Uninfected cell polymerase efficiently transcribes early but not late herpes simplex virus type 1 mRNA


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ABSTRACT The sequences of the DNAs encoding the 5' ends of one early and one late herpes simplex virus type 1 mRNA were analyzed, and the 5' ends of these mRNA species were precisely located. Neither mRNA species is spliced and the noncoding strand of the DNA contains recognizable T-A-T-A and C-A-T boxes upstream from their respective 5' ends. The early mRNA was efficiently transcribed by a commercially available uninfected cell lysate system, but the late mRNA was not. This difference between early and late mRNAs appears to be general in this virus.

Gene expression during herpes simplex virus type 1 (HSV-1) replication is temporally regulated (1, 2; ref. therein). The early phase of gene expression takes place before viral DNA replication and most early viral mRNAs continue to be expressed throughout infection. The early phase of gene expression can be divided into two stages: the α or immediate-early stage, which occurs in the absence of de novo protein synthesis, and the β or true early stage, which requires the action of at least some α genes. The late stage of HSV-1 gene expression can also be divided into two stages: the β or "leaky" late stage, which can be marginally detected before viral DNA replication, and the γ late mRNAs, which can be detected only after viral DNA synthesis.

The question of what factors control the temporal regulation of DNA virus gene expression is an active field of research. The development of faithful uninfected cell transcription systems (3), as well as other techniques, has led to the following observations concerning this regulation in the smaller DNA viruses. In the papovaviruses (3, 4), the uninfected cell polymerase system favors the recognition of promoters for early genes. However, this preference is not absolute; at relatively high template DNA concentrations, the late promoters are efficiently recognized. In adeno virus (5), the situation is somewhat different in that the major late promoter appears to be readily recognized during the early phases of gene expression. Here, the appearance of abundant late mRNA involves changes in processing patterns for the late mRNA precursors.

We have recently reported the detailed characterization of a number of early and late HSV-1 mRNAs (6-8). Interestingly, although HSV-1 mRNAs share general properties of cellular mRNA, splicing of HSV-1 mRNAs is rare. To date, only two well-characterized viral mRNAs, one α (9) and one γ (8), have recognizable splices in them. Such a low incidence of splicing suggests that posttranscriptional processing of intervening sequences does not play a major role in the biogenesis of many HSV-1 mRNAs.

In this study, we have examined the nucleotide sequences of the DNAs encoding the 5' ends of two well-characterized major unspliced HSV-1 mRNAs (6, 7). The DNA sequences upstream of these mRNAs share features with the HSV-1 thymidine kinase (tk) gene (10, 11) and with many other eukaryotic mRNAs (12, 13). The promoter for the 5.2-kilobase (kb) early mRNA was readily recognizable by the "Manley" HeLa cell polymerase system (3), while that for the 6-kb late mRNA was not. Preliminary data indicate that this difference between early and late HSV-1 mRNA recognition is general.

MATERIALS AND METHODS

Cells and Virus. Monolayer cultures of HeLa cells were grown at 37°C in Eagle's minimal essential medium/10% calf serum without antibiotics. Plaque-purified virus of the KOS strain of HSV-1 was used for all infections.

Enzymes. All enzymes were obtained from Bethesda Research Laboratories (Rockville, MD) and digestion was carried out in buffers recommended by that supplier.

Isolation, Labeling, and Size Fractionation of Polyrribosomal RNA. Viral RNA synthesized in the absence of HSV-1 DNA synthesis (early RNA) was prepared as described (6). The drugs were a gift of C. Shipman of the University of Michigan.

Polyribosomes were isolated from the cytoplasm of HSV-1-infected cells by the magnesium precipitation method of Palmiter (14) as described (6-8). Details of the isolation of poly(A)+ RNA have been presented elsewhere (6, 7).

Recombinant DNA and Isolation of Restriction Fragments. All recombinant DNA experiments were covered under the January 1980 National Institutes of Health guidelines. Sal I fragment L (0.527-0.575), Hind III/Bam HI fragment A-N (0.259-0.269), and BamHI fragments A' (0.255-0.266) and F' (0.266-0.270) were cloned in pBR322 in our laboratory using Escherichia coli LE392. The procedure has been described (6, 7) and is essentially that of Bedbrook et al. (15).

Nuclease S1 Mapping of HSV-1 DNA. Our use of nuclease S1 and exonuclease VII to map RNA has been described (6-8) and is essentially the method of Berk and Sharp (16). DNA was 5' labeled with 32P (17). Samples containing 2 μg of DNA and 10 μg of infected cell polyribosomal poly(A+) RNA were hybridized in 50 μl of hybridization buffer/90% recrystallized formamide for 12 hr. When exonuclease VII digests were to be done, a sample containing 0.7 μg of DNA and 4.5 μg of RNA was hybridized separately in 15 μl of hybridization buffer. After nuclease digestion, samples were denatured by incu- bation at 37°C for 30 min in 0.1 M NaOH, neutralized, and fractionated by electrophoresis on Maxam and Gilbert sequence analysis gels (17).

DNA Sequence Analyses. DNA sequence analysis was done by the procedure of Maxam and Gilbert (17). Cloned DNA was 5' or 3' labeled with [32P]ATP and then cut with a second enzyme to allow isolation of unique end-labeled pieces. Sequenc-
ing gels were 30 cm by 80 cm by 0.5 mm. All sequences were done at least in duplicate.

**In Vitro Transcription.** We used a Manley (3) HeLa cell lysate transcription system purchased from Bethesda Research Laboratories. Transcription incubation was carried out in 50-μl volumes that contained 30 μl of HeLa cell lysate. The incubation mix was 15 mM Hepes, pH 7.9/75 mM KCl/10 mM MgCl2/1.5 mM dithiothreitol/1 mM creatine phosphate/210 μM EDTA/500 μM each ATP, GTP, and CTP/50 μM UTP. The mix also contained 10 μCi (1 Ci = 3.7 × 10^{10} becquerels) of [32P]UTP (400 Ci/mmol), 14% (vol/vol) glycerol, and 1.5–4 μg of suitably digested cloned HSV-1 DNA fragments. Incubation was for 60 min at 30°C. The RNA products were isolated by NaDodSO4/phenol extraction and fractionated by denaturing 1.2% agarose gel electrophoresis using 2 mM methylmercury hydroxide as described (6–8).

**RESULTS**

**Sequence Analysis Around the 5’ Ends of HSV-1 mRNA.** Convenient cleavage sites around the 5’ ends of the 5.2-kb early (β) and 6-kb late (βY) mRNAs are shown in Fig. 1. The 5’ ends of these mRNAs were close to cleavage sites so as to allow their very precise localization. Our approach was to 5’ label the DNA at a site just downstream from the 5’ end of the mRNA and use this for both sequence determination and nuclease S1 and exonuclease VII size analysis after hybridization with polyribosomal poly(A)^+ mRNA. The size of the hybrid-protected fragment, run along with the sequencing ladder, provided a precise localization for the 5’ ends of the mRNA in question.

Sol I fragment L (0.527–0.578) was digested with Xho I, and the 5’-end-labeled DNA was hybridized with both early and late HSV-1 poly(A)^+ mRNA. In both cases, the hybridized material yielded a late major band 195 bases long when digested with either nuclease S1 or exonuclease VII (Fig. 2A). Because we have previously shown that any splice in this mRNA must be within 50 bases of its 5’ end (6), this result demonstrates the lack of any splice in the mRNA. Further, the experiment located the 5’ end of this mRNA in the middle of the Kpn I site at 0.565 and sequence analysis was continued beyond this site by labeling DNA at this point. The nucleotide sequence of the non-coding strand of the HSV-1 DNA, starting 120 bases upstream of the 5’ start of the mRNA and extending to the first potential ATG translation start signal 235 bases downstream from the start of this mRNA, is shown in Fig. 3A. Of particular interest are the sequences A-T-A-A-A-A-A starting 27 bases upstream of the 5’ end of this mRNA and C-A-T-C-G-G-C-G-T starting 89 bases upstream of the 5’ end. Both the location of the T-A-T-A box and the C-A-T box are essentially the same number of bases upstream of the 5.2-kb mRNA start, as are similar sequences upstream from the 5’ start of the HSV-1 thymidine kinase (tk) mRNA (10, 11). In this latter gene, the sequences in question

![Fig. 2. Locations of the 5’ ends of the 5.2-kb early and 6-kb late HSV-1 mRNAs. (A) HSV-1 DNA Sol I fragment L was digested with Xho I, and the DNA was 5’ labeled with 32P at this site. Labeled DNA was processed for Maxam and Gilbert (17) sequence analysis, and the chains interrupted at G, A + G, C + T, and C residues were fractionated on an 80 cm by 30 cm by 0.5 mm 8% acrylamide gel. Aliquots of the DNA were also used to hybridize early and late polyribosomal poly(A)^+ mRNA and the hybrids were digested with nuclease S1 (S) or exonuclease VII X. Hybrid-protected DNA was fractionated along the sequence ladder. Residue numbers are counted from the precise 5’ end of the mRNA. (B) HSV-1 DNA HindIII/Bgl I fragment A - N (0.259–0.268; Fig. 1) was digested with BamHI, and the DNA was 5’ labeled with 32P at the BamHI site. Labeled DNA was used for sequence analysis and hybridization as in A. Lane X, DNA protected after exonuclease VII digest of the hybrids. This lane was more heavily exposed than the sequence lanes to emphasize the presence of the minor species nine bases shorter than the major one.**
are A-T-A-T-A-A and C-A-T-T-G-C-G-A. Interestingly, the sequence A-A-A-C-C-A-C-C-C is found 111 bases upstream from the 5' end of the 5.2-kb mRNA compared with the sequence A-A-A-C-C-C-G-C-C-C, which was found (10, 11) this far upstream from the 5' end of the HSV-1 tk gene.

The identity of the location of the 5' end of the 5.2-kb early mRNA, using both early and late poly(A) mRNA, indicated that there is no functional change in the promoter for this mRNA at these two stages of infection. It was also clear that there were other less intense bands of radioactivity 1 to 5 bases removed from the major hybrid band. Such "stutter" has been reported in the localization of the 5' end of the tk mRNA (10) and may be due to heterogeneity in the mRNA population or lack of specificity in the nuclease S1 and exonuclease VII digestions.

There is a significant amount of minor hybrid band migrating at 400 ± 50 bases larger than the major hybrid band (Fig. 2A). This too is reproducibly seen with early and late mRNA, and we have seen it when nuclear RNA was hybridized to this DNA
were seen. However, the most prominent band was -2000 bases long. This 2000-bases-long mRNA product was the size expected because the terminator codon TAG is found in the 5.2-kb early mRNA reported previously (7), indicated that there are no splices anywhere in the 5' end of this mRNA. An example of a typical experiment is shown in Fig. 2B. The nucleotide sequence of the DNA, 110 bases upstream from this 5' end, and extended to the second ATG triplet, which is a potential translation start signal 317 bases downstream from this 5' end, is shown in Fig. 3B. The first ATG triplet is found 13 bases downstream from the 5' end of the 6kb mRNA. This is not a potential translation start site of the major 155,000-dalton translation product of this mRNA because the terminator codon TAG is found in the sequence G-A-T-G-C-C-T-G-C is found 89 bases upstream of the 5' end of this mRNA, DNA 5' to the 5.2-kb early mRNA and the HSV-1 tk mRNA (10, 11) for us to identify it as a C-A-T box.

Some stutter was seen in the 5' end of the 6kb late mRNA as was seen in the localization of the 5.2-kb early mRNA. But interestingly, there is a significant amount of hybrid nine bases shorter than the major band. We suggest that this second band represents a subclass of the 6kb mRNA, which is nine bases shorter than the major one.

**Uninfected HeLa Cell Polymerase Efficiently Transcribes Early but Not Late HSV-1 mRNA.** We used a commercial Manley (3) transcription system to examine the ability of uninfected HeLa cell RNA polymerase to recognize HSV-1 promoters. We carried out most of our experiments with total cloned DNA. When Sal I fragment L (0.527-0.578) cloned in pBR322 was digested with Sal I and used as a transcription template, RNA products ranging in size from 1000 to 5000 bases were seen. However, the most prominent band was -2000 bases long (Fig. 4A, lane 1). Similar results were found using smaller amounts (1.5 µg) of template.

This 2000-bases-long mRNA product was the size expected if the uninfected cell polymerase recognized the promoter for the 5.2-kb early mRNA whose 5' end maps 2 kb to the left of the Sal I site at 0.578 (Fig. 1). We confirmed that this major 2000-base band was indeed due to the transcription of the early 5.2-kb mRNA from the following. (i) Xho I cuts the HSV-1 DNA at a site just 195 bases to the right of the 5' end of the 5.2-kb mRNA (Fig. 1). When Sal I fragment L digested with Xho I/Sal I was used as the transcription template, the 2000-base band was no longer found (Fig. 4A, lane 2). In the gels used, RNA smaller than -500 bases does not resolve, so we did not look for a band 195 bases long. (ii) Templates derived by digestion of Sal I fragment L with BamHI/Sal I yielded an mRNA product migrating with a size of 800 bases as expected (Fig. 4A, lane 3). (iii) We isolated purified Sal I fragment L by agarose gel electrophoresis of Sal I-digested cloned material. This template was eluted by phenol extraction (6, 7). When this was used as the transcription template, a major band of RNA 2000 bases long was synthesized (Fig. 4B). There was also considerable diffusely migrating material, which we feel was due to nicking of the template during its visualization and elution.

We found that the ability of HeLa cell polymerase to initiate transcription of early β mRNAs is not confined to this specific one. We have previously characterized an unspliced 1.8-kb early mRNA whose 5' end maps 1000 bases to the left of a Sal I site at 0.617 (8). In this region, there is a Hpa I site 200 bases upstream from this Sal I site (0.616). When the cloned HSV-1 DNA BamHI/Sal I fragment I - P (0.612-0.617) was cut with BamHI/Sal I and used as a transcription template, a major RNA product 1000 bases long was seen. When the template DNA was also cut with Hpa I, this band disappeared and an 800-base RNA product was found (data not shown). This confirmed the fact that the 1000-base RNA product was indeed due to transcription of the 1.8-kb early mRNA rightward toward the Sal I site at 0.617.

We have found no instance where the uninfected HeLa cell polymerase has initiated transcription of late mRNA with any detectable efficiency. We carefully examined the ability of the HSV-1 DNA HindIII/Bgl II fragment A · N (0.259-0.269)
cloned in pBR322 to serve as a transcription template for the uninfected cell polymerase and found no detectable activity. The 5' end of the 6-kb late (βγ) mRNA lies 950 bases upstream of the HindIII site at 0.259 and 68 bases upstream of the BamHI site at 0.266. When either HindIII-digested or HindIII/BamHI-digested DNA was used as the transcription template, a major band 1100 bases long was seen (Fig. 4C). Because no different RNA products were seen at all with the two different templates, it was clear that no transcription was proceeding leftward through the BamHI site at 0.266.

We confirmed the lack of efficient initiation of late transcription in Sal I fragment L. This region encodes two late mRNAs (6). One is a 7-kb (γ) mRNA whose 5' end is 3700 bases to the left of the Sal I site at 0.578, and the other is a 3.8-kb mRNA (βγ) whose 5' end is 1200 bases to the right of the Sal I site at 0.527. When Sal I-digested Sal I fragment L in pBR322 was used as the template for transcription and the RNA products were compared with those found when this DNA was digested with Sal I/Xho I, only one band was altered: the 2-kb RNA band, which was due to the transcription of the 5.2-kb early RNA (Fig. 4A, lanes 1 and 2). Also, we found no RNA products either 3700 or 1200 bases long when purified Sal I fragment L was used as transcription template (Fig. 4B). Further, when pBR322 itself was digested with Sal I and used as the transcription template, all the minor bands seen in Fig. 4A were present (Fig. 4D). Thus, all these bands were due to transcription initiation within the pBR322 DNA.

**DISCUSSION**

The ability of uninfected cell polymerase to efficiently transcribe early HSV-1 mRNAs is general, as shown by the data presented here for two early mRNAs and by the fact that the HSV-1 tk gene can be efficiently expressed when a clone bearing it is injected into nuclei of amphibian oocytes (18). These results are consistent with earlier data showing that RNA polymerase II can initiate transcription using HSV-1 DNA as a template (19, 20).

Thus, some aspects of the structures of the early promoters for HSV-1 genes must share features with those of cellular ones. Despite this inferred similarity between HSV-1 early promoters and cellular promoters, there are important differences between the structure of early HSV-1 and cellular genes. First, many of the HSV-1 mRNAs are not spliced; therefore, any requirement for splicing in the expression of cellular genes cannot be present here. Second, the HSV-1 (β) early genes are not efficiently expressed in cells in which the α or immediate-early genes are not expressed or are not functional (21-23). One specific 170,000-dalton α HSV-1 protein has been implicated as mediating this modulating effect (23).

The fact that we have not seen efficient transcription of βγ or γ late HSV-1 mRNAs using the commercial uninfected cell system does not mean that there is no low-level transcription but, if present, it must be of considerably lower efficiency than early transcription. The lack of efficient recognition of late HSV-1 genes by the polymerase may be due to unique features of the DNA sequence around late HSV-1 promoters compared with cellular and early promoters, as well as to the action of viral or cellular modulators during normal infection. Unique structural elements of late HSV-1 promoters may define them as "late" and become apparent when further sequence studies are carried out and the data are compared with the growing amount of information concerning similar areas for cellular genes (12, 13, 24).

If modulation is required for the recognition of late promoters, transcription studies using infected cell polymerase systems may give results different from those seen here.

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