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Direct Demonstration That the Abundant 6-Kilobase Herpes Simplex Virus Type 1 mRNA Mapping Between 0.23 and 0.27 Map Units Encodes the Major Capsid Protein VP5

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The two partially colinear 6-kilobase (kb) and 1.5-kb mRNAs mapping between 0.23 and 0.27 map units on the herpes simplex virus type 1 genome were precisely located. The 5′ end of the 6-kb mRNA was located 28 bases downstream of the sequence ATATATT and was 10 bases to the left of the *Bam*HI site at 0.268. This position is ca. 90 bases to the left of our earlier reported sequence (R. J. Frink, K. G. Draper, and E. K. Wagner, Proc. Natl. Acad. Sci. U.S.A. **78**:6139–6143, 1981). We used a polyclonal antibody made against purified herpes simplex virus type 1 VP5 to demonstrate that the 155,000-dalton translation product of the 6-kb mRNA is this capsid protein. The antibody did not react with the 35,000-dalton translation product of the 1.5-kb mRNA. We also confirmed our identification of VP5 as the translation product of the 6-kb mRNA by comparison of tryptic peptides of the in vitro-translated protein and authentic VP5.

Recent work from this and other laboratories (reviewed in reference 22) has shown that herpes simplex virus (HSV) genes are generally encoded by unspliced transcripts under individual promoter control. A high-resolution location for a viral transcript, then, closely approximates the location of a specific gene.

We previously mapped an extremely abundant 6-kilobase (kb) HSV type 1 (HSV-1) mRNA between 0.23 and 0.27 map units on the viral genome (6). We suggested that this is the mRNA for the major HSV-1 capsid protein (VP5) because it encodes a 155,000-dalton (Da) polypeptide in translation experiments in vitro and because it maps near the location for this gene determined from intertypic recombinant studies (17, 19). A second mRNA of 1.5 kb is colinear with the 3′ portion of the 6-kb mRNA and translates a 35,000-Da polypeptide in vitro. The relationship between the two proteins is unknown. Our best available mapping data are summarized in Fig. 1A.

This communication shows that we have carefully determined the position of the 3′ and 5′ ends of these two colinear mRNAs. In the case of the 6-kb mRNA, this positioning revealed that our earlier location for the 5′ end (11) is in error and actually reflected the 5′ end of a very minor transcript of unknown function. Our data demonstrate that the 5′ end of a 3.1-kb mRNA transcribed off the opposite strand is very close to the 5′ end of the 6-kb mRNA. We used a polyclonal antibody prepared against purified VP5 (NC-1; 5) to precipitate the 155,000-Da in vitro translation product of the 6-kb mRNA. This antiserum did not react with the 35,000-Da polypeptide. Finally, we fully confirmed the identity of the 155,000-Da in vitro translation product by comparing its tryptic peptides with those previously reported for purified VP5 (5, 20).

We precisely located the 5′ end of the 6-kb mRNA by S1 nuclease analysis (4, 6). These methods were previously described in detail (1, 6, 7, 12, 14). Briefly, the appropriate cloned restriction fragment of HSV-1 DNA was cleaved with the desired enzyme and was then 5′ end labeled with T4 polynucleotide kinase (Boehringer Mannheim Biochemicals) or 3′ end labeled with the DNA polymerase I Klenow fragment (Boehringer Mannheim). The end-labeled DNA was then strand-separated and isolated by the procedures of Maxam and Gilbert (18). Generally, 5 μg-equivalents of such a DNA fragment were used. A microgram-equivalent is that amount of restriction fragment-specific DNA isolated by digestion of that amount of intact HSV-1 DNA.

The end-labeled DNA was annealed with 10 μg of HSV polyadenylic acid [poly(A)]-containing mRNA. Previous experiments demonstrated that this ratio of DNA to RNA provides great DNA excess. RNA was obtained from HeLa cells grown as described previously (2) in minimal essential medium and infected with a 10-PFU multiplicity of infection of the KOS strain of HSV-1 per cell. Procedures for the isolation of poly(A)-containing mRNA from polyribosomes are standard for this laboratory and were recently described (12). Hybridization was for 8 to 12 h at 65°C in 0.4 M Na^+–0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 8.0)–0.005 M EDTA. After hybridization, the mixture was digested with 5,000 U of S1 nuclease (Boehringer Mannheim), and the size of resistant DNA fragments was measured by electrophoresis on denaturing acrylamide gels against appropriate size standards.

A typical experiment is shown in Fig. 1B. Here, HSV mRNA was hybridized against both strands of a DNA fragment spanning the *Dde*I site at 0.267 map units and the *Sal*I site at 0.271 map units. DNA 5′ end labeled at the *Dde*I site yielded three S1-protected fragments when hybridized with HSV mRNA and digested. The major one was 180 bases long. A minor one was 210 bases long, and a more minor one still was 270 bases long (not visible in Fig. 1B). The major fragment represented the major 5′ end of the 6-kb mRNA, and the very minor fragment 270 bases in size corresponded to the size originally suggested to be the 5′ end of the 6-kb mRNA (11). We located the major 5′ end on the nucleotide sequence of the DNA in this region by methods described previously (11, 12, 14). The best location for the

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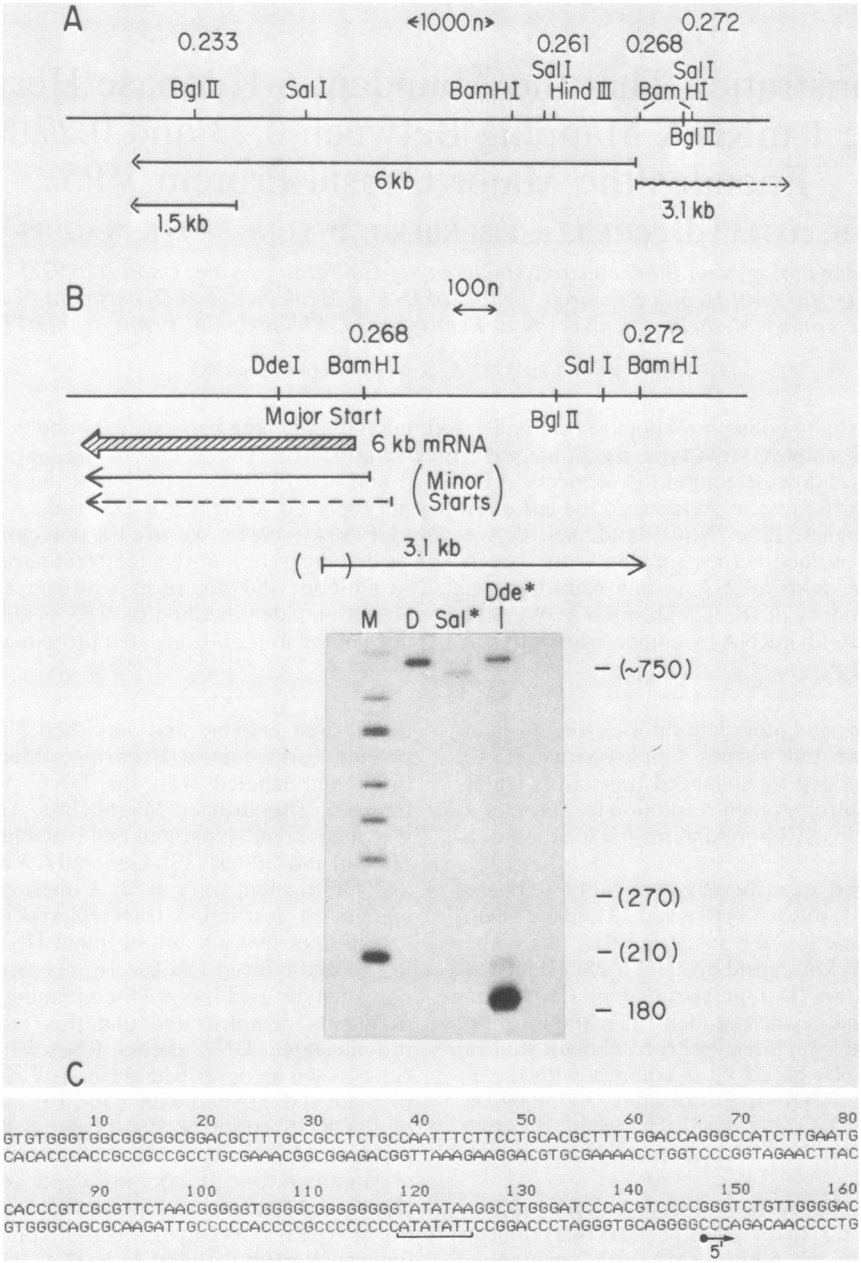

FIG. 1. Localization of the 5′ end of the 6- and 3.1-kb mRNA. (A) Restriction endonuclease sites from 0.233 to 0.272 map units on the prototypic (P) arrangement of HSV-1 (KOS) genome. The diagram illustrates the positions of the 6- and 1.5-kb mRNAs on the restriction map assigned by S1 analysis (see text, Fig. 2, and ref. 6). The arrow shows the position of the 3′ end of the mRNA, and the vertical line shows where transcription begins. Dotted lines at the 3′ end of the 3.1-kb mRNA indicate that the message extends much farther to the right than shown. (B) Major and minor transcription initiation sites for the 6-kb mRNA and approximate location of the 5′ end of the 3.1-kb mRNA. Shown is an S1 digestion with strand-separated DNA 5′ end labeled at both the *Dde*I (0.267 map units) and *Sal*I (0.271 map units) restriction sites obtained from *Sal*I fragment B′ DNA (0.260 to 0.271 map units). Each DNA strand was hybridized to late HSV-1 poly(A)$^+$ mRNA. The S1-digested products were then denatured and size fractionated on a 5% acrylamide–8 M urea gel. The 5′ end-labeled *Hin*fI-*Eco*RI pBR322 (track M) and undigested *Dde*I-*Sal*I DNA (track D) were used as size markers. Tracks marked Sal* and Dde* indicate S1 digest of the hybrids with indicated DNA strands. (C) Nucleotide sequence 5′ to the major start of the 6-kb mRNA. The mRNA start is indicated (arrow), as is the TATA box at −22. Sequence is from ref. 11.

most abundant mRNA was at a position ca. 28 bases downstream of the sequence ATATATT, a good TATA box. The sequence immediately upstream of this 5′ end is shown in Fig. 1C. It shares several features with the nucleotide sequence directly upstream of the 5′ end of gC, another late HSV-1 mRNA (12). These include a TATA box, CATC box (positions 70 to 74), and several relatively AT-rich regions.

Northern blot analysis had previously indicated that a 3.1-kb mRNA mapped in the location under study (6). Hybridization of the DNA strand 5′ -labeled at the *Sal*I site at 0.271 map units yielded a DNA fragment ca. 750 bases long resistant to S1 digestion. Other S1 digestions with DNA 5′ end labeled farther upstream (data not shown) indicated that this protection was due to the 3.1-kb mRNA. Its abundance

on Northern blots was significantly less than that for the 6-kb mRNA, but the relative intensity of the bands of Fig. 1B also reflects the fact that, in this experiment, less radioactivity was recovered in the DNA strand 5′ end labeled at the *Sal*I site.

These mapping data suggest that the 5′ ends of the 6-kb and 3.1-kb mRNAs share complementary overlap. Such has been seen before in other regions of the genome (14). The general size of HSV-1 leader sequences upstream of translational frames is on the order of 150 bases (22). Therefore, no complementary overlap would be needed for the translational frames of the 6-kb mRNA and the 3.1-kb mRNA. If the minor mRNA starts upstream of the 6-kb major start have a specific function, however, then complementarity to the 3.1-kb mRNA must be considered.

We used a similar approach to locate the 5′ end of the 1.5-kb mRNA and demonstrate that its 3′ end is colinear with that of the 6-kb mRNA. HSV-1 poly(A)-containing mRNA was hybridized with the strand-separated *Bgl*II-*Sal*I fragment N-U (0.233 to 0.241 map units) 5′ end labeled at the *Bgl*II site. Three S1-resistant products were obtained (Fig. 2, track ii). The full-length product was the result of S1 protection from the 6-kb mRNA, which is transcribed through this restriction fragment. Another major band at 520 bases represented the position of the major 5′ end of the 1.5-kb mRNA (Fig. 2). The other minor band at 600 bases was not consistently seen. We suggest it was the result of incomplete S1 digestion. We cannot, however, completely exclude its representing a minor start site for the 1.5-kb mRNA.

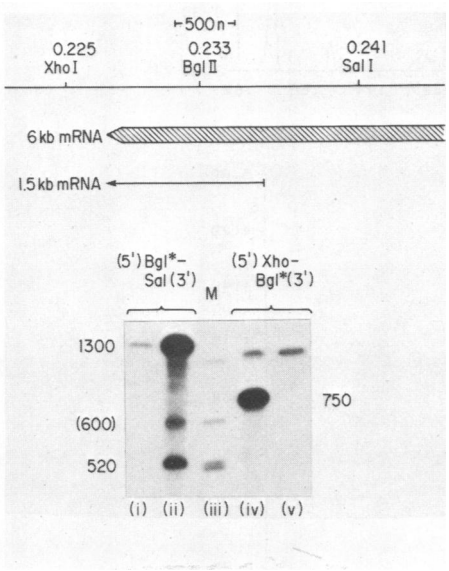

FIG. 2. Location of the 5′ end of the 1.5-kb mRNA and the 3′ ends of the 6- and 1.5-kb species. The diagram illustrates positioning of the colinear 3′ ends of the 6- and 1.5-kb mRNA and the location of the 5′ end of the 1.5-kb mRNA on the HSV-1 restriction map. These data were obtained from S1 nuclease analysis presented below the diagram. Details of the S1 nuclease analysis are described in the legend of Fig. 1 and in the text. DNA size markers used on the denaturing acrylamide gel are as follows: track i, 5′-end-labeled *Bgl*II-*Sal*I fragment N-U (0.233 to 0.241 map units); track iii, 5′-end-labeled *Hin*fI-*Eco*RI pBR322 (sizes from the bottom of the gel, 506, 517, 631, and 1,000 base pairs); track v, 3′-end-labeled *Xho*I-*Bgl*II fragment L-P (0.225 to 0.233 map units). The other two tracks show the fractionation of S1 nuclease-resistant hybrids formed between poly(A)-containing HSV-1 RNA and strand-separated, end-labeled DNA restriction fragments: track ii, S1-protected fragments with *Bgl*II-*Sal*I fragment N-U (0.233 to 0.241 map units) 5′ end labeled at the *Bgl*II site; track iv, S1-protected hybrids with *Xho*I-*Bgl*II fragment L-P (0.225 to 0.233 map units) 3′ end labeled at the *Bgl*II site.

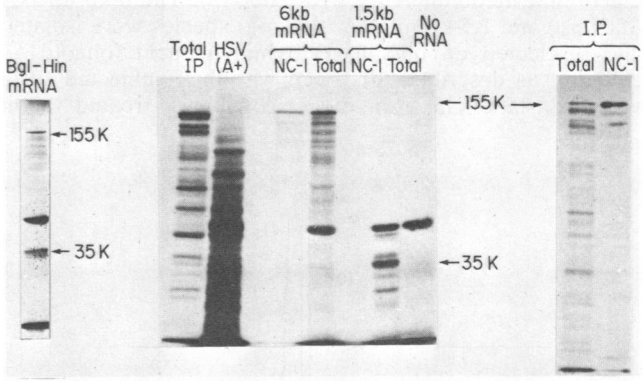

FIG. 3. In vitro translation of the 6- and 1.5-kb mRNAs specific to *Bgl*II-*Hin*dIII fragment N-J DNA (0.233 to 0.261 map units) followed by antibody precipitation with NC-1. Hybrid-selected mRNA was in vitro translated in a micrococcal nuclease-treated rabbit reticulocyte lysate system with [^{35}S]methionine used as a label, and the protein products were fractionated on a sodium dodecyl sulfate–9% polyacrylamide gel (*Bgl-Hin* mRNA track). The 6- and 1.5-kb mRNA species homologous to *Bgl*II-*Hin*dIII fragment N-J DNA (0.233 to 0.261 map units) were size fractionated as described in the text and were translated in vitro as described above. Half of each translation reaction was reacted with NC-1 antibody (NC-1 track), and this was fractionated next to the unreacted half (total track) on a sodium dodecyl sulfate-polyacrylamide gel. Several size standards were included. These were total cytoplasmic [^{35}S]methionine-labeled protein HSV-1 (total IP track), in vitro-translated HSV poly(A)-containing mRNA [HSV(A$^+$) track], and a no-RNA in vitro translation control (no RNA track). The right-hand tracks represent [^{35}S]methionine-labeled cytoplasmic proteins reacted with NC-1 (NC-1 track), with unreacted infected cell protein (total track) used as a size standard.

Only one S1-resistant fragment of 750 bases in size was seen when single-stranded DNA 3′ end labeled at the *Bgl*II site of the *Xho*I-*Bgl*II fragment L-P (0.225 to 0.241 map units) was used as a hybridization probe (Fig. 2, track iv). These data indicate the colinear 3′ ends of the 6- and 1.5-kb mRNAs lie 750 bases to the left of the *Bgl*II site at 0.233 map units. These data, summarized in Fig. 1A and Fig. 2, indicate that the 1.5-kb mRNA is encoded by a 1,270-base segment of the viral genome. Such data are consistent with our measurements of ca. 200 bases for the length of the poly(A) tails of the viral mRNAs (21).

No evidence for a noncontiguous leader sequence for the 1.5-kb mRNA was seen in Northern blot analysis of HSV-1 mRNA and high-specific-activity cDNA probes spanning regions between the located 5′ end and the 5′ end of the 6-kb mRNA. Such experiments demonstrated that the noncontiguous leader for very minor spliced mRNAs was colinear with the HSV-1 gC mRNA (12) and are very strong evidence in favor of the 1.5-kb mRNAs having an independent 5′ end, as has been observed for other partially colinear mRNAs (22).

We isolated both the 6-kb and the 1.5-kb mRNAs from total HSV mRNA by hybrid selection with *Bgl*II-*Hin*dIII fragment N-J DNA (0.233 to 0.261 map units) bound to cellulose as a reagent. These methods have been described (6). We isolated ca. 0.25 μg of region-specific mRNA from 100 μg of ^{32}P-labeled HSV mRNA (ca. 200,000 cpm/μg) by hybridizing it with a 150 μg-equivalent of the specific DNA bound to cellulose. The region-specific mRNA was fractionated by electrophoresis on denaturing agarose gels contain-

ing 10 mM methylmercury hydroxide (3). The 6-kb mRNA (0.05 μg) and 1.5-kb mRNA (0.01 μg) species were isolated and reselected on oligodeoxythymidylic acid [oligo(dT)]-cellulose as described (6). Each was then subjected to in vitro translation in a micrococcal nuclease-treated rabbit reticulocyte lysate system (New England Nuclear Corp.) with [^{35}S]methionine as a label (>800 Ci/mmol). The results are shown in Fig. 3.

Translation of region-specific mRNA (track *Bgl-Hin* mRNA) shows polypeptide products of 155,000 and 35,000

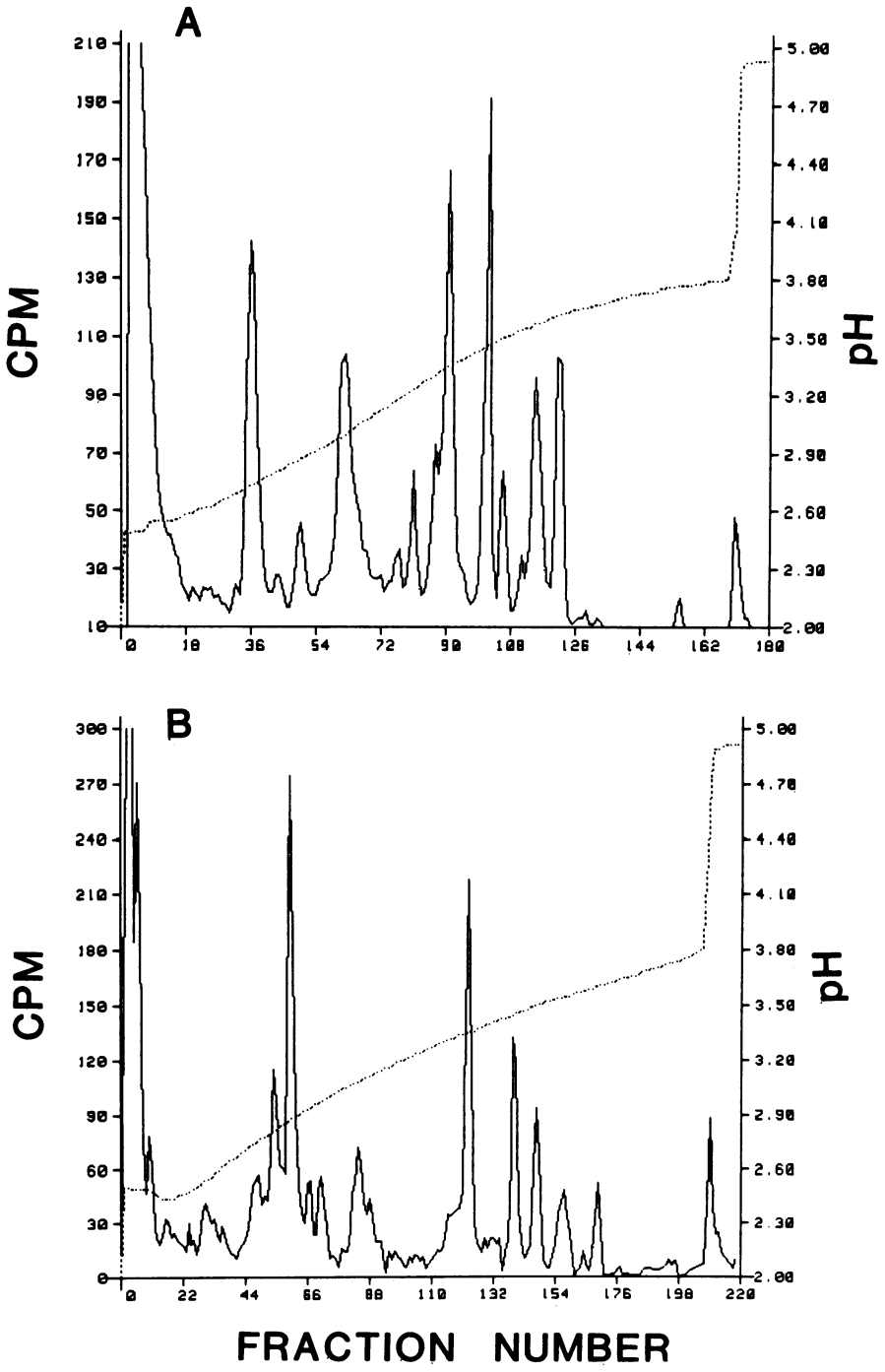

FIG. 4. Tryptic peptide analysis of the major capsid protein (NC-1, 155,000 Da) and the 155,000-Da polypeptide. (A) Nucleocapsids were isolated and purified from [^{35}S]methionine-labeled HSV-1 strain HF-infected cells by methods described previously (5). Capsids were electrophoresed on 10% acrylamide gels cross-linked with *N,N'*-diallyltartardiamide, and the band containing the 155,000-Da protein (NC-1) was dissolved in 2% periodic acid and prepared for trypsinization (see reference 12 for procedures). The tryptic peptides were separated by Chromabeads P cation-exchange chromatography (5, 12, 20). (B) The 155,000-Da polypeptide labeled with [^{35}S]methionine was immunoprecipitated with NC-1 antiserum from an in vitro translation mix by using hybrid-selected mRNA. The precipitate was trypsinized and chromatographed on Chromabeads P by methods described previously (5, 12, 20).

Da, as reported previously (6). The translation products of size-fractionated 6-kb mRNA were incubated with NC-1 antiserum, and the immune complex was bound to protein A-Sepharose 6 MB (Pharmacia Fine Chemicals) as described (12). The 155,000-Da translation product was recovered in good yield (Fig. 2, tract 6-kb mRNA NC-1). The same antiserum did not bind the translation product of the 1.5-kb mRNA (Fig. 3, tract 1.5-kb mRNA NC-1), although unreacted translation product clearly shows the presence of the 35,000-Da polypeptide.

These results suggest that the 155,000-Da polypeptide encoded by the 6-kb mRNA shares no obvious antigenic properties with the 35,000-Da polypeptide encoded by the 1.5-kb mRNA colinear with its 3′ end. We did several control experiments to demonstrate that the NC-1 antiserum binds to the 155,000-Da protein translated from total HSV mRNA and that it exactly comigrated with VP5 immune precipitated from total infected cell extracts. An example is shown in Fig. 3 (track I.P. NC-1). Here, infected cells were labeled from 5 to 7 h postinfection with [^{35}S]methionine (50 μCi/ml) in methionine-free minimal essential medium (4 ml per 6×10^6 cells). A cytoplasmic extract was isolated by previously described procedures (9). This extract is termed infected cell protein. A radioactive band seen in the total protein band migrating with a rate corresponding to 155,000 Da exactly comigrates with the major protein band binding to NC-1. The serum did not react with in vitro translation products encoded by mRNA mapping in another region of the genome, nor did it react with uninfected cell protein (data not shown).

We compared the tryptic peptides of the in vitro translation product and authentic VP5 to confirm our identification further. Viral mRNA homologous to the region encoding the 6-kb mRNA was isolated by hybridizing 200 μg of unlabeled HSV-1 mRNA to *Bgl*II-*Hin*dIII fragment N-J DNA (0.233 to 0.261 map units) bound to cellulose as described above. This hybrid-selected mRNA was translated in vitro and immunoprecipitated with NC-1 antiserum. A single polypeptide was recovered (Fig. 3). The remainder of the immunoprecipitated translation product was trypsinized and fractionated by cation-exchange chromatography (10). The methionine tryptic fingerprint is shown in Fig. 4. This profile compares very favorably with those obtained previously for VP5 isolated from purified HSV-1 nucleocapsids (5, 20). An example is shown in Fig. 4A. The profile of the in vitro product contained the same number of methionine-labeled peaks as the profile of authentic VP5, and the general shape of each peak was quite similar. Further, although the pH gradient for Fig. 4A was somewhat steeper than that for Fig. 4B, the pH at which each peak elutes in both profiles is the same. Finally, it should be noted that, as seen previously with our experiments with gC (12), the absolute peak height of various peptides appears to be somewhat variable. Such variability is seen in individual experiments with in vivo-labeled protein and may reflect some differential recovery of specific tryptic peptides during the workup.

The data presented here remove any reasonable doubt about the location of the transcript encoding HSV-1 VP5. The fact that there is no immunological cross-reactivity between this protein and the 35,000-Da polypeptide encoded by the 1.5-kb mRNA colinear with the 3′ end of the 6-kb mRNA encoding VP5 suggests that the actual translational reading frame for this protein lies in the 4,600 bases between 0.236 and 0.268 map units on the viral genome. This suggests a protein on the order of 1,500 amino acids, a reasonable size for the nominal molecular weight of the protein.

The partial colinearity of HSV mRNA at the 3′ ends is relatively common in HSV. To date, all our data demonstrate that such mRNAs are independent transcription units. Therefore, any coordinate control of expression can be expected to be at the level of transcription and not of processing. It is not known whether such 3′ overlapping transcripts generally encode related functions. In the case of HSV-1 mRNAs examined here and with the gC mRNA family (12), the 3′ overlapping mRNA encodes proteins which share no antigenic determinants as determined by using polyclonal antisera. In the case of the 5.2- and 1.5-kb mRNAs mapping between 0.56 and 0.6 map units in both HSV-1 (1, 7) and HSV-2 (16), it appears that there is a shared epitope between the 140,000-Da (ICP6) and 38,000-Da polypeptides encoded by these mRNAs (13, 15). These proteins appear to be portions of the ribonucleotide reductase complex in HSV-infected cells (8). In another series of experiments (6a), it has been found that the translational reading frame for the early (β) HSV-1 alkaline exonuclease may be shared by a late (βγ) polypeptide encoded by a smaller mRNA partially colinear with the mRNA encoding the early enzyme. Thus, the close proximity of mRNAs suggests that related functions can be encoded even when each is expressed independently. Such data also indicate that we cannot exclude the possibility that other partially colinear mRNAs could encode proteins of related function even if they share no obvious antigenicity.

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