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Detailed Analysis of the Portion of the Herpes Simplex Virus Type 1 Genome Encoding Glycoprotein C

R. J. FRINK,[1] R. EISENBERG,[2] G. COHEN,[3] AND E. K. WAGNER[1]*

Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717[1] and Department of Pathobiology, School of Veterinary Medicine,[2] and Department of Microbiology and Center for Oral Health Research, Dental Medicine,[3] University of Pennsylvania, Philadelphia, Pennsylvania 19104

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We previously showed that the right third of *Hin*dIII fragment L (0.59 to 0.65) of herpes simplex virus type 1 (HSV-1) encodes a family of mRNAs some members of which appear to be related by splicing. In the experiments described in this communication, we determined the nucleotide sequence of the DNA encoding this mRNA family and precisely located the mRNAs associated with this DNA sequence. The major mRNA species is unspliced and encoded by a 2,520-nucleotide region. Just upstream of the 5′ end are TATA and CAT box sequences characteristic of HSV-1 promoters. The 3′ end maps near a region containing a nominal polyadenylation signal. Three minor species (2,400, 2,200, and 1,900 bases, respectively) appear to share a very short leader sequence with the 5′ end of the major mRNA and are then encoded by uninterrupted DNA sequences beginning about 100, 400, and 625 bases downstream of the 5′ end of the major unspliced mRNA. These positions map at or very near positions which agree reasonably well with consensus splice acceptor sequences. The fourth mRNA is encoded by a contiguous 730-nucleotide sequence at the 3′ end of the major unspliced mRNA and has its 5′ end just downstream of recognizable TATA and CAT box sequences. We suggest that this mRNA is controlled by its own promoter. The nucleotide sequence data, in combination with the mRNA localization, demonstrate four potential polypeptides encoded by the region. The largest is 1,569 bases long and defines a 523-amino acid protein with sequence features characteristic of a glycoprotein. This was confirmed to be HSV-1 glycoprotein C by immune precipitation of the in vitro translation product of the major unspliced mRNA, performed with a polyspecific antibody to HSV-1 envelope glycoproteins (anti-env-1 serum), and by comparison of tryptic peptides of this translation product with those of authentic HSV-1 glycoprotein C. Polypeptides encoded by some of the minor species also were tentatively identified.

In a number of previous papers, we reported detailed mapping and characterization of the major mRNAs encoded by about 20% of the herpes simplex virus type 1 (HSV-1) genome (reviewed by E. Wagner, *in* G. Klein, ed., *Advances in Viral Oncology*, vol. 3, in press). Although many HSV-1 mRNAs overlap others and thus form clusters or families, we find detectable splicing to be rare, and the great majority of abundant HSV-1 mRNAs so far characterized appear to be controlled by a promoter region in the DNA, closely juxtaposed to the location of 5′ ends of the mRNAs. The sites of polyadenylation for HSV-1 mRNAs appear similar to those characterized for other eucaryotic mRNAs; these HSV polyadenylation sites often define the 3′ termini of several partially colinear HSV mRNAs.

Despite the relative rarity of detectable splicing during HSV-1 mRNA biogenesis, spliced HSV-1 mRNAs do exist. The spliced family investigated in this report is a prime example. Also, the α (immediate-early) mRNA encoding ICP-25 has a splice in its 5′ region which appears to function in the removal of the transcript of the reiterated terminal portion of the IR_S region where it joins the U_L region (41, 42). We also noted several HSV-1 mRNAs with 5′ ends mapping differently when S1 nuclease and exonuclease VII digestion of DNA-RNA hybrids are compared (15, 18). Splicing also appears to occur in the mRNA expression of other herpesviruses since potential splices have been tentatively identified in certain Epstein-Barr virus mRNAs (22).

To date, the spliced HSV-1 mRNA family mapping in *Hin*dIII fragment L is the only instance where differential splicing can be in-

ferred to play a role in HSV-1 gene expression. It is, of course, a notable curiosity that this mechanism so prevalent in the gene expression of other nuclear replicating DNA viruses appears to be rare with HSV-1. For this reason, we carried out a detailed analysis of these HSV-1 mRNAs related by splicing. By correlating in vitro translation products with the coding capacity of various mRNAs and the predicted amino acid sequences of proteins potentially encoded by these mRNAs, some statement could be made regarding the actual use of splicing in expression of specific HSV-1 gene products.

In addition to this rather general question of the role of splicing in HSV-1 gene expression, the detailed analysis described here was designed to answer specific questions regarding the expression of a known HSV-1 polypeptide, glycoprotein C (gC). Thus, our analysis can be related to the expression of a protein of known biochemical properties. This polypeptide has been located in the general region of interest by intertypic recombinant studies (19, 38). The apparent size of the major translation product (69,000 daltons; 15) of the mRNA encoded by this DNA correlates reasonably well with the size of the unglycosylated precursor for this glycoprotein (9, 34). Further, Lee et al. (24a) have shown that DNA from this precise region can hybrid-select gC mRNA. We used the anti-env-1 serum described earlier (9) to immunoprecipitate the in vitro translation product of the unspliced and most abundant member of the mRNA family, confirming its identification as the mRNA for HSV-1 gC. This antibody was of value in partially defining the translation reading frames of other viral polypeptides which are apparently encoded by this region of the HSV-1 genome.

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. Rabbit skin cells (a gift of J. Stevens of UCLA) were grown in the same medium containing 5% serum. Plaque-purified virus of the KOS strain of HSV-1 was used for all infections.

Enzymes. All restriction enzymes were obtained from Bethesda Research Laboratories, Inc.; digestion was carried out in buffers recommended by that supplier. Phage T4 polynucleotide kinase was used for 5′ phosphate exchange as described by Maxam and Gilbert (26).

Isolation, labeling, and size fractionation of polyribosomal RNA. Monolayer cultures of HeLa cells (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. Polyribosomes were isolated from the cytoplasm of HSV-1-infected cells by the magnesium precipitation method of Palmiter (32). mRNA containing polyadenylic acid [poly(A)] was isolated from total ribosomal RNA by oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc.) chromatography. Details of this procedure have been presented elsewhere (1–4, 11, 15, 18, 20, 21). RNA was size fractionated by electrophoresis on 1.4% agarose gels containing 10 mM methyl mercury hydroxide (5) as previously described.

Recombinant DNA. All recombinant DNA clones described in this paper were derived from either *Hin*dIII fragment L (0.592 to 0.647) or *Bam*HI fragment I (0.602 to 0.643) cloned in plasmid pBR322 (2, 15). We used two subclones: *Sal*I-*Eco*RI fragment T-A (0.621 to 0.633) and *Eco*RI-*Bam*HI fragment I-I (0.633 to 0.643). Procedures for cloning HSV-1 DNA fragments in the pBR322 vector were described previously (2, 6, 11). DNA fragments cloned were named as described previously and located by their map coordinates on the P arrangement of the HSV-1 genome (15).

In situ RNA blots. As described previously, 7-μg samples of infected-cell polyadenylated mRNA were fractionated on methyl mercury gels and dried onto Whatman 3MM paper with vacuum (18). The agarose film was floated off the paper in water and was 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 (12) at 50°C for 36 h. Blots were rinsed at 50°C twice in 50% formamide–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS), then once in 0.1× SSC–0.1% SDS. They were then autoradiographed.

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

Isolation of restriction fragment-specific mRNA. Restriction fragment-specific mRNA was isolated from poly(A) polyribosomal RNA by preparative hybridization to the appropriate DNA, which had been covalently coupled to cellulose. Details of these procedures were as described previously (1–4, 11, 20, 21, 31).

Nuclease mapping of HSV-1 RNA. S1 nuclease and exonuclease VII analysis of RNA was carried out essentially as described by Berk and Sharp (8) and by others (2, 11, 15). Appropriate HSV-1 DNA clones (2 μg) were cleaved at the desired sites with the appropriate restriction enzymes. The DNA was then 5′ labeled with ^{32}P by using polynucleotide kinase (26) to a specific activity of 4,000 to 10,000 cpm/μg of DNA. The DNA fragment then was denatured and strand separated on 5% acrylamide gels as described by Maxam and Gilbert (26). In one set of experiments, DNA was uniformly labeled with $^{32}P_i$ (30 μCi/ml) in low-phosphate MOPS (morpholinepropanesulfonic acid) medium (30, 40).

The separated DNA strand (from 2 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 volume of 30 μl and then was digested with S1 nuclease or exonuclease VII as described. Material was fractionated on a denaturing 5% acrylamide gel with 5′ end-labeled *Hin*fI-digested plasmid pBR322 DNA fragments or with nucleotide sequence gels as size standards. All proce-

dures were based on those of Maxam and Gilbert (26)

Nucleotide sequencing. As described previously (13, 16), nucleotide sequence analysis was carried out by the procedures of Maxam and Gilbert (26).

In vitro translation. Size-fractionated viral mRNA was translated in vitro by using a micrococcal nuclease-treated rabbit reticulocyte system (New England Nuclear Corp.) with [^{35}S]methionine (675 Ci/mmol) as the radioactive amino acid. Details of the procedure and of fractionation of polypeptides in SDS-acrylamide gels by the method of Laemmli (24) have been described previously (10, 13, 15). Gels were dried with vacuum, and radioactive bands were localized by autoradiography with Kodak NS-2T film.

Immune precipitation of in vitro translation products was performed as described by J. T. Matthews, R. J. Eisenberg, and G. H. Cohen (submitted for publication). One-half of the RNAse-treated translation product (14 μl) was diluted with an equal volume of 2× lysis buffer and incubated with 4 μl of anti-env-1 serum, a polyvalent antibody to HSV-1 envelope protein (9, 10). Lysis buffer is 20 mM Tris-hydrochloride (pH 7.4), 50 mM NaCl, 10 mM methionine, 0.5% Nonidet P-40, 0.5% sodium desoxycholate, and 0.1% SDS. After 1 h on ice, 75 μl of a 10% suspension of pro-A Sepharose beads (Pharmacia Fine Chemicals) 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 a further 30 min on ice with frequent mixing. The pro-A Sepharose beads with the immune complex adsorbed 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 followed by recentrifugation. After five washes, the Sepharose pellet was incubated with 20 μl of a buffer containing 0.1 M Tris-hydrochloride (pH 7.0), 3% SDS, 10% β-mercaptoethanol, and 20% (vol/vol) glycerol. The suspension was heated to 95°C for 2 min, and the Sepharose was pelleted by centrifugation. The supernatant containing released immunoglobulin and any translation product that it had adsorbed was then loaded onto SDS-acrylamide gels for size fractionation.

Preparation of samples for tryptic peptide analysis. For isolation of the glycoprotein gC, KB cells infected with HSV-1 (strain HF) were pulse-labeled with [*methyl*-^{3}H]methionine (specific activity, 100 Ci/mmol) as previously described (9). A cytoplasmic extract was prepared and immunoprecipitated with anti-env-1 serum (9). The glycoproteins were separated by SDS-polyacrylamide gel electrophoresis in slabs of 10% acrylamide cross-linked with 0.4% *N,N'*-diallyltartardiamide (9, 39). The gel was stained with Coomassie brilliant blue, dried on filter paper, and exposed to LKB Ultrafilm to locate tritium-labeled bands. The band corresponding to gC (110,000 daltons) was excised, dissolved in 2% periodic acid by the method of Gibson (17), oxidized, trypsinized (9), and chromatographed on a column of Chromabeads P resin (Technicon Corp., Inc.; 9, 14).

For tryptic peptide analysis of the 69,000-dalton protein, hybrid-selected mRNA was translated in vitro in the presence of [^{35}S]methionine. The translation mix was immunoprecipitated with anti-env-1 serum, and the complex was collected with pro-A Sepharose as described above. The antigen-antibody complex was dissociated in SDS disrupting buffer and was centrifuged (13,000 × *g*, 3 min) to remove the Sepharose. Bovine serum albumin was added as a protein carrier (1 mg/0.5 ml), and the protein was precipitated with 25% trichloroacetic acid, oxidized, trypsinized, and chromatographed on Chromabeads P as described previously (14).

RESULTS

The nucleotide sequence of HSV-1 DNA encoding the spliced mRNA family. We previously showed that the DNA between ca. 0.63 and 0.65 in *Hin*dIII fragment L (0.59 to 0.65) encodes a family of mRNAs related by splicing (15). A high-resolution restriction endonuclease cleavage map of this region is shown in Fig. 1A. Sites are shown for the enzymes *Eco*RI, *Sal*I, *Bam*HI, *Pvu*II, *Xba*I, *Sma*I, *Taq*I, *Sst*II, and *Hin*fI. The *Hin*dIII site at 0.647 is not shown, but is ca. 60 bases to the right of the rightmost *Sal*I site. It can be seen that these sites are arranged so as to provide convenient sites for 5', and occasionally 3', end labeling of DNA fragments for Maxam and Gilbert chemical sequencing. Our general method was to use strand-separated DNA fragments, and our DNA sequencing overlaps are shown in Fig. 1A. We numbered the sequence by locating the 5' end of the unspliced and most abundant mRNA at position +1. Our experiments fixing the location of this mRNA with respect to specific restriction sites are described below.

The nucleotide sequence of the noncoding strand of the DNA in the region studied is shown in Fig. 2. This strand has the same sense as the mRNAs encoded by the region. Note the sequence TATAAATT between nucleotides −27 and −19 and the sequence CATTA from −96 to −92. Such putative TATA and CAT boxes are generally found at these positions upstream of the 5' end of unspliced HSV-1 mRNAs (7, 16, 28) and can tentatively define HSV-1 promoters.

The location of polypeptide chain initiation (ATG) and chain termination (TAG, TGA, and TAA) codons are indicated, as are the translation reading frames they define. These data, shown schematically in Fig. 1C, are discussed below in relation to the in vitro translation products of fractionated mRNA species encoded by this DNA.

The coterminal 3' end of the mRNAs was located very near nucleotide 2520, which is in the sequence ATAAAAA. A similar sequence ATAATAAA was found to be very near the coterminal 3' ends of a series of colinear mRNAs mapping between 0.554 and 0.600 (13)

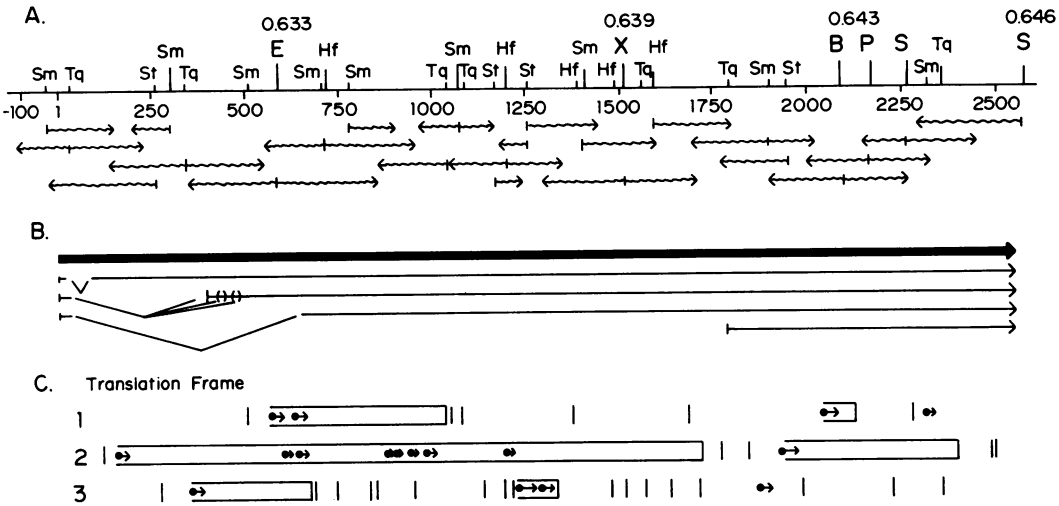

FIG. 1. High-resolution restriction endonuclease cleavage map and sequence strategy for the HSV-1 DNA region encoding the major late 2.7-kilobase (kb) mRNA family. (A) Individual sequence runs (wavey lines with arrowheads showing direction and extent) and restriction sites utilized and listed in the text. Numbering system takes the 5' terminal base of the mRNAs as +1. (B) Overlapping mRNA species described in this report, including major unspliced 2,520-base species (dark bar) and introns for the 2,400-, 2,200-, and 1,900-base spliced species (gaps). The 3' coterminal 730-base mRNA also is shown. (C) Available open translation reading frames, including positions of translation initiation (●→) and termination (|) codons.

and is similar to the putative eucaryotic polyadenylation signal AATAAAA (35). This region between nucleotide ~2,500 and the *SalI* site at position 2575 (not shown) contains the 3' end of mRNAs from both DNA strands (15), and its stretches of AT-rich sequences is reminiscent of a similar structure around position 0.600 noted by us previously (13).

The relative abundance of members of the mRNA family. We used in situ Northern blots to measure the relative abundance of various members of the mRNA family. Polyribosomal polyadenylated mRNA (5 μg) from cells 6 or 11 h postinfection (p.i.) was size fractionated by electrophoresis in 1.4% agarose gels containing 10 mM methyl mercury hydroxide. These gels were dried, and the agarose film was hybridized with [^{32}P]dCTP-labeled DNA from *Eco*RI-*Bam*HI fragment I-I (0.633 to 0.643), which had been cloned in plasmid pBR322 (Fig. 3A). The major mRNA species is indicated as having a size of 2,520 bases, based on our S1 data (see below). It migrates with a rate corresponding to a size of 2,700 bases. This size is expected for an mRNA with a DNA-encoded size of 2,500 bases and a 200-nucleotide poly(A) tail (Wagner, in press). It is clear that this mRNA species is the most abundant at both 6 and 11 h p.i.

Two smaller mRNA species migrate with a rate corresponding to a size of about 2,500 bases, and another with a rate of ca. 2,000 bases is also seen. A very minor band migrating with a rate corresponding to a size of ca. 900 to 1,000 bases can be detected with some difficulty. These species are indicated by the lengths of their encoded sequences (see below) as 2,200, 1,900, and 730 bases, respectively. The relative intensity of the smallest mRNA species is probably an underrepresentation of the abundance of this mRNA because the probe used is homologous to only about 40% of the length of this mRNA.

In a further experiment (data not shown), we found an identical pattern by using polyadenylated mRNA from rabbit skin cells infected with HSV-1. This indicated that the mRNA distribution is generally not dependent on the type of cell infected.

We used a [^{32}P]dCTP-labeled probe covering the region between nucleotides −30 and +256 to demonstrate that the smaller mRNAs of 2,200 and 1,900 bases can be detected with DNA from a region of the genome nominally upstream of their predicted 5' ends (Fig. 3B). Although the 2,200-base species is not clearly resolved in the photo, it can be seen in the original autoradiograph, and the 1,900-base species is readily apparent. The 730-base species could not be detected, even after very long exposure times.

Two faint bands of mRNA migrating with rates corresponding to sizes between 4,000 and 5,000 bases were consistently seen with both probes. The small amounts of these RNA species precluded their further characterization.

Precise location of the mRNAs. We previously reported the gross localization of the mRNAs of

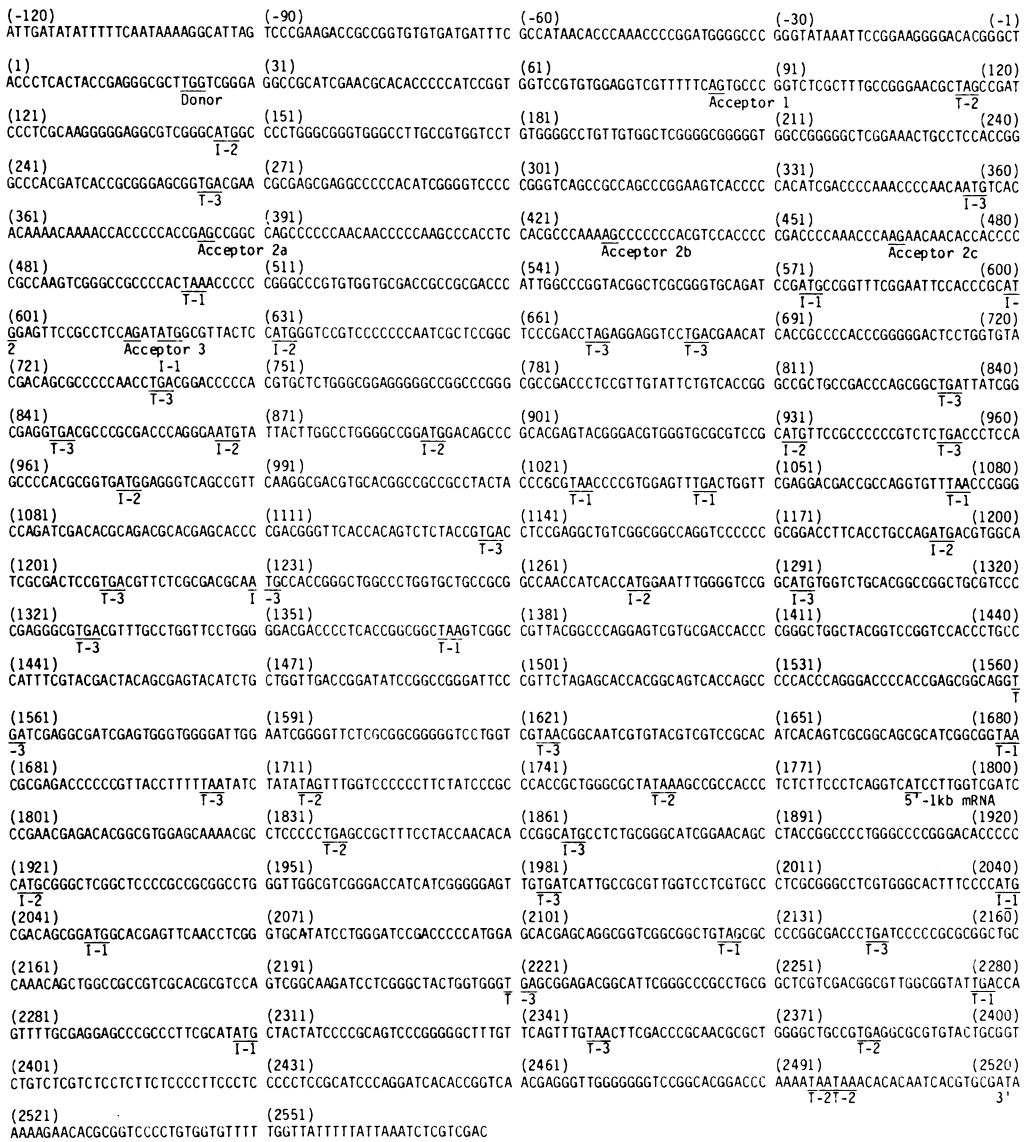

FIG. 2. DNA sequence of the noncoding (message sense) strand of the HSV-1 region encoding the colinear mRNAs under study. The 5′ terminal base of the 2,520-base mRNA is taken as +1. Characterized splice donor and acceptor sites are indicated. Initiation (I) and termination (T) codons are marked for the three potential translation reading frames (see Fig. 1C). Additionally, the 5′ end for the 3′ coterminal 730-base mRNA and the 3′ coterminal end region for all mRNA species are shown.

interest (15). The unspliced mRNA species was found to have its 5′ end about 600 bases to the left of the *Eco*RI site at 0.633 (Fig. 2, nucleotide number 588). The members 2,200 and 1,900 nucleotides long were found to have the 5′ ends of their contiguously encoded regions about 1,400 to 1,500 bases to the left of the *Bam*HI site at 0.643 (nucleotide number 2084). Finally, the 730-nucleotide mRNA was located with the 5′ end of its contiguously encoded region about 500 bases to the left of the *Sal*I site at nucleotide number 2255.

In another experiment (data not shown), we found that DNA 5′ end labeled at the *Hin*fI site at nucleotide 709 and extending about 1,400 bases to the left of this site (corresponding to approximate nucleotide number −700) protected a fragment of DNA about 700 bases long after either S1 nuclease or exonuclease VII digestion. Less abundant DNA fragments of ca. 600 bases

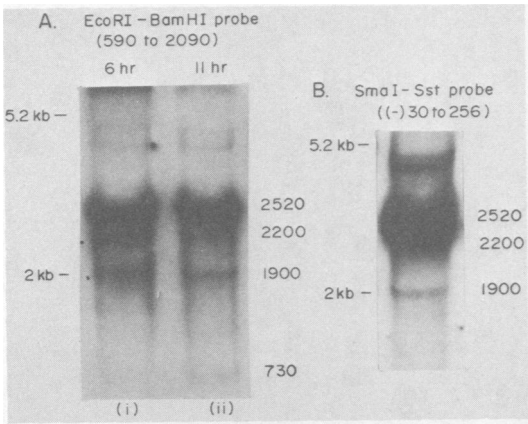

FIG. 3. In situ RNA (Northern) blots. (A) Samples (5 μg) of polyadenylated RNA isolated at 6 h p.i. (track i) and 11 h p.i. (track ii) were fractionated on methyl mercury agarose gels by electrophoresis and prepared for hybridization (see text). The blots were hybridized with cloned *Eco*RI-*Bam*HI fragment I-I (0.633 to 0.643) which had been made radioactive by nick translation with [α-^{32}P]dCTP. (B) RNA (7 μg) from cells 6 h p.i. was hybridized with the *Sma*I-*Sst*II fragment indicated, encompassing only the 5′ terminal portion of the 2,520-base unspliced species, and labeled as above. The positions of these hybridized RNA species are as shown in Fig. 1B. Sizes of mRNAs were based on the migration of 28S and 18S rRNA run in parallel tracks (5.2 and 2.0 kb).

(very minor), ca. 300 bases, and ca. 90 bases were seen with S1 nuclease digestion, but not with exonuclease VII digestion. These data confirmed a common 5′ end for all these members of the mRNA family and also located the splice acceptor sites of the contiguously encoded regions of the 2,200- and 1,900-base-long mRNAs as well as a more minor species about 2,400 bases long.

We precisely located the 5′ end of the 2,520-base-long unspliced and most abundant mRNA by the S1 nuclease and exonuclease VII digestion of hybrids formed by infected-cell polyadenylated mRNA and the DNA fragment extending between the *Pst*I site at nucleotide −350 and the *Sst*II site at nucleotide +256, which was 5′ end labeled at this latter site. The major nuclease-resistant DNA fragment migrated corresponding to a size of ca. 260 bases. Fractionation of such a DNA fragment on a sequence gel localized the 5′ end within the sequence TACC (Fig. 4A). We arbitrarily set the A residue as nucleotide number 1. Such stuttering of nuclease-treated hybrids on sequencing gels has been reported by us and others previously (15, 18, 27) and can reflect both some ambiguity in the exact start of an mRNA and some variability in the extent of digestion. Small amounts of other S1- and exonuclease VII-resistant DNA fragments are also seen; however, for most of them the amounts varied from experiment to experiment, and some could be seen when DNA was incubated without infected-cell RNA. We concluded that such DNA fragments were not due to specific RNA species hybridizing to the DNA. An example is indicated by the question mark (?) in Fig. 4A. One faint S1-resistant band was consistently seen around base numbers 85 to 90 and was only seen in the presence of infected-cell polyadenylated mRNA. This would locate the splice acceptor of the least abundant spliced mRNA species in the sequence TCAGTG. The AG dinucleotide at bases 94 and 95 is indicated as acceptor 1 in Fig. 2. We positioned all splice acceptor sites at AG dinucleotides because this is the canonical splice acceptor site (25, 29).

We carried out a similar experiment with strand-separated DNA 5′ end labeled at the *Eco*RI site at base 587 and extending leftward to the *Sst*II site at base 256. In addition to full-length DNA, a relatively major band about 200 bases long and two less abundant bands about 150 and 120 bases long were seen with S1 nuclease and exonuclease VII digestion. The location of such bands on a sequence ladder extending to the left of the *Eco*RI site at base 587 is shown in Fig. 4B (track HX). In this experiment, exonuclease VII digestion is shown because it tended to produce less stutter. However, S1 nuclease digestion (data not shown) gave fragments about five bases shorter than did the exonuclease VII digestion. In this experiment, we included a track of DNA-protected HSV-infected rabbit skin cell polyadenylated mRNA (Fig. 4B, track RX) to demonstrate that the mRNA species are processed the same in these cells as in cells of human origin. The data of this experiment located three splice acceptor sites for the 2,200-base mRNA. One was within the sequence CCGAGC (acceptor 2A, 381 to 386), one in the sequence AAAAGCC (acceptor 2B, 428 to 434), and one in the sequence CAAGAAC (acceptor 2C, 463 to 469). These sites are indicated in Fig. 2 as the AG dinucleotides at base numbers 384 to 385, 431 to 432, and 465 to 466, respectively.

We used strand-separated DNA 5′ end labeled at the *Hin*fI site at base 710 and extending to the *Hin*fI site at about base −700 to locate the splice acceptor site of the 1,900-base mRNA. This mRNA protected a DNA fragment about 90 bases long from S1 nuclease digestion and located the acceptor within the sequence CCAGAT (613 to 618) (Fig. 4C). We indicated this as acceptor 3 on the AG dinucleotide at positions 615 and 616 in Fig. 2.

We located the 5′ end of the 730-base mRNA about 120 bases to the left of the *Sma*I site at position 1915. We precisely located it within the

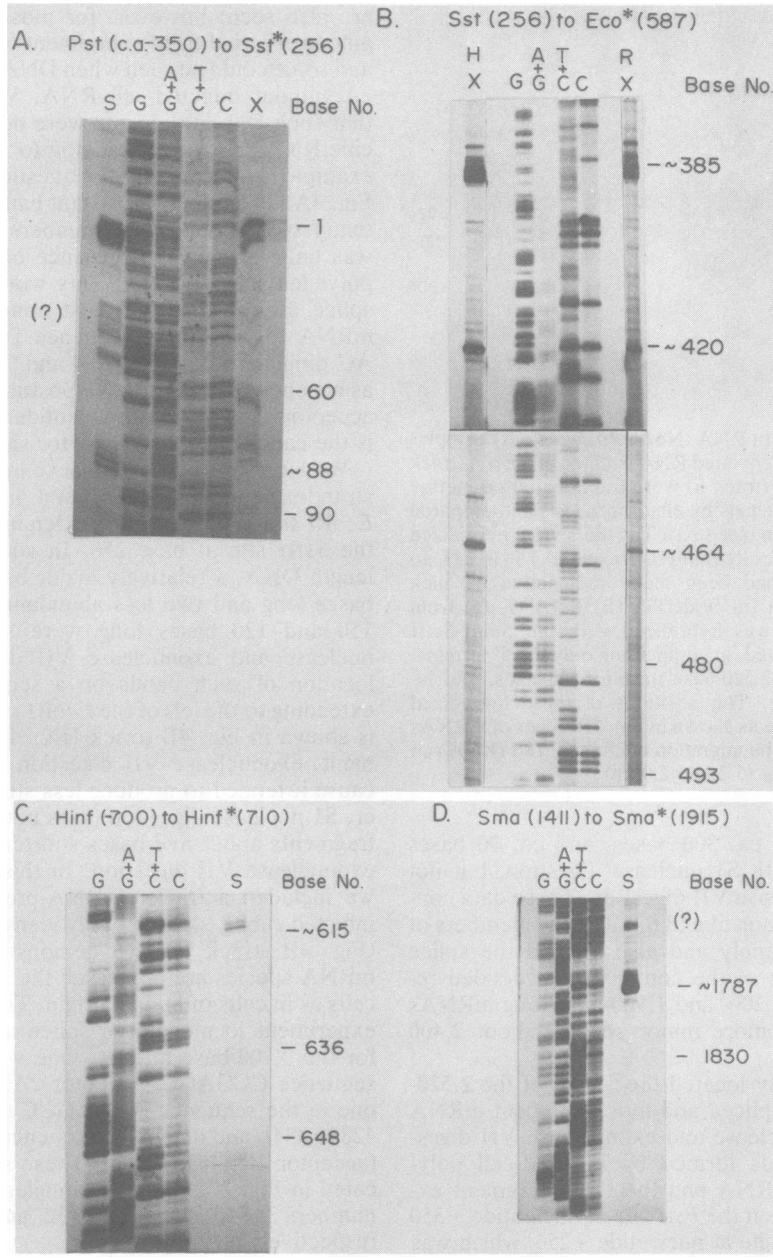

FIG. 4. Precise localization of the 5′ termini of the contiguous regions of the overlapping mRNA species. (A) HSV-1 DNA extending from the *Sal*I site at 0.621 (about 1,250 bases upstream of the mRNA 5′ end) to the *Eco*RI site at base 587 was digested with both *Sst*II and *Pst*II and was size fractionated. The fragment encompassing bases −350 to +256 (Fig. 1A) was eluted, 5′ end labeled, strand separated, and sequenced. Samples of the DNA strand encoding the mRNA were also used to hybridize late polysomal polyadenylated mRNA, and the hybrids were digested with S1 nuclease (S) or exonuclease VII (X). The position of the 5′ end of the 2,520-base unspliced and most abundant mRNA and the splice acceptor of the 2,400-base spliced species were fixed by fractionation of S1- and exonuclease VII-resistant fragments in parallel with the DNA sequence determined from the labeled *Sst*II site. The position of the 5′ end was fixed as position +1 (Fig. 2), and the acceptor site for the 2,400-base mRNA is near position 88. Other bands of radioactivity were not reproducibly seen (see text); one is indicated by (?). The locations of some other specific bases in this sequence ladder are indicated and are complementary to those shown in Fig. 2. (B) HSV-1 DNA as described in the legend to (A) was digested with both *Sst*II and *Eco*RI. The fragment extending from +256 to +587 (Fig. 1A) was used as in (A) for sequence and hybridization analysis. The three splice acceptor sites for the 2,200-base mRNA (positions, ca. 385, 420, and 464) were located with both

sequence CATCCT (1788 to 1791) by S1 nuclease digestion of hybrids of infected-cell polyadenylated mRNA and strand-separated DNA 5′ end labeled at the *Sma*I site at position 1915 and extending leftward to the *Sma*I site at position 1411 (Fig. 4D). This positions the 5′ end about 30 bases below the sequence TATAAA (1755 to 1760). The similarity of these sequences to identified HSV-1 promoters suggests that this mRNA is under its own promoter control. The fact that this mRNA cannot be detected in Northern blots hybridized with probes from the 5′ region of the major mRNA supports this conclusion. We cannot completely exclude the possibility that this mRNA is generated by a splice since there is an AG dinucleotide at nucleotides 1780 and 1781 which could be a splice acceptor site. Again, we cannot exclude the possibility that both mechanisms operate.

We located the 3′ end of the total mRNA family about 260 bases to the right of the *Sal*I site at base number 2255. This was done by S1 nuclease digestion of hybrids between infected-cell polyadenylated mRNA and strand-separated DNA 3′ end labeled at the *Sal*I site at base 2255 and extending rightward to the *Sal*I site at base 2574 (data not shown). Only one S1 nuclease-resistant band was seen, and its size was determined by denaturing acrylamide gel electrophoresis with plasmid pBR322 DNA fragments produced by *Hin*fI digestion as a size standard. As noted above, this locates the 3′ end of the mRNA family at or near the sequence ATAAAAA (2518 to 2524).

Partial characterization of a splice donor sequence. The low abundance of spliced forms has precluded our full characterization of the splice donor. The Northern blot data (Fig. 3) locate a noncontiguous (leader) portion of the 2,200-base and the 1,900-base mRNAs within 250 bases of the 5′ end of the 2,520-base mRNA. Any leader for the 2,400-base mRNA must, of course, be within approximately 100 bases of the 5′ end of the major unspliced mRNA. Our previous data comparing the size of S1 nuclease-protected DNA fragments on neutral and alkaline gels also indicated that the total length of the leader sequences for all spliced mRNA species could be no longer than 100 bases (15). The size of the spliced mRNAs on Northern blots (Fig. 3A), including poly(A) tails, suggests that the leader is even shorter than this.

We used uniformly ^{32}P-labeled DNA spanning the region from −30 to +296 by using *Sma*I to digest ^{32}P-labeled *Sal*I-*Eco*RI fragment T-A (0.621 to 0.633) and isolating the fragment in question by preparative acrylamide gel electrophoresis. The strand complementary to the mRNA was isolated on a strand separation gel and hybridized with infected-cell polyadenylated mRNA. Size fractionation of S1 nuclease-digested hybridized material yielded a major band 310 bases long corresponding to full-length DNA, a minor band due to protection by the 2,400-base mRNA, and a very faint band about 30 bases long. We suggest that this corresponds to DNA protected by the leader sequence of the spliced mRNAs, although the band was not intense enough to photograph.

We performed a primer extension experiment with avian myeloblastosis virus reverse transcriptase to determine that the 1,900-base mRNA contains a noncontiguous fragment ca. 25 bases long at its 5′ end (data not shown). We used the strand-separated DNA fragment contained between the *Hin*fI site at position 710 and the *Sma*I site at 777. This 67-nucleotide DNA fragment with the 3′ end at a position 96 bases below the nominal splice acceptor site was hybridized to infected-cell polyadenylated mRNA. The cDNA produced by avian myeloblastosis virus reverse transcriptase was fractionated on a denaturing acrylamide gel. We consistently found a DNA band migrating with a rate expected for a fragment 120 to 130 bases long. The very small amount of this cDNA precluded further characterization.

We have identified two potential splice donor sites in the region between bases 1 and 100. One has the sequence TTGGT (21 to 25), and the other is GAGGT (71 to 75). We tested the latter site for being a potential splice donor by isolating strand-separated DNA 3′ end labeled at the *Taq*I site at nucleotide 40 and at the *Sst*II site at base 256. In several experiments, the only size of DNA protected by the hybridization was 200 bases long, corresponding to the full length of the fragment (data not shown). Since we would expect a fragment 35 bases long to be protected if the sequence GAGGT were serving as a major

HeLa cell (H) and rabbit skin cell (R) late polysomal polyadenylated mRNA. In the experiments shown, hybrids were digested with exonuclease VII. (C) HSV-1 DNA extending from the *Sal*I site at 0.621 to the *Bam*HI site at 0.643 (Fig. 1A) was digested with *Hin*fI. The fragment between base −700 and base +710 was selected and used as in (A) for sequence and hybridization analysis. The portion of the original autoradiograph shown indicates a band at base number 615, corresponding to the splice acceptor site for the 1,900-base mRNA species. Digestion of hybrids with S1 nuclease is shown. (D) The HSV-1 DNA fragment between nucleotides 587 and 2085 (Fig. 1A) was digested with *Sma*I, and the fragment between bases 1411 and 1915 was selected and used for DNA sequence and hybridization analysis. This fragment located the 5′ end of the 730-base mRNA very near base 1787. The band marked with (?) may be an alternate length form or an artifactual band (see text).

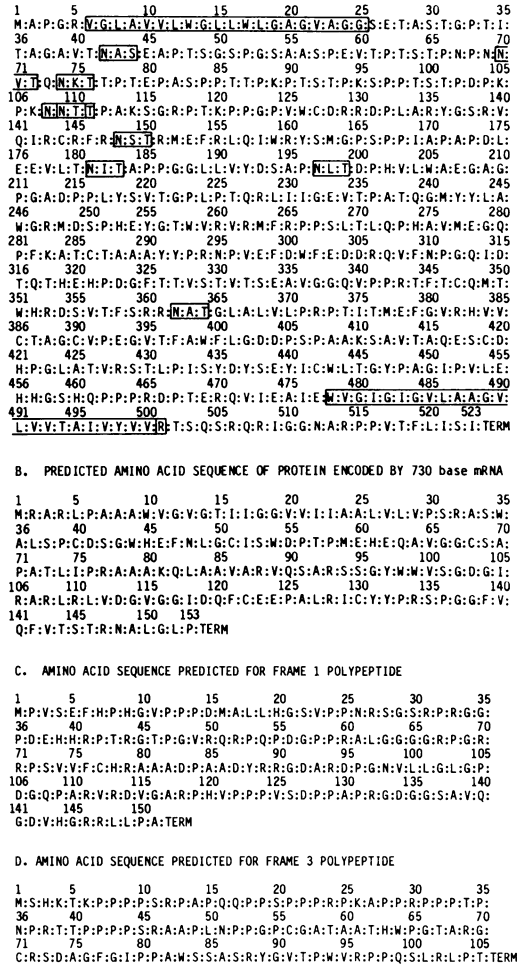

FIG. 5. Predicted amino acid sequences for putative polypeptides encoded by the overlapping mRNA sequences. (A) Predicted amino acid sequence of HSV-1 gC. The 523-amino acid polypeptide contains a membrane insertion sequence (boxed) occurring between amino acids 6 and 25. The membrane-anchoring sequence occurs between amino acids 478 and 501. Potential glycosylation sites of the form N:amino acid:S or T (36) are also indicated. These are features of a glycosylated membrane-associated protein. (B) Amino acid sequence predicted for the 3′ coterminal 730-base mRNA. This mRNA encodes a 17,800-dalton polypeptide by in vitro translation (Fig. 6A, track V). (C) Amino acid sequence predicted for translation reading frame 1. (D) Amino acid sequence predicted for translation reading frame 3.

splice donor, we concluded that this sequence does not, in fact, serve with any frequency.

Taken together, all of these data do not rule out other potential leader sequences or noncontiguous ones functioning in the region upstream of the splice acceptor sites of the spliced mRNA species, but they do suggest that the DNA between nucleotides 1 and 25 can serve to generate a splice donor.

Correlation of translation reading frames with the in vitro translation products of size-fractionated mRNAs. The location of translation initiation and termination signals in the nucleotide sequence of Fig. 2 defines four translation reading frames of appreciable size (Fig. 1C). The longest frame (frame 2) extends from the ATG at bases 146 to 148 to the terminator TAG at bases 1715 to 1717. This 1,569-nucleotide reading frame predicts a polypeptide of 523 amino acids whose sequence is shown in Fig. 5A. The anhydrous molecular size of this polypeptide predicted from its amino acid composition is 60,000 daltons, but its high proline content (12.5%) would cause it to migrate at a rate corresponding to a somewhat larger protein in SDS-acrylamide gels. This polypeptide contains a hydrophobic membrane insertion or signal sequence (23, 33, 34) between amino acids 6 (valine) and 25 (glycine) and a hydrophobic membrane anchoring sequence (37) between amino acid 478 (tryptophan) and 500 (valine) followed at position 501 by an arginine residue. It thus has the form of a membrane-associated protein. Further, there are eight potential glycosylation sites (asparagine-amino acid-serine or threonine; 36), seven of which lie in the N-terminal half of the polypeptide.

These properties suggest that this reading frame encodes HSV-1 gC, and the position of the reading frame predicted that only the unspliced 2,520-base mRNA or the minor spliced 2,400-base mRNA could encode it. We isolated a mixture of the unspliced 2,520-base mRNA and the unresolvable 2,400-base and 2,200-base minor species by size fractionation of mRNA selected by the hybridization of total infected-cell polyadenylated mRNA to *Eco*RI-*Bam*HI fragment I-I (0.633 to 0.643) DNA (see above). This mRNA was translated in vitro and yielded a major product migrating with a rate corresponding to an apparent size of 69,000 daltons on SDS-acrylamide gels (Fig. 6A, track I). A portion of this material was incubated with a polyclonal antibody to HSV-1 envelope glycoproteins previously shown to precipitate HSV-1 gA/gB, gC, gD, and gE (anti-env-1 serum; 9) and the unglycosylated precursors to these glycoproteins isolated from tunicamycin-treated cells. This antiserum specifically reacted with the 69,000-dalton polypeptide (Fig. 6A, track II). A smaller amount of a polypeptide migrating with a nominal size of 50,000 daltons also could be seen, and a very small amount of a polypeptide just smaller than 69,000 daltons could be seen with difficulty after long exposures.

We confirmed that the major translation prod-

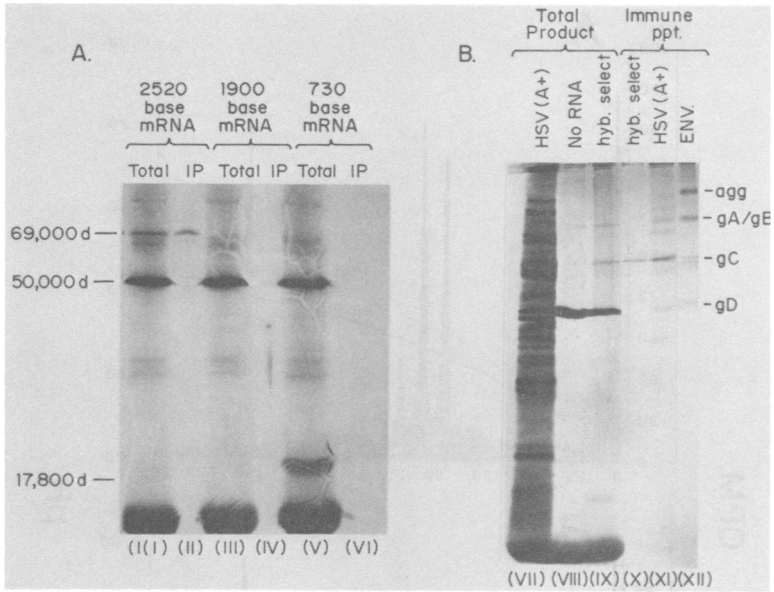

FIG. 6. In vitro translation products of isolated mRNA species identified by immunoprecipitation. (A) Polyribosomal polyadenylated ^{32}P-labeled RNA was hybridized with the HSV-1 DNA fragment extending from base 587 to base 2085 (Fig. 1A) coupled to cellulose. The mRNA selected by hybridization was fractionated by electrophoresis on methyl mercury agarose gels. The gel was sliced, slices were counted, and peaks of radioactivity corresponding to the 2,500-base species plus the unresolvable 2,400- and 2,200-base species, the 1,900-base species, and the 730-base species were eluted and translated. The translation products were equally divided; one half electrophoresed without further treatment, the other half precipitated with anti-env-1 serum, and both portions electrophoresed on a SDS-polyacrylamide gel in parallel. Track I shows the major 69,000-dalton product as well as an endogenous band of 50,000 daltons found after translation of the largest mRNAs. Track II shows that the 69,000-dalton polypeptide reacts with anti-env-1 serum. Smaller amounts of a 50,000-dalton product are also seen. Track III shows only the endogenous 50,000-dalton band found upon translation of the 1,900-base mRNA. A very faint polypeptide band of 50,000 daltons precipitated with anti-env-1 serum is detectable in track IV. Track V demonstrates the presence of a 17,800-dalton polypeptide upon translation of the 730-base mRNA. Track VI shows that this polypeptide does not react efficiently with anti-env-1 serum. Exposure was for 30 days on Kodak XARP film. (B) Comigration of in vitro translated HSV-1 gC and the unglycosylated form of gC isolated from tunicamycin-treated cells is shown. Polyribosomal polyadenylated ^{32}P-labeled RNA was selected by hybridization as in (A) but was not size fractionated. The mRNA then was translated, left untreated, or immunoprecipitated as in (A), and then the translation products were size fractionated by electrophoresis. Track VII shows the translation products of total late HSV-1 poly(A)$^+$ RNA. Track VIII shows the endogenous products present in a control without RNA. Track IX contains the products of hybrid-selected RNA. The two endogenous translation products are seen along with the 69,000-dalton and 17,800-dalton polypeptides expected. Track X shows the translation products of hybrid-selected RNA immunoprecipitated with anti-env-1 serum. We observed the 69,000-dalton polypeptide and a faint band at around 50,000 daltons, which would normally be hidden by the endogenous band. Track XI shows the products of total HSV-1 poly(A)$^+$ RNA translation immunoprecipitated by anti-env-1 serum. Besides the bands for glycoproteins gA/gB, gC, and gD, a number of other bands are present which are characteristically seen with immunoprecipitation of total in vitro translation products. For comparison, track XII shows envelope proteins isolated from tunicamycin-treated HSV-1 infected cells and immunoprecipitated with anti-env-1 serum (9). The doubling of the gC band is due to an artifact from drying the gel.

uct was, indeed, HSV-1 gC by comparing the tryptic peptides of the immunoprecipitated in vitro translation product with tryptic peptides of authentic HSV-1 gC. We used the column chromatography procedures described earlier (9 and above) to do these experiments. Authentic gC was eluted from a polyacrylamide gel before trypsinization, and the recovery of each peptide varied somewhat among experiments. However, the relative elution position of peptides is invariant. A typical profile is shown in Fig. 7A.

We isolated about 0.1 μg of mRNA hybrid selected as described above and immunoprecipitated the in vitro translation product with anti-env-1 serum. When a portion was fractionated on an SDS-acrylamide gel, only the 69,000-dalton polypeptide was efficiently recovered by such treatment (Fig. 6B, track X). We took the

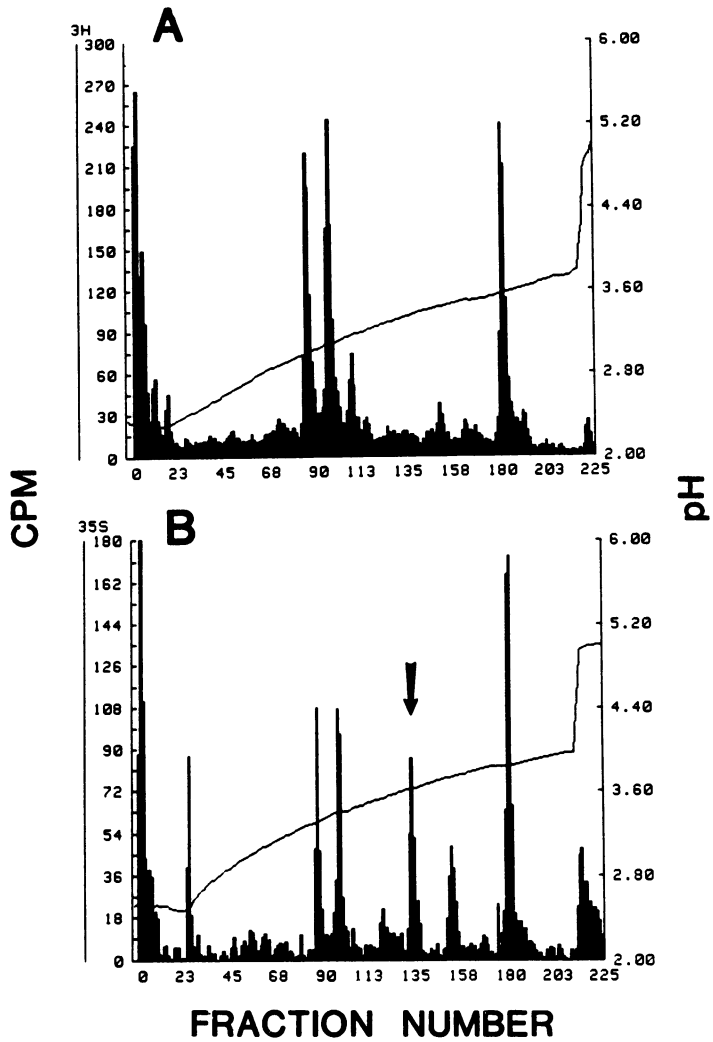

FIG. 7. Tryptic peptide analysis of gC and the 69,000-dalton polypeptide. (A) gC was isolated from a 10% SDS-polyacrylamide gel, trypsinized, and chromatographed on a Chromabeads P cation-exchange column. (B) The 69,000-dalton polypeptide labeled with [^{35}S]methionine was immunoprecipitated with anti-env-1 serum (9) from an in vitro translation mix by using hybrid-selected mRNA. The precipitate was trypsinized and chromatographed on Chromabeads P. The arrow indicates a peptide which is present in the profile for the 69,000-dalton polypeptide and absent from the profile for gC.

remainder of the immunoprecipitated translation product and digested and fractionated it; the profile is very similar to that seen with authentic gC except for one major additional methionine-containing peptide, which probably corresponds to the post-translationally cleaved portion of gC containing the signal sequence (Fig. 7B).

These data confirm the identification of the major translation product of mRNA encoded by this region as HSV-1 gC. We can infer that gC is encoded by the unspliced 2,520-base mRNA because of the relative abundance of this mRNA. We cannot determine whether the minor 2,400-base spliced species also encodes gC or one of the other smaller polypeptides on the basis of its position relative to the translation reading frames. However, the translation reading frame for the glycoproteins shown in Fig. 5A is not completely contained by the 2,200-base spliced mRNA (Fig. 1C), so this mRNA cannot serve as mRNA for gC, although it could encode a truncated immunologically reactive polypeptide.

Although the immunoprecipitation of the 69,000-dalton polypeptide with anti-env-1 serum and the tryptic peptide data demonstrate that it was, in fact, HSV-1 gC, the apparent size of this polypeptide is somewhat at variance with the

size of 74,000 to 85,000 daltons reported for the unglycosylated precursors to gC (33, 34), although the apparent size of 69,000 daltons is consistent with the amino acid composition shown in Fig. 5A. We demonstrated that the in vitro translation product is about the same size as the unglycosylated precursor of gC found in tunicamycin-treated cells. As described, we translated total mRNA isolated by hybrid selection and immunoprecipitated the gC polypeptide with anti-env-1 serum. The immunoprecipitated translation product was subjected to SDS-acrylamide gel electrophoresis along with immunoprecipitated unglycosylated envelope proteins isolated from tunicamycin-treated cells as described previously (9; Fig. 6B, tracks X and XII). Although the envelope protein bands are distorted because of an artifact from drying the gel, the comigration of the in vitro translation product of gC mRNA and the unglycosylated gC is readily apparent.

As an added control, we used anti-env-1 serum to immunoprecipitate the in vitro translation products of total polyadenylated mRNA from HSV-1-infected cells. Translation products comigrating with the unglycosylated forms of gA/gB, gC, and gD were readily apparent (Fig. 6B, track XI). Other faint bands also were seen. Some, such as those marked agg, are probably aggregations of one or more of the nonglycosylated polypeptides also seen in the envelope protein immunoprecipitate. Other bands could result from one or more of the following: (i) internal translation initiation, (ii) premature termination, (iii) translation of mRNAs encoding minor membrane components, and (iv) proteins immunologically cross-reacting with HSV-1 membrane proteins.

There are two other polypeptides potentially encoded by one or another of the spliced mRNA species that are 2,400 and 2,200 bases long. One is defined by a 453-base open reading frame (Fig. 1C, frame 1) between nucleotides 574 and 1027. The sequence predicted for the 151-amino acid polypeptide encoded is shown in Fig. 5C. We would expect such a polypeptide to migrate with an apparent size of 18,000 to 22,000 daltons, but no product this size is obviously apparent in the in vitro translation of the mix of the unspliced 2,520-base and spliced 2,400- and 2,200-base mRNAs shown in Fig. 6A (track I) or in the translation of totally hybrid selected mRNA (Fig. 6B, track IX). A small amount of a polypeptide about this size can be seen in very long exposures of total translation products of hybrid-selected mRNA, but we cannot rule out its being due to the very low level of endogenous translational activity of the commercial reticulocyte lysate.

A third polypeptide of 105 amino acids, encoded by translation reading frame 3 between nucleotides 354 and 669, is also a potential translation product of the mixture of the 2,520-, 2,400-, and 2,200-base mRNAs. Its predicted amino acid sequence is shown in Fig. 5D. Again, however, no obvious polypeptide migrating with a size of 12,000 to 16,000 daltons was apparent in the in vitro translation products of this mixture (Fig. 6A, track II) or in longer exposures. Here, however, the very intense band of radioactivity migrating at about 12,000 daltons could obscure such a product.

From these considerations, it is clear that we cannot determine whether any of the spliced mRNAs of 2,400 to 2,200 bases encodes these alternate reading frame polypeptides. We can state that the 1,900-base spliced mRNA encodes a truncated form of HSV-1 gC. This comes from the fact that a 50,000-dalton polypeptide immunoprecipitable with the anti-env-1 serum was translated by the size-fractionated 1,900-base mRNA. This band is faintly visible in Fig. 6A, track IV. The position of the 1,900-base mRNA in relation to the potential polypeptide initiation signals suggests that this polypeptide is initiated at one of the frame 2 initiators between nucleotides 630 and 980. Its precipitability by the anti-env-1 serum is due to its sharing a sizable C-terminal portion (60 to 70%) of the amino acid sequence of gC and does not imply that this polypeptide is itself a membrane-associated protein. It already has been noted that this 50,000-dalton polypeptide was detectable in the immunoprecipitated in vitro translation products of the mixture of the mRNAs of 2,520, 2,400, and 2,200 bases (Fig. 6A, track II), so one or more of the longer spliced mRNAs could also serve to encode it; however, this suggestion is not directly testable since the polypeptide could be translated by an in vitro interior initiation of the 2,520-base mRNA.

The 730-base mRNA located at the 3′ end of the longer mRNAs encompasses a 459-base reading frame (frame 2) between the ATG sequence at nucleotides 1922 to 1924 and the TGA terminator at nucleotides 2381 to 2383. The predicted amino acid sequence for this 153-amino acid polypeptide is shown in Fig. 5B. The calculated molecular size of this polypeptide is 16,204 daltons, and a polypeptide migrating with an apparent size of 17,800 daltons is readily apparent in the in vitro translation products of the purified 730-base mRNA (Fig. 6A, track V). This polypeptide does not react significantly with the antibody to HSV-1 membrane glycoproteins (Fig. 6A, track VI). This is not surprising since it shares no amino acid sequence homology with HSV-1 gC and has no features of a membrane-associated protein. It is readily detectable when unfractionated mRNA homolo-

gous to this region of HSV-1 DNA is translated (Fig. 6B, track IX), but its function is unknown.

DISCUSSION

The data presented in this report suggest that splicing has a role in late HSV-1 gene expression. They also define rigorously the DNA sequence encoding a known HSV-1 protein, gC.

The fact that the 1,900-base spliced mRNA encodes a truncated form of gC immunoprecipitable with anti-env-1 serum does not rigorously require that this polypeptide be expressed in infected cells, but it is strongly suggestive evidence. This is especially true since this type of differential splicing in generating mRNAs encoding proteins that share amino acid sequences is well proven in other DNA viruses. We have not yet identified the translation products of the 2,200-base spliced mRNA. Its multiple splice acceptor sites could lead to variable mRNA function. At present, however, we cannot definitely claim that either this mRNA species or the rarer 2,400-base species has a defined biological function.

The 730-base mRNA underlying the 3′ end of the longer species appears to be under its own promoter control—a situation analogous to several other regions of the genome (13, 15, 18). Although this suggests that long intervening sequences between the leader and the mRNA body are even more rare than HSV-1 mRNA splicing itself, it should not be taken to imply that HSV-1 must have short introns. Costa and Draper, working in this laboratory, recently characterized an mRNA species encoded by two exons of nearly equal length separated by a 4-kb intron (unpublished results). Further, the fact that the spliced mRNA family members are of low abundance should not be taken to imply that a splice suppression mechanism is operating during HSV-1 infection. In other work (unpublished), Costa characterized an mRNA family of which the most abundant member is spliced. From these considerations, it should be clear that there are no obvious mechanistic reasons for the paucity of detectable splicing in HSV-1 mRNA biogenesis.

The full characterization of an HSV-1 gene whose biochemical properties, if not biological function, are known is clearly of value. Like all well-characterized glycoproteins, HSV-1 gC has hydrophobic membrane insertion and anchoring sequences as well as readily apparent glycosylation sites. The fact that the HSV-1 gC gene is expressed with no detectable splicing makes it potentially convenient for in vitro modification for structure-function studies. Further, the fact that this gene has the capacity for alternate expression via splicing means that it will be useful for manipulating DNA sequences to determine the effects of sequence on mRNA processing.

Although the biochemical properties of HSV-1 gC are known in that it is a membrane-associated glycoprotein, the biological function of this protein is not at all clear. Certainly, in its mature form of about 523 amino acids, it is found only on the membranes of HSV-1 (10). It has been reported that the HSV-1 gC and the immunologically non-cross-reactive HSV-2 protein identified as HSV-2 gC map in different locations on the viral genome (19). Thus, these membrane glycoproteins differ from most HSV proteins, which seem to map in homologous locations on the HSV-1 and HSV-2 genomes. This makes HSV-1 gC a convenient and potentially exploitable type-specific marker. Since it is not found expressed with all strains of HSV-1, it is clearly not required per se for the replication of HSV-1 in cell culture. Certainly our finding that HSV-1 gC comaps with other polypeptides related by mRNA processing will suggest further investigations of the molecular reasons why HSV-1 gC is not expressed in all HSV-1 strains and why it lacks an obviously immunologically cross-reactive homolog expressed during HSV-2 infection.

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