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Characterization of the Genes Encoding Herpes Simplex Virus Type 1 and Type 2 Alkaline Exonucleases and Overlapping Proteins

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A detailed sequence analysis of the herpes simplex virus type 1 (HSV-1) and HSV-2 DNA encoding the alkaline exonuclease mRNA clusters has been completed. Three partially colinear mRNAs (2.3, 1.9, and 0.9 kilobases) are completly encoded within the DNA sequence presented. The putative promoter regions of the transcripts were inserted upstream of a plasmid-borne chloramphenicol acetyl transferase (CAT) gene and assayed for their ability to induce transcription of the CAT gene upon low multiplicity of infection with HSV in transient expression assays. We conclude that the expression of all three transcripts appear to be controlled by individual promoters. The 2.3-kilobase mRNA contains an open translational reading frame sufficient to encode 626 amino acids for the HSV-1 alkaline exonuclease enzyme; this value is 620 amino acids for HSV-2. A comparison of the predicted amino acid sequences of the HSV-1 and HSV-2 alkaline exonuclease enzymes revealed significant amino acid differences in the N-terminal portions of the two proteins; however, computer analyses suggest that the three-dimensional structures of the HSV-1 and HSV-2 nuclease enzymes are very similar. The 0.9-kilobase mRNA contains an open reading frame which shares a small amount of out-of-phase overlap with the C-terminal portion of the alkaline nuclease open reading frame. This open reading frame has the capacity to encode a 96-amino-acid polypeptide (10,500 daltons).

The transcripts of herpes simplex virus type 1 (HSV-1) map in a rather simple manner, as pointed out in two recent reviews (49, 50). In most cases, individual transcripts appear to be controlled by individual promoters. Further, in spite of some individual variations, HSV transcripts encode unspliced open protein translational reading frames. This one-to-one relationship between a viral mRNA and its protein product has allowed the use of transcriptional mapping as a tool for high-resolution localization of genetic markers.

The alkaline exonuclease function of HSV-1 has been localized to a region between 0.16 and 0.18 map units (m.u.), which encodes a set of apparently unspliced, 3′-coterminal mRNAs. We have identified a 2.3-kilobase (kb) mRNA in this set which can be translated in vitro to produce a peptide immunoprecipitable with a monoclonal antibody to the HSV-2 alkaline exonuclease protein (13). The enzyme purified from both virus types migrates with a rate corresponding to 80,000 to 85,000 daltons (Da) in denaturing polyacrylamide gel electrophoresis. Available immunological data suggest that the enzymes encoded by HSV-1 and HSV-2 are clearly related (5, 6). The HSV alkaline nuclease enzyme appears to be important in the replication process of HSV DNA and can be identified within partially purified replication complexes of viral DNA by using monoclonal antibodies (48).

In this communication, we present a complete analysis of the transcripts and DNA sequence encoding the HSV-1 and HSV-2 alkaline nuclease mRNA "families." All transcripts appear to be controlled by specific promoters. This conclusion is based on the fact that chloramphenicol acetyl transferase (CAT) activity can be induced upon low-multiplicity superinfection with HSV in cells transfected with constructs of putative promoter regions linked to the CAT gene. We find a translational open reading frame (ORF) sufficient to encode 626 amino acids (620 for HSV-2) for the HSV-1 nuclease enzyme. We have identified a fifth transcript in this nested set which encodes a small translational ORF that shares a small amount of out-of-phase overlap with the C-terminal portion of the alkaline exonuclease ORF. Comparisons of the predicted strutures of the HSV-1 and HSV-2 enzymes and potential relationships between these and other herpesvirus enzymes are discussed.

MATERIALS AND METHODS

Cells and virus. Monolayer cultures of HeLa cells were grown at 37°C in Eagle minimal essential medium containing 10% calf serum, penicillin, and streptomycin. For HSV-1 RNA preparation, plaque-purified isolates of the KOS strain of HSV-1 were used. HSV-2 RNA was prepared from HeLa cells that had been infected with the HG-52 strain of HSV-2.

Enzymes. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., and New England Biolabs, Inc. Digestions were carried out in buffers recommended by the suppliers. Bacterial alkaline phosphatase and phage T4 polynucleotide kinase (Bethesda Research Laboratories) were used for 5′ end labeling as described by Maxam and Gilbert (37). *Escherichia coli* DNA polymerase I (Klenow fragment; Boehringer-Mannheim Biochemicals) was used for 3′ end labeling by the method of Maniatis et al. (36). *Hin*dIII linkers were obtained from Collaborative Research, Inc. S1 nuclease (Boehringer-Mannheim) was used to localize mRNA termini as described previously (1, 7).

Isolation and size fractionation of polyribosomal RNA. For HSV-1 RNA, monolayer cultures of HeLa cells (1.2×10^7 cells per flask) were infected for 30 min at a multiplicity of 10 PFU of virus per cell in phosphate-buffered saline containing 0.1% glucose and 1.0% fetal calf serum. For HSV-2 RNA, monolayer cultures of HeLa cells were infected for 60 min at a multiplicity of 2 PFU of virus per cell. RNA was isolated from HSV-infected cells by the guanidinium isothiocyanate-

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hot phenol method of Feramisco et al. (23). Poly(A)-containing mRNA was isolated from total RNA by oligo(dt)-cellulose (Collaborative Research, Inc.) chromatography. This is referred to as HSV poly(A) mRNA. Details of this procedure were presented elsewhere (1, 2, 19–21, 24–26, 30, 41). RNA was isolated 6 h postinfection for HSV-1 infection and 20 h postinfection for HSV-2 infection. For isolation of early mRNA, viral DNA synthesis was inhibited by the addition of 1-β-D-arabinofuranosylthymine (Sigma Chemical Co.) to a concentration of 50 μg/ml. RNA was size fractionated by electrophoresis on 1.4% agarose gels containing 10 mM methylmercury hydroxide (4) as previously described (1, 2, 24, 30).

Recombinant DNA. All recombinant DNA clones used in the experiments described here were derived from either *Bam*HI-*Hin*dIII fragment A-IO (0.151 to 0.182 m.u.) of the KOS strain of HSV-1 cloned in pBR322 or *Hin*dIII fragment B (0.065 to 0.260 m.u.) or the HG-52 strains of HSV-2 cloned in pHC-79. The following subclones were used: *Bam*HI-*Bgl*II fragment A-K (0.151 to 0.163 m.u.), *Bam*HI-*Xho*I fragment A-G (0.151 to 0.171 m.u.), *Bgl*II-*Xho*I fragment O-G (0.164 to 0.171 m.u.), *Bgl*II-*Hin*dIII fragment O-IO (0.164 to 0.182 m.u.), and *Xho*I-*Hin*dIII fragment C′-IO (0.171 to 0.182 m.u.) of the KOS strain of HSV-1 or *Bam*HI fragment Q (0.150 to 0.174 m.u.), *Bgl*II fragment P (0.183 to 0.221 m.u.), and *Bam*HI-*Eco*RI fragment D-J (0.174 to 0.222 m.u.) of the HG-52 strain of HSV-2. Procedures for cloning HSV DNA fragments into pBR322 and pHC-49 were described previously (1, 14, 35). *Bgl*II-*Xho*I fragment O-G (0.164 to 0.171 m.u.) was cloned into the m13 phage vector according to published procedures (31, 40, 44). DNA fragments cloned were named as described previously and located by their map coordinates on the P arrangement of the HSV-1 and HSV-2 genomes (14).

Construction of the pSVODori-CAT expression marker vector used in these studies has been described previously (15). The expression marker vectors containing the HSV-1 alkaline exonuclease (AE-CAT) and the HSV-1 VP5(168) [VP5(168-CAT] promoters were described and characterized elsewhere (15).

The expression marker vectors containing the HSV-1 1.9-kb mRNA promoter region [1.9-CAT and 1.9(R)-CAT] were constructed as follows: the DNA fragment (HSV-1 nucleotides 366 through 617; see Fig. 2), which extends from an *Hin*fI site 211 bases 5′ of the primary 1.9-kb cap site to a *Sma*I site 40 bases 3′ of the cap site, was isolated. Flush ends were generated at the *Hin*fI site by using DNA polymerase I (Klenow fragment) as described by Maniatis et al. (36). *Hin*dIII linkers were inserted on both ends of the DNA fragment, and this promoter was ligated into the *Hin*dIII site of the pSVODori-CAT vector. The orientation of the promoter fragment with respect to the CAT gene was determined by diagnostic restriction endonuclease analysis.

The expression marker vectors containing the HSV-1 0.9-kb mRNA promoter region [0.9-CAT and 0.9(R)-CAT] were constructed by using the 241-nucleotide DNA fragment that extends from an *Rsa*I site 230 bases 5′ of the 0.9-kb cap site to an *Nar*I site 11 bases 3′ of this cap site. Flush ends were generated at the *Nar*I site with DNA polymerase I (Klenow fragment). *Hin*dIII linkers were inserted on both ends of the DNA fragment, and the promoter fragment was ligated into the CAT vector.

In situ Northern RNA blots. Samples (10 μg) of HSV poly(A) RNA were fractionated on methylmercury gels and dried onto Whatman 3MM paper under vacuum as previously described (19, 24, 30). The agarose film was floated off the paper in water and hybridized with appropriate ^{32}P-labeled DNA probes in 50% formamide containing 0.4 M Na^+, 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.0), 0.005 M EDTA, and Denhardt solution (17) at 50°C for 36 h. Blots were rinsed at 50°C. The first two rinses were in 50% formamide–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate. The last rinse was in 0.1× SSC–0.1% sodium dodecyl sulfate. Autoradiography was on Kodak XRP film with or without intensifying screens as needed.

In vitro ^{35}P-labeled DNA was made either by nick translating appropriate DNA clones with DNA polymerase I, DNase I (Boehringer-Mannheim), and 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp.) or by primer extension of the M13 hybridization probe primer (New England Biolabs) into *Bgl*II-*Xho*I fragment O-G DNA cloned in the M13lac phage vector with DNA polymerase I (Klenow fragment) and 15 μCi of [α-^{32}P]dCTP by published procedures (40, 43). The ^{32}P-labeled single-stranded M13 probe was nicked with DNase I (200 ng/ml; 4°C, 30 min) before hybridization to in situ Northern blots.

Nuclease mapping of HSV-1 mRNA. S1 nuclease analysis of RNA was carried out essentially as described previously (1, 12–16, 19–21, 24–26, 30). Appropriate HSV-1 and HSV-2 DNA clones (10 μg) were cleaved at the desired site with the appropriate restriction enzyme. The DNA was then 5′ end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; ICN Biochemicals) with T4 polyneucleotide kinase to a specific activity of 100,000 cpm/μg of DNA. Alternatively, the DNA was 3′ end labeled to the same specific activity with DNA polymerase I (Klenow fragment).

The DNA fragments were then denatured and strand separated on 5% acrylamide gels as described by Maxam and Gilbert (37). The strand-separated DNA was hybridized with 10 μg of HSV poly(A) mRNA in 0.1 M Na^+–0.1 M HEPES (pH 8.0)–0.01 M EDTA at 65°C for 6 to 16 h in a 30-μl volume, and the hybrids were subjected to S1 nuclease digestion. S1-resistant material was fractionated on 6% acrylamide–8 M urea sequencing gels.

Nucleotide sequencing. As described previously (12, 13, 15, 19–21, 24, 26, 30), nucleotide sequence analysis was done by the method of Maxam and Gilbert (37).

Transfection and CAT assay. HeLa cells (2×10^6 cells) were passaged 24 h before DNA transfection into 75-cm^2 tissue culture flasks (Corning Glass Works) containing Eagle minimal essential medium supplemented with 10% calf serum. Alternatively, rabbit skin fibroblasts (4×10^5 cells) were passaged 12 h before transfection into 60-mm tissue culture plates containing Eagle minimal essential medium supplemented with 5% fetal calf serum. The cells were plated so that they would reach 50 to 70% confluency before DNA transfection.

The procedure for the calcium phosphate coprecipitation of DNA onto cells was essentially as described by Busslinger et al. (8) and previously (15). Cells that had been transfected with HSV-1 promoter CAT plasmids were either infected with HSV-1 at a multiplicity of 2 PFU/cell or mock infected 12 to 24 h after removal of the medium containing the DNA precipitant.

Protein extracts from transfected cells were made and assayed for CAT activity as described by Gorman et al. (29). The standard CAT assay contained 50 μl of supernatant (equivalent to 2×10^6 cells), 40 μl of 0.25 M Tris (pH 7.6), 20 μl of 4 mM acetyl coenzyme A (P-L Biochemicals, Inc.), and 0.25 μCi of [^{14}C]chloramphenicol (58 mCi/mmol; New England Nuclear Corp.). The reaction products were sus-

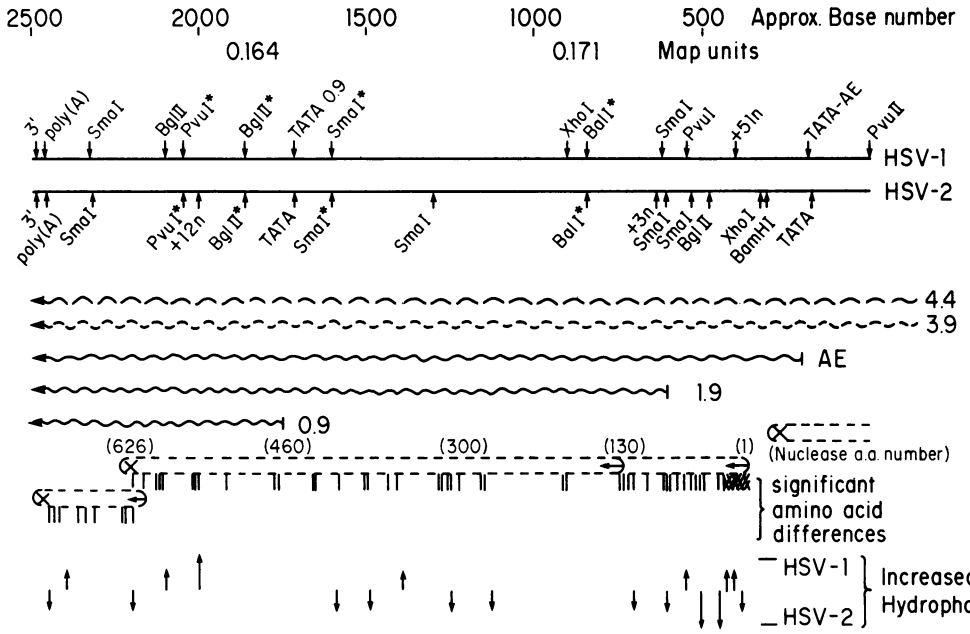


FIG. 1. HSV alkaline exonuclease gene. This is a schematic summary of the genetic expression of the region of HSV-1 and HSV-2 encoding the alkaline exonuclease function(s). Map units localizing the region of the P arrangement of the genomes are given. Some characteristic restriction endonuclease sites are shown. Those which map at identical positions on both genomes are indicated with asterisks. Sites of nucleotide additions are marked (+51 in the HSV-1 genomes, +3 and +12 in the HSV-2 genome). The locations of the 50′ and 3′ termini of mRNAs are shown, where applicable. The overlapping 3.9- and 4.4-kb mRNAs are represented by wavy broken lines. Predicted open translational reading frames are illustrated (←), and stop (×) signals of the three reading frames are derived from DNA sequence analysis. Significant amino acid differences between the putative HSV-1 and HSV-2 polypeptides based on the simplification protocols of Devereux et al. (18) discussed in the text are represented by vertical lines. Regional increases in the predicted hydrophobicity of the HSV-1 and HSV-2 polypeptides are shown graphically by arrows: (↑) small differences, (⇑) large differences.

pended in 20 μl of ethyl acetate and spotted on silica gel thin-layer plates (J. T. Baker Chemical Co.). The products were separated by ascending chromatography with a 95% chloroform–5% methanol solvent mixture. After autoradiography, spots of the different forms of chloramphenicol were localized, cut out, and counted in PCS scintillation fluid (Amersham Corp.).

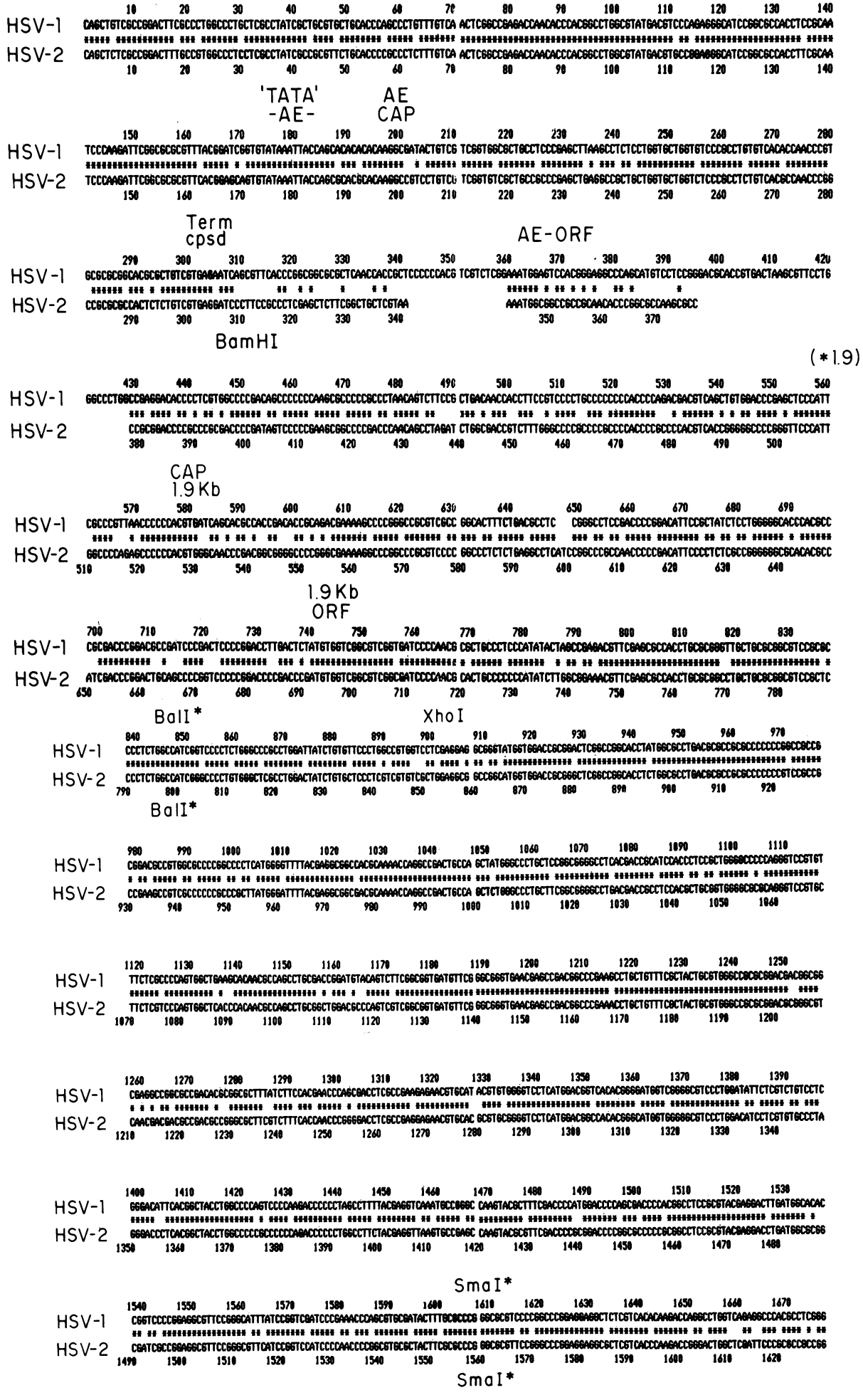
RESULTS

Comparative nucleotide sequence of the region of the HSV-1 and HSV-2 genomes encoding alkaline exonuclease. A partial restriction map of the portion of the HSV-1 and -2 genomes encoding alkaline endonuclease is shown in Fig. 1. This figure also summarizes transcriptional and sequence data reported here as well as previously (13). Five transcripts share a common 3′ terminus at ca. 0.160 m.u. All transcript sizes reported here correspond to the actual number of nucleotides encoded in the DNA; actual migration on denaturing gels is slower because of the presence of the 200-base poly(A) tail (45). The four largest transcripts have been characterized previously (13). In addition, the figure indicates the location of a 0.9-kb transcript detected with a sensitive single-stranded M13 probe.

The nucleotide sequence of the HSV-1 and -2 DNAs encoding these transcripts was determined by use of overlapping cloned DNA fragments. Both strands of both virus DNA types were completely sequenced. As has been seen in previous work in other regions of the genome, there were occasional regions in both HSV-1 and -2 where compressions of bases made sequencing difficult. It was found that increasing the percentage of acrylamide and gel running temperature was sufficient to resolve such uncertainties. The sequences of the HSV-1 and HSV-2 DNA were compared by use of an improved dot matrix program developed by John Coffin of the Tufts University Medical School Cancer Center. The comparative analysis is shown in Fig. 2; the data begin at a point 200 bases 5′ of the cap site of the HSV-1 alkaline exonuclease transcript and continues to ca. 50 bases 3′ of the polyadenylation site for the transcript cluster.

The comparative sequence data showed that four restriction sites occurred in identical locations on the two viral genomes: the *Bal*I site at HSV-1 base 845 (HSV-2 base 797), the *Sma*I site at HSV-1 base 1606 (HSV-2 base 1558), the *Bgl*II site at HSV-1 base 1866 (HSV-2 base 1818), and the *Pvu*I site at HSV-1 base 2061 (HSV-2 base 2025). These sites were used to align Fig. 1 and are indicated by asterisks. There is one unusual site in the HSV-2 sequence at base number 1256 where the sequence was determined to be GGGACCTC by sequencing both DNA strands, yet some lots of the restriction endonuclease *Sau*3AI, whose nominal recognition site is GATC, consistently cleaved at this point, whereas others did not. Careful analysis of the sequence in this region showed no evidence for a "swallowed" or extra base.

The overall homology between the HSV-1 and -2 DNA in this region is approximately 81%. This is similar to levels of homology seen in other regions of the genome (see Discussion). Very high homology was seen between base numbers 1 and 301, which corresponds to the putative translation termination signal of the 50,000-Da putative capsid protein encoded by the overlapping 3.9-kb transcript (13; Draper and Wagner, work in progress). Homology abruptly breaks at this point, and there are a total of 51 fewer bases in the HSV-2 DNA than in the HSV-1 DNA. Computer analysis


HSV-1
HSV-2
'TATA'
-AE-
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Term
cpsd
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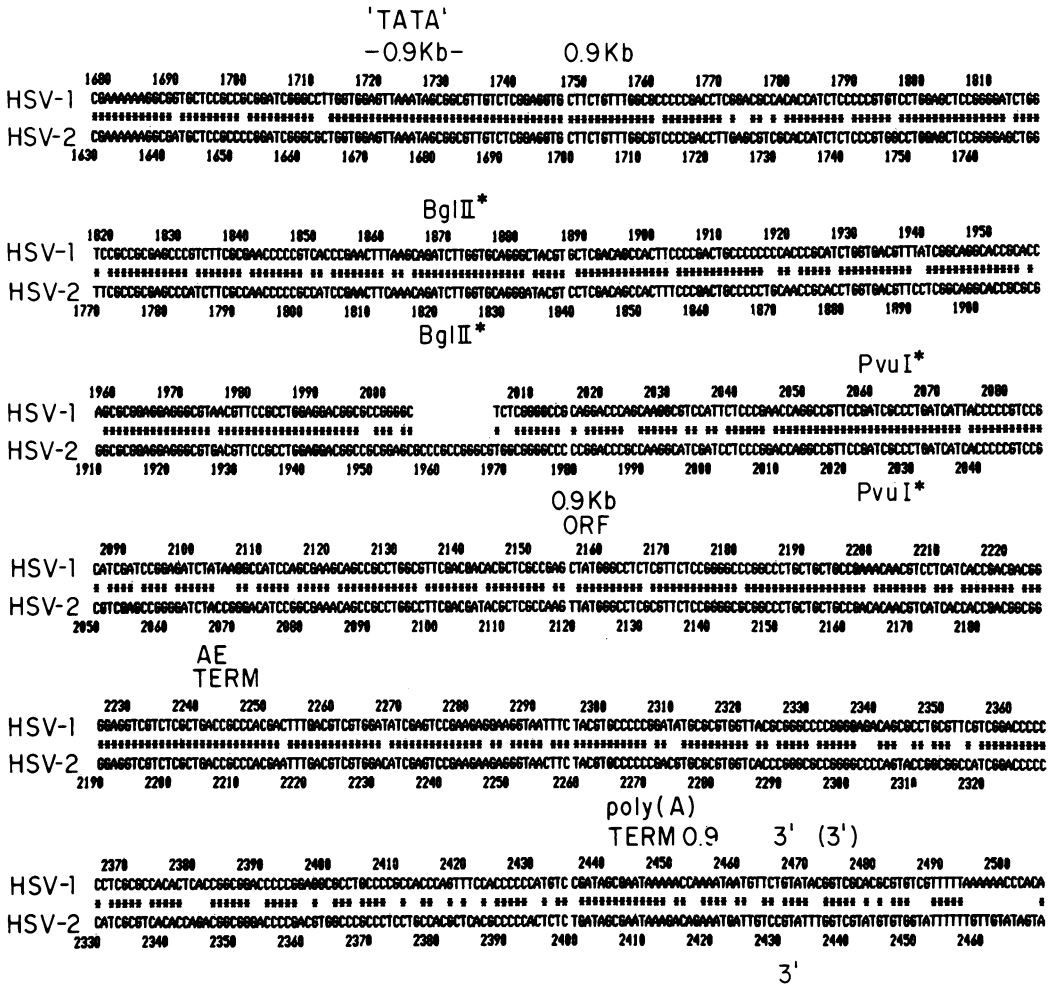

FIG. 2. Nucleotide sequence and homology between HSV-1 KOS DNA and HSV-2 HG-52 DNA encoding the alkaline exonuclease function. HSV-1 and HSV-2 DNA sequences were compared using a dot matrix program as described in the text. Matched bases are designated by asterisks. Nucleotide numbers are listed above and below the HSV-1 and HSV-2 DNAs, respectively. Positions of the mRNA cap sites and 3′ termini of the transcripts were determined by S1 nuclease analysis as discussed in the text. A possible secondary cap site for the 1.9-kb transcript is shown (*1.9). Selected restriction endonuclease sites are listed, including the unique *Bam*HI and *Xho*I sites, as well as the *Bal*I (BalI*), *Sma*I (SmaI*), *Bgl*II (BglII*), and *Pvu*I (PvuI*) sites, which map at identical positions in the two genomes. Putative TATA boxes of the promoter regions for the alkaline exonuclease (AE) and 0.9-kb transcripts and the polyadenylation signal for the transcript cluster are shown. The putative initiation (ORF) and termination (term) sites for the open translational reading frames encoded by this region are predicted from computer analysis of the DNA sequence.

suggests that residual homology is maximized by the arrangement of gaps shown in Fig. 2. Here, it is suggested that there are 33 fewer nucleotides in the HSV-2 sequence just after the translation initiator for the alkaline exonuclease protein (HSV-1 base 362, HSV-2 base 344).

Two other regions show net differences in the number of bases between HSV-1 and HSV-2. In both cases, the HSV-2 DNA contains additions; there are three extra bases at HSV-2 base 598 (HSV-1 base 648) and 12 extra bases at HSV-2 base 1957 (HSV-1 base 2005). In all cases of nucleotide number differences between the two sequences, the ORF for the alkaline exonuclease and colinear 1.9-kb transcripts is conserved.

As has been reported previously (13), the nucleotide sequence 5′ of the alkaline exonuclease transcript has the nominal features of a eucaryotic promoter, whereas the sequence immediately 5′ of the 1.9-kb transcript cap site does not contain such features. The sequence 5′ of the cap site of the 0.9-kb transcript contains an obvious TATA box homology (HSV-1 base 1722, HSV-2 base 1674); however, CAAT box homology is not particularly evident in the region of 60 to 80 bases 5′ of the cap site.

The ORF for the alkaline exonuclease protein terminates at HSV-1 base 2242 (HSV-2 base 2206), providing capacity to encode 626 amino acids (620 for HSV-2). Some comparative properties of the HSV-1 and HSV-2 proteins are summarized in Fig. 1 and discussed below. The 1.9-kb transcript contains a unique translation initiation codon at base 740 (HSV-2 base 692); this ATG codon is in phase with the larger alkaline exonuclease ORF, as was suggested in our earlier report (13). The only ATG triplet encoded by the 0.9-kb transcript occurs ca. 400 bases 3′ of the transcript cap site (HSV-1 base 2158, HSV-2 base 2122) and initiates an ORF which is out of phase with the ORF of alkaline exonuclease. The ORF of the 0.9-kb transcript is sufficient to encode a protein of 96 amino acids.

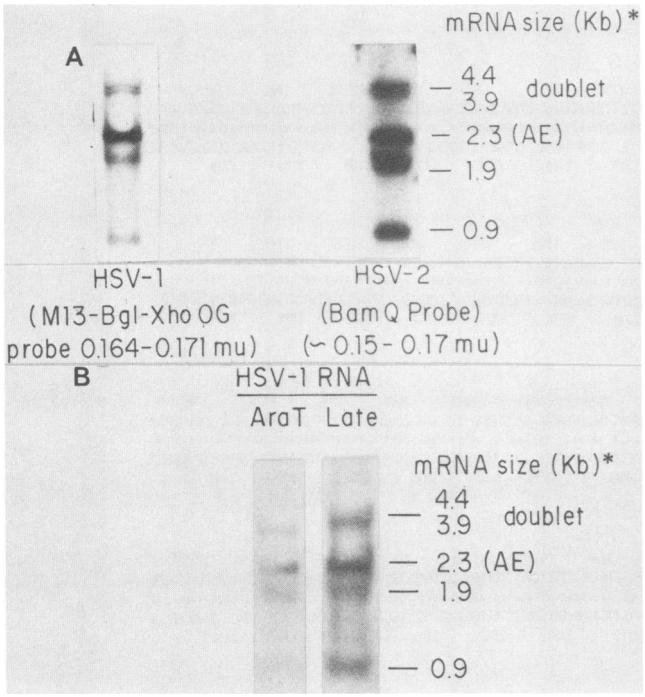

FIG. 3. In situ RNA (Northern) blot analysis of the mRNA encoded between 0.159 and 0.176 m.u. of the HSV-1 and HSV-2 genomes. Samples (10 μg) of the HSV-1 or HSV-2 poly(A) mRNA were frationated on methylmercury-containing agarose gels and immobilized by drying in vacuo. The mRNA species were detected by hybridization with ^{32}P-labeled DNA probes as indicated. Sizes shown were determined by positions of HeLa cell rRNA markers (not shown) as described previously (1, 2, 32, 33, 45). (A) Left panel, KOS strain HSV-1 poly(A) mRNA hybridizing to a single-stranded probe made from *Bgl*II-*Xho*I fragment O-G (0.164 to 0.171 m.u.) DNA cloned in the M13 vector. Right panel, HG-52 strain HSV-2 poly(A) mRNA hybridizing to a nick-translated probe made from *Bam*HI fragment Q (0.154 to 0.174 m.u.) DNA. (B) KOS strain HSV-1 poly(A) mRNA species isolated in the presence (late) or absence (1-β-D-arabinofuranosylthymine; AraT) of viral DNA replication. Hybridization was to the same single-stranded M13 *Bgl*II-*Xho*I fragment O-G DNA probe used above.

There is a polyadenylation signal (AATAAA) at HSV-1 base 2444 (HSV-2 base 2408). As shown below the position of this signal corresponds well with the determined 3′ ends of the transcripts in both HSV-1 and HSV-2. Interestingly, there is a variant of this signal 12 bases 3′ of this site in HSV-1 (AATAA) but not in HSV-2.

Transcription patterns in the HSV alkaline exonuclease gene. Identification of the 0.9-kb transcript colinear within the extreme 3′ end of the alkaline exonuclease transcript cluster was facilitated by the use of single-stranded M13 probes. The 970-base HSV-1 *Bgl*II-*Xho*I fragment O-G (0.164 to 0.171 m.u.) was cloned in M13 to accurately quantitate the relative levels of the alkaline exonuclease and colinear 1.9-kb transcripts during infection. This probe clearly revealed the presence of the 0.9-kb transcript (Fig. 3A); an identical transcript was seen in HSV-2 infection with nick-translated HSV-2 *Bam*HI fragment Q (0.15 to 0.17 m.u.) as a probe. The 0.9-kb transcript was not readily detectable with nick-translated double-stranded *Bgl*II-*Xho*I fragment O-G as a probe, presumably due to the transcript only extending ca. 120 bases beyond the *Bgl*II site at 0.164 m.u. The M13 clone is superior for detecting this transcript because the method used for labeling the single-stranded probe yields the highest specific radioactivity near the *Bgl*II site.

We determined the kinetic relationship between the alkaline exonuclease transcript and the 1.9-kb colinear transcript by using the single-stranded *Bgl*II-*Xho*I fragment OG probe to measure the relative amounts of the two transcripts detectable in the presence or absence of viral DNA replication. A typical experiment is shown in Fig. 3B. Here, based on densitometry, the ratio of alkaline exonuclease mRNA to the 1.9-kb transcript is 2.5 to 3.0 for RNA isolated from cells 6 h postinfection either with or without 1-β-D-arabinofuranosylthymine to inhibit viral DNA replication. The measure of the relative ratios between the two transcripts overcomes any differential efficiency of RNA isolation under the different conditions. Because the M13 probe covers the same regions in both transcripts, the ratio is a valid measure of the comparative steady-state levels of these two RNA species. These data indicate that both the alkaline exonuclease and the 1.9-kb transcripts belong to the same kinetic class, which we have previously classed as early (beta), based on the ease of detection of the alkaline exonuclease transcript in the absence of DNA replication.

We cannot be as precise about the relative abundance of the 0.9-kb transcript compared wih the alkaline exonuclease and 1.9-kb transcripts, since our probe does not cover an equivalent amount of this mRNA. Still, using the M13 probe with HSV-1 RNA, the ratio of the 0.9-kb transcript to alkaline exonuclease transcript increases by a factor of 3 to 5 when DNA replication is allowed to proceed (Fig. 3A). Further, the nick-translated HSV-2 probe indicates that the 0.9-kb mRNA is also a relatively abundant HSV-2 transcript late after infection. We can, therefore, tentatively classify its kinetic class as beta-gamma.

Precise location of the transcripts. The 5′ end of the 1.9-kb mRNA in HSV-1 was previously shown to lie 270 to 280 bases 5′ of the *Xho*I site (base 901, Fig. 2), (13). Here, it was precisely localized by S1 nuclease analysis of HSV-1 poly(A) mRNA hybridized with strand-separated *Pvu*II-*Hin*fI fragment DNA (HSV-1 nucleotides 540 to 722) 5′ end-labeled at the *Hin*fI site at HSV-1 base 722. The S1-protected DNA fragment was size fractionated against a sequence ladder of DNA 5′ labeled at the same *Hin*fI site (Fig. 4A). Note that the sequence ladder is of the DNA strand complementary to the mRNA. Thus, the cap site of this transcript is located at or near HSV-1 base 575 of Fig. 2. This is approximately 30 bases 5′ of our original estimate. A secondary cap site appeared to lie 20 bases 5′ of this position at base 555. The DNA sequence within 50 bases 5′ of the major cap site has no convincing TATA box homolog.

We precisely located the 5′ end of the HSV-1 0.9-kb transcript by carrying out S1 nuclease analysis of HSV-1 poly(A) mRNA hybridized with strand-separated *Bgl*II-*Xho*I fragment O-G DNA 5′ end labeled at the *Bgl*II site at HSV-1 base 1870. These data are shown in Fig. 4B, the transcript has a cap site at HSV-1 base 1748. This site is 27 bases 3′ of the sequence TAAATA, which is an excellent TATA box homolog.

The high homology of HSV-1 and HSV-2 DNA sequences around the HSV-1 cap sites and the fact that the HSV-1 and HSV-2 transcripts encoded in this region are essentially identical (Fig. 3A) indicate that the cap sites for the HSV-2 transcripts can be expected to lie in the same locations as seen for the HSV-1 mRNA species.

The 3′ end of the HSV-1 transcript cluster was precisely located by using S1 nuclease analysis of hybrids between

HSV-1 poly(A) mRNA and a DNA fragment 3′ end labeled at the *Sma*I site at HSV-1 base 2332 and extending to an *Ava*I site 162 baes 3′ of nucleotide 2505. The S1-protected fragment was size fractionated against a sequence ladder of DNA 5′ labeled at the same *Sma*I site. The data are shown in Fig. (4C); there are two major 3′ end sites that are 12 bases apart. We suggest that, in HSV-1, the sequence AATAA at base 2456 serves as a second polyadenylation signal leading to this 3′ "stutter."

The precise location of the 3′ ends of the HSV-2 transcript

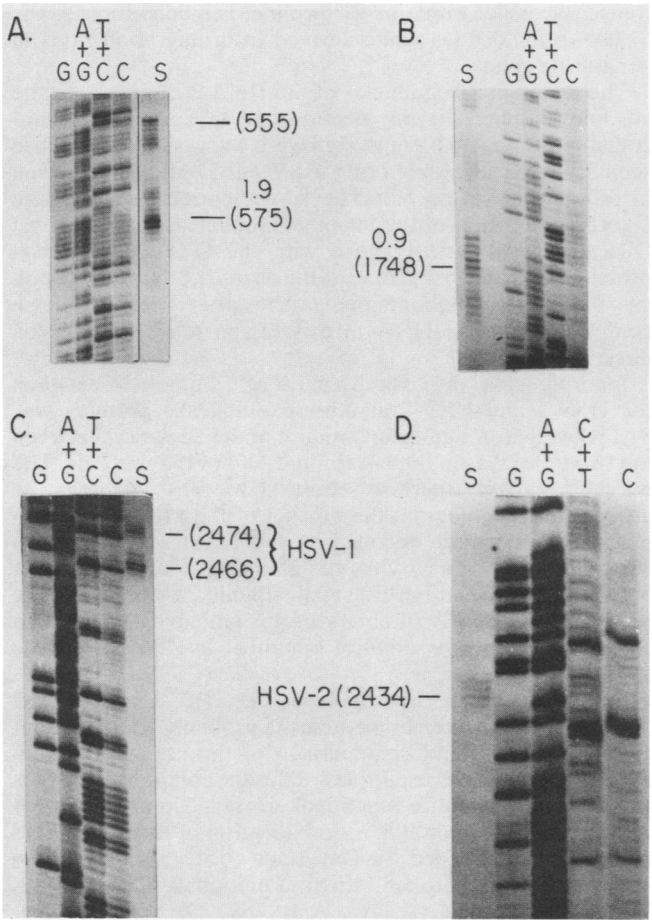

FIG. 4. Precise localization of the 5′ ends of the HSV-1 1.9- and 0.9-kb mRNAs and of the 3′ termini of both the HSV-1 and HSV-2 transcript clusters. HSV poly(A) mRNA was hybridized with 5′ or 3′ end-labeled single-stranded DNA and followed by digestion with S1 nuclease. The S1 nuclease-resistant material was fractionated against a sequence ladder of the DNA fragment 5′ end labeled at the same restriction site. Nucleotide numbers are according to the HSV-1 and -2 DNA sequences of Fig. 2. (A) The 182-nucleotide HSV-1 *Pvu*II-*Hin*fI fragment DNA located in *Xho*I-*Hin*dIII fragment C′-I0 (0.171 to 0.182 m.u.) 5′ end labeled at the *Hin*fI site to localize the mRNA cap site of the HSV-1 1.9-kb mRNA on the DNA sequence. A secondary cap site at base 555 is designated by an asterisk (B) The 970-nucleotide HSV-1 *Bgl*II-*Xho*I fragment O-G DNA (0.164 to 0.171 m.u.) 5′ end labeld at the *Bgl*II site to precisely locate the 5′ terminus of the HSV-1 0.9-kb mRNA. (C) The 335-nucleotide HSV-1 *Ava*I fragment DNA located in *Bam*Hi-*Bgl*II fragment A-K (0.151 to 0.163 m.u.) 3′ end labeled to localize the 3′ termini of the transcripts in this cluster. (D) The 418-nucleotide fragment HSV-2 *Xma*I DNA located in *Bam*HI fragment Q (0.14 to 0.174 m.u.) 3′ end labeled to localize the 3′ termini of the transcripts of this nested set.

cluster was determined by S1 nuclease analysis of hybrids between HSV-2 poly(A) mRNA and a DNA fragment 3′ end labeled at the *Sma*I site at HSV-2 nucleotide 2332 and extending to an *Sma*I site 283 bases 3′ of the nucleotide 2469. The S1-protected DNA fragment was size fractionated against a sequence ladder of DNA 5′ end labeled at the same *Sma*I site. The data are shown in Fig. 4D; there is only one 3′ end site that occurs 22 bases 3′ of the consensus polyadenylation signal of AATAAA at HSV-2 base 2408. This location is in close agreement with the positions of the termini observed in HSV-1.

Determination of promoter activities in transient expression assays. An in vitro assay was used for CAT activity to quantitate the ability of the 0.9-, 1.9-, and 2.3-kb promoters to control the transcription of CAT mRNA in transient expression systems. Fragments of HSV-1 DNA containing the promoter regions of the 2.3-, 1.9- and 0.9-kb transcripts with a limited amount of leader sequence (nucleotides 1 to 230, 366 to 617, and 1518 to 1758, respectively) were inserted immediately 5′ of the bacterial CAT gene of the pSVOD (ori-)CAT vector, which has been described previously (15). These constructs are called AE-CAT (bases 1 to 230), 1.9-CAT (bases 366 to 617), and 0.9-CAT (bases 1518 to 1758). Recombinant clones were selected that contained the desired promoter region in both the forward (HSV-1 sense) and the reverse (HSV-1 antisense) orientations with respect to the CAT gene.

DNA preparations from recombinant clones of AE-CAT and 1.9-CAT were transfected into HeLa cells, which were then mock infected or superinfected at low multiplicities of infection (2 PFU/cell) with HSV-1 KOS strain and assayed for CAT activity as described in Materials and Methods. As shown in Fig. 5A and reported elsewhere (15), the alkaline exonuclease promoter was quite active upon superinfection. We have previously shown that this activity is dependent upon the expression of viral alpha gene products and is independent of viral DNA synthesis.

The atypical promoter for the 1.9-kb tanscript induced significant CAT activity in transfected HeLa cells when superinfected with HSV. The level of CAT activity seen with this construct never reached the levels observed with the alkaline exonuclease promoter constructs. In the experiment shown in Fig. 5A, extracts from HeLa cells transfected with the AE-CAT construct converted 97% of chloramphenicol to the acetylated forms in the standard assay, whereas extracts from cells containing the 1.9-CAT construct converted only about 27%. We have described such activity measurements previously (15). Interestingly, the 4:1 ratio of CAT activity between these promoters (AE-CAT/1.9-CAT) was roughly equivalent to the relative mRNA abundancies of the 2.3- and 1.9-kb transcripts late in viral infection. In both cases, the reversed promoter sequences induced no detectable CAT activity upon superinfection with HSV.

The ability of the 0.9-kb transcript promoter to induce CAT activity upon superinfection with HSV in rabbit skin cells was tested. Although these cells did not support the extremely high levels of CAT activity reported with HeLa cells, they have been more reproducible in their response to superinfection, and smaller amounts of transfecting DNA can be used. A set of transient expression experiments with AE-CAT, 1.9-CAT, 0.9-CAT, and the VP5(168)-CAT construct containing the VP-5 promoter described earlier (15) are shown in Fig. 5B. The data of Fig. 5B indicate that the proper orientation of the 0.9-kb promoter induced levels of CAT activity that are nearly equivalent to those seen with the AE-CAT construct. Again, the reversed orientation of

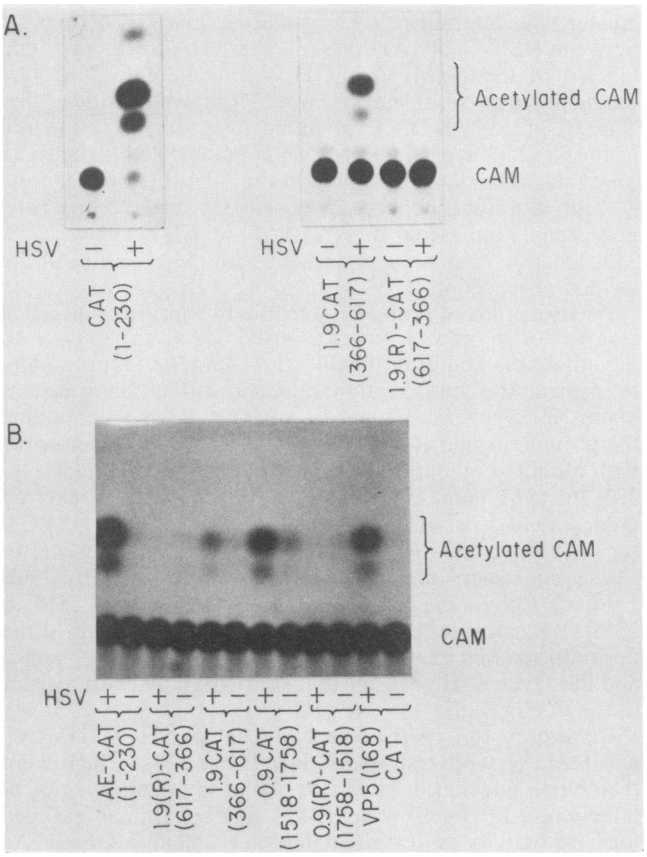

FIG. 5. CAT expression under the control of various HSV-1 promoters. HSV promoter CAT plasmids were transfected into either HeLa cells or rabbit skin fibroblasts, and the transfected cells were either mock infected (−) or superinfected (+) with HSV-1 at a multiplicity of 2PFU/cell. Cell extracts were then assayed for CAT activity 22 h postinfection as described previously (15). Unacetylated (CAM) and acetylated (acetylated CAM) forms of chloramphenicol are shown. Nucleotide numbers of the DNA fragment used for the promoter constructs are given in parentheses (see Fig. 2). (A) CAT expression in HeLa cells transfected with AE-CAT, 1.9-CAT, or 1.9(R)-CAT promoter CAT plasmid constructs. (B) CAT expression in rabbit skin fibroblasts transfected with AE-CAT, 0.9-CAT, 0.9(R)-CAT, or VP5(168)-CAT promoter CAT plasmid constructs.

the 0.9-kb transcript promoter [0.9(R)-CAT] showed little activity. Further, it is clear that the 1.9-CAT-directed activity is less than that of either AE-CAT or 0.9-CAT in these cells, as was the case in HeLa cells.

Comparison of the polypeptides encoded by the alkaline exonuclease transcript cluster. Each of the three transcripts completely contained in the region of HSV DNA sequenced in this report contains a unique translational ORF as is shown in Fig. 1. In HSV-1, the ORF for the alkaline exonuclease enzyme yields a polypeptide of 626 amino acids with a residue molecular weight of 67,000. The HSV-2 ORF is slightly smaller, yielding a polypeptide of 620 amino acids. The predicted comparative sequences of the HSV-1 and HSV-2 enzymes are shown in Fig. 6A. The predicted residue molecular weight is significantly less than the value of 82,000 measured by gel electrophoresis of the HSV-1 and HSV-2 purified enzymes and for the in vitro translation product of the HSV-1 alkaline exonuclease transcript (13). We suggest that the deviation in measured and predicted molecular weight of the alkaline exonuclease protein is a result of the very high proline content of the unique N-terminal 126 (117 for HSV-2) amino acids encoded by the 2.3-kb mRNA. This N-terminal segment of the protein has a predicted proline content of 24% in HSV-1 and 27% in HSV-2. We have previoulsy suggested that the deviation in the predicted and measured molecular weights of the HSV-1 glycoprotein C (50,000-Da residue compared with a migration value of 69,000 to 72,000 Da[19, 24]) is due to the high proline content in that protein, especially in its N-terminal region. Further support for this suggestion is that the residue molecular mass (54,000 Da) of the protein encoded by the 1.9-kb colinear transcript, which contains 9% proline, is much closer to the 57,000- to 60,000-Da value derived from migration rates in denaturing gels.

The codon use frequencies of the DNA encoding both the alkaline exonuclease polypeptide and the polypeptide encoded by the 0.9-kb transcript were very similar to those seen for DNA encoding other HSV polypeptides (reviewed in references 49 and 50). The 84-nucleotide region of the C-terminal portion of the alkaline exonuclease translational ORF, which is out of phase with the ORF of the small protein, deviated from the norm; here, the favored codon use frequency was contained in the latter ORF; the base composition of the DNA in this region tends to blur the deviation, however.

Ignoring gaps, the overall amino acid homology between the HSV-1 and HSV-2 alkaline exonuclease proteins was 84%; however, a significant amount of the deviation between the two types was in the N-terminal 20% of the proteins. The overall homology there was only about 50%, whereas the amino acid homology in the portion of the protein overlapping the polypeptide encoded by the 1.9-kb transcript was 88%. This value approaches the amino acid homology seen between HSV-1 and HSV-2 small subunits of ribonucleotide reductase (21, 27). A summary of the available comparative amino acid homology data for a number of HSV proteins is shown in Table 1.

Functional divergence between the HSV-1 and HSV-2 alkaline exonuclease polypeptides is probably less than that suggested by a simple comparison of the predicted amino acid sequences. We used three different computer analyses to assess the potential functional significance of the amino acid variation. The first is a simplification of the amino acid composition described by Devereux et al. (18). Here, the amino acids were broken into six functional types: neutral, weak hydrophobic (A-Pro, Ala, Gly, Ser, Thr), small hydrophilic (D-Gln, Glu, Asn, Asp), large hydrophilic (H-His, Arg, Lys), small hydrophobic (I-Leu, Ilu, Val, Met), large hydrophobic (F-Phe, Tyr, Trp), and cross-linking (C-Cys). A sumamry of the divergence between the HSV-1 and -2 proteins seen with this simplification protocol is shown in Fig. 7 and summarized in Figure 1. As discussed below, the analysis suggested that overall functional amino acid homology between the portion of the alkaline exonuclease enzymes encoded by the HSV-1 and HSV-2 1.9-kb transcripts was on the order of 93%. Such simplication values for the amino acid differences for other HSV-1 and HSV-2 proteins are also included in Table 1.

The close functional relationship between the HSV-1 and HSV-2 alkaline exonuclease enzymes was also seen with a computer analysis of the average degree of hydrophobicity throughout the predicted amino acid sequences of the HSV-1 and HSV-2 enzymes. The computer program used calculates the average hydrophobicity over groups of nine amino acids (42); the results (not shown) are summarized in Fig. 1. Here,

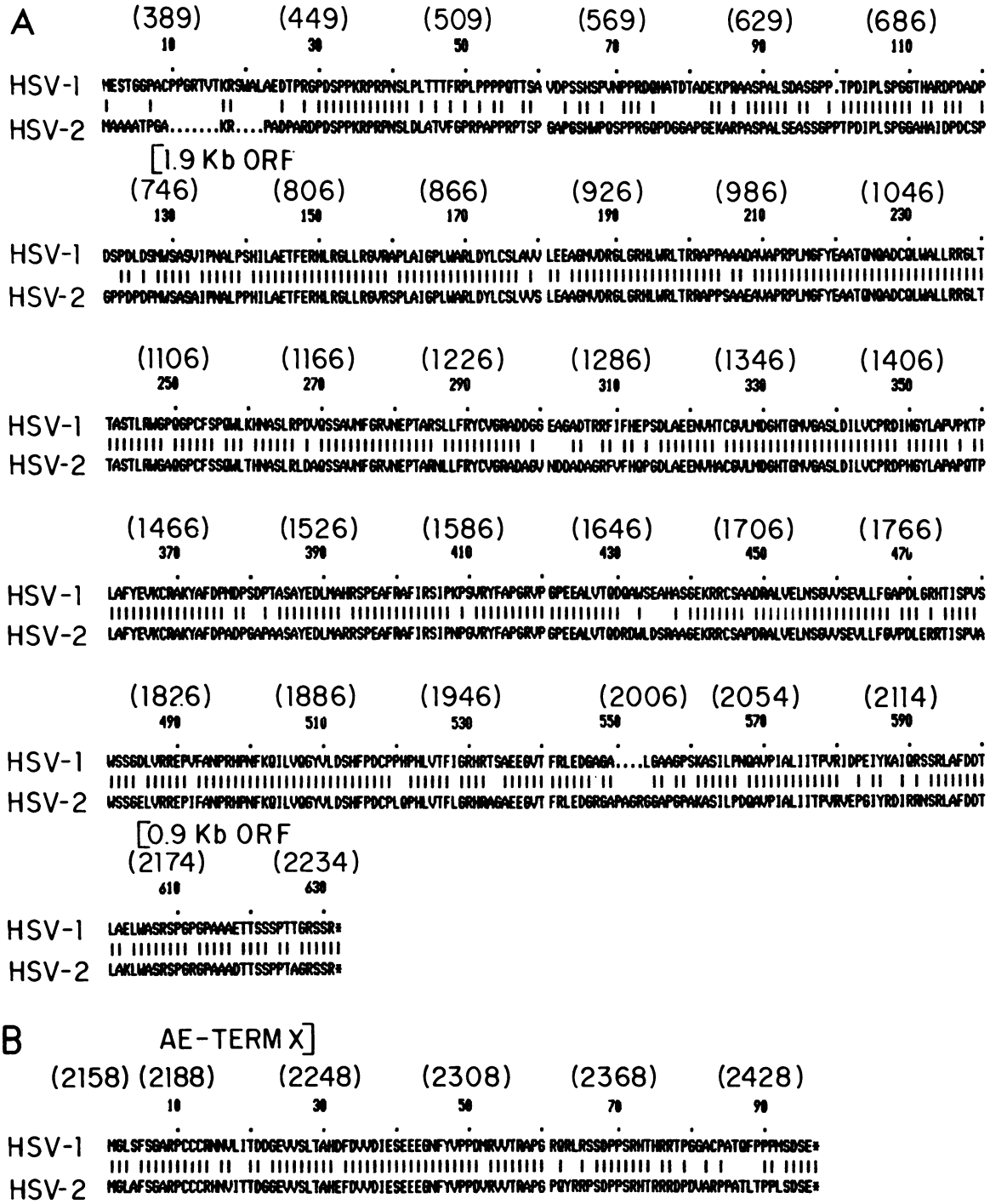

FIG. 6. Comparative amino acid sequence analysis between the putative proteins encoded by alkaline exonuclease (2.3-kb), 1.9-kb, and 0.9-kb transcripts of HSV-1 and -2. HSV-1 nucleotide numbers are shown in parentheses (see Fig. 2). Matched amino acids are designated by vertical lines; regions where gaps were introduced by the computer homology program to maximize overall sequence fit are indicated (•). (A) Predicted amino acid sequences of the HSV-1 and HSV-2 alkaline exonuclease proteins. Also shown are the predicted initiation sites for the in-phase ORF of the 1.9-kb mRNA and for the out-of-phase ORF of the 0.9-kb mRNA. (B) The putative amino acid sequence of the HSV-1 and HSV-2 10.5-kDa proteins encoded by the 0.9-kb mRNA. The site of the termination codon for the out-of-phase alkaline exonuclease ORF is shown.

regions of minor increases of hydrophobicity in either the HSV-1 or HSV-2 enzyme were found throughout their predicted lengths (small arrows). Large differences (large arrows) were only seen in two regions of the proteins, however. One of these was in the N-terminal region of the alkaline exonuclease enzyme. The other major difference was seen in the C-terminal addition of four amino acids in the HSV-2 enzyme. This resulted in a significant loss of hydro-

TABLE 1. Summary of homology between predicted polypeptides encoded by HSV-1 and HSV-2

Protein	Residue mol wt		Amino acid homology			
			Overall		Significant[a]	
	HSV-1	HSV-2	Matching/total[b]	%	Matching/total	%
Alkexo	67,440	66,100	507/626	81	568/626	91
Alkexo[c]	54,300	54,600	429/500	86	466/500	93
3′ protein	10,500	10,500	74/96	77	84/96	88
gC[d]	54,900	51,500	371/511	73	420/511	82
gC 3′ protein[d]	18,200	18,100	134/172	78	155/172	90
tk[e]	40,800	40,400	274/376	73	322/376	86
RRDT[f]	37,900	37,600	302/340	89	317/340	93

[a] Simplified as described in the text and in reference 18.
[b] Number of matching amino acids/total, ignoring gaps.
[c] Protein of protein encoded by 1.9-kb transcript.
[d] Data from references 19, 24, and 47.
[e] Data from references 39, 46, and 51.
[f] Data from references 21 and 27.

phobicity of the HSV-2 enzyme in this region compared with its HSV-1 counterpart.

The third analysis performed was a comparison of the simplified computer predictions of secondary structure and hydrophobicity of the proteins. This analysis, which utilized the parameters of Chou and Fasman (9, 10), has been described by G. Cohen, R. Eisenberg, and E. Golub of the University of Pennsylvania and their colleagues (11, 22). The graphic comparisons were supplied to us by R. Eisenberg and are shown in Fig. 8. Specific features are described in the figure legend, and the general properties of the proteins are similar. Variations in the overall hydrophobicity (indicated by the scaled hexagons) in the N-terminal region and around amino acids 550 to 600 as described above are also shown in this analysis. Other differences in the "structure" of the two proteins with this analysis were the differences in beta-turns around amino acids 50, 130, 325, and 425. This analysis does suggest, however, that there are some significant differences in the overall shapes of the proteins despite their general amino acid homology.

A functional analysis of the 96-amino-acid polypeptide encoded by the 0.9-kb transcript and sharing the out-of-phase translational reading frame with the larger transcripts indicated that this protein has 82% homology in predicted sequence (Fig. 6B). The simplification comparison shown in Fig. 7B and summarized in Table 1 indicated that this protein is more divergent than the portion of the alkaline exonuclease enzymes encoded by the 1.9-kb transcripts of HSV-1 and HSV-2. A hydrophobicity plot summarized in Fig. 1, however, suggested that the overall conformation of the HSV-1 and HSV-2 polypeptides is rather similar.

DISCUSSION

Complete analysis of the transcript family encoding the HSV-1 alkaline exonuclease and neighboring proteins reveals some variant aspects of herpesvirus gene packaging and expression, although the overall transcriptional patterns are consistent with HSV data outlined in several reviews (49, 50). Variants include the extreme density of packaging of protein translational reading frames within the transcripts, the atypical promoter sequence for the 1.9-kb transcript, and the very long (ca. 400-base) leader length for the translational reading frame unique to the 0.9-kb transcript.

Dense packing of protein translational reading frames within the genome is a general feature of viruses. However, based on available transcription mapping data (49, 50), the present instance is rather extreme for the long unique region of HSV. Such density is approached in the genes partially encoded in the DNA contained in *Xho*I fragment W (0.690 to 0.703 m.u. [30]), and in the short unique region (38). The translational ORF for the 50,000-Da putative capsid protein occupies two-thirds of the unique leader for the alkaline exonuclease polypeptide for both HSV-1 and HSV-2. The out-of-phase overlap of translational reading frames between the C-terminal region of alkaline exonuclease and the N-terminal region of the 10,000-Da polypeptide encoded by the 0.9-kb transcript is another example of this compression of translational information. This is the first reported instance of such an occurrence in the long unique region of HSV, although it does occur in the short unique region (38).

Sequence analysis of the DNA encoding the overlapping 3.9- and 4.4-kb transcripts of Fig. 1 indicates that there is another out-of-phase overlap between the N-terminal portion of the ORF of the 50,000-Da protein encoded by the 3.9-kb mRNA and an ORF unique to the 4.4-kb mRNA (Draper and Wagner, work in progress). These data indicate that there is some constraint on the amount of untranslated information in this part of the long unique region. Whatever the reason for the slight increase in information density, there is no obvious compression of nontranscribed DNA in this region of the genome. This conclusion is based on the observation that the extent of untranscribed DNA between the 3′ terminus of the transcripts discussed in this report and the 3′ end of a presently uncharacterized transcript encoded from the opposite DNA strand, immediately to the left of this region, is only about 170 bases in HSV-1 and 140 bases in HSV-2 (data not shown). There is essentially the same amount of DNA between the 3′ ends of the transcripts terminating at 0.6 and at 0.645 m.u. (19, 20, 24, 47). Interestingly, in both cases, the HSV-2 genome contains significantly less bases than is found in HSV-1.

The data of Fig. 3 demonstrate that both the 2.3-kb and the colinear 1.9-kb transcripts are expressed with very similar, if not identical, kinetics. It is therefore interesting that the "promoter" for the 1.9-kb transcript is so atypical for HSV promoters. There is no compelling TATA box homology in the promoter region, and the characteristics of some early promoters, such as seen in the promoter for the alkaline exonuclease mRNA, are missing. The fact that the promoter of the 1.9-kb transcript can be activated in transfection assays is evidence that this region of DNA acts as an independent promoter, especially since we have found no evidence of a splice between the cap site of the 1.9-kb transcript and the cap site for the 2.3-kb alkaline exonuclease mRNA (13). Indeed, no convincing candidate sequence for a splice acceptor is seen in the HSV-1 and HSV-2 comparative data.

The 400-base leader in the 0.9-kb transcript is significantly longer than the average value of 150 bases seen with most well-characterized HSV transcription units (49, 50). However, the length is not at great variance with the extreme values seen in other regions of the HSV genome. Several leaders in excess of 250 bases have been seen in the short region of the HSV-1 genome (38). In addition, the spliced transcript, which we characterized previously and mapped at 0.19 m.u., appears to have a translational leader of approximately 330 bases (16; Draper and Wagner, unpublished data). Finally, the 6-kb transcript encoding the major capsid protein VP-5 appears to have a leader sequence at least 240 bases in length (Draper, Costa, and Wagner,

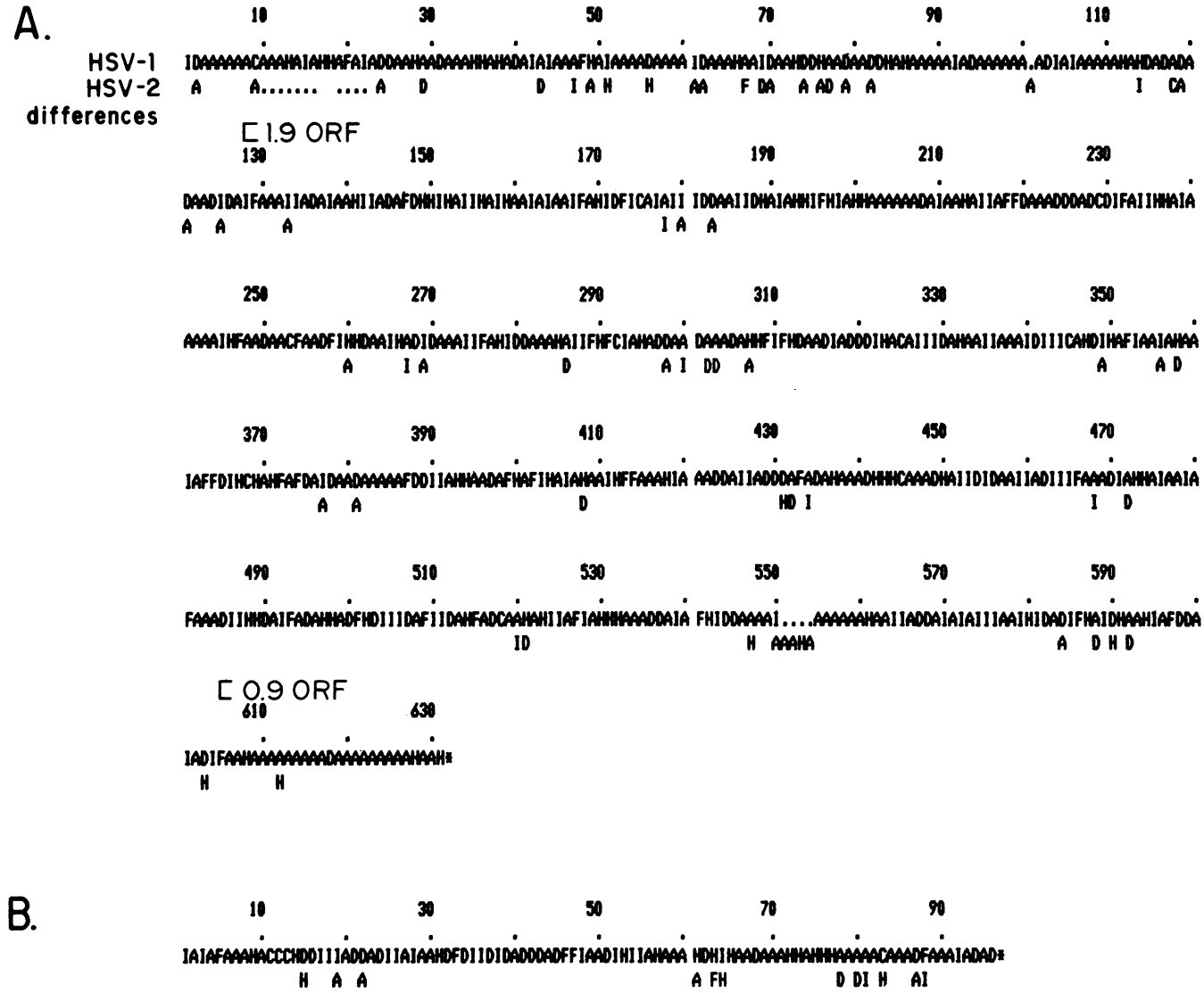

FIG. 7. Significant amino acid differences between the predicted HSV-1 and HSV-2 polypeptides encoded by the alkaline exonuclease and 0.9-kb transcripts. A simplified amino acid sequence was obtained by assigning each amino acid residue to one of six categories, according to the charge of the amino acid, the strength of that charge, or the ability of that residue to form cross-linkages in the protein structure. Symbols of the charge assignments given each amino acid group are explained in the text (and reference 18). The simplified amino acid sequences of the predicted HSV-1 polypeptides are given. Differences in the HSV-2 polypeptides are shown below the HSV-1 sequences. (A) Amino acid charge profiles of the HSV-1 and HSV-2 alkaline exonuclease proteins. Gaps generated by the computer program to maintain the best overall sequence fit are shown (•). (B) The putative amino acid charge profiles of the HSV-1 and HSV-2 polypeptides encoded by the 0.9-kb transcripts.

unpublished data). Such long leader sequences may reflect coding constraints imposed by overlapping transcripts; however, they may also be important in the post-transcriptional regulation of expression of certain herpesvirus proteins.

The fact that the C-terminal 80% of the alkaline exonuclease polypeptide is expressed as an independent polypeptide via its own transcript suggests that conservation of functions contained in this region of the protein are especially important for replication of the virus. The homology between the HSV-1 and HSV-2 DNA within the region of the genome encoding this portion of the alkaline exonuclease gene (87%) is essentially as great as that seen between the 38,000-Da ribonucleotide reductase genes of HSV-1 and HSV-2 (88%). This is the highest reported among the HSV-1 and HSV-2 polypeptides (21, 27; Table 1). The analysis summarized in Fig. 8 demonstrates that this conservation of sequence does not, however, preclude the possibility that the HSV-1 and HSV-2 proteins will have some rather different structural features.

Davison has recently announced the complete nucleotide sequence of the DNA of herpes zoster virus, which has a 46% G+C content compared with a 67% G+C content in HSV (A. Davison, unpublished). Interestingly, there is significant homology between the predicted amino acid sequences of the proteins described in this report and proteins encoded by herpes zoster virus. Indeed, the arrangement of ORFs in the homologous regions of the two genomes are quite similar.

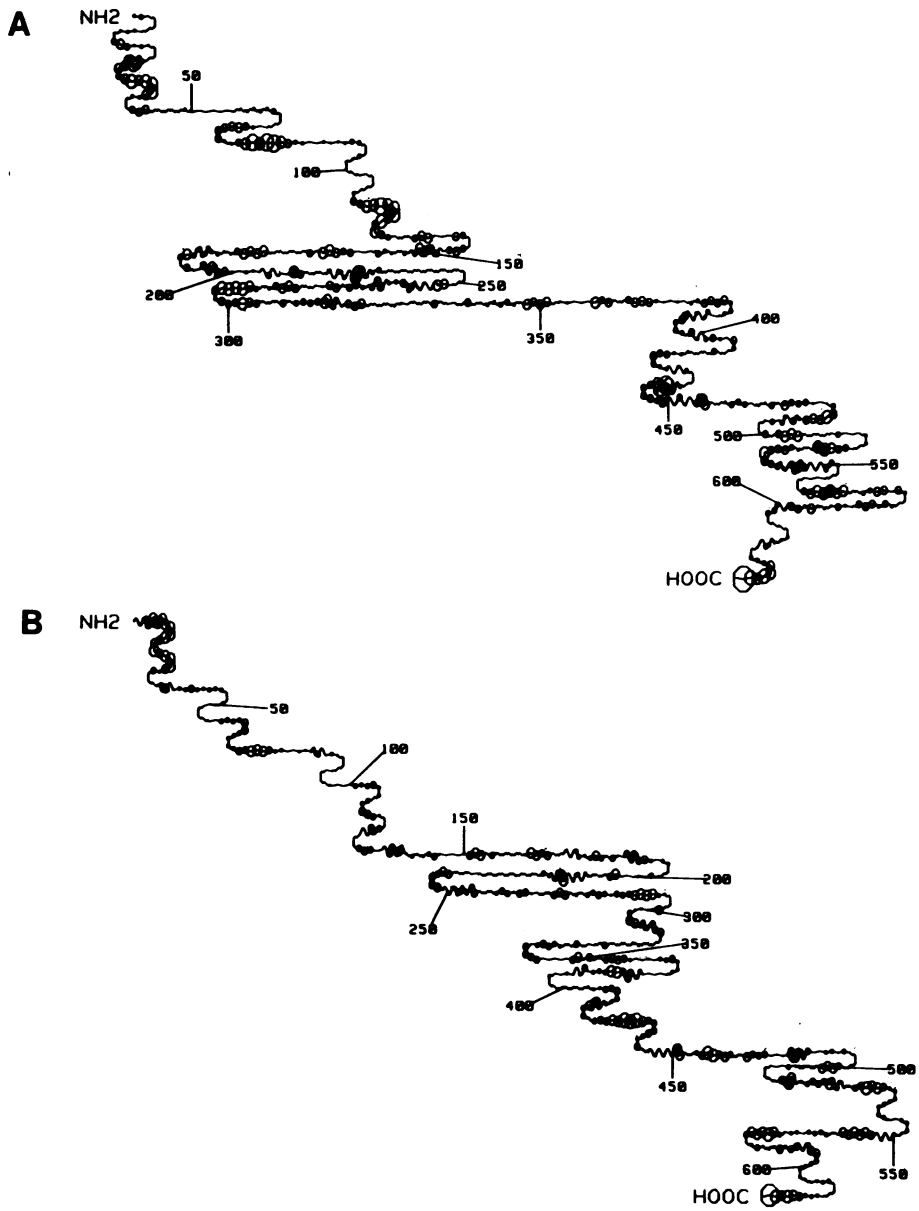

FIG. 8. Predicted secondary structure and hydrophilicity maps of HSV-1 (A) and HSV-2 (B) alkaline exonuclease proteins. Secondary structures were predicted by a computer program, according to the rules of Chou and Fasman (9, 10). Probabilities for the occurrence of helix (P_a), pleated sheet (P_b), and beta turns (P_t) were evaluated using modified coditions: $P_t > 7.5 \times 10^{-5}$ or $P_t > 5 \times 10^{-5}$; $P_t > P_a$ and $P_t > P_b$. Shaded hexagons represent hydrophobic regions; scaled open hexagons indicate hydrophilic areas. The dimension of the hexagons over a residue is proportional to the mean hydrophilicity calculated for that residue and the next five residues (34). The value is therefore distorted at the C-termnal end. Sine waves are indicative of alpha helixes, and pleated sheet regions are indicated by zig-zag liens.

Despite this conservation of sequence between some herpesviruses, the DNA sequence encoding the alkaline exonuclease gene is not strongly conserved between HSV and Epstein-Barr virus. Homology searches of the Epstein-Barr virus genome (3) with the sequence data presented here do not show the length of homology seen either with the ribonucleotide reductase gene or with the spliced HSV-1 transcript (16, 28). Some limited amino acid homology has been seen between the HSV nuclease and two predicted proteins encoded by Epstein-Barr virus (E. Littler, K. G. Draper, E. Wagner, A. McBride, K. L. Powell, and J. R. Arrand, manuscript in preparation). Possible reasons for the duplication in the Epstein-Barr virus genome are discussed in that report. All of these data, when taken together, strongly suggest that the enzymatic activity encoded by the herpesvirus alkaline exonuclease genes is important to the biology of the viruses as a group.

The divergence of the amino acid sequence in the unique 120 N-terminal amino acids of the alkaline exonuclease protein suggests that a lengthy contiguous amino acid sequence in this region is not directly involved in the enzymatic activity of the protein. The very high proline content (ca. 25%) for both HSV-1 and HSV-2 and the comparative analysis of Fig. 8 indicate, however, that the three-dimensional structure of the enzyme is conserved in this region. This may be involved in its enzymatic activity. In this light,

it may be significant that a large change in the hydrophobicity of the protein in this region is only seen at one site (Fig. 6 and 7; summarized in Fig. 1).

We have no idea concerning the possible function of the 96-amino-acid polypeptide encoded by the 0.9-kb transcript. The out-of-phase overlap of the first 18 amino acids with the C-terminal region of the alkaline exonuclease polypeptide would tend to put some severe constraints on divergence in this region of the protein. Despite this, there is one significant amino acid change in the predicted HSV-1 and HSV-2 sequences.

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