RNAs Transcribed from a 3.6-Kilobase *Sma*I Fragment of the Short Unique Region of the Herpes Simplex Virus Type 1 Genome

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A 3.6-kilobase (kb) *Sma*I subclone of the *Bam*HI J fragment of herpes simplex virus type 1 (KOS) DNA was utilized to characterize the mRNAs transcribed from the genome segment (0.91 to 0.93 map units) that encodes glycoprotein D mRNA. RNA blotting demonstrated two major RNA species of 2.3 and 1.5 kb. 5' and 3' mapping with ³²P-end-labeled DNA fragments indicated that these RNAs are a nested set, each having its own promoter and 3' terminus. Less abundant RNA species with discrete 5' ends were also observed. Precise 5' mapping and sequence data located the initiation sites and demonstrated TATA boxes, CAT boxes, and AC-rich regions in the appropriate positions. 3' mapping located a common end for both mRNAs, but the 2.3-kb mRNA was reduced in size by splicing at a point near the RNA terminus. In vitro runoff transcription experiments confirmed the location of the two promoters and showed that an uninfected cell extract initiated faithfully at both sites. Despite the similarities in DNA structure and the apparent equal efficiency of promoter utilization in vitro, the 2.3-kb mRNA appeared in the cytoplasm early (1 h) after infection, whereas the 1.5-kb mRNA was delayed until 3 h after infection.

The regulation of transcription of the herpes simplex virus (HSV) genome displays several interesting characteristics. These include both temporal controls regulating the production of specific RNA species during the infectious cycle and additional controls regulating the quantity of each mRNA synthesized (37; E. Wagner, in G. Klein, ed., *Advances in Viral Oncology*, Vol. 3, in press). Some temporal controls have been shown to involve viral proteins; in the best-studied case, the proteins produced immediately after infection (immediate-early proteins) are required for transcription from additional segments of the genome (1, 9, 21, 45). To date, there is only limited information available on the mechanisms by which immediate-early and other viral proteins influence RNA synthesis (33, 42, 43) and on regulatory signals for immediate-early RNA encoded in HSV DNA (24-26; Wagner, in press).

In general, nucleotide sequences are found in HSV DNA that correspond to previously identified eucaryotic mRNA initiation and control sites, e.g., TATA boxes, CAT sequences, etc. (see Wagner, in press, for review). Of the HSV mRNA transcripts examined so far, very few are

spliced (14, 16, 46), and there are many examples of multiple mRNA transcripts arising from a DNA segment (2, 12, 14, 17, 30). Detailed characterization of additional early and late HSV genes and their mRNAs are needed to understand how RNA polymerase transcribes viral genes and whether virus-encoded factors or modifications of host enzymes are involved in gene selection as well as transcription efficiency.

We have examined the transcription of a region of the HSV type 1 (HSV-1) genome known to encode a biologically important protein, glycoprotein D (gD), and another protein of unknown function (23). In a previous communication (6), we described a restriction map of this DNA region, the *Bam*HI J fragment (0.900 to 0.945 map units). RNA transfer (Northern) analysis of the steady-state levels of polyadenylated [poly(A)⁺] mRNA identified three major mRNA species of 2.5, 1.3, and 0.9 kilobases (kb) hybridizing to this segment of the HSV-1 genome. Our data did not establish which of the abundant mRNAs seen by Northern analysis is the RNA for gD, nor did they locate the positions of the 5' ends of the transcripts. J. Matthews, G. Cohen, and R. Eisenberg (personal communication) have demonstrated by in vitro translation that the mRNA for gD is somewhat less than 2.5 kb

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in size. While our experiments were in progress, Watson and co-workers (47) reported the DNA sequence of a segment of the HSV genome containing the gD gene and recently reported the localization of a 3-kb mRNA on this DNA segment and the expression of this protein after microinjection of the DNA (44). All of these results, as well as kinetic experiments which showed that gD protein is produced early in infection (10, 31), indicate that the 2.5-kb mRNA we detected encodes the gD protein. The translation products of the 1.3- and 0.9-kb mRNAs remain unknown.

In this paper, we further characterize the two 3'-colinear mRNAs of 2.5 and 1.3 kb transcribed from the region of the HSV-1 genome encoding gD (0.91 to 0.93 map units). We located the 5' and 3' ends of these mRNAs and established at what point after infection they were synthesized. We did not detect splicing at the 5' end of either mRNA but have observed splicing at the 3' end of the gD message.

MATERIALS AND METHODS

Cells and virus. The adenovirus-transformed human cell line 293-31 was used as the source of infected-cell RNA; the procedure used to infect these cells with HSV-1 (KOS) has been previously described (38). Details of the methods used to propagate virus and its plaque assay on rabbit skin cells are described in the same paper (38). A previous report describes the infection of pyBHK cells (32).

Preparation of RNA and DNA. The isolation of cytoplasmic RNA was carried out by a modified Holmes and Bonner technique (19, 38). Cells were harvested at 5 or 6 h after infection with HSV-1 at a multiplicity of 10 to 20 PFU per cell, and the RNA was isolated from the cytoplasm. The RNA was routinely treated with RNase-free DNase; poly(A)⁺ RNA was selected on oligodeoxythymidylate cellulose (3). The recombinant plasmid pJB3 was constructed by subcloning a 3.6-kb *Sma*I fragment of HSV-1 DNA from a plasmid containing the 6.2-kb *Bam*HI J fragment of HSV-1 (KOS) (6). This *Sma*I fragment contains the

2.7-kb *Sac*I (*Sst*I) fragment which encodes gD mRNA (23). The *Sma*I fragment was ligated into the bacterial plasmid pACYC177 (8) at its single *Sma*I site and used to transform *Escherichia coli* HB101. Ampicillin-resistant transformant bacteria were selected; those sensitive to kanamycin contained inserts in the *Sma*I site of the plasmid. Plasmid DNA isolated by standard techniques (35) was routinely purified by two cycles of cesium chloride centrifugation. The restriction map derived for the *Sma*I fragment is shown in Fig. 1.

RNA mapping techniques. RNA transfer (Northern) analyses of infected-cell RNAs were performed as previously described (6; R. M. Stenberg, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1981). To locate the positions of the 5' and 3' ends of mRNA on the HSV genome, we used the hybridization procedure of Berk and Sharp (5) as modified by Villarreal and Jovanovich (40). DNA fragments were labeled at the 5' end by exchange kination with [γ -³²P]ATP and T4 polynucleotide kinase (29). DNA fragments were labeled at the 3' end with [α -³²P]dGTP with the Klenow fragment of DNA polymerase I (27). Routinely, 50 to 100 μ g of cytoplasmic RNA was alcohol precipitated with 100 to 500 ng of DNA containing 1×10^4 to 5×10^4 cpm. The DNA-RNA precipitate was dissolved in 100% formamide and heated at 80°C for 2 min, and then buffered salts were added to give 80% formamide (final concentration). After 10 min at 78°C, hybridization was continued for 3 h at 50°C. The reaction was terminated by dilution into S1 or exonuclease VII (*Exo*VII) buffer, and 200 U of nuclease S1 or 10 U of *Exo*VII was added. Nuclease digestion was carried out for 45 min at 37°C. The undigested DNA was size fractionated by electrophoresis on either an alkaline agarose gel (5) or an 8 M urea-polyacrylamide gel (29); restriction fragments of simian virus 40 (SV40) were used as molecular weight markers (7). After neutralization and fixation with 5% glutaraldehyde, the gels were dried and subjected to autoradiography with either Kodak XRP-5 or XAR-5 film. Cronex intensifying screens were routinely employed, and development was carried out at -70°C.

DNA sequencing. The sequencing method of Maxam and Gilbert (29) was employed with 8 M urea-8 or 20% polyacrylamide gels. For precise location of the 5' end of mRNAs, the method of Weaver and Weissman (48) was used with known sequence ladders of DNA as

FIG. 1. Restriction map of *Bam*HI J fragment (19). Selected enzymes and fragment designations and sizes in kbp for fragments used in hybridizations are indicated.

markers (29; details are given in the legends to Fig. 7 and 8).

In vitro transcription. The in vitro transcription system described by Manley et al. (28) was purchased from New England Nuclear Corp., Boston, Mass. The HeLa cell lysate and other reagents were used as described by the supplier, except the reaction volume was increased to 50 μ l and 40 μ Ci of [α - 32 P]GTP (specific activity, 760 Ci/mmol) was used as the labeled nucleotide. Duplicate reactions, one of which contained α -amanitin at a concentration of 2 μ g/ml, were carried out. To each reaction was added 3 μ g of plasmid DNA that had previously been cut with an appropriate restriction enzyme, extracted with phenol-chloroform (1:1), and precipitated with ethanol. Incubation was carried out for 60 min at 30°C, and the reaction was terminated with 250 μ l of 7.5 M urea containing 0.5% sodium dodecyl sulfate–10 mM EDTA (pH 7.5). The above mixture was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (1:1:0.05, vol/vol), and the aqueous phase was reextracted with chloroform. Carrier tRNA (20 μ g) was added to the aqueous phase, followed by sodium acetate, to give a final concentration of 0.2 M. The RNA was precipitated with 2.5 volumes of ethanol, redissolved, and reprecipitated before being dissolved in buffer and denatured with 12.5 mM methylmercury hydroxide. The RNAs were separated on a 1.5% agarose gel containing 7.5 mM methylmercury hydroxide (4). The gels were stained with ethidium bromide to locate the major DNA and RNA bands, dried, and autoradiographed.

Reagents. Enzymes were purchased from Bethesda Research Laboratories, Gaithersburg, Md., or New England Biolabs, Beverly, Mass., and used under the conditions described by the supplier. Radioactive nucleotides were purchased from ICN Pharmaceuticals, Irvine, Calif., or New England Nuclear Corp.

RESULTS

RNA sizes and direction of transcription from the 0.91- to 0.93-map unit region of the HSV genome. Lee and co-workers (23) utilized a 2.7-kb *Sac*I (*Sst*I) segment of the *Bam*HI J fragment to select mRNA that could be translated to give the gD polypeptide; a second protein with a molecular weight of approximately 55,000 was also observed. No information was provided on the mRNAs themselves. We analyzed viral RNAs immobilized on diazobenzylloxymethyl paper with radioactive DNA fragments and concluded that, although several mRNAs of different sizes were transcribed from this region of the *Bam*HI J fragment, two species of 2.5 and 1.3 kb predominated (6).

To simplify later detailed mapping, we repeated the Northern analyses with RNA isolated from an independent infection. The RNA blots were probed this time with DNA specifically labeled at a single 3' end. The first probe was labeled at the 3' end of the large *Hind*III fragment and cut with *Ava*I to give an 850-base pair (bp) fragment labeled uniquely at the left-hand end. This probe hybridized only to a 2.3-kb

FIG. 2. Analysis of steady-state RNAs by transfer to diazobenzylloxymethyl paper (Northern analysis). Analysis of nuclear and cytoplasmic poly(A)⁺ RNA from infected and uninfected baby hamster kidney cells was carried out with 32 P-labeled DNA fragments. (A) RNAs were probed with the 3'-labeled *Hind*III fragment which had been secondarily cut with *Ava*I. Lanes 1 and 2, nuclear and cytoplasmic RNAs, respectively, from uninfected cells; lanes 3 and 4, nuclear and cytoplasmic RNAs, respectively, from cells 5 h after infection. (B) RNAs were probed with the 3'-labeled *Ava*I 2 fragment which had been secondarily cut with *Sst*I. The internal 850-bp fragment was used. Lanes 1 and 2, nuclear and cytoplasmic poly(A)⁺ RNA, respectively, from infected cells. Markers indicated on the left-hand side were *Hind*III restriction fragments of SV40 of known nucleotide sequences (7) which had been treated with glyoxal before electrophoresis. (C) Diagram of 3'-labeled probes used to analyze the immobilized RNAs. The 3' label is indicated by an asterisk.

RNA present in the nucleus and cytoplasm of infected cells (Fig. 2A, lanes 3 and 4). The second probe was constructed by 3' labeling of the *Ava*I 2 fragment, followed by secondary cutting with *Sst*I to give a 850-bp fragment. Hybridization with this fragment detected two RNA molecules of 2.3 and 1.5 kb (Fig. 2B, lanes 1 and 2). Because both DNA fragments were labeled uniquely at the left-hand 3' end (Fig. 2C), we concluded that both RNAs were copied from the same DNA strand and that transcription of the gD region proceeds from left to right.

FIG. 3. Mapping of the 5' ends of mRNAs within the *AvaI* 1 fragment. Total cytoplasmic RNA from infected and uninfected cells was hybridized with the intact 5'-labeled *AvaI* 1 fragment or with the *AvaI* 1-*SstI* subfragment. (A) After S1 digestion. Lane 1, probe; lane 2, markers (*HindIII* digest of SV40); lane 3, hybridization with non-infected-cell RNA; lane 4, hybridization of infected-cell RNA with probe labeled at both ends; lane 5, hybridization of infected-cell RNA with probe labeled only at the right-hand end. (B) After S1 or *ExoVII* digestion. Lane 1, probe (*AvaI* 1 fragment labeled at both ends); lane 2, S1 digest; lane 3, *ExoVII* digest; lane 4, markers as in (A). (C) Diagram of labeled probes and calculated locations of transcripts. The abundant species is indicated by the heavy line.

The 2.3- and 1.5-kb RNAs shown in Fig. 2 correspond to the 2.5- and 1.3-kb RNAs we reported earlier (6). The differences in sizes (200 nucleotides) probably reflect the precision of the RNA transfer method.

Mapping the 5' ends of the mRNAs. The position of the 5' end of the 2.3-kb mRNA was established with the *AvaI* 1 DNA fragment labeled at both 5' ends. The results of the S1 nuclease analysis of DNA-RNA hybrids (Fig. 3A, lane 4) indicated that the major mRNA starts approximately 840 nucleotides from an *AvaI* site. Minor RNA species with their 5' ends, 1,050, 500, and 300 nucleotides upstream from an *AvaI* site, were also observed. The same patterns were observed when the *AvaI* probe was cut with *SstI* (Fig. 3A, lane 5) to remove the left-hand label. We therefore concluded that all the RNAs copied from this segment of the

genome that accumulate in the cytoplasm are transcribed from left to right and that the major transcript has its 5' end at a position close to the single *HindIII* site.

The possibility should be noted that other transcripts present in the nucleus fail to enter the cytoplasm and the RNA abundancies measured in the cytoplasm reflect regulatory processes rather than relative rates of transcription.

When *ExoVII* was used to digest the *AvaI* 1 DNA-RNA hybrid, the major protected DNA fragments were the same size as those found after S1 digestion (Fig. 3B). This result indicates that the 5' region of the mRNA was not spliced from segments of RNA copied from discontinuous stretches of DNA.

S1 hybridization mapping with a 5'-labeled *AccI*-1 probe confirmed the above results. In addition to the major 860-nucleotide band running from left to right, low-abundance RNAs of 1,080-, 500-, and 300-nucleotide stretches were found hybridizing to the *AccI* probe (data not shown). The differences in sizes of protected fragments are in agreement with the map positions of the *AvaI* and *AccI* sites (Fig. 1). A diagram of the organization of these RNAs is shown in Fig. 3C.

To locate the 5' end of the shorter (1.5-kb) mRNA seen on the RNA Northern blot, we repeated S1 mapping with 5'-labeled *AvaI*-3 DNA and *AccI*-3 DNA (Fig. 1). The results of the experiment with 650-bp *AvaI*-3 are shown in Fig. 4. In addition to protection of the full-length DNA fragment, presumably by the 2.3-kb transcript, a major band of 260 nucleotides was observed (Fig. 4, lane 4), together with a less abundant species 350 nucleotides in length. Secondary cutting of the *AvaI*-3 5'-labeled DNA with *Sau3A* resulted in a 500-nucleotide fragment with only the right-hand end labeled. Mapping with this subfragment gave the same three bands, thereby eliminating any ambiguity in the direction of transcription of the smaller RNA. The *AccI*-3 probe (data not shown) hybridized to RNAs protecting the full-length DNA, which was 1,200 nucleotides long, as well as molecules which were 780 and 670 nucleotides in length.

In summary, we located the 5' ends of the major mRNAs identified by Northern analysis. One species, most likely the 2.3-kb RNA, is transcribed starting near the unique *HindIII* site, and the other (1.5 kb) begins near the middle of the *AvaI* 3 fragment, roughly 1,400 bp downstream of the *HindIII* site. All the mRNAs from this region of the HSV-1 genome accumulating in the cytoplasm, including the minor species observed, are transcribed from left to right.

Mapping the 3' ends of mRNAs. From the mapping information on the 5' ends and the sizes of the major mRNAs, we calculated that both

FIG. 4. Mapping the mRNA 5' ends within the *AvaI* 3 fragment. (A) Total cytoplasmic RNA from infected or uninfected cells was hybridized with the *AvaI* 3 fragment labeled at both 5' ends, and the hybrids were digested with S1 nuclease. Lane 1, markers (*HindIII* digest of SV40); lane 2, probe; lane 3, hybridization with non-infected-cell RNA; lane 4, hybridization with infected-cell RNA. (B) Diagram of the labeled probe and the calculated location of the transcripts. Abundant species are indicated by heavy lines.

mRNAs should terminate near the *SstI* site 2,400 bp downstream of the *HindIII* site. To locate the 3' ends of the RNAs, the 1,250-bp *AvaI* 2 fragment was labeled at its 3' ends and either used directly or cut with *SstI* to give an 850-bp fragment labeled only at the left end.

Preliminary experiments with both 3'-labeled DNA fragments indicated a unique end for each RNA transcript. To explain this result, we considered two possibilities: either there are two termination sites, one for the 2.3-kb RNA and another for the 1.5-kb RNA, or there is a single termination site with the second 3' end generated by splicing. To test the latter possibility, the 3'-labeled *AvaI*-2 fragment was hybridized to RNA, and the hybrid was subjected to *ExoVII* and S1 nuclease digestion. The *ExoVII*-resistant

DNA gave a single band of 1,100 nucleotides (Fig. 5A, lane 2), whereas S1 digestion produced an additional band of 460 to 500 nucleotides (Fig. 5A, lane 3). Two additional minor bands were also observed.

From the sizes of mRNAs, it appears that gD mRNA is spliced from a donor site 400 to 500 bp downstream from the *AvaI* site to an acceptor site near the 3' terminus, giving an mRNA of about 2.1 kb before the addition of poly(A). The second mRNA is not spliced and terminates 1,100 bp downstream from the *AvaI* site, giving a 1.3- to 1.4-kb mRNA before the addition of poly(A). Figure 5B shows the results obtained with the *AvaI* 2-*SstI* fragment. A main band of approximately the same size as the DNA probe (Fig. 5B, lane 2) and a second smaller band of about 460 nucleotides were seen. This result is consistent with those described above and establishes that neither of the 3' ends detected with the *AvaI* 2 fragment arises by transcription from right to left. Figure 5C shows the 3'-labeled

FIG. 5. Mapping of 3' ends of mRNAs within the *AvaI* 2 fragment. Total cytoplasmic RNA from infected cells was hybridized with the *AvaI* 2 fragment labeled at both ends (A) or only at the left end (labeled *AvaI* 2 fragment secondarily cut with *SstI*) (B). (A) Lane 1, probe; lane 2, *ExoVII* digest; lane 3, S1 digest. (B) Lane 1, probe; lane 2, S1 digest. The positions of the SV40 markers are indicated between (A) and (B). The sizes of the labeled DNA fragments are indicated alongside each panel. (C) Diagram of the 3'-labeled probe.

FIG. 6. Summary of the 5' and 3' RNA mapping data for the transcripts from the *SmaI* subfragment of the *BamHI* J fragment. Abundant species are indicated by the heavy lines; the "V" indicates a splice. The minor species that have undefined 3' ends are indicated by dashed lines. A, *AvaI*; H, *HindIII*; N, *NruI*; S, *SstI*; Sm, *SmaI*.

DNA probe, and the scheme shown in Fig. 6 summarizes the 5' and 3' mapping data.

Precise mapping of 5' ends. The DNA sequence around the *HindIII* site was determined from uniquely end-labeled fragments obtained by cutting with *HindIII* (Fig. 1), end labeling the DNA, and secondarily cutting with *PvuII*. The labeled fragments of 250 and 220 bp were separated by polyacrylamide gel electrophoresis. Base modification and cleavage reactions and

sequencing gels were performed and prepared as described by Maxam and Gilbert (29). The sequence we determined for the 193 bp around the *HindIII* site of HSV-1 (KOS) (Fig. 7A) agrees very closely with the sequence recently published by Watson et al. for HSV-1 (Patton) (47). The two positions at which the sequences differ are indicated; we found a *HinfI* site at position -113 to -117 and the corresponding sequence 5' GATTC, where their sequence was 5' GAAC

FIG. 7. DNA sequence around the initiation site of the 2.3- and 1.5-kb mRNAs. The most probable 5' end(s) of the messages are indicated by the asterisks (defined as position +1). Restriction enzyme sites are underlined. Putative regulatory sequences are indicated by the wavy lines. (A) 2.3-kb mRNA. Progressing in the 5' direction (upstream), we found a TATA box (23 to -28), an AT-rich CAAT region (-69 to -77), and a CAT box (-93 to -95). An AC-rich region from -107 to -132 is underlined with dotted lines. Other potential TATA boxes are indicated by dashed wavy lines. Where our sequence differs from that of Watson et al. (47), their sequence is indicated below the line. (B) DNA sequence around the initiation site of the 1.5-kb mRNA. The most probable 5' end of the message (from *ExoVII* data; see Fig. 8B) is indicated by an asterisk (position +1). Underlinings are defined in (A); the *NruI* site from which the complementary strand was sequenced is at position 75 on this sequence (not shown).

FIG. 8. Precise mapping of the 5' ends of the 2.3- and 1.5-kb mRNAs. (A) 2.3-kb mRNA. Infected-cell RNA was hybridized with a gel-purified *Sau*3A fragment labeled at its 5' ends. Fragments were analyzed on an 8 M urea–20% polyacrylamide sequencing gel (29). Lanes 1, 2 and 3, respectively, Maxam and Gilbert C, A > C, and G reactions on a *Bst*NI–5'-labeled fragment of pBR322; the positions of oligonucleotides relative to the label are indicated. Lanes 4 and 5, S1 and *Exo*VII digest, respectively. Lane 6, undigested probe. (B) 1.5-kb mRNA. The appropriate fragment from an *Nru*I digest of plasmid pJB3 was gel purified and kinased by the blunt-end procedure of Maxam and Gilbert (29). Secondary cutting with *Ava*I yielded a 450-bp, 5'-labeled fragment which was sequenced by the Maxam and Gilbert procedure (29), and fragments were analyzed on an 8 M urea–8% polyacrylamide gel (lanes 1 through 5). The fragment was also hybridized to infected-cell RNA and treated with S1 (lane 7) or *Exo*VII (lane 8). Lane 6, 44-base-per-strand *lac* operator DNA marker.

(47); at position 11 we found a C in place of a T. Both sequence changes are in the nontranscribed region or in the leader mRNA and hence would have no effect on the gD protein (47).

Our earlier experiments shown above (Fig. 3) indicated that the 5' end of the 2.3-kb mRNA was near the *Hind*III site. However, the 3'-labeled *Hind*III fragment (Fig. 2C) was not protected by HSV-1 RNA from S1 digestion (data not shown), indicating that the 5' end of this mRNA starts downstream of the *Hind*III site. For precise location of the 5' end of the gD mRNA, the 470-bp *Pvu*II fragment from pJB3 was isolated (see Fig. 1), cut with *Sau*3A, and 5' labeled with [γ - 32 P]ATP. The 200-bp radioactive *Sau*3A subfragment was purified by electrophoresis and hybridized with infected-cell mRNA. Figure 8A shows the resulting sizes of DNA fragments after S1 and *Exo*VII digestion. The S1 digestion (Fig. 8A, lane 4) shows, in addition to an overloaded band near the salt front, two

bands corresponding to oligonucleotides of lengths 6 and 7. The *Exo*VII digestion shows a main band at position 9 and several satellite bands. Heterogeneity of mRNA start, or fragment protection, has also been reported for 5'-labeled polyomavirus and SV40 mRNA (11, 13) as well as for HSV-1 mRNA (Wagner, in press).

Our conclusion from this experiment is that the gD mRNA most likely initiates at the A residue 9 nucleotides from the *Sau*3A site. This A residue (indicated by the asterisk on Fig. 7A) has been taken as position +1. The ATG initiation codon is 52 nucleotides downstream from this point. Examination of the DNA sequence upstream of the mRNA start reveals possible regulatory elements. A putative TATA box was found at –23 to –28, although similar sequences were found at –52 to –59 and –67 to –75. The latter sequence encodes the regulatory AT-rich CAAT box described for several eucaryotic messengers by Shenk (36). A CAT box was

found at -93 to -95 and an AC-rich region was found at -107 to -132. These regulatory sequences and their positions relative to the 5' end of the mRNA were similar to those described by Wagner and colleagues (see Wagner, in press, for review).

The 5' end of the 1.5-kb mRNA was precisely located and the adjacent DNA was sequenced with a fragment uniquely labeled at the *Nru*I site roughly 60 bases downstream from the start of transcription (Fig. 6) (47). After secondary *Ava*I cutting and electrophoretic separation, the fragment 5'-labeled at the right-hand end was both hybridized to infected-cell RNA and sequenced (29). The sequence ladders and the labeled DNA protected from *Exo*VII and S1 nuclease are shown in Fig. 8B. The major bands seen after S1 or *Exo*VII digestion are 71 to 75 nucleotides from the labeled end, indicating that the 1.5-kb mRNA is unspliced. A minor band corresponding to the minor transcript initiating on the *Ava*I 2 fragment is observed about 100 nucleotides farther up the sequence ladder.

The most likely start of transcription for the nucleotide sequence shown in Fig. 7B, as determined from *Exo*VII data, is indicated as position 1, and that of a putative TATA sequence is at -23 to -18. This sequence is in complete agreement with that published by Watson et al. (47) for HSV-1 (Patton).

Time course of mRNA appearance in infected cells. We next determined at what point after infection the two messages (2.3 and 1.5 kb) transcribed from the 0.91- to 0.93-map unit segment of the HSV genome appear in the cytoplasm and how their abundances alter as infection proceeds. Cytoplasmic RNA was extracted from infected cells at different times and hybridized to 5'-labeled *Ava*I-3 DNA (Fig. 1). After S1 digestion, the resistant [³²P]DNA was separated by electrophoresis and autoradiographed. By 1 h after infection, RNA in the cytoplasm protected the full-length probe (Fig. 9A). This RNA corresponds to the 2.3-kb mRNA for gD. The quantity of this RNA increased from 1 to 4 h and appeared to remain at the 4-h level at least until 6 h postinfection. The shorter mRNAs with 5' ends within the *Ava*I 3 fragment did not appear until after the 2-h time point. These RNAs were abundant by 4 h and increased in amount by 6 h. It therefore appears that the 2.3-kb mRNA belongs to the early (β) mRNA class and the 1.5-kb mRNA belongs to the late ($\beta\gamma$) class. The appearance of the 2.3-kb gD mRNA early after infection is consistent with the kinetics of gD protein synthesis (10, 31).

To confirm the results of the time course and to establish that the 1.5-kb mRNA is indeed made late in infection, we used a temperature-sensitive mutant blocked in the production of

FIG. 9. Time course of mRNA appearance and analysis of 5' ends of the mRNAs. Cytoplasmic RNA was isolated from infected cells 1, 2, 4, and 6 h after infection and utilized to protect 5'-labeled *Ava*I-3 DNA from S1 digestion. Two concentrations of RNA (40 and 80 μ g) were used for each time point. Lane 1, probe; lanes 2 and 3, 40 and 80 μ g of RNA obtained 1 h postinfection; lanes 4 and 5, 2-h RNA; lanes 6 and 7, 4-h RNA; lanes 8 and 9, 6-h RNA; lane 10, standard *Hind*III digest of SV40 DNA as markers.

late mRNA and proteins (18). Cells were infected at 39°C with HSV-1 (KOS) or HSV-1 (KOS) *ts*B2 at the nonpermissive temperature for 5 h, and then RNA was prepared. Equivalent amounts of RNA were hybridized with the 5'-labeled *Ava*I-3 probe, and the hybrids were digested with S1 nuclease. The results of subsequent gel electrophoresis and autoradiography are shown in Fig. 10. Infection with wild-type virus resulted in the appearance of both RNAs, but infection with the *ts*B2 mutant leads to only the larger (gD) mRNA. It is worth noting that cells infected with the mutant virus contained more total RNA, but the amount of gD mRNA was lower than that for cells infected with wild-type virus. It appears that not only the synthesis of the late mRNA, but also the amplification of the gD mRNA, normally seen at later times, are blocked in mutant-infected cells.

In vitro transcription. In vitro transcription of plasmid pJB3 DNA has confirmed the in vivo mapping of promoter sites. Figure 11, lane 2, shows the two transcripts obtained when *Sst*I-cut plasmid DNA was used as the template. The 2.4-kb transcript corresponds to the 2.3-kb in vivo product detected by Northern analyses (Fig. 2). The size of this transcript indicates that termination occurred at the *Sst*I site and that splicing that reduces the size of this RNA in the cell does not occur in vitro. The 1.1-kb runoff transcript corresponds to the 1.5-kb mRNA found by Northern analyses (Fig. 2) interrupted at the *Sst*I site. The in vitro transcripts from *Ava*I- and *Acc*I-cut DNA (Fig. 11, lanes 4 and 6)

FIG. 10. HSV mRNA produced after infection at 39°C. Cytoplasmic RNA obtained from cells 5 h postinfection at 39°C with HSV-1 (KOS) or HSV-1 (KOS) *tsB2* was used to protect 5'-labeled *Ava*I-3 DNA from S1 digestion. Lanes 1, 2, and 3, results obtained with 20, 40, and 80 μ g of RNA, respectively from HSV-1 (KOS)-infected cells; lanes 4, 5, and 6, results with comparable RNA from mutant virus-infected cells; lane 7, SV40 markers.

also agree in size with those measured by *in vivo* mapping data presented earlier and confirm that the polymerase faithfully recognizes two initiation sequences *in vitro*. The transcripts of 840 and 900 nucleotides in length copied from the *Ava*I- and *Acc*I-cut DNAs, respectively, start at the promoter for the 2.3-kb mRNA, whereas the 270-, 330-, and 750-nucleotide transcripts initiate

at the internal promoter for the 1.5-kb mRNAs. It is of interest that the RNA polymerase from uninfected cells appears to transcribe from both promoters with equal facility; in contrast, the different times of mRNA appearance in the infected cell indicates temporal regulation of transcription.

DISCUSSION

A number of general transcription features from the HSV genome have been identified through detailed mapping of mRNAs in the U_L region (see Wagner, *in press*, for review). When individual segments of the genome were analyzed, families of RNAs were found that share common 5' initiation or 3' termination sites. DNA sequence data around the 5' initiation sites indicate the typical eucaryotic TATA and CAT sequences (36; Wagner, *in press*); in addition, homologous sequences (possible regulatory elements) are found upstream of genes expressed at similar times in the infectious cycle (12, 15–17, 30, 41). We have studied a segment of the short unique region of the HSV genome (0.91 to 0.93 map units) that encodes the glycoprotein gD. Our interest in the transcription of the gD gene stems from the importance of this protein as type-common antigen and as another example of an early protein (10, 31).

The results reported in this paper show that two major mRNAs of 2.3 and 1.5 kb in length are transcribed from the DNA segment known to encode the gD message. As discussed above, the 2.3-kb mRNA is most likely the gD mRNA (23, 44, 47). From our 5' and 3' mapping data, we

FIG. 11. Analyses of *in vitro* transcripts from plasmid pJB3 DNA. *In vitro* transcription products separated on a 1.5% agarose gel were autoradiographed with Kodak XRP-5 film and a Cronex intensifying screen. Lane 1, SV40 markers; lanes 2 and 3, transcription from *Sst*I-cut pJB3 DNA (exposure was for 2 h at 20°C); lanes 4 and 5, transcription pattern with *Ava*I-cut pJB3 DNA (exposure was for 12 h at –70°C); lanes 6 and 7, transcription pattern with *Acc*I-cut pJB3 DNA (exposure was for 2 h at 20°C); lane 8, control reaction without added DNA (exposure was for 12 h at –70°C). The reactions shown in lanes 3, 5, and 7 contained 2 μ g of α -amanitin per ml. The bands in lanes 2, 4, and 6 that were absent in the corresponding α -amanitin control are marked by arrows, and their calculated sizes are indicated.

conclude that these RNAs are a nested set, each having its own promoter, with 3' termini that are close together. Because the coding sequence for gD is near the 5' end (47), this gD mRNA correspondingly has a trailer sequence of more than 1 kb in length. Neither mRNA is spliced near the 5' end. From the positions of the 5' ends of the two mRNAs and their sizes, we calculated that the 2.3-kb gD mRNA either terminates, or is reduced in size by splicing, near the 3' end. The demonstration of splicing (Fig. 5) now enables us to account for the 2.3-kb message without requiring transcription through its termination signal to synthesize the 1.5-kb message. The sizes of the mRNAs derived from transcription mapping agree well with those derived by Northern analysis described in this paper; a slightly larger size for gD mRNA (2.5 kb) was reported earlier from our laboratory (6). The 5' end of the 1.5-kb mRNA, whose translation product is unknown, is located approximately 100 bp downstream from the presumed translation termination site for the gD protein (47). These two proteins, therefore, do not share any of the same coding sequence.

The location of the 5' end for the gD mRNA was determined by precise S1 and *ExoVII* mapping data. The most likely initiation site for transcription (position +1) is an A flanked by pyrimidines, an arrangement that is a common feature of several herpesvirus genes (34; Wagner, in press). Examination of DNA sequences that are 5' to (upstream of) the transcription initiation sites for the gD RNA revealed typical eucaryotic regulatory sequences at the appropriate spacing: a TATA box at position -23 to -28 and a CAT box at -93 to -95 (Fig. 7). Also, this RNA has the AT-rich CAAT box at -69 to -77 and the AC-rich region farther upstream (-107 to -132 nucleotides) found for nine β mRNAs but not for γ mRNAs (Wagner, in press). Watson and co-workers (44, 47) place the gD mRNA initiation site upstream from the *HindIII* site, between nucleotides -25 to -33 on Fig. 7. Upstream of the initiation site presented by Watson et al. is an excellent TATA box at the correct location, but the other regulatory sequences are too close to the 5' end of the mRNA. We were unsuccessful in detecting a major RNA initiating upstream from the *HindIII* site, using a 3'-labeled *HindIII* fragment which had been used previously to probe the RNA blot (Fig. 2). This result, as well as the good agreement between the spacing of the regulatory sequences upstream of the gD gene with published data on other herpesvirus genes, led us to conclude that the gD message initiates downstream from the *HindIII* site (Fig. 7). In addition to the major regulatory signals, additional putative TATA boxes occur near the 5' ends of both

the 2.3- and 1.5-kb mRNAs. The multiplicity of putative regulatory sequences suggests some flexibility in promoter selection, which might account for our finding of several low-abundance RNAs having different 5' termini transcribed from this region of the genome.

The 293-31 cells used for the experiments described in this paper replicate HSV rapidly, with viral DNA synthesis and structural proteins being detected between 3 to 5 h after infection (39). There is, therefore, a rapid transition between the early and late stages in RNA synthesis; however, the 2.3-kb gD mRNA appears in the cytoplasm early in infection and reaches a high level after DNA synthesis starts, whereas the 1.5-kb mRNA appears later after infection. Preliminary examination of the DNA sequences upstream from the two RNAs fails to show differences that could account for the delay between the appearance of the 2.3-kb mRNA and the 1.5-kb mRNA. In addition, our *in vitro* runoff transcription studies showed that an uninfected cell extract initiates faithfully at both major promoters on this HSV-DNA fragment; further, the amounts of RNA synthesized indicate equal efficiency of utilization of the two promoters. Whether the differences in time of appearance observed for the 2.3- and 1.5-kb mRNAs in infected cells (Fig. 9) are due to activator or inhibitor factors not present in the cell extract remains to be explored. An alternative possibility is that both RNAs are transcribed with equal efficiency and that posttranscriptional processes control the time at which each of the mRNAs appears in the cytoplasm as an abundant species. We can detect traces of the 1.5-kb late RNA in cytoplasmic RNA extracted from cells infected for 1 and 2 h with HSV-1 and from cells infected with the *tsB2* mutant at 39°C, but only if exposure times for the autoradiographs are considerably longer. These observations suggest that the 1.5-kb RNA may not belong to the true late class as typified by the gC mRNA (16) but rather is an earlier RNA that accumulates at low levels early after infection and becomes amplified at later times. This situation would be consistent with reports that early genes are transcribed *in vitro* by uninfected cell polymerase (15, 34).

Our difficulty in detecting the 1.5-kb mRNA at early times after infection is probably due in part to the conditions used for S1 mapping. In these experiments, a large molar excess of ³²P-labeled DNA was used, so in a typical experiment, less than 1% of the input DNA becomes resistant to nuclease digestion. Even though the DNA is in excess, the time of hybridization is relatively brief, and therefore hybridization does not go to completion. Autoradiographs of the S1-resistant DNA, although reflecting RNA abundancies, do

not give a linear response to RNA concentration, and low-abundance RNA could be detected only after long hybridization times or overexposure of the gel to X-ray film.

Our RNA mapping data show that, in addition to the two major mRNAs, several less abundant RNAs are transcribed from the segment of the genome investigated (0.91 to 0.93 map units). All of the RNA species detected are transcribed from left to right and therefore compose two families of RNAs starting in different regions. Preliminary data (not shown) indicate that the transcripts from the DNA fragment to the right of the 3' ends of these mRNAs are also copied from left to right; it therefore appears that self-complementary cytoplasmic mRNA is not produced from this segment of the genome. These results are consistent with those obtained by Wagner and co-workers for other segments of the HSV-1 genome, for example, the region that encodes glycoprotein C (16). The presence of multiple promoters near the 5' end of a major message, as well as internal promoters, is a common feature, as is the presence of long trailer sequences following the sequences that code for the protein (2, 16, 17, 30). The long trailer sequences indicate that the signals for termination or polyadenylation or both are infrequent in HSV-infected cells. Transcription from both DNA strands, coupled with infrequent termination, would result in the high level of self-complementary RNA found in the nucleus after HSV infection (20, 22). We have not investigated the nuclear RNAs transcribed from the segment of the genome described in this paper but plan to do so in the future; we also plan to identify the polypeptide encoded by the 1.5-kb message.

Our experiments to date, although not uncovering new principles of how HSV mRNA synthesis is initiated, add to the accumulated information on HSV genome expression and demonstrate a role for RNA splicing in modifying transcript size. Preliminary data (not shown) suggest that this splicing pattern changes with time after infection. This type of detailed analysis of the transcripts from different regions of the genome should uncover the mechanism by which the large complex HSV genome is expressed in a coordinated way.

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