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Related functional domains in virus DNA polymerases

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Analysis of the lesions in several drug-resistant DNA polymerase mutants of herpes simplex virus along with comparative analysis of the published polymerase sequences of other human herpesviruses has shown that most lesions (five out of six) are substitutions at amino acid residues conserved in all four polymerases. Furthermore, the majority of lesions are in regions of the polypeptide where there are marked clusterings of conserved residues. On the basis of these data we have identified several domains within the polypeptide which we believe may have important functional roles in the action of the enzyme. The apparent restriction in the potential sites of lesions conferring drug resistance may explain the difficulty in selecting such mutants using acyclovir (ACV) in culture and their failure to emerge so far during ACV therapy. Extension of the comparative analysis to the polymerases of adenovirus type 2, vaccinia virus and phage ϕ29 suggests that these enzymes also possess domains homologous to those most conserved in the herpes polymerases (regions I-III) and that these domains have a similar linear spatial distribution on the polypeptides. The results are discussed in relation to the known function of the DNA polymerases.

Key words: DNA polymerase/drug resistance/functional domains/herpes simplex virus/sequence analysis

Introduction

A key enzyme in the multiplication of all human herpesviruses is the virus-coded DNA polymerase. Not only does this protein play a central role in the replication of the virus genome, it also provides us with an ideal target for antiviral drugs (see Larder and Darby, 1984). Despite the importance of DNA polymerase, relatively little is known about the interaction of this protein with its various substrates, its DNA template, or other proteins involved in the DNA replication complex. Information of this nature would be of fundamental interest and would also aid the future design of more effective anti-herpes drugs.

Recently, the DNA polymerase genes from several of the human herpesviruses have been sequenced and it is clear from these studies that they are closely related (Baer *et al.*, 1984; Gibbs *et al.*, 1985; Quinn and McGeoch, 1985; Davison and Scott, 1986; Kouzarides *et al.*, 1987). Furthermore, the herpesvirus polymerases are partially homologous with other apparently unrelated polymerases such as those of vaccinia, adenovirus type 2 (Ad2) and the bacteriophage ϕ29 (Gibbs *et al.*, 1985; Quinn and McGeoch, 1985; Davison and Scott, 1986; Kouzarides *et al.*, 1987; Argos *et al.*, 1986; Earl *et al.*, 1986). These observations have of course led to speculation that areas of homology may represent important functional regions in the polypeptide.

While comparison of sequences derived from different virus species can generate useful information, it can be equally informative to compare variants derived from a single virus strain. This type of approach has been used to identify several putative components of the active centre of the HSV-1 thymidine kinase (TK) (Darby *et al.*, 1986) and it was expected that a similar approach would provide insight into the functional regions of the HSV-1 DNA polymerase.

We have recently described a collection of drug-resistant mutants all derived from the HSV-1 *wt* strain SC16 using selection in acyclovir (ACV) in a TK-transformed baby hamster kidney (BHK) cell line (Larder and Darby, 1985). Both biochemical data and the patterns of drug resistance of these variants suggested that they had lesions in DNA polymerase (Larder and Darby, 1985, 1986). They could be broadly divided into three groups based on their sensitivity to the pyrophosphate analogue, phosphonoacetic acid (PAA) and within each group, individual variants showed different levels of resistance to ACV. Such a collection of mutants provided the opportunity to examine the effects of alterations in the gene on the functional properties of the protein.

The aim of the present study was therefore to identify the specific lesions in representative variants by nucleotide sequencing, to relate the predicted changes in amino acid sequence to observed changes in the properties of the enzymes and to identify functionally important regions of the herpesvirus DNA polymerase.

Results

Mapping drug resistance mutations by marker rescue

The mutants used in this study are shown in Table I together with ACV and PAA sensitivity data. The variant RSC-26 was

Table I. Properties of ACV-resistant mutants

		Fold changes in sensitivity[a]		Recombination frequency[b]		
		ACV	PAA	ACV (4.4 μM)	ACV (13.3 μM)	PAA (730 μM)
wt	SC16	1	1	0.024	0.03	0.003
PAA^r	RSC-26	10	8	–	–	–
	TP2.4	19	10	–	–	–
	TP2.5	47	20	0.37	–	0.25
PAA^s	TP1.3	3	1.3	–	–	–
	TP2.7	29	2.6	0.17	–	–
	TP4.4	233	0.6	–	0.2	–
PAA^{hs}	TP3.2	59	0.18	–	0.42	–
	TP4.1	187	0.24	–	0.23	–

[a]These values were derived from plaque reduction assays and represent ED_{50} for mutant per ED_{50} for *wt*. These data were adapted from Larder and Darby (1985) and Larder *et al.* (1986).
[b]Recombination frequency values were calculated as (plaque number with inhibitor present per total plaque number) × 100. The concentration of either ACV or PAA used in each experiment is shown in brackets at the top of each column.

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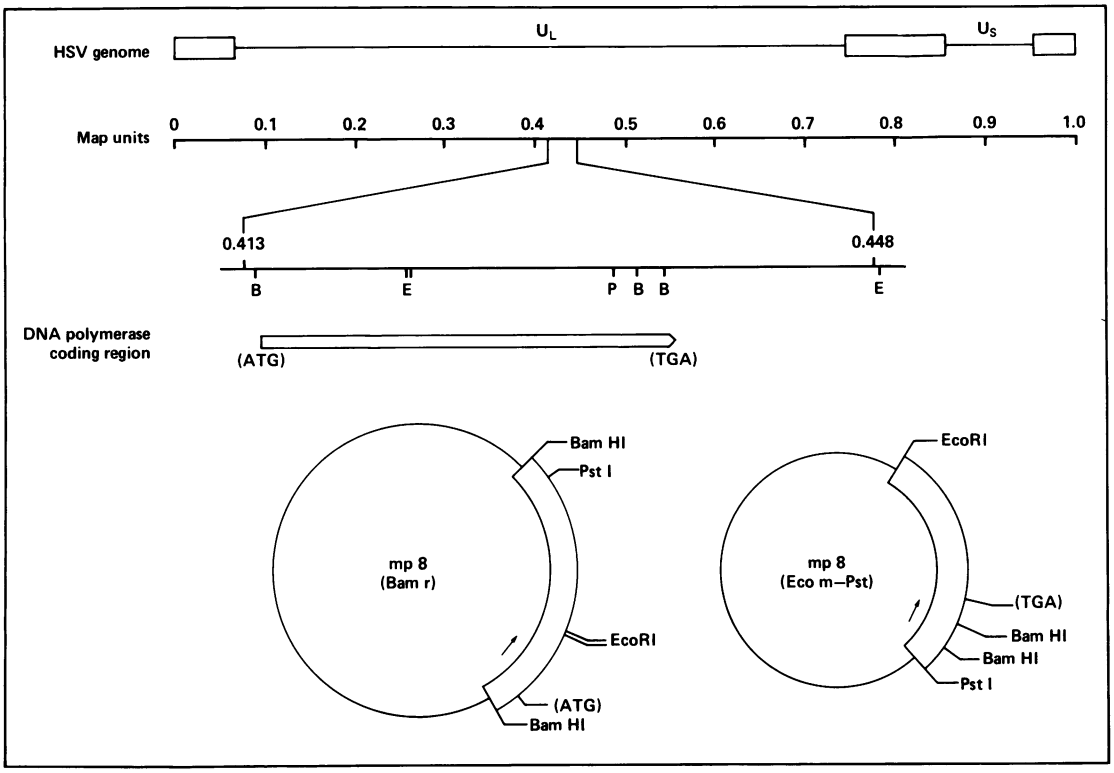

Fig. 1. Cloning HSV DNA polymerase gene fragments. The HSV prototype genome is shown indicating the map position of the DNA polymerase gene. An expanded region of the genome depicts a number of restriction enzyme sites within and around the polymerase gene coding region, B = *Bam*HI, E = *Eco*RI and P = *Pst*I. The lower part of the figure indicates the M13mp8 clones constructed containing the *Bam*r and *Pst*I–*Eco*RI fragments used to obtain sequence data. The arrows show the orientation (5′ → 3′) of the polymerase gene coding region in the constructs.

included since it had been established previously by marker transfer experiments that this virus, derived from the *wt* strain SC16, had a lesion in the coding region of the DNA polymerase (Larder *et al.*, 1986) and some characterization of the mutant enzyme had been performed (Darby *et al.*, 1984). The remaining mutants (the TP series) were all isolated from TK-transformed BHK cells in the presence of ACV, and while there was good evidence suggesting that they had polymerase lesions this had not been formally proved. To obtain more direct evidence we performed marker rescue experiments using the *Bam*HIr (*Bam*r) restriction fragment cloned from selected viruses (see Figure 1). This fragment contains about 87% of the polymerase gene coding region. Each *Bam*r fragment was mixed with infectious wt genomic DNA and the mixture was used to transfect BHK cells. Progeny virus was harvested and titrated in the presence and absence of either ACV or PAA and recombination frequencies were determined (Table I). In all viruses tested, the recombination frequencies indicated drug resistance markers mapping to the *Bam*r fragment.

Nucleotide sequencing

The overlapping restriction fragments *Bam*r and *Eco*RIm (*Eco*m) span the entire DNA polymerase coding region which comprises 3705 bp (Figure 1). These fragments were derived from the *wt* virus and from each of the mutants. The *Bam*r fragments were cloned directly into M13mp8 digested with *Bam*HI and the *Eco*m fragments were further digested with *Pst*I, and then the larger of the resulting fragments (2.5 kb) was cloned into M13mp8 digested with *Eco*RI and *Pst*I (Figure 1). Single-stranded DNA derived from the bacteriophage clones was sequenced by the dideoxy chain termination method using a set of 20 oligonucleotides (17-mers) as primers spaced at intervals of ~180 nucleotides along the gene. The sequences of the primers were based on published data (Gibbs *et al.*, 1985; Quinn and McGeoch, 1985).

The entire nucleotide sequence of the *wt* DNA polymerase gene (strain SC16) and the predicted amino acid sequence of the polymerase polypeptide is shown in Figure 2.

Predicted amino acid substitutions

The nucleotide sequence of the polymerase gene of each of the eight ACV-resistant mutants shown in Table I was determined. The only changes in sequence observed were base-substitutions expected to result in amino acid substitutions. The changes are summarized in Table II. Six mutants had single amino acid substitutions and three of these (TP3.2, TP4.1 and TP4.4) had an identical change (Asn → Ser at residue 815). The most PAA-resistant mutant (TP2.5) had two amino acid substitutions 369 residues apart (Gly → Asp at nucleotide 355 and Ser → Asn at 724). The change at residue 355 in TP2.5 was closest to the N terminus of the polypeptide, most of the others being clustered in the carboxy-terminal region between residues 719 and 841. The sequence at residue 355 was also the only one affecting the overall charge on the molecule. No mutation was found in the polymerase gene from TP1.3, which had a sequence identical to *wt*.

Amino acid homology between the human herpesvirus DNA polymerases

The amino acid sequences of the human herpesvirus DNA polymerases were aligned to generate maximum cross-homology. The data used for this comparison were published EBV (Baer *et al.*, 1984), HCMV (Kouzarides *et al.*, 1987) and VZV (Davison and Scott, 1986) sequences, along with the HSV-1 SC16 sequence described in this paper. Following alignment, the residues in HSV-1 which were identical in the other three polypeptides were

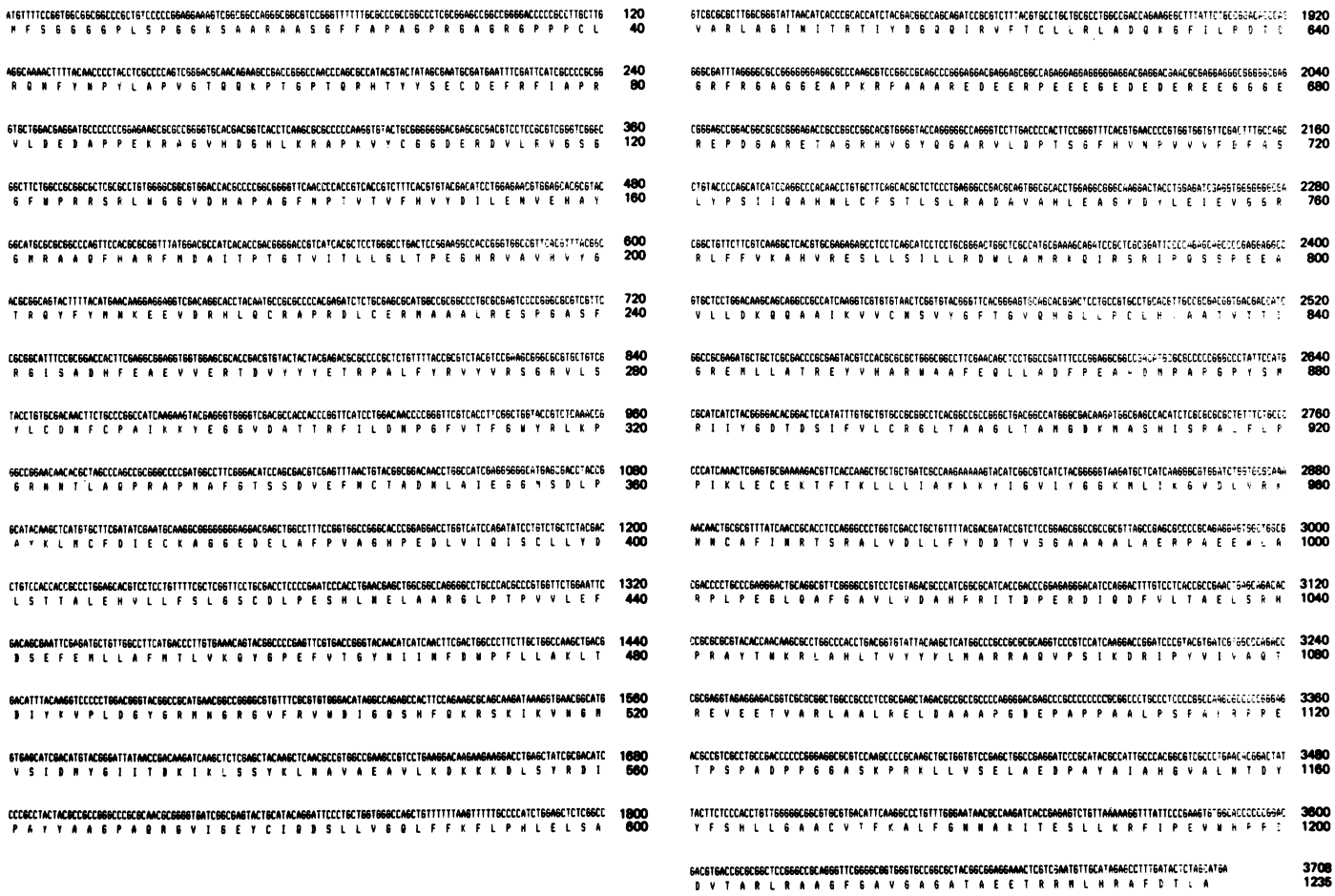

Fig. 2. HSV-1 strain SC16 DNA polymerase gene. The nucleotide sequence of the HSV-1 strain SC16 DNA polymerase gene coding region is shown with the predicted amino acid sequence illustrated beneath.

identified and this information was then used to construct the amino acid conservation plot shown in Figure 3. The striking feature which emerged from this analysis was that the conserved residues (18% of the total residues in the HSV-1 polypeptide) were not distributed randomly but clustered in specific regions. The four regions most conserved (labelled I−IV) are all located between residues 700 and 1000. Three other regions (V−VII) are of particular interest because they have an excess of basic amino acids (Table III). In these regions the ratio of basic:acidic residues is considerably higher (in the range 4−5:1) than the average over the whole polypeptide (1.2:1). Region IV is included in Table III as it also has an excess of basic amino acids (25%) and a high ratio of basic:acidic residues (6). The amino acid substitutions in the mutant polypeptides are indicated on Figure 3. Five out of the six changes are in conserved amino acids, the exception being the change at residue 355 in TP2.5. Furthermore, two of the changes are in close proximity within region II and two more are in region III, both highly conserved domains in the polypeptide.

Secondary structure predictions

Secondary structure predictions on the HSV-1 DNA polymerase polypeptide were performed using the Robson algorithm (Garniea *et al.*, 1978), and a program devised by Dr C.Hodgman. Although such predictions taken in isolation may be of limited reliability, a number of interesting features emerged. Specifically, the highly conserved regions I−III are all flanked by regions strongly predicted to be α-helices (see Figure 4). Within the domains themselves there is little predicted α-helix and they appear to consist predominantly of β-pleated sheet and turns. Possible implications of these findings are discussed below.

Table II. Nucleotide changes and predicted amino acid substitutions in mutant virus DNA polymerases

	Virus	Nucleotide residue number	Nucleotide change	Amino acid residue number	Amino acid substitution
PAA^r	RSC-26	1790	G → T	597	Glu → Asp
	TP2.4	2155	C → T	719	Ala → Val
	TP2.5	1063	G → A	355	Gly → Asp
		2170	G → A	724	Ser → Asn
PAA^s	TP2.7	2520	G → A	841	Gly → Ser
	TP4.4	2443	A → G	815	Asn → Ser
PAA^{hs}	TP3.2	2443	A → G	815	Asn → Ser
	TP4.1	2443	A → G	815	Asn → Ser

Amino acid homology with other virus DNA polymerases

Several groups have recently noted limited homology at the amino acid level between one or other of the herpesvirus DNA polymerases and those of vaccinia, Ad2 and ϕ29 (Gibbs *et al.*, 1985; Quinn and McGeoch, 1985; Davison and Scott, 1986; Kouzarides *et al.*, 1987; Argos *et al.*, 1986; Earl *et al.*, 1986). We have extended these analyses looking particularly at the highly conserved regions in the herpes polymerases (I−IV), searching for

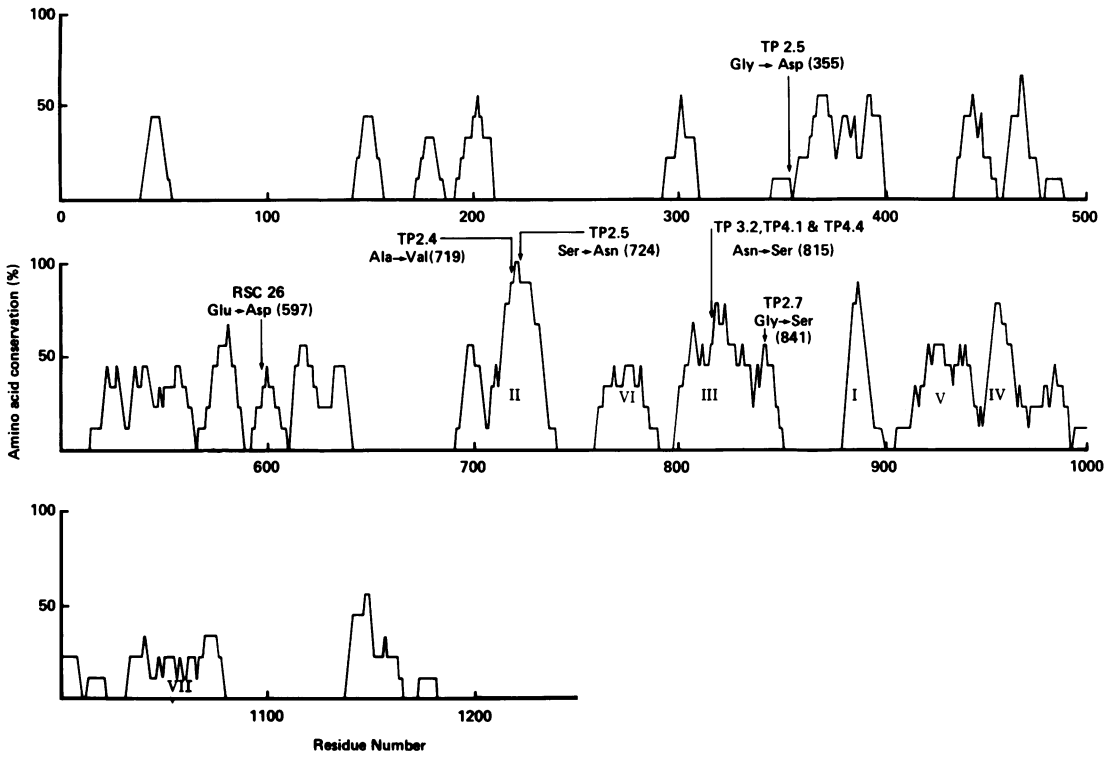

Fig. 3. Amino acid homology between human herpesvirus DNA polymerases. The amino acid sequence of the four human herpesvirus DNA polymerases were used to generate maximum cross-homology and amino acids conserved in all four identified. Conserved regions in the HSV-1 polymerase are illustrated, the amino acid sequence is represented on the plot with the N terminus at the top left hand side. The value plotted for a particular residue represents the proportion of conserved amino acids in a stretch of nine, centred on that residue. The position and nature of the amino acid substitutions found in each mutant DNA polymerase are illustrated. The properties and putative function of each region, numbered I–VII, are discussed in the text.

homologies in the other polymerases and analysing the spatial arrangement of such homologies. The regions of homology identified are illustrated in Figure 5. We could find no regions homologous to region IV of the herpes polymerases but there were marked homologies to the other three regions (I–III).

In these analyses we considered only amino acids conserved in all four herpesviruses and we looked for conservation of these residues in the other polypeptides. There was remarkable homology with all three conserved regions, the only doubtful region being the Ad2 sequence homologous to region II (Figure 5). If we look for residues present in not less than six of the seven polymerases considered we can identify consensus sequences associated with each of these regions (Figure 5). Taken alone these sequences may appear of little significance. However their importance is suggested when their location in the polypeptides are also considered (Figure 6). Firstly the three regions are in the same linear arrangement on each polypeptide (II–III–I), and secondly the distances between consensus sequences are remarkably similar (~100 amino acid residues in each case).

Discussion

The sequence data recently obtained for the human herpesvirus DNA polymerases demonstrate that these enzymes possess clusters of highly conserved amino acids (Figure 3), and this poses the question as to whether the conservation is in any way a reflection of the functional importance of these regions. There are compelling reasons for believing this to be the case, certainly for the most conserved regions I–III. We and others have shown that these domains appear to have homology with domains in the apparently unrelated DNA polymerases of vaccinia virus, adenovirus and phage ϕ29 (Gibbs *et al.*, 1985; Quinn and McGeoch, 1985; Argos *et al.*, 1986; Kouzarides *et al.*, 1987; Earl *et al.*, 1986). Furthermore these domains are arranged in the same sequence and are similarly spaced in all the polymerase polypeptides (Figure 6). Additional evidence for the functional importance of regions II and III is that each of these regions contains the sites of mutations which alter the drug sensitivity of the enzymes (Table I and Figure 3). It is interesting that secondary structure predictions for the herpes simplex virus polypeptide suggest that regions I–III may be composed predominantly of β-pleated sheet flanked by α-helices (Figure 4), and it is attractive to speculate that these regions may be in close proximity on the native protein to provide the catalytic and substrate binding surface of the molecule.

It is difficult at this stage to assign specific functions to the conserved regions. Quinn and McGeoch (1985) suggested that region I (residues 883–896) may exist as a consequence of a common interaction between this part of the polypeptide and a host cell protein involved in the replication complex. However an alternative is that it may contribute to the catalytic or substrate binding regions of the active site. The lack of amino acid substi-

Table III. Basic domains in the polymerase polypeptide

Region	Amino acid residues	Proportion basic residues (%)	Ratio basic/acidic residues (%)
Whole polypeptide	1–1235	12	1.2
IV	949–971	25	6
V	912–946	22	4
VI	760–790	29	4.5
VII	1032–1080	20	5

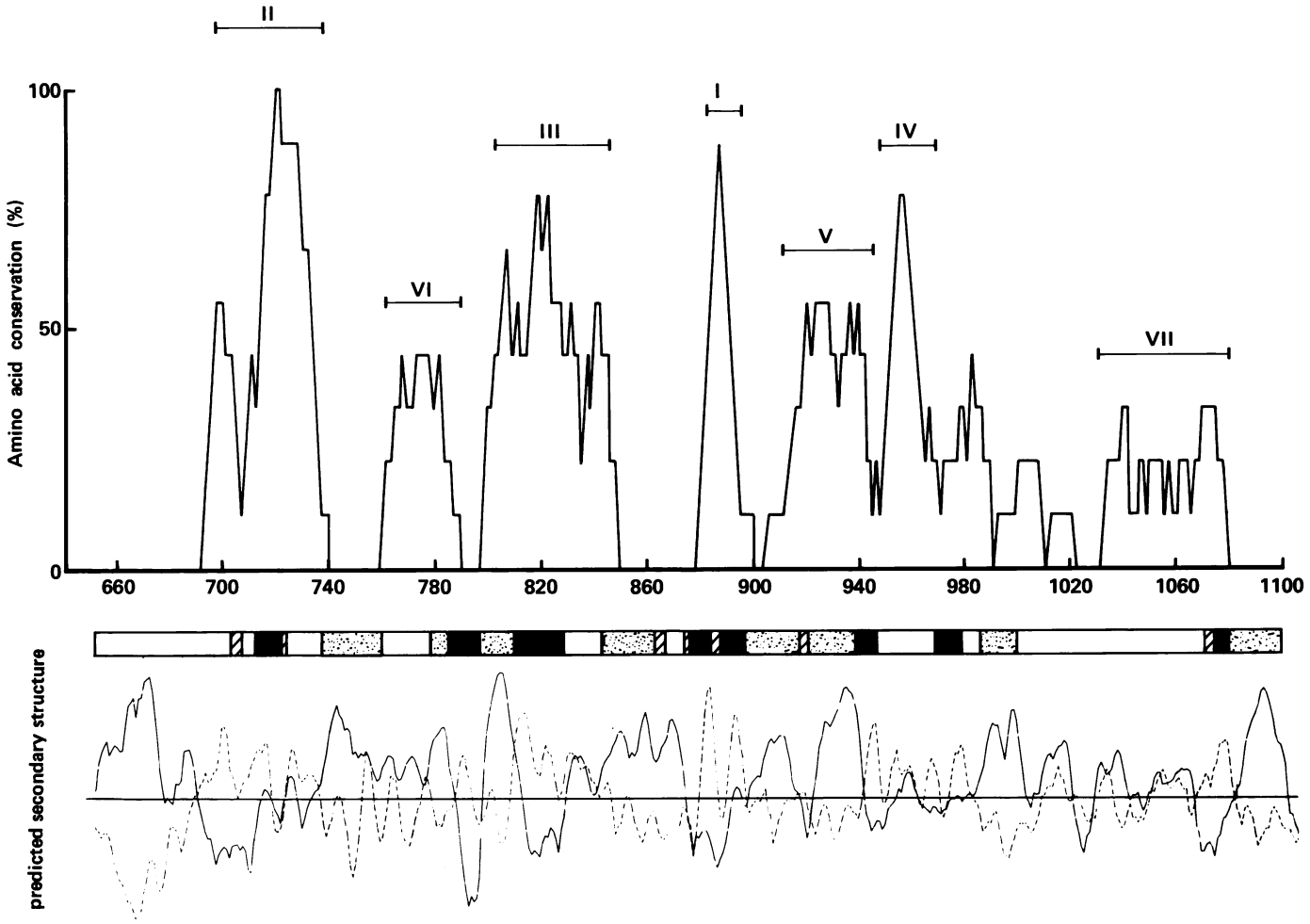

Fig. 4. Amino acid homology and predicted secondary structure of HSV-1 DNA polymerase between residues 660–1100. The top part of the figure illustrates amino acid conservation between the four human herpesvirus DNA polymerases between residues 660–1100 of the HSV-1 polypeptide. This plot is an expanded version of that shown in Figure 3, and depicts the conserved regions I–VII. The lower part of the figure shows a secondary structure prediction plot for the HSV-1 polymerase between residues 660–1100, constructed using the Robson algorithm (Garniea *et al.*, 1978). The solid line predicts α-helix and the dotted line β-pleated sheet. Positive values (in centinats) are plotted above the mid-line and negative values below. The blocked regions above the secondary structure prediction plot summarizes data derived from the predictions. Dotted areas (▨) represent likely α-helix, heavy blocked areas (■) represent likely β-pleated sheet and hatched areas (▨) represent likely turns.

tutions in this region weighs slightly in favour of the former hypothesis. Other clues to function are the effects of lesions in these regions on the properties of the enzyme. Both TP2.4 and TP2.5 have lesions in region II (residues 696–736) and as well as showing resistance to ACV these mutants are also resistant to PAA which is known to interact with the pyrophosphate binding site on the enzyme. Region II must therefore be considered a strong candidate for this site. One argument against this idea is the conspicuous absence of basic amino acids not only in the consensus sequence (Figure 5) but also, for herpes and vaccinia polymerases, in the surrounding residues. A further problem in this interpretation is that TP2.5 contains a second amino acid substitution outside region II (Gly → Asp at residue 355) and this could account for the PAA-resistant phenotype of the enzyme. However, since the lesion in region II (at residue 724) is separated by only four amino acid residues from the only lesion in TP2.4 (at residue 719) it is tempting to argue that it is the region II lesions which confer on the mutants their strikingly similar phenotypic characteristics.

In contrast to lesions in region II, those in region III have little effect on PAA sensitivity although they still reduce ACV sensitivity. They may therefore have a direct role in nucleotide binding rather than in pyrophosphate exchange. One of the residues in the region III consensus sequence is the basic amino acid lysine (Figure 5) and this may interact with the acidic phosphate moiety of the nucleotide. We have no direct evidence for the functions of the basic regions (IV–VII) but we can speculate that they may interact with DNA, either binding the double-stranded helix or single-stranded template.

A number of interesting points regarding ACV resistance have emerged from analysis of the sequence data presented here. Firstly it is clear that a single amino acid substitution can confer considerable resistance to ACV. However since the majority (five out of six) of the substitutions were in amino acid residues conserved on all four herpesvirus polymerases it is possible that there are a restricted number of sites at which such substitutions can occur. It is also interesting in this respect that all five substitutions in conserved amino acids were themselves conservative. The non-conservative change (Gly → Asp at residue 355) observed in TP2.5 is a change whose significance is questionable (see above). Apparent constraints on substitutions conferring ACV resistance may explain why it is relatively difficult to isolate such mutants using ACV (Larder and Darby, 1984, 1985). However it is not clear why the same arguments should not apply to PAA and phos-

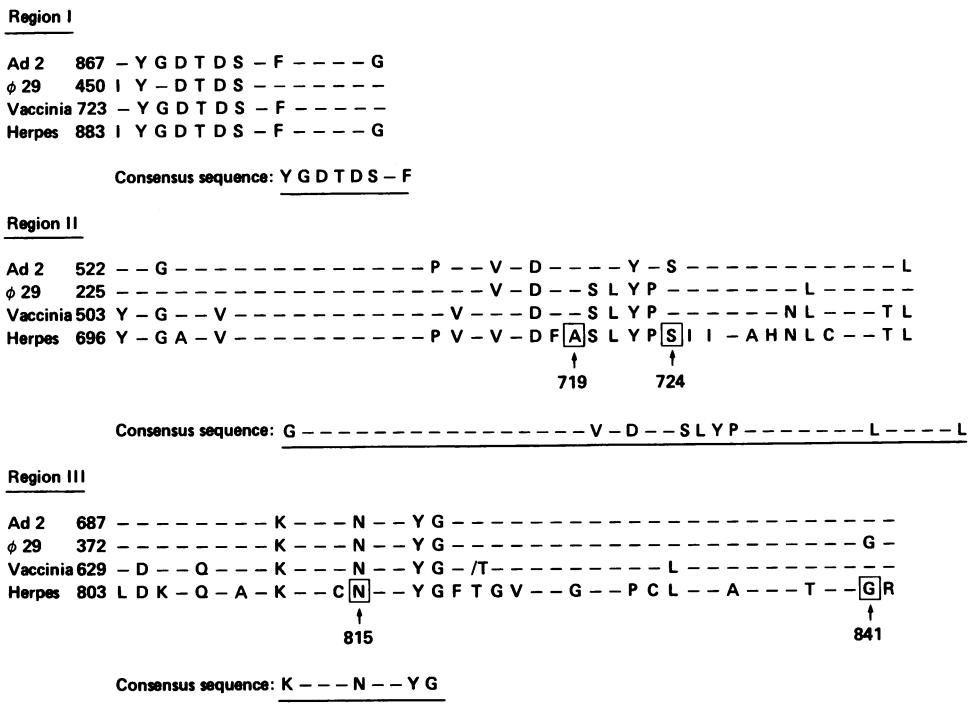

Fig. 5. Regions of homology between the human herpes polymerases and those of vaccinia, Ad2 and φ29. This figure shows amino acid residues conserved between all four human herpesviruses in regions I–III. Alignment of the vaccinia, Ad2 and φ29 polymerase sequences have allowed identification of homologous regions in those polypeptides and the amino acids in common with the herpes polymerases are indicated. A dash (–) indicates no absolute conservation but no attempt was made to distinguish conservative and non-conservative changes. Also indicated on this figure are the numbers of the first residue in each sequence, the positions of lesions identified in these regions of the herpes simplex polymerase (↑) and consensus sequences derived from the observed homologies. The consensus sequences represent residues conserved in the herpes polymerases and at least three of the other four polypeptides. The slash (/) in region III of the vaccinia polypeptide indicates an insertion of 17 nucleotides relative to HSV.

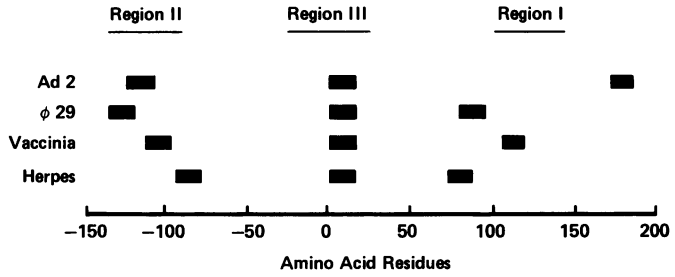

Fig. 6. Spatial relationships between consensus sequences. The relationships between the positions of the consensus sequences (black bars) in each of the polypeptides are shown. In each case the first residue in the region III consensus is taken as residue 1.

phonoformate (PFA) where it is relatively easy to select resistant mutants (Klein and Friedman-Klein, 1975; Hay and Subak-Sharpe, 1976; Honess and Watson, 1977; Eriksson and Oberg, 1979; Derse *et al.*, 1982). One possible explanation is that PAA and PFA exert additional effects resulting in an increased mutation rate. This could simply be less effective inhibition of DNA synthesis allowing more rounds of DNA replication and thus more opportunity for mutations to appear.

Finally, our data suggest that there may be mutations in genes other than polymerase which modulate the sensitivity of the replication complex to ACV triphosphate or PAA. Three mutants (TP3.2, TP4.1 and TP4.4) have identical amino acid substitutions in the polymerase gene and yet they were originally distinguished on the basis of differences in their sensitivity to ACV and PAA. Furthermore TP1.3 had a *wt* polymerase gene but showed a small consistent decrease in ACV sensitivity relative to *wt* virus. In fact previous reports have demonstrated that mutations in the major DNA binding protein can confer slight changes in sensitivity to other drugs targetted to DNA replication such as PAA and aphidicolin (Honess *et al.*, 1984; Chiou *et al.*, 1985) and so this would therefore be a strong candidate gene for such a lesion. This possibility is currently under investigation.

Materials and methods

Cells and virus strains

Baby hamster kidney cells (BHK-21) were used in this study. They were maintained in Glasgow modified Eagle's medium supplemented with 10% newborn calf serum and 10% tryptose–phosphate broth. The *wt* HSV-1 strain SC16 (Hill *et al.*, 1975) and drug-resistant mutants derived from it were used. Construction and properties of the ACV-resistant recombinant, RSC-26, have been described previously (Darby *et al.*, 1984). The ACV-resistant 'TP' collection of mutants, which were all isolated using ACV during the same series of experiments, have been described previously (Larder and Darby, 1985, 1986).

Virus stocks were made by infection of BHK cells at low multiplicity.

Plasmid construction and DNA manipulation

The source of DNA for marker-rescue experiments came from recombinant plasmids constructed with the virus *Bamr* fragment inserted into the *Bam*HI site of pBR322. This fragment contains about 87% of the HSV-1 DNA polymerase gene coding region. Virus DNA, isolated from infected BHK cells essentially as described by Pignotti *et al.* (1979), was digested with *Bam*HI and fragments separated by electrophoresis in 0.8% Tris–acetate–EDTA agarose gels. The *Bamr* fragment from each virus was purified and ligated with *Bam*HI cleaved pBR322. This mixture was used to transform *Escherichia coli* (strain TG-1) made competent by the method of Hanahan (1983). Ampicillin-resistant, tetracycline-sensitive colonies were identified and screened for plasmids containing the *Bamr* insert, by the alkaline-lysis method of Birnboim and Doly (1979). Large-scale isolation of plasmid DNA was carried out using a modification of the alkaline-lysis procedure as described by Maniatis *et al.* (1982).

Recombinant M13 clones were constructed in essentially the same way as described above for pBR322 plasmids. Briefly, purified *Bamr* fragments were

ligated with *Bam*HI digested M13mp8 RF, and *Eco*m fragments (containing the 3′ end of the polymerase gene coding region) were digested with *Pst*I, and the larger of the resulting fragments (~2.5 kb) was ligated with M13mp8 RF (digested with *Eco*RI and *Pst*I). These mixtures were used to transform TG-1 cells, resulting in the formation of recombinant bacteriophage plaques. (See Figure 1 for M13mp8 constructs and genome location of the *Bam*r and *Eco*m fragments.)

Marker-rescue experiments

Plasmid DNA (2 μg) containing virus *Bam*r inserts was digested with *Bam*HI and following extraction with phenol and ethanol precipitation, was mixed with 5 μg of infectious SC16 DNA. This mixture was used to transfect monolayers of BHK cells, in 6 cm dishes, by the calcium phosphate precipitation method (including DMSO treatment) as described previously (Graham and Van der Eb, 1973; Stow and Wilkie, 1976). Control transfections were performed using SC16 DNA with no added plasmid. Progeny virus was harvested from these transfections and titrated in BHK cells in the presence or absence of various concentrations of ACV or PAA. Recombination frequencies were calculated as follows: (plaque number obtained with drug present per total plaque number) × 100.

DNA sequencing

Single-stranded DNA was isolated by PEG-precipitation from the M13mp8 clones described above and the inserts were sequenced by the dideoxy chain termination method of Sanger *et al.* (1977) using a set of 20 oligonucleotides (17-mers) as primers (made with a Biosearch 8600 synthesizer) and ^{35}S-labelled dATP. It was sometimes necessary to substitute dITP for dGTP in sequencing reactions to resolve artefacts which usually occurred in highly G-C rich regions of the gene. The buffer gradient gel system of Biggin *et al.* (1983) was used, although gels containing additional urea, to 9 M, were run when sequencing reactions contained dITP.

Restriction enzymes and other reagents

All restriction enzymes used in this study, in addition to T4 DNA ligase and the Klenow fragment of DNA polymerase I, were purchased from Boehringer Corporation Ltd., FRG. ^{35}S-Labelled dATP (10 mCi/ml, 1000–1500 Ci/mmol) was purchased from N.E.N. Research Products. Antibiotics were purchased from Sigma, Poole, UK.

Secondary structure predictions

Secondary structure predictions were kindly performed by Dr C.Hodgman, M.R.C. Laboratory of Molecular Biology, Cambridge, UK. The Robson algorithm was used (Garniea *et al.*, 1978) with a program devised by Dr Hodgman.

Amino acid sequences of polymerase polypeptides

The DNA polymerase sequences used to derive the amino acid sequences of the polymerase polypeptides were obtained from the following sources:

Herpes simplex virus	—	This study
Epstein–Barr virus	—	Baer *et al.* (1984)
Varicella zoster virus	—	Davison and Scott (1986)
Human cytomegalovirus	—	Kouzarides *et al.* (1987)
Adenovirus type 2	—	Gingeras *et al.* (1982)
Vaccinia virus	—	Earl *et al.* (1986)
Phage φ29	—	Yoshikawa and Ito (1982)

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