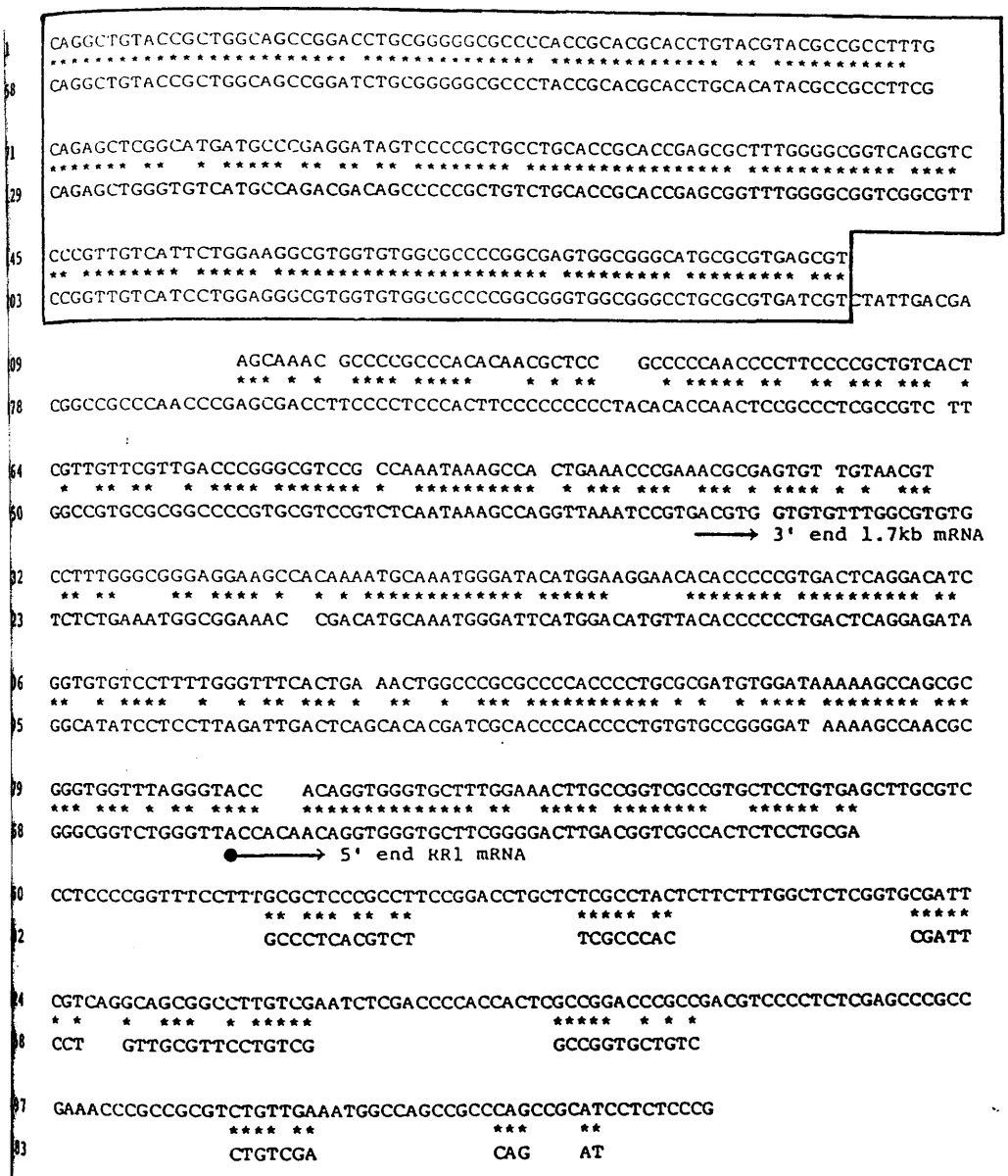

Figure 71. Nucleotide sequence comparison of the HSV-2 DNA region which was subcloned into pGEM-2, in order to produce the RNA probe specific for the HSV-2 RR1 mRNA, with the corresponding HSV-1 DNA region. The 3' end of the HSV-2 1.7kb mRNA and the 5' end of the RR1 mRNA are indicated. The nucleotide sequences sharing extremely high homology are boxed and the HSV-2 sequence is numbered as in Fig. 67.

(4ug). Track 2 also contains a minor band (indicated by o) with an approximate size of 400nuc. This band is likely to represent pRR-specified transcripts which are not proccessed at the 3' co-terminal polyadenylation signal for the RR1 and RR2 mRNAs; possibly, these transcripts run throught this signal and terminate at the polyadenylation signal of the 1.7kb mRNA which is present in pRR. This is based on the fact that the size of the band approximates the size of the probe region that corresponds to the HSV-1 DNA region specifying the 3' end of the 1.7kb mRNA. A similar 400nuc band appears in the HSV-2-infected control sample (track 3 in Fig. 70) and this probably results from the probe hybridising to the 3' end of the L 1.7kb mRNA specified in HSV-2-infected cells. Further, this band appears as a major species because cytoplasmic RNA was isolated at L times p.i. (16h; see Materials and Methods, Page 69). Finally, the band appearing in the HSV-2 control sample (indicated by p; track 3) most probably represents the full-length pR45-produced probe hybridising to the L 6.4kb mRNA which, as shown in Fig. 66, overlaps entirely with the RR1 and RR2 mRNAs.

As can be seen in Fig. 70 the pRR-specified constitutive levels of RR1 mRNA are readily detectable while after induction by HSV-1 infection a moderate increase is observed; this increase is approximately 5-fold (S. Simpson, personal communication).

b) <u>pRR-borne constitutive and induced RR2 mRNA levels</u>.

For the detection of the RR2 mRNA, equal amounts of cytoplasmic mRNA to those used for the detection of the RR1 mRNA were hybridised to the RNA probe produced from plasmid pR12. The results obtained after hybridisation are shown in Fig. 72 where the order of RNA samples analysed is similar to that of Fig. 70. The expected ribonuclease T2-resistant hybrid should be 144nuc (see Fig. 67); however, due to the higher mol. wt. of RNA, the bands resulting from hybridisation of the pR12-produced probe to RR2 mRNA migrate between the 147nuc and 160nuc bands of the DNA size standards (Fig. 72).

As mentioned on Page 113, the HSV-2 ribonucleotide

reductase locus specifies a family of three 3' co-terminal mRNAs, comprising the RR1, RR2 and 6.4kb mRNAs. As a result of the overlapping nature of the transcripts specified by this locus, a probe specific for RR2 mRNA will also hybridise to RR1 and 6.4kb mRNAs. This can clearly be seen in tracks 1, 2 and 3 of Fig. 72 where bands of size greater than 622nuc were detected (indicated by p). In track 1, the band represents the probe hybridising to pRR-borne RR1 mRNA. In track 2 the probe hybridises to pRR-borne RR1 mRNA and, in addition, to mRNA specified by the infecting virus; this is proposed on the basis of the high homology between the HSV-1 and HSV-2 regions upstream from the RR2 mRNA cap site (Fig. 73). Finally, in the HSV-2-infected control sample (track 3 in Fig. 72) the probe hybridises, as expected to the HSV-2 specified RR1 and 6.4kb mRNAs. Other bands of different sizes appearing on track 2 probably arise from the probe hybridising to HSV-1 infecting virus mRNA; these bands appear also in track 5 although, as already noted, their intensity is low due to the small amount of RNA analysed on this track (0.5ug) as compared to that analysed on track 2 (4ug).

As can be seen in Fig. 72, the pRR-borne constitutive RR2 mRNA levels were barely detectable (track 1) whereas, after induction by HSV-1, elevated RR2 mRNA levels were observed (track 2).

18. Trans-activation of Ribonucleotide Reductase Gene Transcription by HSV-1 IE Polypeptides.

The rationale behind these experiments was to study effects of the HSV-1 IE polypeptides Vmw175 and Vmw110 on mRNA basal levels specified from pRR. For this purpose pRR-transfected HeLa cells were co-transfected with plasmids: i) p175, which contains the coding sequences of Vmw175 under the control of the SV40 E promoter and enhancer (Perry et al., 1986) and ii) p111, which encodes Vmw110 (Perry et al., 1986).

Sets of four sub-confluent 90mm dishes of HeLa cells were transfected each with 10ug of pRR. From each set, in addition, the second was transfected with 10ug of p111, the

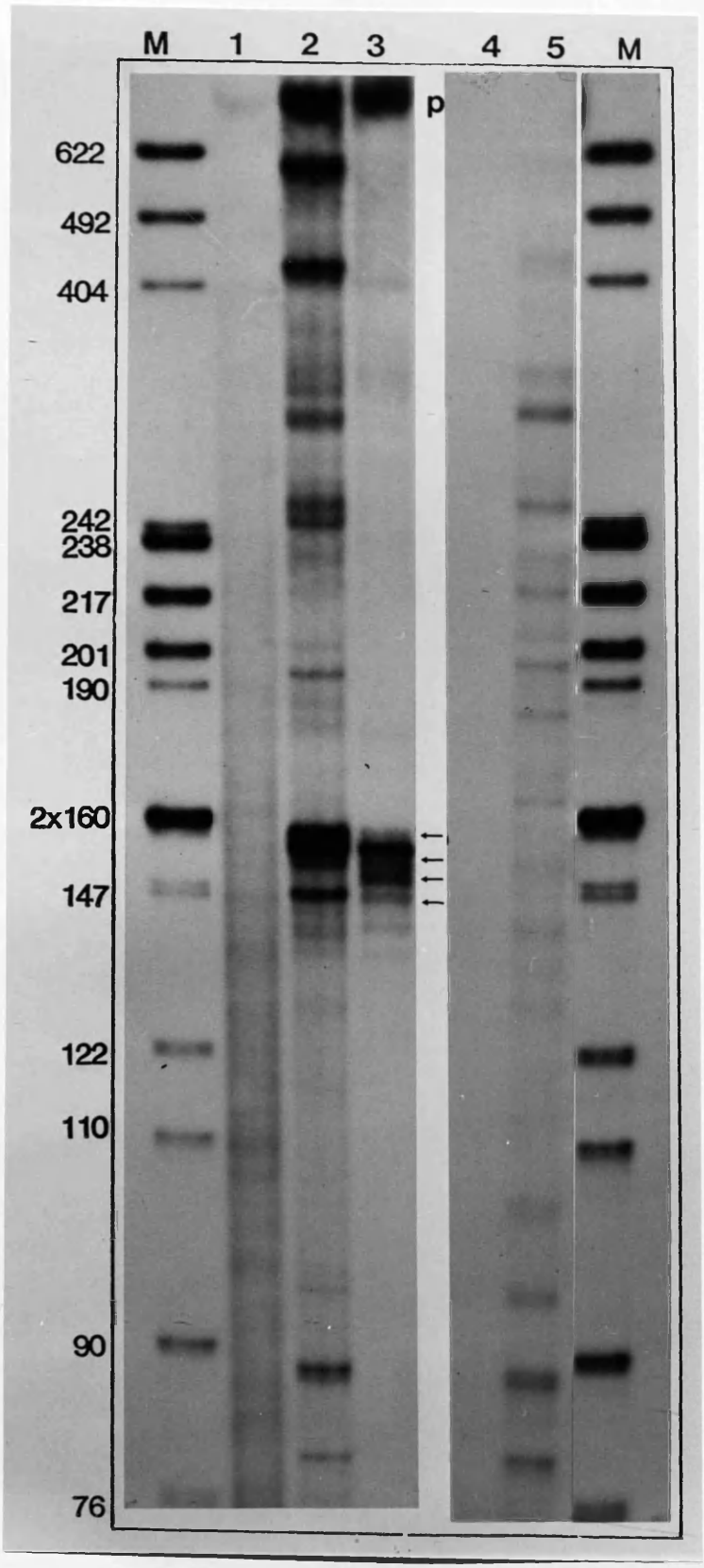
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Figure 72. Ribonuclease T2 analysis of pRR-borne constitutive and HSV-1-induced RR2 mRNA levels with the pR12-produced RNA probe. Cytoplasmic RNA samples were from HeLa cells transfected with plasmid pRR1 which were either mock-infected (track 1) or HSV-1-infected (track 2). The negative and positive control cytoplasmic RNA samples were: HSV-2-infected HeLa RNA (track 3), mock-infected HeLa RNA (track 4) and HSV-1-infected HeLa RNA (track 5). Following hybridisation at 50^{o}C, samples were digested with ribonuclease T2 and the T2-resistant products were analysed on 8% denaturing polyacrylamide gels. The position of the RR2 mRNA main hybrids is indicated by arrowheads and the full-length protected pR12-produced probe is indicated by (p). The DNA size standards (M) were pAT153 DNA digested with Hpa II.

```
First  sequence:  HSV-1
Second sequence:  HSV-2

1     CTCGGACGTCAGCGAGGGCTTTGCCCCCCTGTTCACCAACCTGTTCAGCAAGGTGACCCGGGACG
      ************************************************************ ******
3770  CTCGGACGTCAGCGAGGGCTTTGCCCCCCTGTTCACCAACCTGTTCAGCAAGGTGACCAGGGACG

65    GCGAGACGCTGCGCCCCAACACGCTCCTGCTAAAGGAACTGGAACGCACGTTTAGCGGGAAGCGCCTCCTGGAG
      **************************** **** ******** ** ********  ********** ********
3835  GCGAGACGCTGCGCCCCAACACGCTCTTGCTGAAGGAACTCGAGCGCACGTTCGGCGGGAAGCGGCTCCTGGAC

139   GTGATGGACAGTCTCGACGCCAAGCAGTGGTCCGTGGCGCAGGCGCTCCCGTGCCTGGAGCCCACCCACCCCCT
      * ******* * ***** ************** ***** ***** ** ** ******** *** **********
3909  GCGATGGACGGGCTCGAGGCCAAGCAGTGGTCTGTGGCCCAGGCCCTGCCTTGCCTGGACCCCGCCCACCCCCT

213   CCGGCGATTCAAGACCGCGTTTGACTACGACCAGAAGTTGCTGATCGACCTGTGTGCGGACCGCGCCCCCTACG
      ****** ******** ** ** ************ *  ******************** ************** *
3983  CCGGCGGTTCAAGACGGCCTTCGACTACGACCAGGAACTGCTGATCGACCTGTGTGCAGACCGCGCCCCCTATG

287   TCGACCATAGCCAATCCATGACCCTGTATGTCACGGAGAAGGCGGACGGGACCCTCCCAGCCTCCACCCTGGTC
      * ** ** ************** *********** ***************** ***** ***************
4056  TTGATCACAGCCAATCCATGACTCTGTATGTCACAGAGAAGGCGGACGGGACGCTCCCCGCCTCCACCCTGGTC

361   CGCCTTCTGGTCCACGCATATAAGCGCGGACTAAAAACAGGGATGTACTACTGCAAGGTTCGCAAGGCGACCAA
      ******** ******************** ** ** ** ************************************
4141  CGCCTTCTCGTCCACGCATATAAGCGCGGCCTGAAGACGGGGATGTACTACTGCAAGGTTCGCAAGGCGACCAA
                                                  ●————→ 5' end RR2 mRNA

435   CAGCGGGGTCTTTGGCGGCGACGACAACATTGTCTGCATGAGCTGCGCGCTGTGACCGACAAACCCCCTCCGCG
      ********* ** * *************** *******  ************* * * ***    * *****
4205  CAGCGGGGTGTTCGCCGGCGACGACAACATCGTCTGCACAAGCTGCGCGCTGTAAGCAACA   GCGCTCCGAT

509   CCAGGCCCGCCGCCACTGTCG          TCGCCGTCCCACG
      *  ** * * ** * ** ***          ***** *    *
4275  CGGGGTCAGGCGTCGCTCTCGGTCCCTCATATCGCCATGGATCC
```

Figure 73. Nucleotide sequence comparison of the HSV-2 DNA region which was subcloned into pGEM-2, in order to produce the RNA probe specific for the HSV-2 RR2 mRNA, with the corresponding HSV-1 DNA region. The 5' end of the RR2 mRNA is indicated and the HSV-2 sequence is numbered as in Fig. 67.

third with 10ug of pl75 and the fourth with 10ug each of pl11 and pl75. The total amount of transfected plasmid DNA in all four dishes was 30ug, and this was made up by the addition of an appropriate amount of pUC8 plasmid DNA. As a positive control, subconfluent monolayers of HeLa cells were infected with HSV-2 strain HG52 at a multiplicity of 10 p.f.u./cell. After a 24h incubation, the medium was replaced with fresh medium, and the dishes were incubated for a further 16h at 37^{o}C (see Materials and Methods, Page 69). At the end of the second incubation, cells were harvested and cytoplasmic RNA was prepared. Then, 4ug of RNA from co-transfected cells and 0.5ug of RNA from HSV-2-infected cells were hybridised to probes produced from plasmids pR45 and pR12; the hybrids were treated with ribonuclease T2 and analysed on 8% denaturing polyacrylamide gels.

The autoradiographs of Figs. 74 and 75 show the results obtained after hybridisation of cytoplasmic RNA to the pR45- and pR12-produced probes respectively. The RNA samples analysed on these gels were isolated from HeLa cells co-transfected with: pRR and pUC8 (track 1), pRR, pl11 and pUC8 (track 2), pRR, pl75 and pUC8 (track 3), pRR, pl11 and pl75 (track 4). The RNA sample analysed on track 5 was from HeLa cells infected with HSV-2. As can be seen, the bands due to the probes hybridising to the RR1 (Fig. 74) and RR2 (Fig. 75) mRNAs migrate at positions corresponding to those observed in Figs. 70 and 72 respectively. These bands were quantitated as described in Materials and Methods (see Page 62), although, cells were not transfected with a control plasmid, to allow standardisation of the transfection procedure; this was due to the limited amount of DNA that can be taken up by transfected cells (30ug per 90mm Petri dish; R.D. Everett, personal communication). The results of this analysis, shown on Table V, demonstrated that Vmw110 had no detectable effect on RR1 mRNA basal levels, whereas, Vmw175 or a combination of Vmw110 and Vmw175 resulted in a 2- to 3-fold increase. In contrast, Vmw175 substantially increased the RR2 mRNA levels by 12-fold In the presence of both of these IE polypeptides, the highest increase was observed resulting in

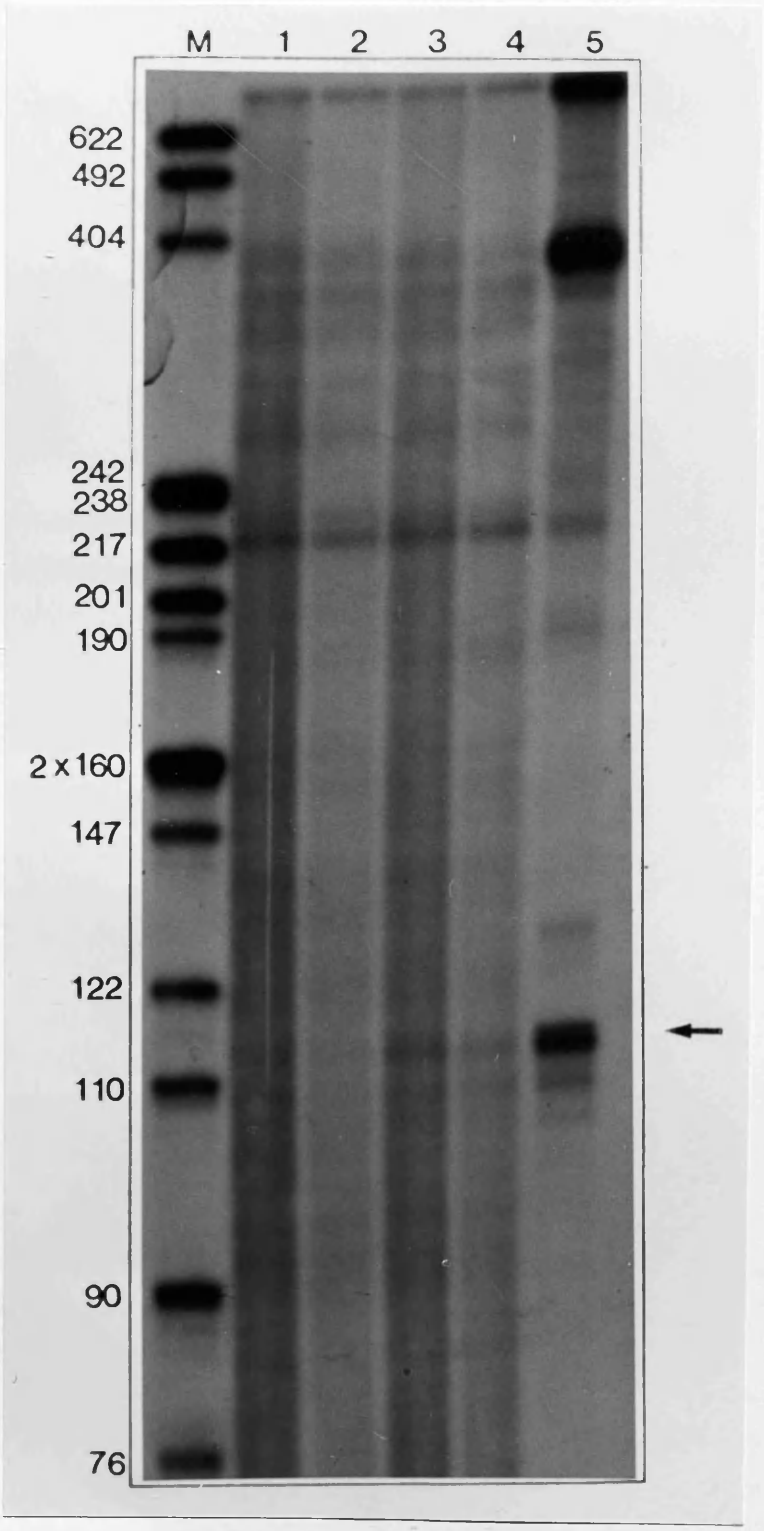
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Figure 74. Transcriptional activation of pRR-borne RR1 mRNA levels by plasmid-borne HSV-1 IE Vmw175 and Vmw110 polypeptides. Cytoplasmic RNA samples were from HeLa cells co-transfected with plasmids:

pRR and pUC8 (track 1)
pRR, p111 and pUC8 (track 2)
pRR, p175 and pUC8 (track 3)
pRR, p111 and p175 (track 4)

As a positive control, HeLa cells were infected with HSV-2 virus (track 5). Samples were hybridised to pR45-produced probe at 50°C, digested with ribonuclease T2 and the T2-resistant products were analysed on 8% denaturing polyacrylamide gels. The position of the RR1 mRNA hybrids is indicated by an arrow. The DNA size standards (M) were pAT153 DNA digested with Hpa II.

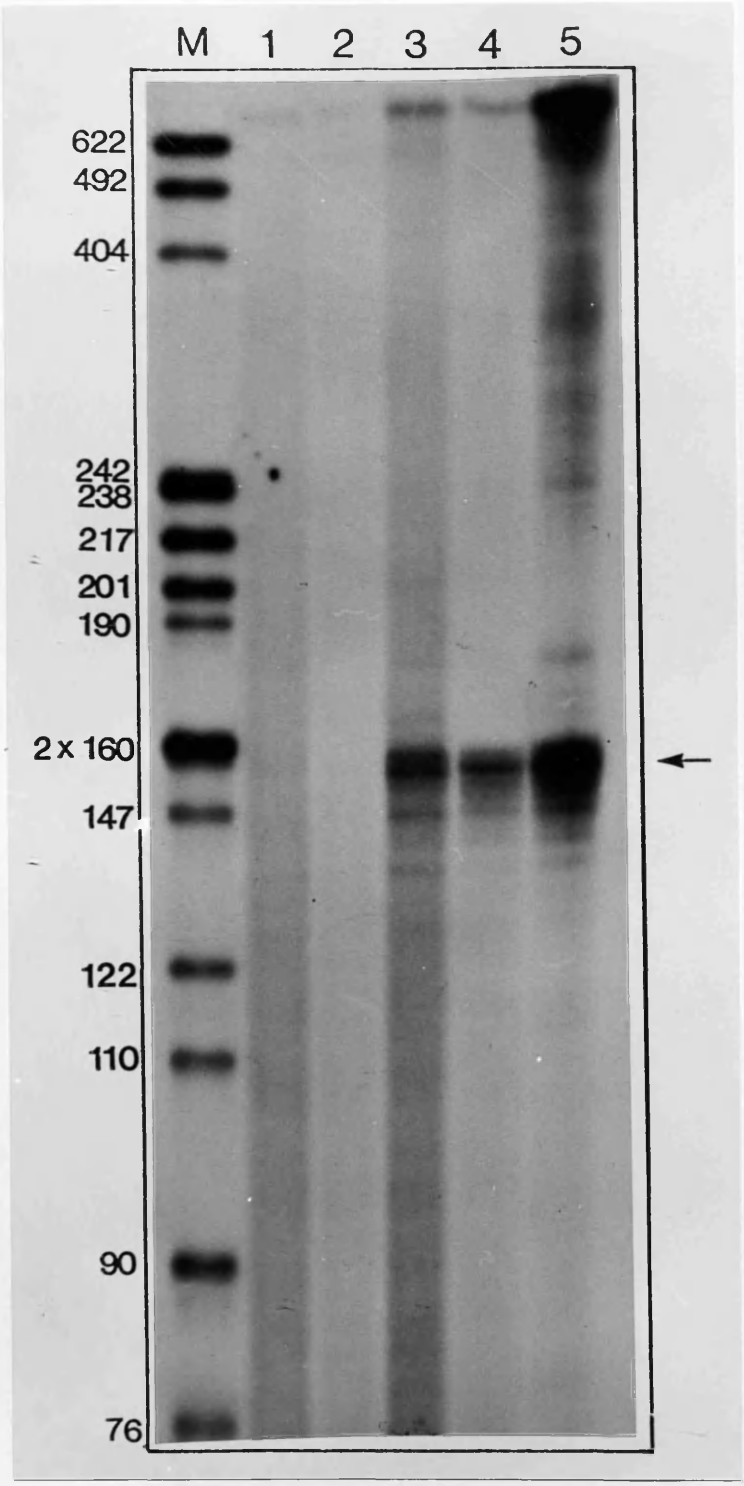
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Figure 75. Transcriptional activation of pRR-borne RR2 mRNA levels by plasmid-borne HSV-1 IE Vmw175 and Vmw110 polypeptides. Cytoplasmic RNA samples were from HeLa cells co-transfected with plasmids:

pRR and pUC8 (track 1)
pRR, p111 and pUC8 (track 2)
pRR, p175 and pUC8 (track 3)
pRR, p111 and p175 (track 4)

As a positive control, HeLa cells were infected with HSV-2 virus (track 5). Samples were hybridised to pRI2-produced probe at 50°C, digested with ribonuclease T2 and the T2-resistant products were analysed on 8% denaturing polyacrylamide gels. The position of the RR2 mRNA main hybrids is indicated by an arrow. The DNA size standards (M) were pAT153 DNA digested with Hpa II.

TABLE V

RELATIVE INCREASES OF THE RR1 AND RR2 mRNA CONSTITUTIVE LEVELS AFTER TRANS-ACTIVATION WITH THE HSV-1 IE POLYPEPTIDES Vmw110 AND Vmw175

	Vmw110	Vmw175	Vmw110/ Vmw175
RR1 mRNA	1	2	2-3
RR2 mRNA	1	12	17

17-fold elevated RR2 mRNA levels.

DISCUSSION.

In this Section, *in vitro* expression of the RR1 and RR2 genes was studied using transient expression assays. Further, studies were performed on regulation of expression of these genes using HSV-1 infection or *trans*-activation with plasmid-borne IE polypeptides. In the Discussion, the results obtained from these experiments will be combined with other available data on the regulation of ribonucleotide reductase gene expression.

19. Regulation of HSV-2 RR1 Gene Transcription.

The main observations on HSV-2 RR1 gene expression were: i) RR1 mRNA basal levels are readily detectable in the absence of HSV-1 *trans*-activating functions, ii) HSV-1 infection or *trans*-activation with the HSV-1 IE polypeptide Vmw175 results in a 5- and 3-fold increase of RR1 mRNA basal levels respectively and iii) the HSV-1 IE polypeptide Vmw110 has no detectable effect on RR1 mRNA basal levels.

a) Constitutive transcription from the HSV-2 RR1 gene promoter.

A striking observation on RR1 gene expression was that RR1 mRNA basal levels were readily detectable in the absence of viral *trans*-activating functions. The RR1 gene has been classified as an E gene because studies on the kinetics of appearance of the RR1 mRNA in the cytoplasm of HSV-infected cells demonstrated its detection by 2h p.i. and levels continued to accumulate by 6h to 8h p.i. after which there was no increase in abundance (McLauchlan and Clements, 1982; McLauchlan, 1986). As an E gene, activation of RR1 expression should require the presence of functional IE polypeptides (see Introduction, Page 22). However, further to the data presented in this Section, a number of reports

have identified either the RR1 mRNA or the RR1 polypeptide present under IE conditions: in fact, certain of these reports have classified the RR1 gene as an IE gene. First, Clements et al. (1977) detected, by blot hybridisation to viral DNA fragments, low levels of the RR1 mRNA under IE conditions; following reversal of the cycloheximide block, RR1 mRNA was rapidly and abundantly synthesised and its synthesis preceded that of other E mRNAs such as the DNA polymerase or the TK. Second, Watson et al. (1980) detected the RR1 polypeptide after in vitro translation of pooled IE mRNA. Third, the RR1 polypeptide is expressed by HSV ts-Vmw175 or ts-Vmw110 mutants grown at the NPT (Marsden et al., 1976; Preston, 1979; DeLuca et al., 1985; Stow and Stow, 1986; Sacks and Schaffer, 1987). Fourth, infection with HSV-1 or HSV-2 result in the expression of the RR1 polypeptide at very early times (Pereira et al., 1977; Easton et al., 1980; Frame et al., 1986b; A. Cross, personal communication). Finally, by nuclease S1 analysis, RR1 mRNA was detected after infection with HSV-1 mutant tsK at the NPT or HSV-1 wild-type virus infection under IE conditions, although, its levels were lower than those of other IE mRNAs (J. McLauchlan, personal communication).

Since E gene promoter activity is generally low in the absence of HSV trans-activating functions (reviewed in Everett, 1987b) the readily detectable pRR-specified RR1 mRNA levels would suggest that the RR1 gene promoter (P1) is activated by cellular transcription factors. For this reason, the upstream HSV-2 P1 DNA sequences were analysed for the presence of sequence elements known to bind cellular transcription factors.

i) Potential binding sites of the Sp1 cellular transcription factor within the P1 sequences. Initially, P1 sequences were examined for potential binding sites of the Sp1 transcription factor known to enhance transcription from RNA polymerase II promoters (see Introduction, Page 19; reviewed in Kadonaga et al., 1986). This analysis revealed the presence of a C-rich sequence located between -50 to -40nuc relative to the RR1 mRNA start site (+1) whose complement exhibits reasonable homology to the Sp1 consensus

(Fig. 76). Further upstream from the putative Sp1 binding site, there is a G-rich tract on the complementary strand at position -110 relative to the RR1 mRNA start site. G-rich motifs like the latter were observed in the E gD promoter and share similarities with the Sp1 binding site in the human immunodeficiency virus long terminal repeat (see Introduction, Page 22). Further, the complement of this motif has been found located at a similar distance from the cap sites of several HSV mRNAs and, although not all E promoters contain these sequences, they appear to be absent from most L promoters (reviewed in Wagner, 1985). The highly conserved nature of this motif would indicate that it may represent a functional component of P1. Interestingly, both of these elements are well conserved in the promoter of the HSV-1 RR1 mRNA although their distances from the HSV-1 mRNA start site differ from those observed in HSV-2.

ii) A potential binding site of the cellular 'octamer motif binding factor' in the P1 sequences. Further analysis of the P1 sequences revealed the existence of a 5'-ATGCAAAT-3' sequence (octamer) at position -136. This sequence element is well conserved in the SV40 enhancer, in the enhancer and the promoter of both heavy- and κ light-chain immunoglobulin genes (Falkner and Zachau, 1984), in different H2B histone promoters (Harvey et al., 1982) and in the enhancers or promoters of several other genes (Falkner et al., 1986). Studies with different octamer motifs demonstrated that each gave rise to complexes with fractionated HeLa cell nuclear extracts which had identical mobilities in gel retardation assays and that different motifs were almost equally competitive for binding to the same factor of the complex (Bohmann et al., 1987). Purified 'octamer motif binding factor' has a stimulatory effect on transcritpion in vitro (Bohmann et al., 1987; Flether et al., 1987). The protein factors that bind the octamer motifs have been designated either OTF-I, or OBP100, or IgNF-A (reviewed in Jones et al., 1988), and they appear closely related to each other and to NF-III (see Introduction, Page 20; reviewed in Hames and Glover, 1988).

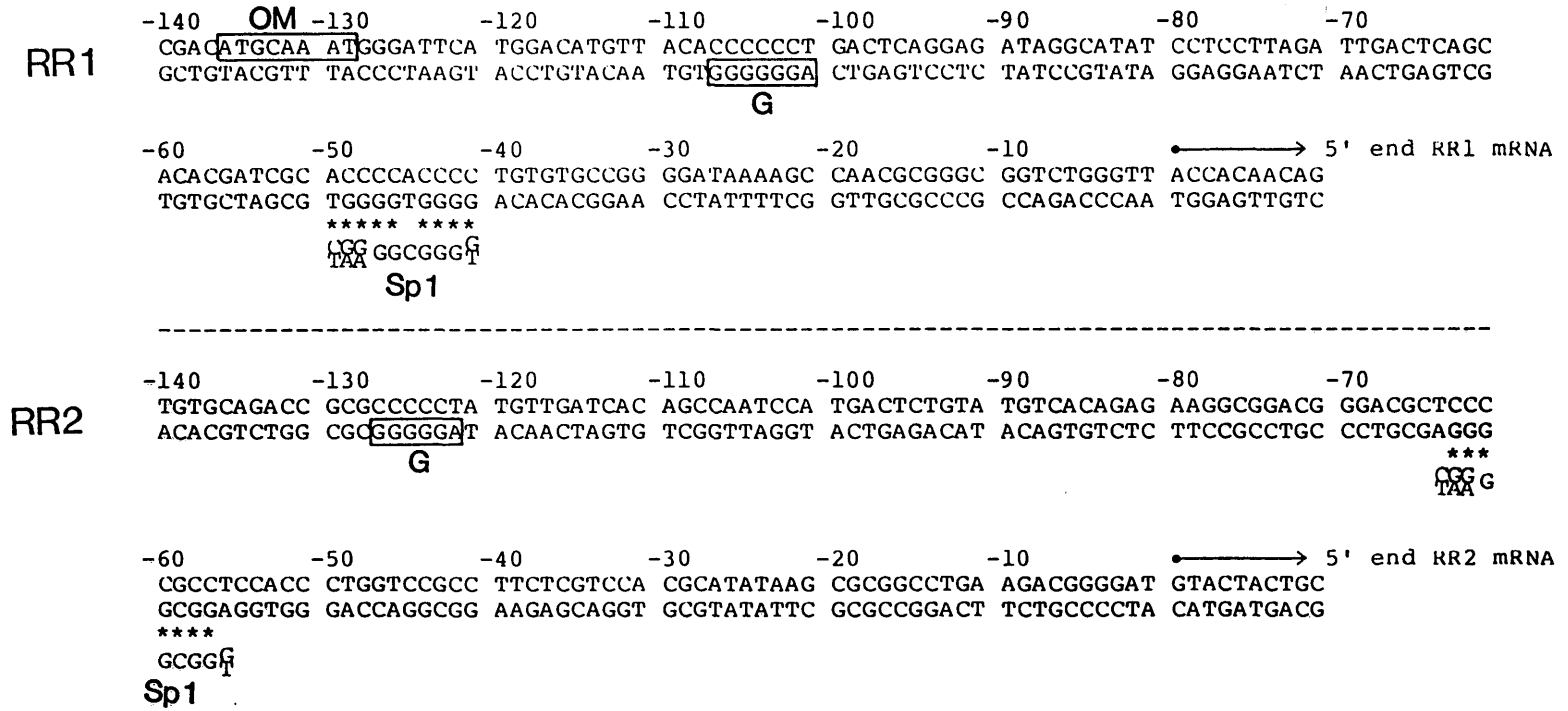
RR1
-140
OM
-130
-120
-110
-100
-90
-80
-70
CGACATGCAA ATGGGATTCA TGGACATGTT ACACCCCCCT GACTCAGGAG ATAGGCATAT CCTCCTTAGA TTGACTCAGC
GCTGTACGTT TACCCTAAGT ACCTGTACAA TGTGGGGGGA CTGAGTCCTC TATCCGTATA GGAGGAATCT AACTGAGTCG
G
-60
-50
-40
-30
-20
-10
5' end RR1 mRNA
ACACGATCGC ACCCCACCCC TGTGTGCCGG GGATAAAAGC CAACGCGGGC GGTCTGGGTT ACCACAACAG
TGTGCTAGCG TGGGGTGGGG ACACACGGAA CCTATTTTCG GTTGCGCCCG CCAGACCCAA TGGAGTTGTC
Sp1
RR2
-140
-130
-120
-110
-100
-90
-80
-70
TGTGCAGACC GCGCCCCCTA TGTTGATCAC AGCCAATCCA TGACTCTGTA TGTCACAGAG AAGGCGGACG GGACGCTCCC
ACACGTCTGG CGCGGGGGGAT ACAACTAGTG TCGGTTAGGT ACTGAGACAT ACAGTGTCTC TTCCGCCTGC CCTGCGAGGG
G
-60
-50
-40
-30
-20
-10
5' end RR2 mRNA
CGCCTCCACC CTGGTCCGCC TTCTCGTCCA CGCATATAAG CGCGGCCTGA AGACGGGGAT GTACTACTGC
GCGGAGGTGG GACCAGGCGG AAGAGCAGGT GCGTATATTC GCGCCGGACT TCTGCCCCTA CATGATGACG
Sp1

Figure 76. Cis-acting transcriptional elements within the promoter sequences of the HSV-2 RR1 and RR2 genes. The nucleotide sequences of both DNA strands are shown and the locations of the mRNA 5' ends are indicated. The consensus sequence of the Sp1 cellular transcription factor binding site is given in the 3' to 5' direction and is aligned with homologous RR1 and RR2 promoter sequences. Residues on the mRNA coding strands which have homology to the Sp1 consensus are indicated by asterisks. The two G-rich motifs (G) are boxed as is the putative 'octamer motif binding factor' consensus.

Analyses of the P1 sequences for potential binding sites of other cellular transcription factors, such as the CTF/CBP (see Introduction, Page 19), were negative. It is therefore suggested that the readily detectable pRR-specified RR1 mRNA constitutive levels of expression are mainly due to the interaction of a complex of cellular transcription factors, one of which is the 'octamer motif binding factor', with the P1 sequences. This is based on the fact that, in analogy to P1, the RR2 gene promoter (P2) sequences contain both a potential Sp1 binding site and a G-rich motif (see Fig. 76). However, P2 lacks the octamer sequence and this may possibly account for the extremely low pRR-borne RR2 mRNA levels observed (see Page 118). This suggestion is currently being investigated by means of deletion mutagenesis (J. McLauchlan, personal communication).

b) Effects of HSV-1 trans-activating functions on RR1 mRNA transcription.

Activation of pRR-specified RR1 mRNA levels with Vmw110 had no detectable effect. As has been noted previously, an internal control plasmid was not included in the co-transfection experiments due to the limited amount of DNA that can be taken up by transfected cells. Nevertheless, Vmw110 is most probably present in cells transfected with pRR and p111, since the pRR-borne RR2 mRNA levels are increased (see Table V). Thus, the failure of Vmw110 to increase RR1 mRNA levels may reflect a real inability, rather than a failure of transfection. On the contrary, Vmw175, either independently or in combination with Vmw110, induced RR1 mRNA constitutive levels by 3-fold. This is in good agreement with the increase in CAT activity observed after co-transfection of a P1-CAT construct with Vmw175 (S. Simpson, personal communication). Equally, induction by HSV-1 infection resulted in an increase of RR1 mRNA levels which, however, was higher than that observed with Vmw175 (5-fold; S. Simpson, personal communication). This result suggests that perhaps one or more of the remaining HSV-1 IE functions may account for this. The most likely candidate

appears to be Vmw12, which is thought to augment the effect of Vmw175 and Vmw110 in activation of E gene expression (O' Hare and Hayward, 1985b).

20. Constitutive and Induced Transcription Levels from the HSV-2 RR2 Promoter.

The main observations on HSV-2 RR2 gene expression were: i) RR2 mRNA basal levels are extremely low in the absence of HSV-1 trans-activating functions, ii) HSV-1 infection or trans-activation with HSV-1 V_{mw175} results in a substantial increase of RR2 mRNA basal levels

The observation that the RR2 mRNA basal levels are barely detectable in the absence of viral trans-activating functions is consistent with the regulation pattern of HSV E genes whereby, in the absence of these functions, E gene promoter activity is generally low (reviewed in Everett, 1987b). In the presence of HSV-1 IE functions, however, a substantial increase of RR2 mRNA levels was observed (see Table V). From the two IE products used in this study, Vmw175 activates P2 more efficiently than Vmw110 and this is in good agreement with other reports on E gene regulation of expression (O'Hare and Hayward, 1985b; reviewed in Everett, 1987b; S. Simpson, personal communication). However, the effect of these polypeptides in combination was additive, whereas, in a study by O'Hare and Hayward (1985b), it resulted in a synergistic increase of P2-CAT-specified CAT levels. Analysis of the trans-activation efficiency of the gD promoter under a variety of experimental conditions demonstrated that quantitative differences, like the one described above, may be due to different cell types and/or transfection methods used (Everett, 1987c). For example, it was shown that co-transfection of a gD promoter-CAT plasmid with Vmw175 and Vmw110 in WSHeLa cells had a very strong synergistic effect whereas in Flow HeLa or BHK cells the effect of these IE products was additive rather than synergistic.

GENERAL DISCUSSION

The work presented in the Results and Discussion Sections has identified a number of points concerning the HSV-1-encoded ribonucleotide reductase, namely:

i) structural and potential functional features resulting from the predicted primary and secondary structures of the large and small subunit polypeptides

ii) the conservation of these features in homologue herpesviral polypeptides and in the equivalent subunit polypeptides of the eukaryotic and prokaryotic enzymes and,

iii) the transcriptional regulation of the HSV-induced ribonucleotide reductase mRNAs.

This Section is concerned with the evolution and the function of the RR1 N-terminal region and with unique features of the RR1 and RR2 subunits which could represent targets for antiviral compounds. Further, the enzyme's transcriptional regulation during the IE and E stages of the HSV lytic cycle is also discussed.

1. The HSV-1 RR1 N-terminal Region.

a) On the evolution of the HSV-1 N-terminal region.

The most interesting feature resulting from the analysis of the HSV-1 RR1 predicted amino acid sequence is the N-terminal region, whose amino acid composition differs from the remainder of RR1, and which is absent from other RR_L polypeptides; as already mentioned (see Results and Discussion, Page 81), this region contains a run of prolines, two stretches of roughly alternating aspartic acid and serine residues and seven sets of tandemly repeated amino acid sequences.

On the basis of these amino acid composition features a hypothetical scheme of evolutionary events leading to the formation of this region can be proposed.

i) It appears likely that an ancestral RR1 gene, which would be similarly sized to the other herpesviral RR_L,

acquired part of the DNA sequences coding for the N-terminal region by a recombination event; this step is suggested by the absence of the RR1 N-terminal region from other RR_L polypeptides. The acquired sequences may have originated from a second virus in the cell or from the host cell.

ii) Second, the DNA regions coding for certain amino acid sequences were duplicated by a gene duplication event whereby the DNA regions corresponding to certain amino acid sequences were duplicated. This step is proposed on the basis of the repeated amino acid sequences observed within this region (see Fig. 29). A number of proteins appear to have evolved by gene duplication (reviewed in Doolittle, 1981), a process resulting in increased stability of the protein or in the enhancement of its functions (reviewed in Li, 1983). One example of this evolutionary mechanism is the ovomucoid protein of bird egg white which is involved in the inhibition of trypsin. It appears that this protein evolved by triplication of the DNA sequences coding for a primordial domain because it consists of three domains exhibiting between them 33% amino acid homology (Kato et al., 1978). As a result of this event, each of the three domains is capable of binding one molecule of trypsin or other serine protease thus enhancing the catalytic activity of the protein.

iii) Finally, it appears that mutations accumulated within the duplicated DNA sequences, and this is reflected by the observed insertions and deletions within the N-terminal regions of the HSV-1 and HSV-2 RR1 polypeptides (see Figs. 33 and 55). It has been proposed that accumulation of mutations within duplicated regions of proteins is due to a relaxation of evolutionary restraints imposed by natural selection (reviewed in Doolittle, 1981).

This evolutionary scheme, however, does not exclude other possible events which lead to the formation of the RR1 N-terminal region (D.J. McGeoch, personal communication). For example it is possible that, instead of this region being acquired by a recombination event, it evolved by mutation of sequences which were originally located upstream from the coding sequences of the ancestral RR1 gene. Further, the evolutionary events might not have occured in

the order described above such that the DNA sequences acquired by the ancestral RR1 gene might have already been duplicated; therefore, the duplication event might have preceded the recombination event.

b) A possible function for the RR1 N-terminal region.

Although the RR1 N-terminal region appears not to be directly involved in ribonucleotide reductase activity (see Results and Discussion, Page 100), it is thought that it has an additional function. This is primarily based on the retention of this region in RR1 and on its distinct amino acid composition as compared to the remainder of RR1. A similar case exists between the HSV-1 Vmw65 IE *trans*-inducing virion polypeptide which contains a C-terminal region of 80 amino acids not present in its VZV homologue Vmw54 (Dalrymple *et al.*, 1985). Studies concerning the function of this Vmw65 region demonstrated that it is required for the *trans*-induction of IE gene expression as an HSV-1 Vmw65 insertion mutant in this region displayed strongly reduced ability to *trans*-induce IE polypeptides (Ace *et al.*, manuscript in preparation). On the other hand, Vmw54, which does not contain a corresponding region, appears to be unable to *trans*-induce the VZV IE polypeptides (T. McKee, personal communication).

It is interesting to propose that certain portions of RR1 Region 1, which comprises the N-terminal region (see Results and Discussion, Page 98), are involved in nuclear localisation of the polypeptide. This suggestion arises from analysis of the amino acid sequences of RR1 Region 1 for motifs indicative of a certain function, whereby, a sequence of KRVPPR was identified (at HSV-1 position 356; see Fig. 55). This sequence is also present in HSV-2 RR1 and closely resembles the proposed nuclear localisation signal for the yeast MATα2 which comprises three hydrophobic amino acids, one of which is a proline, flanked by basic amino acids (Hall *et al.*, 1984). However, it has been proposed that multiple signals may be present in polypeptide domains involved in nuclear localisation (Dingwall *et al.*, 1987). In that respect, another highly conserved HSV-1 and

HSV-2 sequence of RPAAS was also identified (at HSV-1 RR1 position 4; see Fig. 55). Interestingly, this sequence is quite similar to one of the proposed signals for nuclear localisation of nucleoplasmin (RPAAT; Dingwall et al., 1987). Analysis of other HSV-1 polypeptides known to translocate to the nucleus of infected cells identified at the C-terminus of the DNA polymerase two sequences of KRPR and KPRK which resemble the proposed signal for nuclear localisation of the adenovirus E1A gene products (KRPR; Krippl et al., 1985). As can be observed, these signals are homologous to the first of the potential RR1 signals.

Large protein molecules appear to accumulate in the nucleus by an ATP-dependant selective entry mechanism which is mediated by the part of the protein containing the nuclear localisation signals (Feldherr et al., 1984; reviewed in Dingwall and Laskey, 1986). This is evidenced by the fact that the rate of nuclear uptake is faster than can be accounted for by diffusion. Feldherr et al. (1984) studied the selective entry mechanism of nucleoplamin, a 165,000 mol. wt. nuclear protein of the Xenopus laevis oocyte. These authors proposed that nucleoplasmin binds to proteins, termed the annular material, at the cytoplasmic surface of the nuclear pores and that this binding occurs only when the domain comprising the nuclear localisation signals is present. Then, nucleoplasmin is transported in the nucleus through the centre of the nuclear pore where it is dissociated from the annular material.

If RR1 is transported to the nucleus, one could speculate that binding of RR1 to the annular material and subsequent transport in the nucleus is mediated by part 1 of the N-terminal region (RR1 positions 1 to 150, see Fig. 55). The GARNIER program demonstrated that this part of the N-terminal region is punctuated by seven stretches of hydrophobic amino acids, predicted to adopt β-strand conformation, while the intervening sequences were predicted as unstructured. Hence, part 1 may adopt an ordered three-dimensional structure consisting of a hydrophobic core of seven β-strands which are joined by unstructured or loop regions and which are arranged in one or more β-sheets (Nikas et al., 1986). This structure resembles the

three-dimensional structure of the immunoglobulin V_L domains; these consist of seven β-strands which are arranged in two β-sheets and which are joined between them by hypervariable loops (Poljak et al., 1974). On the basis of the similarity of the three-dimensional structures of part 1 with the immunoglobulin V_L domains and in conjunction with the fact that the latter are involved in antigen binding, it is possible that part 1 of the RR1 N-terminal region is involved in protein-to-protein interactions (W.R. Taylor, personal communication). These could possibly be interactions with the annular material of the nuclear pore in order to transport RR1 into the nucleus. In that respect, it is interesting to note that the mammalian M1 subunit, which lacks the N-terminal region, is exclusively localised in the cytoplasm of cells as deduced from studies using monoclonal antibodies directed against M1 (Engstrom et al., 1984). Clearly, the involvement of the N-terminal region in RR1 nuclear localisation requires rigorous investigation.

It is unclear as yet why the RR1 polypeptide should localise to the nucleus of infected cells. A strong possibility is that nuclear localisation of RR1 is not related to ribonucleotide reduction as dNTPs are formed in the cytoplasm of cells (reviewed in Reichard, 1988). As reported on Page 81, the N-terminal region contains two aspartic acid and serine rich stretches which are similar in amino acid composition to stretches present at the N-terminal regions of all the IE polypeptides with the exception of Vmw12. One could therefore speculate that the RR1 and the equivalent IE polypeptide stretches may be involved in similar processes. In an attempt to elucidate the function of the aspartic acid and serine rich stretch of Vmw175, Paterson and Everett (1988) have shown that deletion of these sequences reduces, althought not drastically, the ability of Vmw175 to trans-activate E gene expression in short-term transfection assays.

2. Ribonucleotide Reductase as a Target for Antiviral Compounds.

As reported in the Introduction (see Page 53), studies with the synthetic oligopeptide YAGAVVNDL demonstrated that, *in vitro*, it selectively inhibited the HSV-specified activity by subunit dissociation. Thus, it became apparent that inhibition of subunit interaction may have important therapeutic implications. However, exposure of infected cell monolayers to various concentrations of the nonapeptide did not reduce the yield of infectious virus most probably because it was too large to enter the cell (Dutia *et al.*, 1986). As a result, effort is currently being put into the development of antiviral compounds designed to mimic the mode of function of the nonapeptide; these compounds should have a three-dimensional structure similar to the nonapeptide and be able to enter the infected cell.

The RR_L alignment identified a number of amino acid sequences which are not conserved in the prokaryotic or eukaryotic polypeptides and which may be contact regions of the RR1 and RR2 subunits or may represent regions related to the activity of the HSV-1-induced enzyme. These sequences are retained in blocks 1, 5, 12 and 13 of the RR_L alignment (see Fig. 56). From these, of particular interest is block 5 which is missing from the mammalian polypeptide and is predicted to map within a hydrophobic region of the HSV-1 RR1 polypeptide (see Fig. 27); therefore, it is quite possible that it may be located at the interface of the RR1 and RR2 subunits and be involved in their association. Further, as block 5 is highly conserved between the herpesviral RR_L, compounds that prevent its function could prove general inhibitors of the herpesviral enzymes. Another region of interest is the N-terminal region which is unique to the HSV RR1 and may be required for translocation to the nucleus. As postulated above, any nuclear localisation of RR1 may be unrelated to ribonucleotide reduction but instead related to some as yet unknown function. If this was an essential HSV function, compounds preventing nuclear localisation of RR1 may prove potential antivirals.

In contrast to the RR_L polypeptides, the majority of the RRS blocks are present in

the mammalian enzyme (McLauchlan, 1986). The only exception appears to be block 16 which, along with its flanking amino acid sequences, is conserved only in the HSV and VZV polypeptides (see Fig. 57). However, this region may not be involved in subunit association as it was predicted to map within a hydrophilic region of HSV-1 RR2 (data not shown).

3. Regulation of the HSV Ribonucleotide Reductase Activity.

The results reported in Results and Discussion Section E, in conjunction with the unpublished data of J. McLauchlan and S. Simpson, allow the proposal of a general scheme for the transcriptional regulation of the ribonucleotide reductase genes under IE and E conditions.

a) Transcriptional regulation of the RR1 and RR2 genes under IE conditions.

On the basis that RR1 mRNA constitutive levels are readily detectable in the absence of functional IE polypeptides (see Results and Discussion, Page 117), it is proposed that during viral infection, RR1 gene expression is activated under IE conditions. This is most probably achieved by the interaction of cellular and/or viral transcription factors with target sequences positioned in the P1 promoter. One of the cellular factors may possibly be Sp1, potential binding sites of which have already been identified in the P1 sequences (see Results and Discussion, Page 121). Second, RR1 expression could be activated by a complex containing Vmw65 and cellular transcription factors. Vmw65 is considered as a P1 activator because co-transfection of a Vmw65-expressing plasmid with a P1-CAT construct resulted in a significant increase of CAT levels (S. Simpson, personal communication). However, as Vmw65 does not bind HSV DNA directly (see Introduction, page 20), it is expected to act as a constituent of a complex with cellular transcription factors. One of these may be related to the 'octamer motif binding factor', this suggestion being based on the presence of the octamer in the P1 sequences (see Results and Discussion, Page 122). Recent data indicate that HSV-2 DNA fragments containing the P1 octamer

are retarded in gels by Vmw65/cellular transcription factors complexes (J. McLauchlan, personal communication). Interestingly, these complexes have similar mobility in gels with the IEC complex formed between DNA fragments containing the TAATGARAT motif of the regulatory region of the IE gene promoters (see Introduction, Page 20).

In contrast to RR1, under IE conditions, the RR2 gene is not expressed. This suggestion is clearly evidenced by the extremely low contitutive RR2 mRNA levels observed: i) in the absence of functional IE products (see Results and Discussion, Page 119) and ii) after infection with the HSV mutant tsK in the presence of cycloheximide (J. McLauchlan, personal communication).

b) Transcriptional regulation of the RR1 and RR2 genes under E conditions.

In the presence of functional IE gene products the RR1 mRNA levels increase further as compared to the levels observed under IE conditions; upon reversal of the cycloheximide block and in the presense of actinomycin D, tsK specified elevated levels of RR1 mRNA as compared to those specified under IE conditions (J. McLauchlan, personal communication). Likewise, under E conditions, RR2 gene expression is also activated and this is evidenced by the substantial increase of RR2 mRNA levels in the presence of Vmw175 and/or Vmw110 (see Results and Discussion, Page 119).

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