High-Resolution Characterization of Herpes Simplex Virus Type 1 Transcripts Encoding Alkaline Exonuclease and a 50,000-Dalton Protein Tentatively Identified as a Capsid Protein


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Four partially overlapping mRNAs (1.9, 2.3, 3.9, and 4.5 kilobases [kb]) were located between 0.16 and 0.19 map units on the herpes simplex virus type 1 genome. Their direction of transcription was found to be from right to left. The 2.3-kb mRNA was found to be early (β), whereas the others were late (βγ). Partial sequence analysis of the DNA encoding these genes indicated that the promoter for the 2.3-kb mRNA shares structural features with other early (β) promoters. In vitro translation of hybrid-selected mRNA indicated that among the proteins these mRNAs encode are an 82,000-dalton (d) polypeptide reactive with a monoclonal antibody against herpes simplex virus type 2 alkaline exonuclease and a 50,000-d polypeptide weakly reactive with a polyclonal antibody made against the capsid protein VP19C. Further experiments suggested that the 2.3-kb mRNA encodes the 82,000-d polypeptide, whereas one (or both) of the larger mRNAs encodes the 50,000-d protein. A novel finding was that the 1.9-kb mRNA appears to share part of the translational reading frame for alkaline exonuclease, but any polypeptide it encodes does not react with the monoclonal antibody to this enzyme.

We have established in a number of recent publications (reviewed in reference 35) that herpes simplex virus type 1 (HSV-1) mRNAs map relatively simply on the viral genome. Furthermore, the low frequency and relatively short span of the characterized HSV-1 introns suggest that the correlation of specific transcripts with genetic markers and known viral proteins will be generally straightforward. Thus, HSV transcription maps add a valuable further dimension to high-resolution genetic marker localization.

As described in the most recent available genetic mapping report (S. K. Weller, W. R. Sacks, D. M. Coen, and P. A. Schaffer, Virology, in press), five complementation groups map in the region between 0.1 and 0.2 map units on the P arrangement of the HSV-1 genome. Details of the data mapping these groups are presented in that publication. Two characterized viral proteins have been located in this region. The first is alkaline exonuclease, an important enzyme of HSV-induced DNA replication (10, 16, 25, 26, 33). Preston and Cordingley (29) mapped this by measuring enzyme levels of in vitro translation products of hybrid-selected mRNA and by performing hybrid-arrested translation. The second viral protein characterized is a 50,000-d protein (VP19C) located by Lemaster and Roizman (20), using intertypic recombinants.

Recent work (L. Banks, D. J. M. Purifoy, R. A. Killington, and K. L. Powell, J. Gen. Virol., in press) has led to the isolation of several monoclonal antibodies against HSV-2 alkaline exonuclease, some of which are cross-reactive with the HSV-1-induced enzyme. Previously reported work (5) described the preparation of polyclonal rabbit antisera reactive with a 50,000-d capsid protein (NC-2 in that publication), which can be inferred to be VP19C. This protein appears to be located on the capsid vertices (34).

In the present study, we used these antisera to identify the mRNA encoding the alkaline exonuclease as a β (early) one and, tentatively, a βγ (late) mRNA as encoding the capsid protein. High-resolution mapping of these mRNAs indicated that all are partially colinear. Correlation of transcript location with nucleotide sequence data demonstrated that the 5′ end of the β mRNA lies downstream of a 120-base re...
sharing certain features with promoters for other β HSV-1 mRNAs. Another mRNA was found to be colinear with the 3' portion of the alkaline exonuclease mRNA. It appeared to have its own independent 5' end and was temporally a βγ mRNA. Preliminary data suggested that this mRNA can be translated in vitro to yield a polypeptide smaller than alkaline exonuclease. Such a polypeptide was not reactive with the Q1 antibody, but partial sequence analysis suggested that it is encoded by the same translation frame as the alkaline exonuclease.

**MATERIALS AND METHODS**

**Cells and virus.** For RNA preparation, plaque-purified isolates of the KOS strain of HSV-1 were used to infect HeLa cells. Monolayer cultures of HeLa cells were grown at 37°C in Eagle minimal essential medium containing 10% calf serum, penicillin, and streptomycin. HSV-2 alkaline exonuclease was prepared from human epidermoid carcinoma no. 2 (HEp-2) cells grown in the same medium. HSV-2 was the 186 strain.

**Enzymes.** All restriction enzymes were obtained from Bethesda Research Laboratories. Digestions were carried out in buffers recommended by the supplier. Phage T4 polynucleotide kinase (Bethesda Research Laboratories) was used for 5' end labeling as described by Maxam and Gilbert (23). *Escherichia coli* DNA polymerase I (Klenow fragment, Boehringer-Mannheim) was used to generate 3'-end-labeled restricted DNA by procedures described by Maniatis et al (21).

**Isolation, labeling, and size fractionation of polyribosomal RNA.** Monolayer cultures of HeLa cells (2 × 10⁷ 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. Polyribosomes were isolated from the cytoplasm of HSV-1-infected cells by the magnesium precipitation method of Palmer (27). Polyadenyllic acid-containing [poly(A)] mRNA was isolated from total rRNA by oligodeoxynucleotide acid-cellulose (Collaborative Research, Inc.) chromatography. This is referred to as HSV poly(A) mRNA. Details of this procedure were presented elsewhere (13). RNA was isolated at 6 h postinfection except when early (β) RNA was required. Then cells were preincubated for 1 h and treated for 5 h with 1.5 × 10⁻⁴ M adenosine arabinoside and 3.7 × 10⁻⁶ M pentostatin to inhibit viral DNA replication, as described previously (15). RNA was size fractionated by electrophoresis on 1.4% agarose gels containing 10 mM methylmercury hydroxide (3) as previously described (1, 2).

**Recombinant DNA.** All recombinant DNA clones described in this paper were derived from either HindIII fragment I-O (map units 0.082-0.182), HindIII fragment J (0.182-0.261), Xho-I HindIII fragment C'-IO (0.171-0.182), BamHI-HindIII fragment A-IO (0.152-0.182), or HindIII-Sall fragment J-D (0.182-0.195), cloned in pBR322. Procedures for cloning HSV-1 DNA fragments in the pBR322 vector were described previously (1, 6). Cloned DNA fragments were named as described previously and located by their map coordinates on the P arrangement of the HSV-1 genome (6).

**In situ Northern RNA blots.** As described previously, samples (5 μg) of HSV poly(A) mRNA were fractionated on methylmercury gels and dried onto Whatman 3mm paper with vacuum (15). The agarose film was floated off the paper in water and hybridized with appropriate nick-translated, 32P-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 (7) at 50°C for 36 h. Blots were rinsed at 50°C; the first two rinses were in 50% formamide-2× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS). The last rinse was in 0.1× SSC-0.1% SDS. Autoradiography was on Kodak XRP film with or without intensifying screens as needed.

In vitro 32P-labeled DNA was made by nick-translating appropriate DNA clones with DNA polymerase I, DNase I (Boehringer-Mannheim), and 50 μCi of [α-32P]dCTP (3,000 Ci/mmol, Amersham).

**Isolation of restriction fragment-specific mRNA.** Restriction fragment-specific mRNA was isolated from HSV poly(A) mRNA by preparative hybridization to the appropriate DNA covalently coupled to cellulose. Details of coupling of DNA to cellulose and preparative hybridization were as described previously (2, 6).

**Nuclease mapping of HSV-1 mRNA.** S1 nuclease and exonuclease VII analysis of RNA was carried out essentially as described by Berk and Sharp (4) and as described previously (1, 6, 8, 11-13, 15). Appropriate HSV-1 DNA clones (10 μg) were cleaved at the desired site with the appropriate restriction enzyme. The DNA then was 5' labeled with [γ-32P]ATP (3,000 Ci/mmol, ICN), using polynucleotide kinase (Bethesda Research Laboratories), to a specific activity of 4,000 to 10,000 cpm/μg of DNA. Alternatively, the DNA was 3' labeled to the same specific activity by using DNA polymerase I-Klenow fragment (Boehringer-Mannheim).

The DNA fragments then were denatured and strand separated on 5% acrylamide gels as described by Maxam and Gilbert (23). The strand-separated DNA (from 10 μg of cloned DNA) was hybridized with 10 μg of infected-cell 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. Hybrids were subjected to S1 nuclease (Boehringer-Mannheim) or exonuclease VII (Bethesda Research Laboratories) digestion as described (11, 12). Material was fractionated on a denaturing 5% acrylamide gel with 5'-end-labeled, *HaeIII- or HindIII-digested* pBR322 DNA fragments as size standards. When the nuclease-protected material was to be fractionated on sequence ladders, mung bean nuclease (PL Biochemicals) was used instead of S1 nuclease. All procedures were based on those of Maxam and Gilbert (23).

**Nucleotide sequencing.** As described previously (8, 13), nucleotide sequence analysis was carried out by the procedures of Maxam and Gilbert (23).

**In vitro translation.** Translation of size-fractionated viral mRNA was carried out in vitro by using a micrococcal nuclease-treated rabbit reticulocyte system (New England Nuclear Corp.) with [35S]methionine (>800 Ci/mmol) as the radioactive amino acid. Details of the procedure and fractionation of polypeptides in SDS-acrylamide gels by the method of Laemmli (19) were described in several previous papers. Gels were treated with En3Hance (New England Biosystems).
Nuclear Corp.) and dried with vacuum at 60°C, and radioactive bands were localized by autoradiography with Kodak XRP film. Exposure was for 3 to 5 days at -70°C.

Immunoprecipitation of in vitro translation products was performed as described previously (13). One half of the RNase-treated translation product (14 µl) was diluted with an equal volume of 2X lysis buffer and incubated with 4 µl of polyclonal NC-2 serum, a polyclonal antibody to the HSV-1 50,000-d capsid protein (VP19C), or with 1 µl of Q1, a monoclonal antibody made against purified HSV-2 alkaline exonuclease. Lysis buffer is 20 mM Tris-hydrochloride (pH 7.4)-50 mM NaCl-10 mM methionine-0.5% Nonidet P-40-0.5% sodium deoxycholate-0.1% SDS. After 1 h on ice, 50 µl of a 10% suspension of Pro-A Sepharose beads (Pharmacia) in 50 mM Tris-hydrochloride (pH 7.5)-150 mM NaCl-5 mM disodium EDTA-0.2% sodium azide was added, and the suspension was incubated for a further 30 min on ice with frequent mixing. The Pro-A Sepharose beads with the immune complex absorbed were then deposited by 1 min of centrifugation in an Eppendorf microcentrifuge, and the pellet was suspended in 200 µl of lysis buffer and centrifuged for 5 min through a 1 ml pad of lysis buffer containing 1 M sucrose. The Sepharose was then washed by suspension in 0.5 ml of 10 mM Tris-hydrochloride (pH 7.4)-150 mM NaCl-10 mM methionine-0.2% Nonidet P-40-0.1% SDS and recentrifugation. After five washes, the Sepharose pellet was suspended in 40 µl of a buffer containing 0.075 M Tris-hydrochloride (pH 6.8)-3% SDS-10% β-mercaptoethanol. The suspension was heated to 95°C for 2 min, and the Pro-A Sepharose was pelleted by centrifugation. After this the supernatant, containing released monoclonal antibody with any translation product with which it had reacted, was loaded onto SDS-acrylamide gels for size fractionation.

In one set of experiments, we used washed, Formalin-killed *Staphylococcus aureus* (IgGorb, The Enzyme Center, Inc., Boston, Mass.) to precipitate the immune complex. This method resulted in a considerably larger amount of protein precipitated but a much higher background of nonspecific radioactive proteins. This procedure, however, yielded interpretable results.

**Preparation of radioactive protein markers.** [35S]methionine-labeled, cytoplasmic infected-cell protein was isolated by the basic procedure of Edwards and Fan (9). Cultures (6 x 10⁶ cells) of HeLa cells were incubated from 6 to 8 h postinfection with 200 µCi of [35S]methionine (400 Ci/mmol; New England Nuclear Corp.) in 4 ml of methionine-free Eagle minimal essential medium.

In vitro [35S] labeling of purified HSV-2 alkaline exonuclease was carried out by the method of Greenwood et al. (14). Samples of the purified protein (0.05 µg) were labeled with carrier-free [35S]methionine (400 Ci/mmol; New England Nuclear Corp.) in 4 ml of methionine-free Eagle minimal essential medium.

**Preparation and characterization of monoclonal antibodies against HSV alkaline exonuclease.** Details of monoclonal antibody preparation are described in the paper by Banks et al. (in press). Briefly, HSV-2 enzyme was purified from high-salt extracts of HSV-2-infected Vero cells (28). Steps included DEAE-cellulose, phosphocellulose, and DNA-cellulose chromatography. Enzyme activity was measured by the method of Purifoy and Powell (30). Purity can be assessed from the data in Fig. 3A.

Monoclonal antibodies were prepared by two inoculations of purified enzyme (2 to 3 µg) into the footpads of BALB/c mice during a 2-week interval. Cell fusion was by the method of Kenneth et al. (17). Hybrid cell colonies were grown in microtiter plates (Linbro) and tested for production of antibody with an enzyme-linked immunosorbent assay (horseradish peroxidase-conjugated anti-mouse immunoglobulin G; DAKO, Denmark) using detergent extracts of HSV-2-infected cells as antigen. Peroxidase assay was done with 0.01% orthophenylendiamine and 0.003% hydrogen peroxide.

The antibody used in this paper (Q1) was shown to be able to immunoprecipitate and neutralize the HSV-2 alkaline exonuclease. Furthermore, it has been used in an immunosorbent column to purify the enzyme. It is strongly cross-reactive with the HSV-1-induced enzyme (K. Powell, unpublished data).

**RESULTS**

**Four partially collinear mRNA species map between 0.16 and 0.19 on the HSV-1 genome.** We used a number of cDNA probes made to small (1.0- to 1.5-kilobase [kb]) fragments of HSV-1 DNA mapping between 0.16 and 0.19 map units to determine the number and time of appearance of abundant mRNAs. Four readily detectable mRNA species of approximately 2.1, 2.5, 4.1, and 4.7 kb were seen in Northern (RNA) blots of HSV-1 mRNA isolated from cells 6 h postinfection. We could infer that the actual mRNA sizes were 1.9, 2.3, 3.9, and 4.5 kb, since the average poly(A) tail length of HSV-1 mRNA is ca. 200 bases (32). Nick-translated DNA probes mapping between ca. 0.16 and 0.17 map units hybridized to all mRNA species. Probes containing DNA mapping between ca. 0.174 and 0.19 hybridized to only the three larger species. Examples of such experiments are shown in Fig. 1.

A Northern blot of viral mRNA isolated from cells in which DNA synthesis was inhibited is shown in Fig. 1A. Here the RNA blot was hybridized with 32P-labeled DNA made by nick-translation cloned *BglII*-XhoI fragment O-G (0.164-0.171 map units). The 2.3-kb mRNA was a major band early, whereas the 1.9-, 3.9-, and 4.5-kb mRNAs were present in low abundance. The same probe revealed the 2.3-kb mRNA in equivalent abundance in Northern blots of late viral mRNA (Fig. 1A). In this RNA, however, the other species were present in relatively greater abundance. This relative abundance difference with time after infection suggested that the 2.3-kb mRNA is an early (β) mRNA, whereas the 1.9-, 3.9-, and 4.5-kb species are late (β′) species. Criteria for temporal classification of specific mRNA species have been described in several recent reviews (31, 35, 36).

We used two nick-translated probes to determine how far the 1.9-kb mRNA extended be-
beyond the XhoI site at map unit 0.171. A probe extending to a DdeI site 490 bases to the right of the XhoI site (see sequence data below) hybridized to all four mRNA species (Fig. 1B). A DNA probe extending from this DdeI site rightward to the HindIII site at 0.182 did not hybridize detectably with the 1.9-kb mRNA (not shown).

The two larger β mRNA species were readily shown to extend beyond the HindIII site at map unit 0.182 since a 32P-labeled DNA probe made from HindIII-EcoRI fragment J-D (0.182–0.190 map units) hybridized to them in Northern blots of late mRNA (Fig. 1C). As mentioned above, other probes were used to show the precise extent of these mRNAs. Heavier exposures of Northern blots hybridized with HindIII-EcoRI fragment J-D (0.182–0.190 map units) also revealed a relatively low-abundance mRNA ca. 2.7 kb in size. This mRNA extended to the right of the area of interest presented in this paper and is not characterized here.

The data of the next section demonstrated that the 3' end of the four mRNA species lies to the left of the BglII site at map unit 0.164. Therefore, the BglII-XhoI fragment O-G (0.164–0.171 map units) DNA probe could be expected to hybridize with the four mRNAs colinear through that region in amounts reflecting their relative abundance. The data of Fig. 1A, then, indicate that the 2.3-kb (β) mRNA is the most abundant mRNA in this region, whereas the 1.9-kb mRNA is somewhat less so and the 3.9- and 4.5-kb mRNAs are considerably less abundant than the two smaller mRNAs.

High-resolution localization of the 5' and 3' ends of the overlapping mRNA species. We used 5'- and 3'-end-labeled single-stranded DNA as hybridization probes for precise S1 and exonuclease VII mapping of the four mRNAs located in Fig. 1. Basic methods have been reviewed recently (35) and are outlined above. These data are summarized in Fig. 2.

The 2.85-kb BamHI-XhoI fragment A-G (0.152–0.171 map units) was 3' end labeled at the XhoI site at map unit 0.171. Strand-separated probe was hybridized with viral mRNA, the hybrids were digested with SI nuclease, and protected DNA was fractionated on a denaturing acrylamide gel. The major protected species was 1,600 bases long (Fig. 2A, track S), indicating a major mRNA 3' end 1,600 bases to the left of the XhoI site at map unit 0.171. Minor amounts of other bands were seen with long exposures. However, none contained more than 1 to 2% of the total radioactivity seen. Furthermore, similar experiments (not shown) using DNA 3'-labeled at the BglII site at map unit 0.163 gave a major SI protected band 400 bases long. These data, when taken with experiments below, indicated that the four mRNAs share a coterminal 3' end ca. 400 bases to the left of the BglII site at 0.163. We did not exclude the presence of small amounts of other RNA species terminating at other sites.

The 5' ends of the 1.9- and 2.3-kb mRNA species were located by hybridizing viral mRNA with strand-separated XhoI-HindIII fragment C'-IO DNA (0.171–0.182 map units) 5' end labeled at the XhoI site at map unit 0.171. Exonuclease VII digestion of hybrids (Fig. 2B, track X) gave three protected species. One was 1,650 bases long, corresponding to the full length of the DNA. This was due to the protection of the 3.9- and 4.5-kb mRNAs (see below). The second protected fragment was 700 bases long, suggesting that the 2.3-kb mRNA extends 700 bases to the right of the XhoI site at 0.171. The third species was 270 to 280 bases in length. This suggested that the 5' end of the contiguous portion of the 1.9-kb mRNA extends this far to the right of the XhoI site. In the exonuclease VII digestion track of Fig. 2B (track X), some other (faint) intermediate-sized bands were seen. However, these were not consistently present in several repeat experiments. We suggest these were digestion intermediates of the nuclease reaction. SI nuclease digestion of hybrids between viral mRNA and strand-separated XhoI-HindIII fragment C'-IO DNA (0.171–0.182 map units) 5' end labeled at the XhoI site yielded the same three fragments (Fig. 2B, track S). These mapping data, the close correlation of the size of

FIG. 1. In situ RNA (Northern) blots of HSV-1 mRNA encoded by DNA mapping between map units 0.164 and 0.190. Samples of HSV poly(A) mRNA from cells in which DNA synthesis was inhibited (early) or allowed to proceed (late) were fractionated on methyl-mercury-containing agarose gels and immobilized by drying in vacuo. The region-specific RNA was detected by hybridization with nick-translated DNA probes as indicated. Sizes shown were determined by the position of HeLa cell rRNA markers (not shown) as described previously (1,6). (A) mRNA species hybridizing to a cDNA probe from BglII-XhoI fragment O-G (0.164–0.171 map units) (B) mRNA species hybridizing to a probe from the 490 bases between the XhoI site at 0.171 and a DdeI site indicated in Fig. 3 and 4 (below). (C) mRNA species hybridizing to a probe made from HindIII-EcoRI fragment J-D (0.182–0.190 map units).
FIG. 2. Localization of the mRNAs by S1 nuclease and exonuclease VII mapping. Single-stranded DNA 5' or 3' labeled at specific restriction sites was hybridized with HSV poly(A) mRNA, and the DNA protected from nuclease digestion was size fractionated on denaturing acrylamide gels as described previously (13, 15). (A) BamHI-XhoI fragment A-G DNA (0.152–0.171 map units) 3' labeled at the XhoI site. S, S1 nuclease-resistant material. D, Undigested DNA. M, Size marker of HindIII-digested pBR322 DNA; sizes are (in nucleotides from bottom): 220/221, 298, 345, 396, 506/517, 600, and 999. (B) XhoI-HindIII fragment C'-IO DNA (0.171–0.182 map units) 5' labeled at the XhoI site. D, Undigested DNA. X, Exonuclease VII-digested material. M2, Marker of HaeII-digested pBR322 DNA; sizes are (in nucleotides from bottom): 227, 280 (partial digestion fragment), 370, 430/439, 622, and 1876. S, S1 nuclease-digested material. M, Size markers as described for (A). (C) HindIII-SalI fragment J-D DNA (0.182–0.195 map units) 5' labeled at the HindIII site. M, Size marker as described for (A); S, S1-digested material; X, exonuclease VII-digested material; D, undigested DNA.

the S1-resistant hybrid (ca. 1,880 nucleotides) compared with the total size of the mRNA (2 to 2.1 kb), and the lack of hybridization of DNA probes to the right of the DdeI site to the 1.9-kb mRNA all strongly suggest that this mRNA has a distinct 5' end. Certainly any intron must be less than 50 bases long in total length and must be unable to hybridize efficiently with the DNA under our standard conditions.

The 5' ends of the generally colinear 3.9- and 4.5-kb mRNAs were located by using as a hybridization probe strand-separated HindIII-SalI fragment J-D (0.182–0.195 map units) 5' end labeled at the HindIII site at map unit 0.182. Here late viral mRNA protected fragments 650 and 1,250 bases long from both S1 nuclease and exonuclease VII digestion (Fig. 2C, tracks S and X). Thus, the 5' ends of these mRNAs map 650 and 1,250 bases to the right of the HindIII site at map unit 0.182. The fact that the ratio of radioactive activities in the two bands is similar with both nuclease digestion regimens suggested that each mRNA has its own discrete 5' end.

Further characterization of the regions containing the 5' end of the 2.3-kb β and 1.9-kb βγ mRNAs. The 1,000-base nucleotide sequence of the DNA from ca. 30 bases 3' of the XhoI site at map unit 0.171 to just beyond the AvaI site at 0.177 is shown in Fig. 3. A summary of the results of the following experiments and the sequence strategy are shown in Fig. 4.

We precisely located the 5' end of the 2.3-kb mRNA by doing nuclease protection experiments and fractionating the mRNA-protected DNA fragment on a DNA sequencing gel. This procedure has been documented in several previous publications (8, 12, 13). It was done by hybridizing HSV poly(A) mRNA with single-stranded DNA, spanning the two HindIII sites at nucleotides 625 to 780, which was 5' end labeled at nucleotide 625. Hybrids were digested with mung bean nuclease. Protected DNA was fractionated against a sequence ladder of DNA 5' labeled at the same HindIII site (Fig. 5A). Note that the sequence ladder is of the DNA strand complementary to the mRNA. The 5' end of the mRNA fell within the sequence GTATC, and thus the mRNA begins within the region between nucleotides 273 and 277.

The 5' end of this mRNA fell ca. 28 to 30 bases 3' to the sequence TATAAATTA, an excellent TATA box. The 120 or so bases 5' upstream of this 5' end share several features with promoters for other HSV-1 β mRNAs (reviewed in reference 35). These include a TATA box about 30 bases upstream of the transcript and an AC
the 2.3- and 1.9-kb PvuII, and sequence is was Sequencing 596 COSTA E) and FIG. 3.

The locations (Z) indicated.

FIG. 3. Nucleotide sequence of DNA between the XhoI site at map unit 0.171 and the AvaI site at 0.177. Sequencing was by the method of Maxam and Gilbert (23), and the strategy is shown in Fig. 4. The locations of the 2.3- and 1.9-kb mRNA 5' ends (●●○), in-phase translation initiators (●), and several other features of the sequence are indicated.

FIG. 4. Sequencing strategy and summary. High-resolution restriction maps for XhoI, Hinfl, Smal, AvaI, PvuII, and Ddel sites are shown, as well as the length of sequence ladders from DNA 5' labeled at such sites ( → ). The locations of the 5' ends of the 2.3-kb (β) and the 1.9 kb (βγ) mRNAs and of the translational start (●) and stop (△) codons are shown.
The sequence data in Fig. 3 were analyzed for potential translational initiation and termination codons. The 5' end of the 2.3-kb (β) mRNA is 160 bases upstream of the canonical eucaryotic translation initiation sequence AAATG (nucleotides 432 to 437), as described by Kozak (18). This translation frame (frame 1 of Fig. 4) stays open throughout the region sequenced and contains methionine codons 379, 553, and 643 bases downstream. It is a reasonable assumption that this frame defines the N-terminal region of a protein. A second ATG codon is seen in reading frame 3 at position 457-459, but this frame is terminated 24 bases downstream with the TAA triplet at position 481-483.

All reading frames contain multiple terminators upstream of the potential translation frame for the 2.3-kb mRNA, so any protein encoded by the 3.9- or 4.5-kb mRNAs would not share any translational frames with a protein encoded by the 2.3-kb mRNA.

We hybridized single-stranded DNA, 5' end labeled at the XhoI site (base 28; Fig. 3) and extending to the DdeI site at base 520, with viral mRNA and carried out 5' nuclease analysis. Two protected species were seen. One migrated with a size of 290 bases, and one migrated along with undigested DNA (Fig. 5B). The shorter DNA species corresponded to the 270- to 280-base band seen in Fig. 2B (tracks S and X). The full-length band indicated that no other major species was seen in the region extending up to 520 bases upstream of the XhoI site at 0.271.

Control experiments (not shown) indicated that 5' nuclease-protected DNA was entirely dependent on the presence of viral mRNA in the hybridization. We precisely located the mRNA

![FIG. 5. (A) Precise localization of the 5' end of the 2.3-kb alkaline exonuclease mRNA. HSV poly(A) mRNA was hybridized with single-stranded DNA 5' end labeled at the HindIII site at nucleotide 622 (Fig. 3) and extending to the HindIII site at nucleotide 778. This was then digested with mung bean nuclease, and the protected DNA was fractionated against a sequence ladder of DNA 5' end labeled at nucleotide 622. (B) Precise localization of the 5' end of the 1.9-kb mRNA. DNA spanning from the XhoI site at nucleotide 28 (Fig. 3) to the DdeI site at nucleotide 520 was 5' end labeled at base 28 and strand separated. It was hybridized with viral mRNA, the hybrids were digested with 5' nuclease, and protected DNA was fractionated on a denaturing acrylamide gel using HaeIII-cut pBR322 DNA fragments as a size standard.](image_url)

![FIG. 6. Sequence comparison for the promoters of HSV-1 β mRNAs. The 2.3-kb mRNA promoter region from the data of Fig. 3 is shown compared with data for other HSV-1 β mRNAs. The data for HSV-1 thymidine kinase are from McKnight (24), those for the 5.2-kb mRNA are from Frink et al. (13), those for the 1.2-kb mRNA are from Draper et al. (8), and those for the 1.3-kb mRNA are from Hall et al. (15).](image_url)
end (seen above) to around base 320 by using high-resolution sequencing gels against sequence ladders (not shown). This putative cap site does not lie 25 to 30 bases downstream of any exceptionally appealing TATA box sequence. However, the sequence between bases 350 and 345 (TGATC) could conceivably serve as an equivalent feature. The cap site does lie 131 bases to the right of the ATG triplet at position 187–189. This could serve as a translational initiator. If so, the polypeptide encoded should share the same reading frame as that encoded by the 2.3-kb mRNA.

Evidence that the 2.3-kb early mRNA encodes alkaline exonuclease. We used a monoclonal antibody against HSV-2 alkaline exonuclease to demonstrate that the 2.3-kb mRNA encodes the cross-reactive HSV-1 protein. Denaturing gel electrophoresis showed that this antibody (Q1) specifically binds to purified [125I]-labeled HSV-2 alkaline exonuclease (82,000 molecular weight) (Fig. 7, track Q1). Another monoclonal antibody (T2/T1), which is directed against other specific sites on the HSV-2 enzyme, did not precipitate it (Fig. 7A, track T2/T1). This demonstrated the specificity of the Q1 precipitation. Q1 reacted with the 82,000-d HSV-1 enzyme from [35S]methionine-labeled, HSV-1-infected cell extracts (Fig. 7A, track Q1-I.P.). Other faint protein bands were also visualized. No specific protein band could be seen when Q1 was reacted with uninfected cell extract (data not shown). Therefore, it is not known whether these represent nonspecific binding or binding of antigenically related, infected-cell proteins or both.

We purified mRNA encoded in this region of HSV-1 DNA by using BamHI-HindIII fragment A-I0 (0.152–0.182 map units) and Xhol-HindIII fragment C'-I0 (0.171–0.182 map units) bound to cellulose. Both fragments hybridized to all mRNAs (see Fig. 1), but the former fragment also encodes the 3' region of another 2-kb mRNA whose 5' end maps near 0.147 and whose 3' end maps near 0.160 (data not shown). We subjected a sample of purified mRNA (ca. 0.1 μg of total mRNA) to in vitro translation by using [35S]methionine and incubated the total translation mix with Q1. Reactive protein was size fractionated on a denaturing SDS-acrylamide gel (Fig. 7B, tracks C'-I0 and A-I0). Both mRNA preparations translated the same 82,000-d polypeptide. In vitro translation of HSV-1 mRNA purified by using DNA fragments from other regions of the genome did not yield any detectable protein reactive with Q1 antibody (data not shown).

In another experiment, we purified mRNA by using Xhol-HindIII fragment C'-I0 (0.171–0.182 map units) and translated it in vitro. Translation products were reacted with Q1 antibody. This

FIG. 7. In vitro translation and identification of HSV-1 alkaline exonuclease. All autoradiographs are of denaturing SDS-acrylamide gels (19). Sizes of proteins (×10^6 d) were determined by comigration with adenovirus mRNA translation products (not always shown) as described previously (1). (A) Q1. Immuno precipitation of [125I]-labeled HSV-2 alkaline exonuclease with monoclonal antibody Q1. T2/T1. Lack of precipitation with monoclonal antibody T2/T1. Enzyme, Authentic enzyme. Q1-I.P., Immunoprecipitation of cytoplasmic, [35S]-labeled, infected-cell protein with monoclonal antibody Q1. I.P., Cytoplasmic infected-cell protein. (B) Immunoreactivities of in vitro translation products. C'I0-Q1. Products of translation of Xhol-HindIII fragment C'-I0 DNA (0.171–0.182 map units) specific mRNA immunoprecipitated with Q1. AIO-Q1. The same immunoprecipitation, except that BamHI-HindIII fragment A-I0 DNA (0.152–0.182 map units) specific mRNA was used for the in vitro translation. Ad. In vitro translation products of adenovirus mRNA. (C) Comigration of HSV-2 enzyme and HSV-1 in vitro translation product. HSV, HSV-1 poly(A) mRNA (1 μg) in vitro translation products. Enzyme-Q1. [125I]-labeled HSV-2 alkaline exonuclease precipitated with Q1. C'I0-Q1. In vitro translation product of Xhol-HindIII fragment C'-I0 DNA (0.171–0.182 map units) specific mRNA precipitated with Q1. (D) Translation of size-fractionated HSV poly(A) mRNA and precipitation with Q1. Sizes of mRNA are indicated above the appropriate tracks. I.P., [35S]-labeled, cytoplasmic infected-cell protein.
VOL. 48, Hindlll (Fig. contains number these tionated to alkaline exonuclease. We
mRNA. The translation products were then
reacted with Q1 antibody. It was clear (Fig. 7D)
that major synthesis of the 82,000-d alkaline
exonuclease is mediated by mRNA in the size
range of 2.9 to 2.5 kb. These data demonstrate
that the 2.3-kb β mRNA does, indeed, encode
the enzyme. [Note: as in Fig. 1, this mRNA
migrates with a rate corresponding to a size of
2.5 kb because of the poly(A) tail.] The radioac-
tivity migrating at ~50,000 d in each track is an
endogenous band from the reticulocyte system.
Its presence here is due to the fact that we used
IgGorbs instead of Pro-A Sepharose to react with
the immune complex because of the former’s
greater ability to bind to immune com-
plexes.

Identification of a potential translation product
for the 1.9-kb mRNA. Partial sequence data (Fig.
3) indicated that the 1.9-kb mRNA collinear
with the 3’ region of the 2.3-kb mRNA could encode
a polypeptide about 130 amino acids shorter than
the alkaline exonuclease. We used BamHI-
HindIII fragment A-IO (0.152-0.182 map units)
bound to cellulose to isolate all the mRNAs
encoded in the region of interest. We size frac-
tionated these region-specific mRNAs and trans-
lated both the 2.3-kb and 1.9-kb mRNAs in vitro. We
used methods described in detail in several
recent publications (see reference 13) for the
size fractionation of the hybrid-selected
mRNA. Translation of the 2.3-kb mRNA yielded
a number of polypeptides, but only the 82,000-d
alkaline exonuclease was reactive with Q1 anti-
body (Fig. 8, tracks T-1 and Q1-1).

The mRNA isolated as the 1.9-kb species
actually contains both this mRNA and a 1.9-kb
mRNA which is homologous to the other DNA
strand and has a 3’ region homologous to the
region between 0.152 and 0.160 map units (see
the preceding section). Translation of this
mRNA mixture consistently gave two polypep-
tide products distinct from endogenous bands
found in the translation system. The larger
migrated with a rate corresponding to a molecular
weight of 60,000, and the smaller migrated with
a rate corresponding to 54,000 (Fig. 8, track T-2).
We suggest that one of these polypeptides is the
translation product of the 1.9-kb mRNA in ques-
tion. Since, however, neither product reacted
with Q1 antibody (Fig. 8, track Q1-2), our identi-
fication of a translation product for the 1.9-kb
mRNA described in this report is only tentative.

Tentative evidence that a 50,000-d HSV-1 capsid
protein is encoded by either the 3.9- or 4.5-kb
mRNA. We used a polyclonal rabbit antiserum
made against the SDS gel-purified 50,000-d
HSV-1 capsid protein (VP19C) to tentatively
identify it as a translation product of the 3.9- or
4.5-kb mRNA. This antiserum (NC-2) specifically
reacts with a 50,000-d protein from [35S]meth-
ionine-labeled extracts from HSV-1-infected
cells. A denaturing acrylamide gel size fraction-
ation of infected-cell protein reactive with NC-2
is shown in Fig. 9A (track NC2-I.P.). Some
other proteins are also detectable, especially
with long exposures (ca. 2 weeks). A prominent
one is the 155,000-d capsid protein, ICP5, which
may have been a contaminant of the original
size-fractionated protein antigen.

We prepared purified region-specific viral
mRNA, using BamHI-HindIII fragment A-IO

FIG. 8. In vitro translation of the 1.9-kb mRNA.
The 2.3-kb and 1.9-kb hybrids, selected by using
BamHI-HindIII fragment A-IO (0.152-0.182 map
units) bound to cellulose, were fractionated by electo-
phoresis on a denaturing agarose gel containing meth-
ylmercury hydroxide. Each was translated in vitro;
half of each translation product was fractionated with-
out further treatment (tracks T), and half was reacted
with Q1 monoclonal antibody (tracks Q1). Details of
the translation and fractionation of products are as
described for Fig. 7. A track of the translation product
of rabbit globin mRNA is included as a control.
FIG. 9. Immunoprecipitation of a 50,000-d protein with antiserum NC-2. Details are as for Fig. 7. (A) Comigration of protein precipitable from cytoplasmic infected-cell protein and in vitro translation product. I.P., 35S-labeled cytoplasmic infected-cell protein. NC2-I.P., Cytoplasmic infected-cell protein precipitated with NC-2 antiserum. NC2-AIO, In vitro translation products of BamHI-HindIII fragment A-I0 DNA (0.152–0.182 map units) specific mRNA precipitated with NC-2 antiserum. (B) Antiserum NC-2 precipitation of a 50,000-d translation product of HindIII-Sall fragment J-D (0.182–0.195 map units) specific mRNA. I.P., Cytoplasmic infected-cell protein. NC-2 No RNA, Material precipitable with NC-2 and IgGsorb from the translation results from no added RNA. NC-2 HS-JD, Proteins precipitated using NC-2 and IgGsorb from the translation products of HindIII-Sall fragment J-D specific mRNA. The band migrating at ~65,000 d (?) was not identified.

(0.152–0.182 map units) and Xho-HindIII fragment C’-I0 (0.171–0.182 map units) DNA bound to cellulose as described in the previous section. We used this mRNA as a template for in vitro translation and used the NC-2 antiserum as a reagent to detect the 50,000-d capsid protein. Both fragments consistently gave positive results. However, the amount of radioactivity in the 50,000-d protein isolated was considerably less than that in the 82,000-d protein isolated by using Q1 antibody against alkaline exonuclease. This conclusion was based on the fact that autoradiographs required three to five times more exposure to obtain equivalent exposures of the 50,000-d protein compared to the 82,000-d one. A typical experiment using BamHI-HindIII fragment A-I0 (0.152–0.182 map units) DNA for hybrid selection is represented in Fig. 9A (track NC-2-AIO).

The band of radioactivity migrating at 82,000 d is alkaline exonuclease, which is also translated from this mixed mRNA preparation (see above). We could not determine whether this ability of NC-2 antiserum to react with this enzyme was due to specific immunological cross-reactivity. Long exposures of immunoprecipitates of 35S-labeled infected-cell protein extracts, such as shown in Fig. 9, did reveal a band of protein migrating at a rate corresponding to 82,000 d in size. However, nonimmune rabbit serum bound purified 125I-labeled alkaline exonuclease unless large amounts of carrier protein were present. Therefore, the precipitation of the enzyme seen with the in vitro translation could be nonspecific binding. Q1 did not appear to react with the 50,000-d protein synthesized in vitro as judged by very long exposures (3 months) of the autoradiographs seen in Fig. 3.

The relative specificity of the NC-2 antiserum, as well as the comigration of immunoprecipitated infected-cell 50,000-d capsid protein and the in vitro translation product (compare Fig. 9A, tracks NC2-I.P. and NC2-AIO), suggested that the mRNA for this protein is either the 3.9-kb or the 4.5-kb (βγ) mRNA mapped in Fig. 2. This identification must be regarded as tentative, however, since the amount of radioactivity isolated in the 50,000-d translation product precluded tryptic peptide comparison with the purified capsid protein. The relatively small amount of protein recovered from the in vitro translation product could be due to any combination of three factors. First, total translation product contains a relatively small amount of the 50,000-d protein, equivalent to endogenous products of translation (data not shown). This reflects the fact that neither of the two mRNAs is highly abundant. Second, the methionine content of the protein is unknown and may be low. Third, the NC-2 antiserum was made against denatured 50,000-d capsid protein and may not react efficiently with protein translated in vitro.

We confirmed our inference that either the 3.9-kb or 4.5-kb (βγ) mRNA encoded a 50,000-d polypeptide in two ways. First, we used HindIII-Sall fragment J-D (0.182–0.192 map units) DNA bound to cellulose to purify the 3.9-kb and 4.5-kb mRNAs away from the 2.3-kb mRNA for alkaline exonuclease (see Fig. 1). Such hybrid purified mRNA, when translated in vitro, yielded the 50,000-d protein immunoreactive with NC-2 antiserum (Fig. 4B). Here, the use of IgGsorb led to the recovery of other bands due to endogenous translation products, but the presence of the 50,000-d protein specific for the viral mRNA is clear. Second, we used size-fractionated total HSV poly(A) mRNA to translate this protein. This experiment (data not shown) was done as described in the preceding section, except that mRNA ranging from a size of 5.0 to 4.5 kb was translated and then reacted with NC-2 antiserum. Here other protein tracks were also seen, but the 50,000-d protein was clearly translated by mRNA of the size range between 3 and 4 kb. Larger and smaller size fractions did not translate this protein.

DISCUSSION

The moderate-resolution transcription map presented in Fig. 2 is entirely consistent with the
picture of HSV-1 gene expression and gene packaging developed in the last several years (reviewed in reference 35). The fact that the mRNAs characterized can be correlated with specific viral genes demonstrates, again, the genetic resolution available through HSV transcription mapping. We should note at the outset, however, that none of the data presented here precludes the presence of small amounts of other transcripts in this region being generated via splicing from other promoters or by use of other polyadenylation sites.

Our confidence in the identification of the 2.3-kb β mRNA as encoding alkaline exonuclease is quite high. The immune reactivity with the highly specific monoclonal antibody Q1 is, itself, strong evidence, especially when migration of the in vitro translation product is compared with that of authentic enzyme and with that seen in the infected cell (Fig. 7). The fact that Preston and Cordingly (29) also located the gene for this important enzyme in this region adds to the already strong case. It should be noted, however, that these workers estimated the mRNA for the enzyme to be in the size range of 3.6 kb, considerably greater than that seen here. Their result could be due to the procedures used to fractionate the RNA (sucrose gradients). It could be due, however, to the amphibian oocyte system they used for translation being able to initiate translation in the interior of the partially colinear 3.9- and 4.5-kb mRNAs. Based on the present data, we conclude that these mRNAs contain all the information for the alkaline exonuclease downstream of their translational reading frames. Such patterns of long untranslated regions of some large HSV-1 mRNAs have been described by us previously.

Our confidence in the conclusion that the 50,000-d capsid protein VP19C is encoded by either the 3.9- or 4.5-kb mRNA is not as high. Certainly, these mRNAs encode a protein migrating at 50,000 d, but the relatively weak reaction between the NC-2 antiserum and the in vitro translation products must indicate some caution. It is conceivable (although unlikely) that the precipitation of the translation product by this antiserum is nonspecific. The fact that the capsid protein has been mapped in this general location (20) appears to make this possibility even less likely.

The partial sequence analysis presented here, and further sequence analysis currently under way in this laboratory, are useful in defining potential promoters and translational reading frames. Our location of the 5' end of the 2.3-kb alkaline exonuclease mRNA is quite solid and places this mRNA immediately downstream of a 120-base sequence containing features of HSV-1 β promoters. We first noted the similarity be-

between another β promoter (for the 5.2-kb mRNA mapping in HindIII fragment K) and that for HSV-1 ts several years ago (12). Subsequent studies have tended to confirm the general features noted. These studies have been reviewed, but the data of Fig. 6 are the most complete comparison available at this time.

It is clear from the analysis of the potential reading frames summarized in Fig. 4 that there is no apparent shared reading frame for the 82,000-d alkaline exonuclease and the 50,000-d VP19C. This is consistent with results from other regions of the genome containing partially colinear mRNAs. We cannot be absolutely certain that translation frame 1 of Fig. 4 does indeed encode the alkaline exonuclease, nor that frame 3 (which initiates further upstream) encodes VP19C. We are certain, however, of our finding of translational stop signals in all three frames before the start signal of frame 1. Furthermore, sequence data not shown indicate that frame 1 stays open for at least several hundred bases downstream of the data shown and that frame 3 stays open for at least the same amount of bases upstream.

Given the size of 2.3 kb as the actual transcript size for alkaline exonuclease and translation frame 1 as its proper frame, we can infer that the total mRNA coding capacity is on the order of 2,100 nucleotides. Such would encode a protein of 700 amino acids, somewhat small for the measured size of alkaline exonuclease (82,000 d). However, there are 37 prolines in the first 236 positions in the predicted amino acid sequence. Such a high proline content (ca. 15%) would lead to a protein migrating more slowly than predicted from its amino acid residue weight. We have cited this as the reason for the discrepancy between the predicted amino acid residue molecular weight of HSV-1 gC and its migration rate (60,000 d versus 69,000 d; 13).

Partial sequence data (not presented here) around the 5' ends of the 3.9- and 4.5-kb mRNAs suggest that the larger of the two mRNAs encodes a short reading frame which terminates in the leader of the 3.9-kb mRNA. Furthermore, data suggest that the reading frame seen in the 3.9-kb mRNA is open for a significant stretch of nucleotides. Such data indicate that the 3.9-kb mRNA, then, actually encodes the 50,000-d protein that we have suggested is VP19C. This situation would be similar to that seen by Hall et al. (15) for two partially colinear and complementary overlapping mRNAs in the region about map unit 0.7.

The most disturbing aspect of our conclusion that the 50,000-d translation product is actually VP19C is the low abundance of the mRNA. We should state, however, that the amount of [35S]methionine-labeled VP19C from purified vi-
rions is very much less than that of VP5 (5). Also, we have found that the total amount of VP19C recoverable from infected-cell extracts is very much less than that of VP5 by using immune precipitation (Costa and Wagner, unpublished data). Therefore, the amount of VP19C in the infected cell may be low and the abundance of the mRNA encoding it may also be low.

A striking result in terms of HSV-1 mRNA expression of our present study is the finding of a smaller mRNA underlying the 3' three-quarters of the 2.3-kb mRNA and apparently sharing a translational reading frame with it. Other examples of shared translational reading frames are found in the HSV-1 thymidine kinase gene as shown by Marsden et al. (22) and, potentially, in the gC gene (13).

The sequence data suggest that any protein encoded by the 1.9-kb mRNA should contain the 570 C-terminal amino acids of alkaline exonuclease. This prediction is certainly consistent with the size for either in vitro translation product seen in Fig. 8. We can assume that the type-common epitope recognized by the Q1 antibody is dependent upon the N-terminal 130 amino acids of the alkaline exonuclease. The properties and possible biological function of any truncated form of the alkaline exonuclease which would lead to its being required at late times after infection are unknown. Indeed, it should be emphasized that such a protein has not been described as being present in normal lytic infection by HSV. Further sequence analysis, as well as further mRNA and protein fractionation procedures currently being undertaken in our various laboratories, may lead to more specific predictions regarding this putative protein. The data do suggest, however, that the partial colinearity of mRNAs under independent temporal or promoter control can be a result of related features of the proteins encoded.

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LITERATURE CITED

ERRATUM

High-Resolution Characterization of Herpes Simplex Virus Type 1 Transcripts Encoding Alkaline Exonuclease and a 50,000-Dalton Protein Tentatively Identified as a Capsid Protein


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