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Structures of Herpes Simplex Virus Type 1 Genes Required for Replication of Virus DNA

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Recently, a method has been developed to identify regions in the genome of herpes simplex virus type 1 (HSV-1) which contain genes required for DNA synthesis from an HSV-1 origin of DNA replication, and seven genomic loci have been identified as representing the necessary and sufficient gene set for such replication (C. A. Wu, N. J. Nelson, D. J. McGeoch, and M. D. Challberg, J. Virol. 62:435–443, 1988). Two of the loci represent the well-known genes for DNA polymerase and major DNA-binding protein, but the remainder had little or no previous characterization. In this report we present the DNA sequences of the five newly identified genes and their deduced transcript organizations and encoded amino acid sequences. These genes were designated UL5, UL8, UL9, UL42, and UL52 and were predicted to encode proteins with molecular weights of, respectively, 99,000, 80,000, 94,000, 51,000, and 114,000. All of these genes had clear counterparts in the genome of the related alphaherpesvirus varicella-zoster virus, but only UL5 and UL52 were detectably conserved in the distantly related gammaherpesvirus Epstein-Barr virus, as judged by amino acid sequence similarity. The sequence of the UL5 protein, and of its counterparts in the other viruses, contained a region closely resembling known ATP-binding sites; this could be indicative, for instance, of a helicase or primase activity.

In the last 25 years, replication of herpes simplex virus (HSV) DNA has been studied by biochemical characterization of the virus-specified enzymes and other proteins involved, by genetic methods, and through analyses of the structure of replicating DNA molecules.

It is now known that the virus genome encodes a replicative DNA polymerase (6, 23). Another protein species, the major DNA-binding protein (DBP), is also clearly implicated in DNA replication, although its precise role is not well defined (8, 49). A virus-coded DNase, possessing both exo- and endonuclease activities, is highly active in extracts of infected cells (21, 22). The requirement for this enzyme in replication is a matter of controversy at present (18, 31, 32). Two enzymes of nucleotide anabolism, thymidine kinase and ribonucleotide reductase, are also encoded by the virus genome (7, 15, 16). Finally, biochemical experiments have suggested the possible involvement of several other proteins or enzyme activities in the replicative process. These include a species which copurifies with HSV DNA polymerase (46), topoisomerase activities (3, 33), and site-specific DNA-binding proteins (9, 17).

Studies on HSV genetics have characterized a number of temperature-sensitive mutants in about 10 complementation groups, which are considered defective in virus DNA synthesis at nonpermissive temperatures (38). These include mutations in the DNA polymerase gene (*pol*) (6, 23), the DBP gene (*dbp*) (8, 49), and the gene encoding the larger subunit of ribonucleotide reductase (34). Of the remaining DNA-negative complementation groups, some represent situations in which DNA synthesis is affected indirectly as part of a pleiotropic effect, but others may correspond to proteins involved directly in DNA replicative processes.

Analyses of DNA extracted from infected cells late in infection have demonstrated the presence of extensive head-to-tail concatemers of HSV DNA, which are thought to be the product of a rolling-circle mode of replication (24, 25). Three *cis*-acting regions in HSV DNA have been defined as necessary for replication and are thought to be sites for initiation of new DNA synthesis (42, 50). These presumptive origins of replication are termed ori_S (two copies per genome; see Fig. 1) and ori_L (one copy per genome). Analysis of ori_S in particular has been greatly advanced by development of an assay that uses ori_S sequences in a circular plasmid. When this is introduced into an HSV-infected cell, replication functions are supplied in *trans*, so that the plasmid sequences are replicated and amplification of the non-HSV part of the plasmid can be assayed with extracted DNA (42–44).

Recently, this plasmid amplification assay has been used in a method for identifying genes of HSV-1 necessary for DNA replication. It was found that a set of large plasmid clones, representing most of the HSV-1 genome, could be transfected into culture cells and there expressed to supply functions needed to replicate an *ori*-containing test plasmid (5). Systematic subcloning then identified the genomic regions necessary for plasmid amplification (52).

In the Medical Research Council Virology Unit in Glasgow, we are close to completing determination of the 153,000-base-pair (bp) sequence of HSV-1 DNA. The primary objective of this work is to deduce the genetic organi-

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zation in as much detail as possible. Our present view is that the genome contains 72 genes. Refinement of the genomic loci needed for replication of an *ori*-containing plasmid, in light of our sequence data, has now allowed unambiguous identification of seven HSV-1 genes which are necessary and sufficient for plasmid replication. These include the well-known *dbp* and *pol* genes. However, little was previously known about the other five genes, although it appears that all are the loci of DNA-negative mutants (V. G. Preston, personal communication; S. K. Weller, personal communication). The purpose of this paper is to present the structures of these previously uncharacterized genes and to evaluate the proposed amino acid sequences of their encoded proteins.

MATERIALS AND METHODS

DNA sequence analysis. Determination of DNA sequences was done by the M13 dideoxy shotgun method, with plasmid-cloned fragments of HSV-1 DNA (27, 28, 37). For the sequences reported here, the following fragments were used: *Kpn*I-b for gene UL5, *Kpn*I-f for genes UL8 and UL9, *Hin*dIII-l for gene UL42, and an *Xho*I-*Bam*HI subfragment of *Xho*I-b for gene UL52. Plasmids were obtained from our colleagues A. J. Davison and V. G. Preston.

Computing. Sequence analysis, interpretation, and comparison were carried out with a DEC PDP11/44 computer under RSX11M as described previously (27, 28, 35, 39, 45). Searches of the NBRF protein sequence library used the program WORDSEARCH (14), run on a VAX computer under VMS.

RESULTS

Interpretation of DNA sequences. This report is concerned with the DNA sequences of four separate regions, containing five genes, within the long unique (U_L) region of the genome of HSV-1 strain 17. The data presented are part of a study of the complete U_L sequence (in preparation). Following determination of the DNA sequence in each region, an extensive effort was made to interpret it in terms of locations of reading frames for encoded proteins and of probable transcriptional control signals. We have not yet undertaken mRNA mapping analyses for the genes described here, and precise mapping data are presented for only one of them. It was therefore of primary importance to employ critical tests in evaluating candidate open reading frames (ORFs).

First, the working assumption was made that we were dealing only with intronless genes. This is reasonable in light of current information about HSV gene structures (47). The sequences were scanned for ORFs starting with a potential ATG initiator codon. As is well known, the high G+C content of HSV DNA results in a low frequency of out-of-frame, adventitious stop codons, since these are A+T-rich (27, 28). Hence, there exist many large ORFs other than those finally considered to be genuinely polypeptide coding, and additional tests were desirable. The main approach was to examine the codon usage of candidate ORFs by the method of Staden and McLachlan (40). Authentic HSV ORFs characteristically have a pronounced bias in base composition with respect to the positions within their codon set; most notably, the third positions exhibit a particularly high G+C content. This means that evaluation by codon usage generally gives a strong indication of reading frame authenticity. In addition to reading frame analysis, we used locations of potential transcriptional polyadenylation signals (AATAAA and ATTAAA) to indicate possible transcript organization.

External data were used to validate further the proposed gene organization. First, the layout of genes was almost completely congruent to that obtained by similar methods for the genome of varicella-zoster virus (VZV) (10), and all of the proposed HSV-1 amino acid sequences reported were detectably similar to their VZV counterparts. Since the genomes of these two alphaherpesviruses have diverged to the extent that they differ by 22 percentage points in overall base composition, this is a powerful indicator of correctness of interpretation for both genomes. In addition, certain of the proposed ORFs have recognizable homologs encoded by the very widely diverged gammaherpesvirus Epstein-Barr virus (EBV) (1). Next, the results of Wu et al. (52) define regions of the HSV-1 genome as composing active functional units for supply of replication factors, and the limits of these regions correspond well with the limits of proposed genes.

In conclusion, we are confident that we have located genes correctly and have identified their protein-coding sequences. Loci of mRNA polyadenylation have been predicted. There remain two areas of uncertainty in our interpretation. First, it is not feasible to predict a functional translational initiator codon, ATG, with high confidence. This means that some of our ORFs could be shown as starting at an ATG upstream of the true start. Second, the 5′ termini of mRNAs cannot be proposed realistically in the absence of mapping data.

Structures of HSV-1 genes required for DNA replication. This section describes in turn each of the five previously uncharacterized genes implicated by Wu et al. (52) in replication of HSV-1 DNA. These genes are all located in the U_L component of HSV-1 DNA, and on the basis on our sequence data, we have called them UL5, UL8, UL9, UL42, and UL52. (A description of the complete gene set of U_L is in preparation.) Figure 1 shows the locations of the genes on the standard map of the HSV-1 genome, and Fig. 2 shows more details of their locations in the genome, their orientations, and proposed transcript structures. The sequences of the genes are listed in Fig. 3 to 6.

On the prototype HSV-1 genome map, the leftmost locus identified by Wu et al. (52) corresponds closely to our UL5 ORF, shown in Fig. 3. No detailed mRNA mapping data are available for this region. However, a clear prediction of transcript organization emerged from the layout of ORFs and AATAAA sequences. We consider that UL5 must be transcribed as the upstream member of a leftward-oriented, 3′-coterminal family, whose distal member is UL4. An

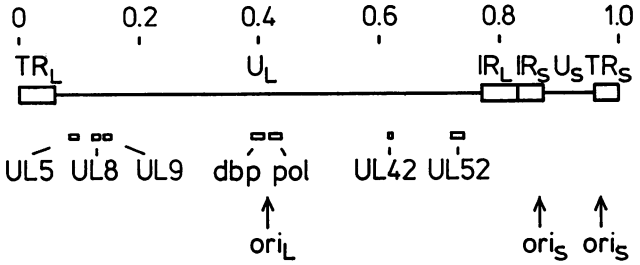

FIG. 1. Locations of DNA replication genes in the genome of HSV-1. The prototype arrangement of the HSV-1 genome is depicted with the long and short repeat regions (IR_L and IR_S, respectively) as open boxes and the long and short unique regions as solid lines. The scale represents sequence map units. The locations of origins of DNA replication and of ORFs proposed to encode proteins involved in virus DNA synthesis are shown.

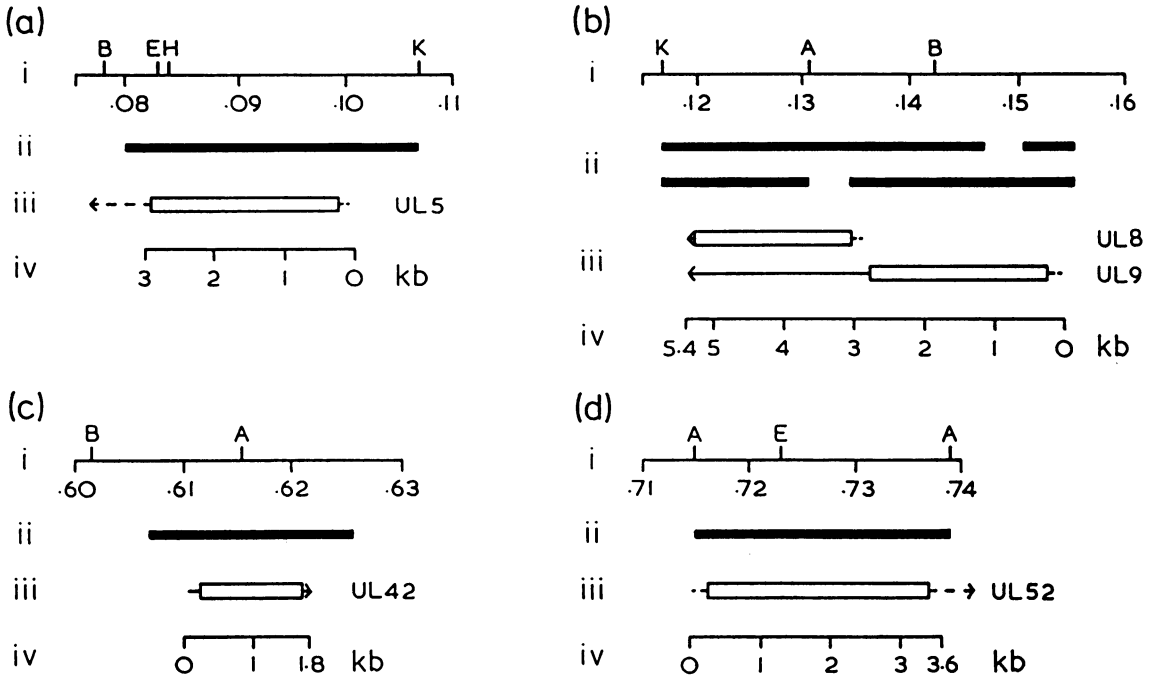

FIG. 2. Organization of HSV-1 DNA replication genes. Sections a to d give data on the locations and layouts of the five newly characterized DNA replication genes of HSV-1: (a) UL5; (b) UL8 and UL9; (c) UL42; and (d) UL52. In each section, part i shows the location, in sequence map units, and landmark restriction sites; part ii indicates by a solid bar the locus of each gene as mapped by Wu et al. (52); part iii shows the proposed location and orientation of the gene's transcript, with the proposed ORF as an open box; and part iv indicates the numbering system used in the subsequent sequence-listing figures. Restriction enzyme abbreviations: A, *Hpa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I.

unresolved point regarding expression of the plasmid-borne UL5 gene used by Wu et al. (52) is that the gene's proposed polyadenylation site is outside the HSV-1 DNA fragment used. The ORF to the right of UL5 is the rightward-oriented UL6, whose start codon is predicted to overlap with that for UL5 (Fig. 3). The predicted amino acid sequence for UL5 contains 882 residues, constituting a protein of M_r 98,710.

The next two genomic loci identified by Wu et al. (52) correspond to the adjacent ORFs UL8 and UL9 (Fig. 4). Again, no transcript maps are available, but we consider that these leftward-oriented genes must be expressed as 3′-coterminal transcripts terminating next to the sequence AATAAA downstream of UL8 (Fig. 4). To the left of UL8 lies UL7, proposed to be the distal member of the rightward-transcribed 3′-coterminal pair of UL6 and UL7. To the right of UL9 lies the rightward-oriented UL10, which is a gene previously described by us and predicted to encode a hydrophobic protein (29). As shown in Fig. 3, the ORFs for UL9 and UL10 overlap by 19 codons. If UL10 translation were initiated at the second available ATG, then the ORFs would overlap only at the first codon of each (as proposed above for UL5 and UL6). We cannot at present discriminate between these possibilities. UL8 is predicted to encode a protein of 750 amino acids with an M_r of 79,921, and UL9 is predicted to encode a protein of 851 amino acids with an M_r of 94,246.

The fourth and fifth genomic loci required for DNA replication represent the *dbp* and *pol* genes (Fig. 1), whose sequences have already been published (20, 36). The next replication locus, to the right of these, corresponds to our UL42 ORF (Fig. 5). This lies in a region where transcripts have been mapped by Frink et al. (19). Those workers characterized an abundant early mRNA species, which they termed RNA 2, and which can now be seen clearly to correlate with ORF UL42. A minor complication is that they mapped the positions of RNA 2 termini from a *Sal*I site internal to the gene, and our sequence contains two such sites, at residues 1045 and 1392 (Fig. 5). At the 3′ extremity, we presume that the mRNA terminates downstream of the AATAAA sequence at residue 1761 in Fig. 5. The best candidate for the mRNA 5′ terminus is near residue 50, downstream of a potential TATA box at residue 33. UL42 is predicted to encode a protein of 488 residues and M_r 51,156. The UL42 protein has recently been shown to correspond to a species which is abundant in HSV-1-infected cells, has an estimated M_r of 62,000 or 65,000, and binds to DNA (2, 26; D. S. Parris, A. Cross, L. Haarr, A. Orr, M. C. Frame, M. Murphy, D. J. McGeoch, and H. S. Marsden, J. Virol., in press).

The final DNA replication gene corresponds closely to our ORF UL52 (Fig. 6). No precise mRNA mapping data are available, although the UL52 transcript has probably been visualized by Northern (RNA) blot analysis (47). The UL52 ORF is rightward oriented. To its right, UL53 is a rightward-oriented gene sequenced by Debroy et al. (13). The first potential polyadenylation-associated sequence (ATTAAA) lies downstream of UL53, so we propose that transcripts of both genes terminate there. This means that the HSV-1 DNA fragment used by Wu et al. (52) to supply this gene in their assay lacked an appropriate polyadenylation site. To the left of UL52, UL51 is a leftward ORF. The UL52 ORF encodes a protein of 1,058 residues, M_r 114,416. At its downstream end, the UL52 ORF overlaps by 15 codons with the start of the UL53 ORF.

Properties and relationships of the sequences of HSV-1 DNA replication proteins. We have examined several aspects of the predicted amino acid sequences encoded by the DNA

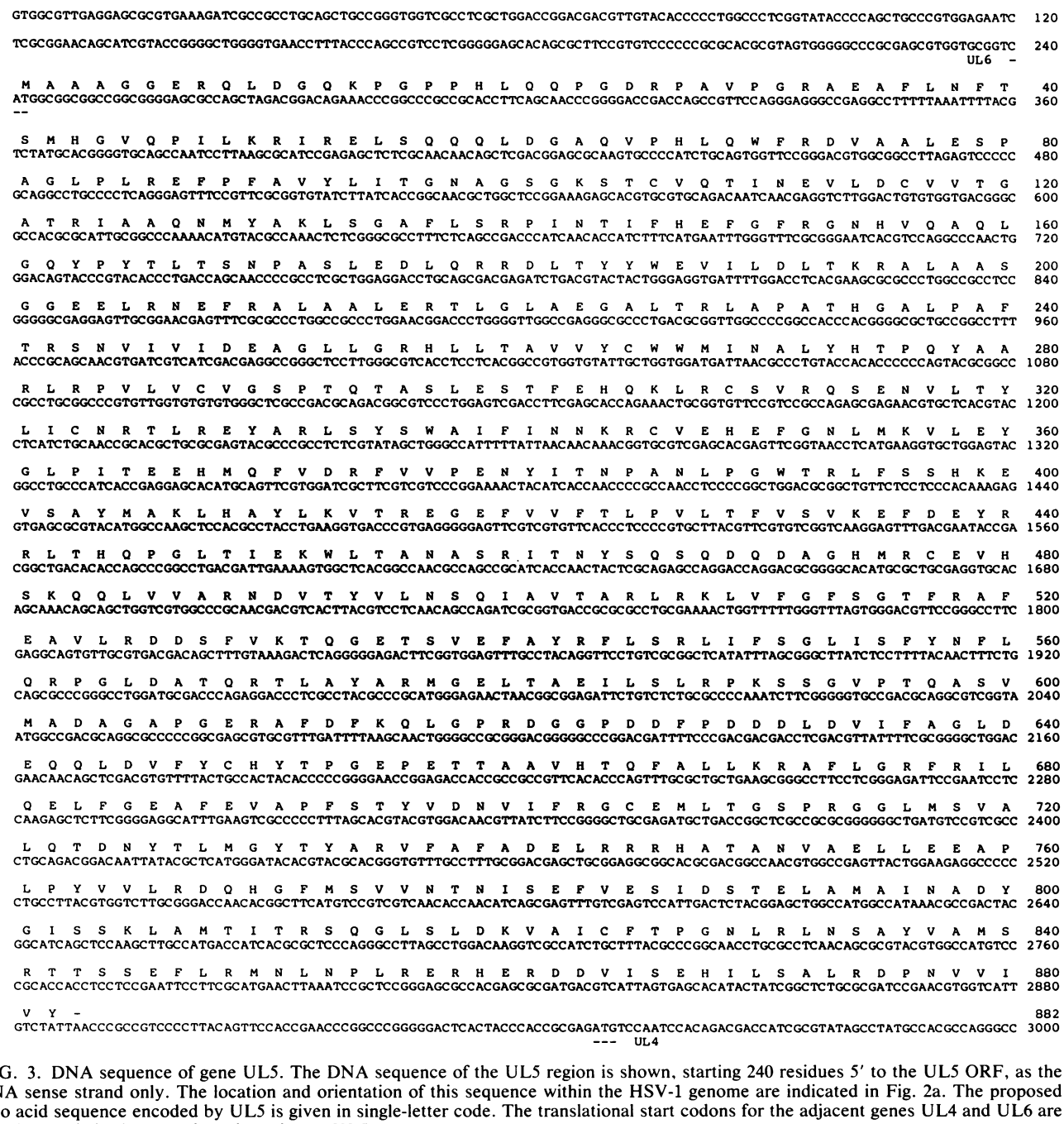

FIG. 3. DNA sequence of gene UL5. The DNA sequence of the UL5 region is shown, starting 240 residues 5′ to the UL5 ORF, as the mRNA sense strand only. The location and orientation of this sequence within the HSV-1 genome are indicated in Fig. 2a. The proposed amino acid sequence encoded by UL5 is given in single-letter code. The translational start codons for the adjacent genes UL4 and UL6 are marked (UL6 is in the opposite orientation to UL5).

replication genes. None exhibit any extreme features of composition or sequence; there are, for instance, no histone-like species. No extended similarities were found between the sequences of the seven replication proteins. We searched for homologous proteins with the NBRF data base, but none were found apart from proteins of other herpesviruses (see below). Thus, no ideas on functions have yet emerged from this approach.

A clear homolog of each HSV-1 DNA replication protein was found in the set of proteins predicted from DNA sequence analysis to be encoded by the related alphaherpesvirus VZV (10). Each pair of predicted amino acid sequences was aligned by using the HOMOL program (45), and values for overall homology were calculated (30) (Table 1). The strengths of these relationships spanned a wide range. HSV-1 gene UL5 and VZV gene 55 were strongly conserved, to a degree comparable to that found with the *pol* genes of the two viruses, while UL8 and VZV gene 52 and UL42 and VZV gene 16 showed much weaker similarities.

Davison and Taylor (11) presented a systematic comparison of the complete gene set of VZV with that of the widely diverged gammaherpesvirus EBV. Transferring their results to the newly characterized HSV-1 DNA replication proteins, we found that the UL5 and UL52 proteins had EBV coun-

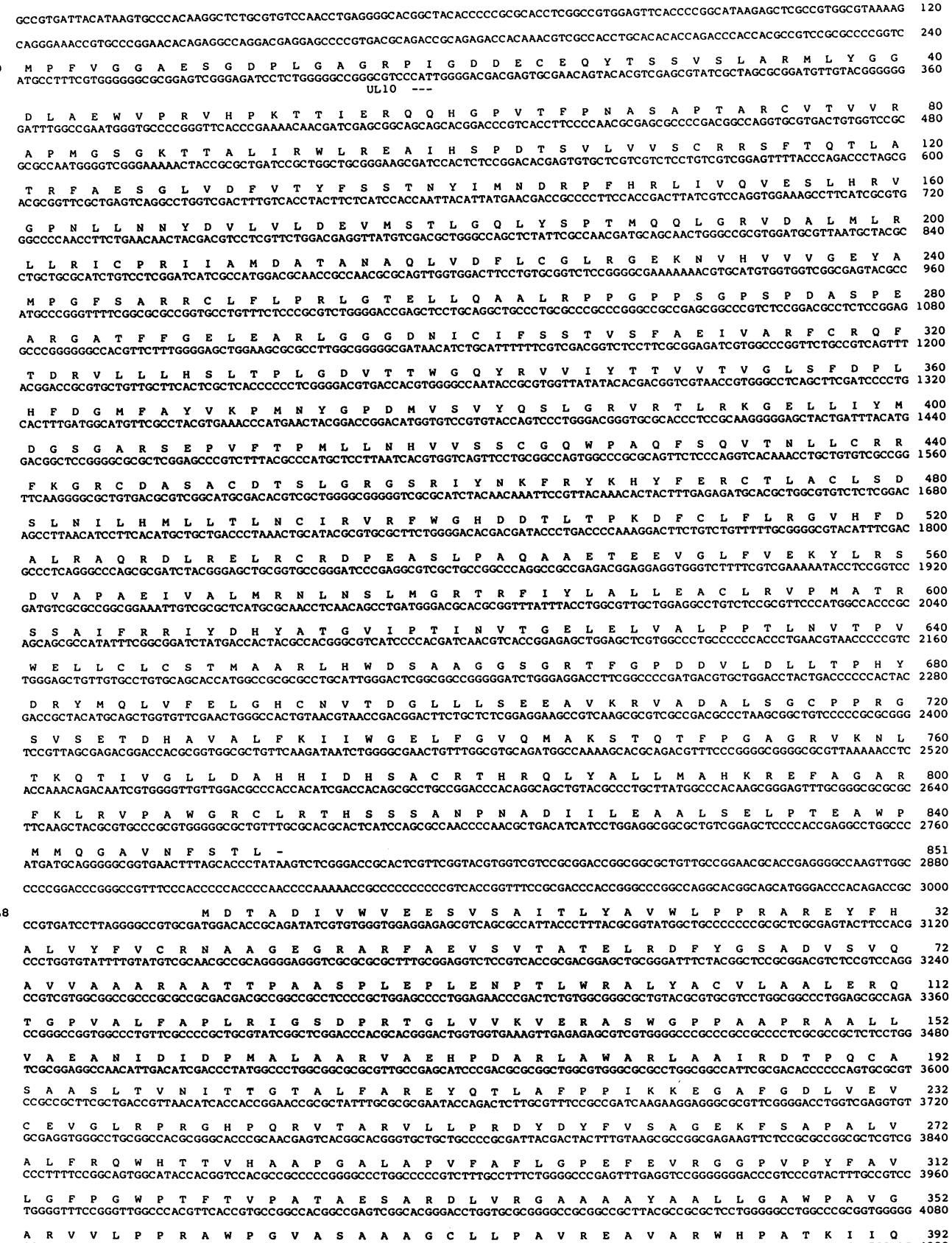

FIG. 4—*Continued.*

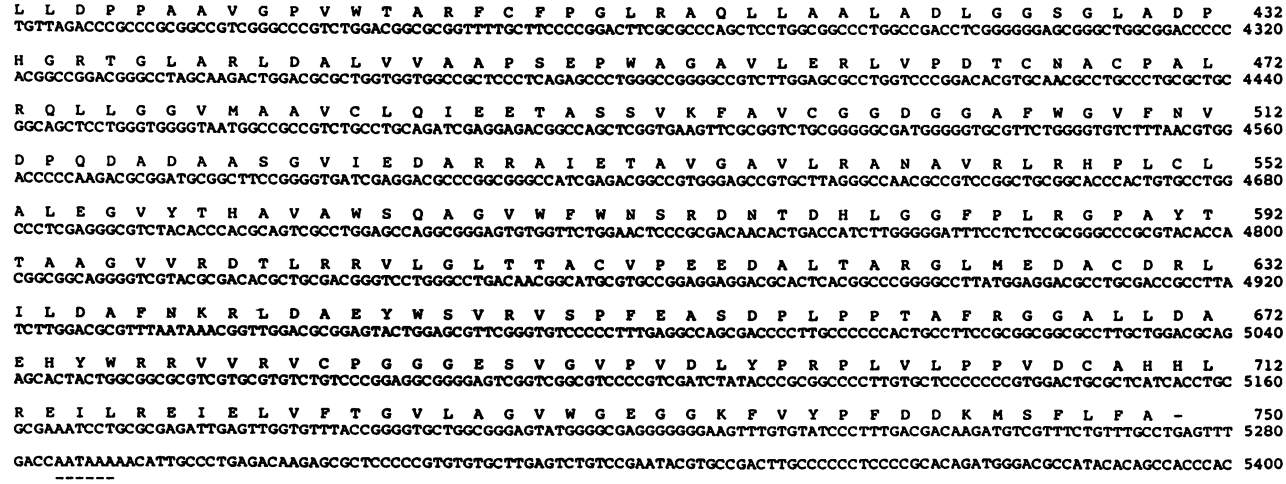

FIG. 4. DNA sequence of genes UL8 and UL9. The DNA sequence of UL8 and UL9 is shown, as the mRNA sense strand only. The location and orientation of this sequence within the HSV-1 genome are indicated in Fig. 2b. Proposed amino acid sequences are given in single-letter code. The start codon for UL10 (in the opposite orientation) is marked. It is proposed that UL8 and UL9 transcripts end downstream of AATAAA (underlined) at residue 5285.

terparts with clear sequence similarity (Table 2). Counterparts were previously identified for HSV-1 DNA polymerase and DBP (36). The strongest homologies were seen with the UL5 protein and DNA polymerase. However, the UL8, UL9, and UL42 proteins had no EBV counterparts with detectable amino acid sequence homology. Possible counterparts can be proposed on the basis of genomic locations only, and for UL8 and UL9 these were markedly different in size.

We examined the predicted sequences of the HSV-1 DNA replication proteins for the presence of several consensus elements, associated with DNA binding, nuclear entry, and nucleotide binding. The only finding of note was with a consensus sequence derived by Walker et al. (48) associated with ATP-binding sites in many proteins. This consensus contains completely conserved elements and other, partially conserved elements. We searched the complete predicted protein sets of HSV-1, VZV, and EBV by using the core

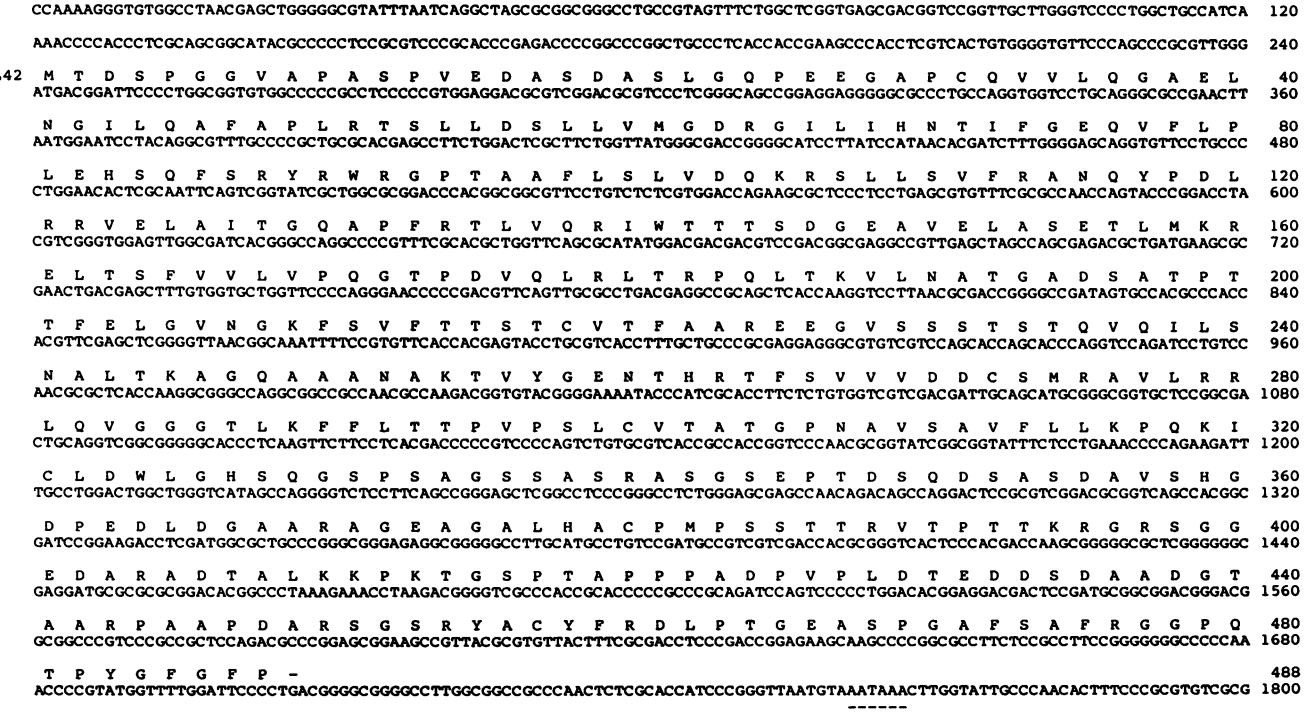

FIG. 5. DNA sequence of gene UL42. The DNA sequence of UL42 (mRNA sense strand) and encoded amino acid sequence are given, from the genomic region indicated in Fig. 2c. The proposed polyadenylation signal AATAAA (residue 1761) is underlined.

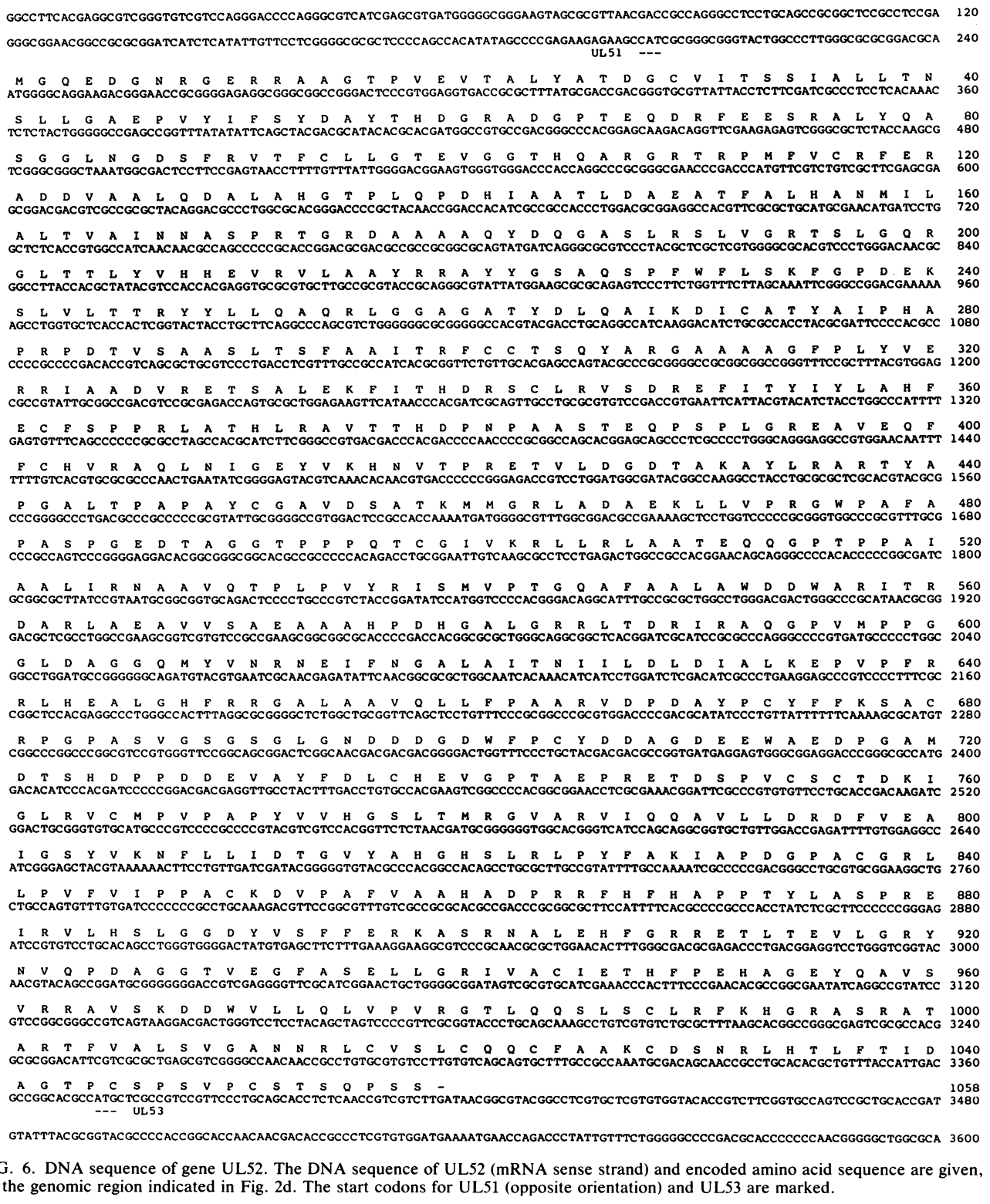

FIG. 6. DNA sequence of gene UL52. The DNA sequence of UL52 (mRNA sense strand) and encoded amino acid sequence are given, from the genomic region indicated in Fig. 2d. The start codons for UL51 (opposite orientation) and UL53 are marked.

consensus and found six occurrences in HSV-1 sequences, six in VZV, and eight in EBV. When comparing the HSV-1 and VZV examples, only three loci were common to both—in the UL5 protein, in thymidine kinase, and in DNA polymerase. These loci were conserved also for EBV. The thymidine kinase site has been remarked previously (11) and represents a reasonable finding in view of that enzyme's activity. The DNA polymerase example may be involved in deoxynucleoside triphosphate rather than ATP binding. The UL5 locus is shown in Table 3, together with the VZV and

TABLE 1. Comparisons of the amino acid sequences of HSV-1 and VZV DNA replication proteins

HSV-1[a]		VZV[b]		Length after alignment[c] (residues)	No. of aligned identical residues	% Homology[d]
Gene product	No. of residues	Gene product	No. of residues			
UL5	882	55	881	890	505	56.7
UL8	750	52	771	800	226	28.3
UL9	851	51	835	864	387	44.8
DBP	1,196	29	1,204	1,220	612	50.2
Pol	1,235	28	1,194	1,271	667	52.5
UL42	488	16	408	505	109	21.6
UL52	1058	6	1,083	1,130	425	37.6

[a] Data for DBP and Pol are from Quinn and McGeoch (36).
[b] Data from Davison and Scott (10).
[c] Aligned lengths of sequences after introduction of gaps by HOMOL program (45).
[d] Number of aligned identical residues as a percentage of aligned length.

EBV counterparts and other recognized examples. As well as the minimum consensus, the UL5 sequence and its VZV and EBV counterparts also possessed partially conserved features of the consensus. From the criteria of, first, closeness of fit to the consensus and, second, conservation in three distinct herpesviruses, we conclude that the UL5 protein probably does contain a functional ATP-binding site.

DISCUSSION

Our sequence data, in conjunction with the analyses of Wu et al. (52), have clearly identified seven HSV-1 genes whose products are required for the replication of viral DNA. Studies of papovavirus and adenovirus DNA replication have implicated one and three virus-coded proteins, respectively; host factors are also required (reviewed by Campbell [4]). Thus, HSV-1 supplies more factors for the replication of its genome than do these smaller DNA viruses. Three of the HSV-1 DNA replication proteins (DBP, DNA polymerase, and the UL42 protein) have been characterized in extracts of infected cells. The UL42 gene product has recently been shown to be a previously recognized DNA-binding protein found in extracts of HSV-1-infected cells; its function is not known (Parris et al., in press). The remaining four species do not correlate with any previously known proteins. This could well point to their being present only at low abundance. Recently, we prepared antisera against fusion proteins corresponding to parts of these polypeptides, and for two of them (against the UL5 and UL9 products) we found that the antiserum reacted with an appropriately sized protein in extracts of infected cells (P. D. Olivo and M. D. Challberg, unpublished data).

A priority now in analyzing HSV-1 DNA replication is to assign functions to these proteins. To this end, we pursued comparative analyses with the predicted amino acid sequences. Comparisons with nonherpesvirus protein sequences did not yield any interpretable similarities. We found that all of the HSV-1 DNA replication genes had homologs in VZV, while only four (*dbp*, *pol*, UL5, and UL52) had EBV counterparts which exhibited amino acid sequence similarity. One of the newly identified genes, UL5, was particularly well conserved between HSV-1, VZV, and EBV. Since no functions are assigned for the VZV or EBV genes, these comparisons do not give direct information on the functions of the HSV-1 genes. However, we suggest that recognizable preservation of genes between the alphaherpesviruses and EBV may correlate with their products having roles in central, invariant parts of the DNA replicative process. We also note that the HSV-1 DNA replication proteins are quite large and could thus well be multifunctional.

The final datum gained from comparative analyses was that the UL5 protein contained a convincing candidate for an ATP-binding site. Several components of the DNA replicative machinery could be expected to utilize ATP, in particular primase and helicase. As shown in Table 3, the large T antigen of simian virus 40 possesses a closely similar ATP-binding sequence: in the case of T antigen, this is in a part of the molecule which functions as a helicase, in opening the strands of duplex DNA, perhaps both at the origin of replication prior to initiation of DNA synthesis and at the replication fork (12, 41, 51). We lean towards favoring a similar role for the UL5 protein.

TABLE 2. Comparisons of the amino acid sequences of HSV-1 and EBV DNA replication proteins

HSV-1[a]		EBV[b]		Length after alignment[c]	No. of aligned identical residues	% Homology[d]
Gene product	No. of residues	Gene product	No. of residues			
UL5	882	BBLF4	809	903	307	34.0
UL8	750	BBLF2?[e]	541			
UL9	851	BBLF3?[e]	182			
DBP	1,196	BALF2	1,128	1,219	310	25.4
Pol	1,235	BALF5	1,015	1,251	417	33.3
UL42	488	BMRF1?[e]	404			
UL52	1,058	BSLF1	874	1,098	260	23.7

[a] Data for DBP and Pol are from Quinn and McGeoch (36).
[b] Data from Baer et al. (1).
[c] Aligned lengths of sequences after introduction of gaps by HOMOL program (45).
[d] Number of aligned identical residues as a percentage of aligned length.
[e] Possible EBV counterparts which exhibited no quantifiable sequence similarity and which were proposed only on genomic locations of reading frames (45).

TABLE 3. Possible ATP-binding sites in herpesvirus proteins

Virus and protein	Residues	Sequence
HSV-1 UL5	83–112	L P L R E F P F A V Y L I T G N A G S G K S T C V Q T I N E
VSV gene 55[a]	76–105	M Q R N E L P F S V Y L I S G N A G S G K S T C I Q T L N E
EBV BBLF4[b]	58–87	S E P P F L P F S A V V I T G T A G A G K S T S V S C L H H
Bovine ATPase β[c]	144–173	L A P Y A K G G K I G L F — G G A G V G K T V F I M E L I N
Adenylate kinase[c]	1–30	M E D K L K K S K I I F V V G G P G S G K G T Q C E K I V Q
RecA protein[c]	52–84	G A G G L P M G R I V E I Y G P E S S G K T T L T L Q V I A
SV40 antigen[c]	415–588	M V Y N I P K K R Y W L F K G P I D S G K T T L A A A L L E
Polyomavirus T antigen[c]	559–588	L T E N V P K R R N I L F R G P V N S G K T Q L A A A L I S
Consensus[d]		G — — g — G K t

[a] From Davison and Scott (10).
[b] From Baer et al. (1).
[c] Extracted from the NBRF database.
[d] From (48). Completely conserved residues are shown as uppercase and partially conserved are shown as lowercase letters.

We expect that progress in biochemical and genetic characterization of HSV DNA replicative processes will now be significantly facilitated by these structural analyses of the genes involved.

ACKNOWLEDGMENTS

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