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Detailed Characterization of an Apparently Unspliced β Herpes Simplex Virus Type 1 Gene Mapping in the Interior of Another

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We precisely localized the coding region and determined the nucleotide sequence of a 1.2-kilobase β herpes simplex virus type 1 mRNA which underlies the 3′ region of the 5.2-kilobase β mRNA mapping in *Hin*dIII fragment K. This mRNA, which lacks readily detectable splices, has its own promoter by the criteria of identification of putative herpes simplex virus type 1 control sequences and in vitro transcription by a Manley polymerase system.

Previously, we characterized the major mRNA species of herpes simplex virus type 1 (HSV-1) mapping in *Hin*dIII fragments K (0.527 to 0.592) and L (0.592 to 0.647) and *Eco*RI fragment I (0.633 to 0.721) (1, 9, 11a). Many of these mRNAs overlap each other. As a good example, consider the 6.9-, 5.2-, 1.7-, and 1.2-kilobase (kb) mRNAs mapping between 0.555 and 0.600 in *Hin*dIII fragments K and L. The 5.2-kb (β) mRNA has its 5′ end in the interior of the 6.9-kb (γ) mRNA juxtaposed to the 3′ end of the 1.7-kb (γ) mRNA (see Fig. 1A). The 6.9-kb mRNA, then, is a result of an inefficient transcription termination at the end of the "gene" for the 1.7-kb mRNA. We previously showed that the promoter for the 5.2-kb mRNA maps just upstream of the 5′ end of this mRNA and shares certain features with the promoter of another β HSV-1 mRNA, thymidine kinase (tk). The 5′ end of the 1.2-kb β mRNA underlying the 3′ end of the 6.9- and 5.2-kb mRNAs could be generated in the same way or could result from using the 5.2-kb mRNA promoter and splicing. As shown in this note, the region just upstream of the 5′ end of the 1.2-kb mRNA has sequence similarities to the other HSV-1 promoters previously characterized. Further, transcription of this mRNA can be initiated by using a Manley uninfected cell lysate transcription system (10, 13), indicating that this mRNA is under its own promoter control and lacks detectable splicing.

We located the 5′ end of the 1.2-kb (β) mRNA to be approximately 300 bases upstream of the *Hin*dIII site at 0.592 and the common 3′ end of all three mRNAs to be 900 bases downstream of this site (1). A high-resolution restriction map of the region in question is shown in Fig. 1B. Here, *Hin*fI, *Sal*I, *Ava*I, *Hin*dIII, *Pvu*II, and *Dde*I sites are indicated. We used two HSV-1 DNA clones for these studies, *Bam*HI-*Hin*dIII fragment O-K (0.576 to 0.592) and *Hin*dIII-*Bam*HI fragment L-O (0.592 to 0.602). Details of our cloning procedures were described elsewhere (1, 8). We located the 5′ end of the 1.2-kb mRNA by S1 nuclease digestion of hybrids between the *Sal*I-*Ava*I fragment 5′ end labeled at the *Ava*I site and infected-cell polyadenylated mRNA (viral mRNA). The basic details of isolation of viral mRNA via the use of the Palmiter Mg^{2+} precipitation of polyribosomes has been described previously (1, 18). We also have described our methods for end labeling viral DNA fragments (9, 10, 15). In this case, *Bam*HI-*Hin*dIII fragment O-K was digested with *Ava*I, 5′ end labeled, and redigested with *Sal*I, and the labeled fragment was strand separated on a nondenaturing acrylamide gel as described by Maxam and Gilbert (15). S1 nuclease digestion of hybrids gave two distinct bands (Fig. 2A); one was 370 bases long, owing to protection of the full length of the DNA by the 6.9- and 5.2-kb mRNAs, and one was 250 bases long, owing to protection by the 1.2-kb mRNA. The relative intensity of the two bands (Fig. 2A) provides a good measure of the abundance of the 1.2-kb mRNA relative to the two larger species. We precisely located the 5′ end of the mRNA to be 251 bases upstream from the *Ava*I site (data not shown) by running the S1 nuclease-protected fragment against a Maxam-Gilbert sequence ladder. The 5′ end was located in the sequence TGTACT at the A residue (see sequence data below). We similarly located the 3′ end of the three colinear mRNAs to be approximately 100 bases downstream (3′) of the rightmost *Dde*I site by using strand-separated DNA from *Hin*dIII-*Bam*HI fragment L-O (0.592 to 0.602) 3′-labeled at the *Dde*I site. Details of 3′ end labeling have been described

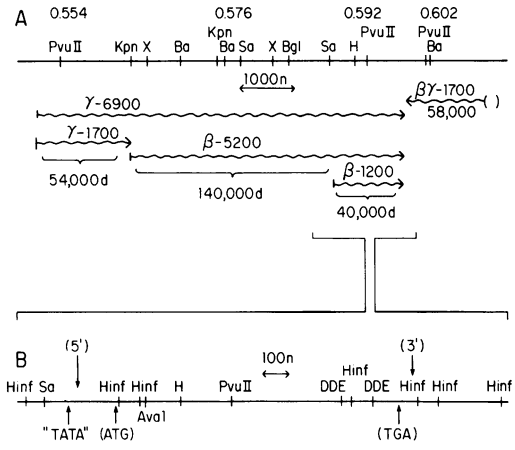

FIG. 1. (A) Schematic localization of mRNA species mapping between 0.567 and 0.620 on the P arrangement of the HSV-1 genome. Values are based on S1 nuclease analysis and in vitro translational studies (1). Arrows indicate direction of transcription and approximate 5′ and 3′ termini of mRNA species. The total length of the mRNAs and their relative times of appearance are shown above the arrows, and the size of the polypeptide encoded by each species is shown below the bracket. (B) High-resolution map of the HSV-1 region (0.587 to 0.602) encoding the 1,200-base β mRNA, with flanking regions. Specific sites are based on results described in the text. Note (i) the location of the 5′ and 3′ termini of the 1,200-base mRNA, (ii) the presence of the canonical TATA box, and (iii) the putative site of the initiation (ATG) and termination (TGA) codons of the 40,000-dalton polypeptide encoded by this mRNA. The restriction sites used to derive the sequence found in Fig. 3 are shown; enzymes used were *Hin*fI (Hinf), *Sal*I (Sa), *Ava*I, *Hin*dIII (H), *Pvu*II, and *Dde*I (DDE).

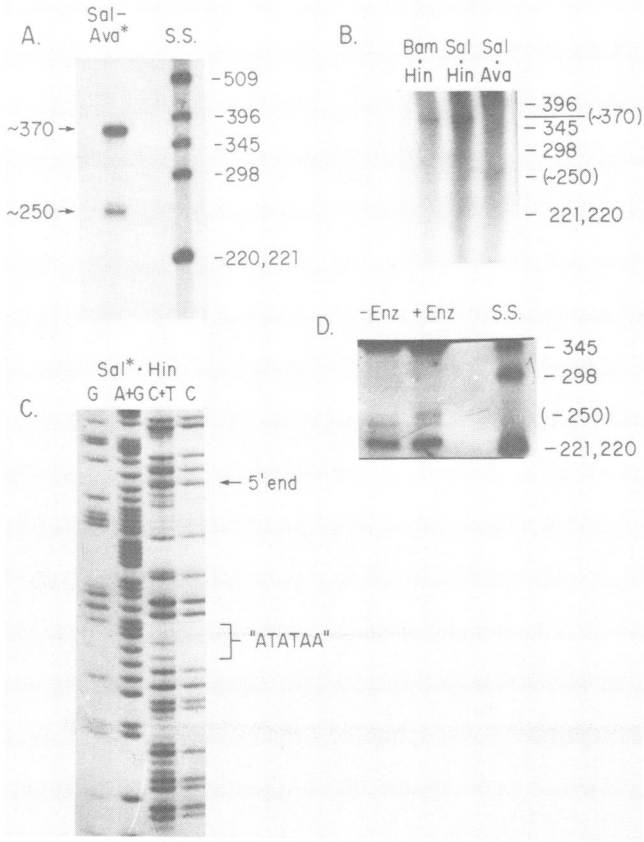

FIG. 2. (A) S1 nuclease analysis of mRNA species mapping proximal to the 5′ terminus of the 1,200-base β mRNA of HSV-1. Five micrograms of the HSV-1 clone *Bam*HI-*Hin*dIII fragment O-K (0.576 to 0.592) was digested with *Ava*I, 5′ labeled with [^{32}P]ATP, redigested with *Sal*I, and electrophoresed on a 6% acrylamide gel by the method of Maxam and Gilbert (15). The 370-base piece representing the HSV-1 region of 0.588 to 0.591 was further purified, after denaturation, by electrophoresis on a nondenaturing 5% acrylamide (1:50 cross-link) gel. Samples containing the resultant 370-base single strand of DNA and 10 μg of viral polyadenylated RNA were hybridized in 50 μl of hybridization buffer (80% formamide, 0.4 M Na^+, 0.1 M HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 8.0], 0.005 M EDTA) for 16 h. S1 nuclease analysis was done essentially by the method of Berk and Sharp as described previously (4–6, 9). After nuclease digestion, samples were

previously (9; data not shown). The DNA fragment ranging between the second *Dde*I site and the *Bam*HI site at 0.602 was separated from a digest by using a nondenaturing 6% acrylamide fragment separation gel as described by Maxam and Gilbert (15).

The precise localization of the 5′ end of the 1.2-kb (β) mRNA allowed us to examine the nucleotide sequence upstream of it (see below) and suggested that this mRNA is unspliced, since a TATA box and a CAT box sequence could be identified. Uninfected-cell RNA polymerase II recognizes the promoter for the β tk gene, since this gene is expressed in biochemically transformed cells and in micro-injected amphibian oocytes (17, 24). The early (β) alkaline exonuclease of HSV-1 is similarly expressible in amphibian oocytes (19), and we suggested that the ability of uninfected-cell polymerase to recognize β promoters is general, since we found that the Manley polymerase recognized major β promoters mapping in *Hin*dIII fragments K and L (10). The situation with the Manley system is not absolutely clear, since several workers have reported that multiple transcription initiation sites are seen when HSV-1 *Bam*HI fragment P (0.298 to 0.318), which encodes tk, is used as a template. Our rather more clear-cut results suggested that we can use the uninfected system to identify early HSV-1 promoters.

We used three templates in a transcription runoff experiment (10, 21) to determine whether accurate initiation at the 5′ end of the 1.2-kb mRNA occurred. We used a commercial system, in a 50-μl total volume, with α-[^{32}P]UTP (400 Ci/mmol; Amersham Corp.) and 4 μg of template made by appropriately digesting the *Bam*HI-*Hin*dIII fragment O-K (0.576 to 0.592) in the pBR322 vector. Use of template formed by digestion with *Bam*HI and *Hin*dIII gave a radioactive band 370 bases long (Fig. 2B, upper panel), a size expected from the position of the 5′ end of the 1.2-kb mRNA (371 bases upstream of the *Hin*dIII site; see below). This same band was seen when the template was cut with both *Sal*I and *Bam*HI-*Hin*dIII, suggesting that any recognition sites for polymerase are between the *Sal*I site (120 bases upstream of the 5′ end of the mRNA; see below) and the 5′ end of the mRNA. When the template was formed by digestion of the template with *Ava*I, *Bam*HI, and *Hin*dIII, a product 250 bases long was seen. Several other size bands were also seen, notably one about 150 bases long and one 600 bases long; these sizes were unaffected by the enzymes used to cut the template, and we suggest they were due to the presence of promoters on the pBR portion of the

suspended in 30 μl of 98% formamide–10 mM HEPES (pH 8.0), heat denatured at 95°C for 2 min, and electrophoresed in a 5% acrylamide (1:40 cross-link), 8 M urea gel. The results of such an analysis are shown in the column marked Sal-Ava*. Size standard (S.S.) was prepared by digesting 5 μg of pBR322 with *Hin*fI and *Eco*RI restriction endonucleases and subsequent 5′ labeling with [^{32}P]ATP. Marker DNA was also denatured before electrophoresis. Numbers represent sizes of DNA fragments in nucleotides. Exposure was overnight without intensifying screens. (B) In vitro transcription, using the HSV-1 clone *Bam*HI-*Hin*dIII fragment O-K as a template. Four micrograms of the HSV-1 clone *Bam*HI-*Hin*dIII fragment O-K was suitably digested and used as a template in transcriptional runoff experiments, using a Manley HeLa cell lysate system (13). Incubations in a 50-μl total volume were performed as described previously (10). RNA was isolated and analyzed by electrophoresis at 600 V for 2.5 h on denaturing 5% acrylamide (40:1), 8 M urea gels (1.8 by 40 by 200 mm). Column 1, Template DNA digested with *Bam*HI and *Hin*dIII; column 2, template DNA digested with *Bam*HI, *Sal*I, and *Hin*dIII; column 3, template DNA digested with *Bam*HI, *Ava*I, and *Hin*dIII. Size markers are based on comigration with 5′ end-labeled fragments produced by *Hin*fI digestion of pBR322. The 250- and 370-base RNA transcription products are indicated. Exposure was for 5 days with intensifying screens (22). (C) Analysis of HSV-1 DNA sequence proximal to the 5′ end of the 1.2-kb β mRNA. Ten micrograms of the HSV-1 clone *Bam*HI-*Hin*dIII fragment O-K was digested with *Sal*I and *Hin*dIII. The 490-base DNA fragment was 5′ end labeled, strand separated, and sequenced from both ends as descried by Maxam and Gilbert (15). Chains interrupted at G, G+A, C+T, and C residues were fractionated on an 8% acrylamide gel (80 by 30 cm by 0.5 mm). The sequence shown represents a portion of the strand which was labeled at the *Sal*I site (0.588) and proceeds through the 5′ end of the 1.2-kb mRNA toward the *Hin*dIII site. Note the presence of the ATATAA sequence 29 bases 5′ to the transcription initiation site of the mRNA. (D) Hybridization of unlabeled in vitro transcription product to the coding strand of HSV-1 DNA. In vitro transcriptions were performed as described above, except equimolar concentrations (0.5 mM) of all four base triphosphates were used, and no labeled nucleotide was present. Template was 4 μg of cloned HSV-1 DNA from *Bam*HI-*Hin*dIII fragment O-K digested with *Bam*HI and *Hin*dIII. Thirty microliters of dilution buffer (10) was substituted for the HeLa cell lysate in the "no enzyme" reactions. Transcription products were purified from template DNA as described in the text. After extensive dialysis, each set of transcription products was hybridized with 5-μg equivalents of the single-stranded, 5′-labeled, 370-base HSV-1 (*Ava*I-*Sal*I) DNA fragment described in the legend to Fig. 2A. Hybridizations were carried out in 10 μl of aqueous buffer (0.2 M Na^+–0.1 M HEPES [pH 8.0]–0.005 M EDTA) in sealed capillary tubes at 65°C for 16 h. Reactions were quenched by dilution into 400 μl of S1 buffer, and S1 nuclease digestion was performed (1, 9). Products of the digestion were denatured and analyzed on denaturing gels as shown in panel B. 5′ End-labeled fragments of *Hin*fI-digested pBR322 were used as size standards. Exposure was for 4 days with intensifying screens (22).

template, such as those which have been described previously (10).

We confirmed the synthesis of an RNA product with a 5′ end very near the location of the 1.2-kb mRNA by using S1 nuclease analysis of in vitro transcription products (Fig. 2D). The Manley polymerase lysate was incubated with *Bam*HI-*Hin*dIII-digested template and unlabeled base triphosphates (0.5 mM each); a second incubation with no enzymes was carried out as a control. Radioactive carrier RNA (100,000 ^{32}P cpm; 10 μg) was added to the products of reaction, and the RNA was separated from the template by 36 h of centrifugation in a 1-ml CaCl gradient of a starting density of 1.6 g/cm^3. Centrifugation was carried out in an SW60 Ti rotor at 50,000 rpm at 17°C. These conditions were found adequate to remove essentially all of the template DNA from the RNA pellet in separate experiments. The pelleted RNA was redissolved in water, dialyzed versus 0.1 M NaCl–0.01 M Tris (pH 7.4)–0.001 M EDTA, and then hybridized under aqueous conditions with strand-separated HSV-1 DNA 5′ end labeled at the *Ava*I site as described above and in the legend to Fig. 2D. Hybridization for 18 h yielded several S1-resistant bands, caused by the structure of the radiolabeled DNA probe, as well as some undigested material, as shown by the bands of 220, 240, and 370 bases in both the control and experimental tracks. However, a band of 250 bases was consistently seen in the enzyme incubation but was missing in the control. We concluded that this was owing to synthesis of RNA initiating 250 bases upstream of the *Ava*I site (1).

The in vitro transcription data indicated that the 1.2-kb mRNA has a functional promoter within 120 bases upstream of its 5′ end. We carried out Maxam-Gilbert DNA sequence analysis of the DNA encoding this mRNA. We sequenced both strands of DNA end labeled at the restriction sites indicated in Fig. 1B. The methodology in which strand-separated DNA was used was exactly as described by Maxam and Gilbert and previously by us (10, 15). An example of the sequence data proceeding downstream from the *Sal*I site at 0.588 is shown in Fig. 2C. The sequence is of the same sense as the mRNA, and the sequence ATATAA starting 29 bases upstream of the 5′ end of the mRNA is indicated. The full mRNA sense sequence of the DNA beginning 313 bases upstream of the 5′ end of the 1.2-kb mRNA and going 1,244 bases downstream is shown in Fig. 3.

The 5′ end of the mRNA is, as noted, 29 bases downstream from a putative TATA box sequence. The sequence TCAC is seen 90 bases upstream. In the 5.2-kb β mRNA, the sequence ACATC is seen at −90 bases (10), and in tk, the sequence TCATT is seen at −88 bases (16, 23). We suggest that this is a CAT box sequence (2, 3, 14). More significantly, the region between −110 and −97 is mainly A's and C's, which was also seen in the two other β mRNAs characterized, but not with βγ or γ mRNAs. Further comparative studies in progress will indicate how significant and general this finding is.

The only translation start signal in the mRNA sequence is seen at position 151. The sequence around this translation start (GCCATGG) is a favored one for eucaryotic translation starts (12). The reading frame defined by this start signal is open for 1,017 bases, defining a 339 amino acid polypeptide whose predicted composition would give a molecular weight of 37,970, a value in good agreement with our in vitro translation value of 40,000.

The 3′ end of the 6.9-, 5.2-, and 1.2-kb mRNAs is very near the sequence ATAATAAA. The sequence ATAAAA is found 69 bases downstream of this sequence (data not shown), and the sequence TAATTTTATT is downstream another 60 bases from that (data not shown). Since this region encodes the 3′ end of mRNAs on both strands, we must regard it as an efficient polyadenylation region. The sequence AATAAAA has been implicated as the 3′ stop signal of both HSV-1 tk (16, 23) and other eucaryotic mRNAs (20). Whether the departure from this nominal sequence seen in the present case is significant is as yet unknown, but AT-rich regions are seen in the area encoding the 3′ ends of several HSV-1 mRNAs around the *Hin*dIII site at 0.647 (unpublished data), so the general character of the polyadenylation signal seems well established.

In light of the fact that a similar size polypeptide immunologically cross-reacting with a 140,000-dalton HSV-2 polypeptide is encoded by the analogous region of HSV-2 and can be detected in some HSV-2-transformed cells (11), it is interesting to ask what the relationship is between the HSV-2 polypeptides and the one encoded by HSV-1. The following facts are clear: in HSV-1, there is no open reading frame upstream of the 5′ end of the 1.2-kb mRNA that would reasonably allow an in-phase fusion protein to be synthesized from the 5.2-kb mRNA. The frame that is used as the phase for the reading of the 1.2-kb mRNA is terminated many times upstream of the translation initiator in the mRNA. Similar results have been reported by Clements and McLauchlan (7). Another area of difference between HSV-1 and HSV-2 is that the 1.2-kb mRNA in HSV-2 appears to be a major mRNA (11), whereas the protein analogous to the 140,000-dalton product of the major 5.2-kb β HSV-1 mRNA is not readily seen, and then only late. Therefore, some interesting differences in

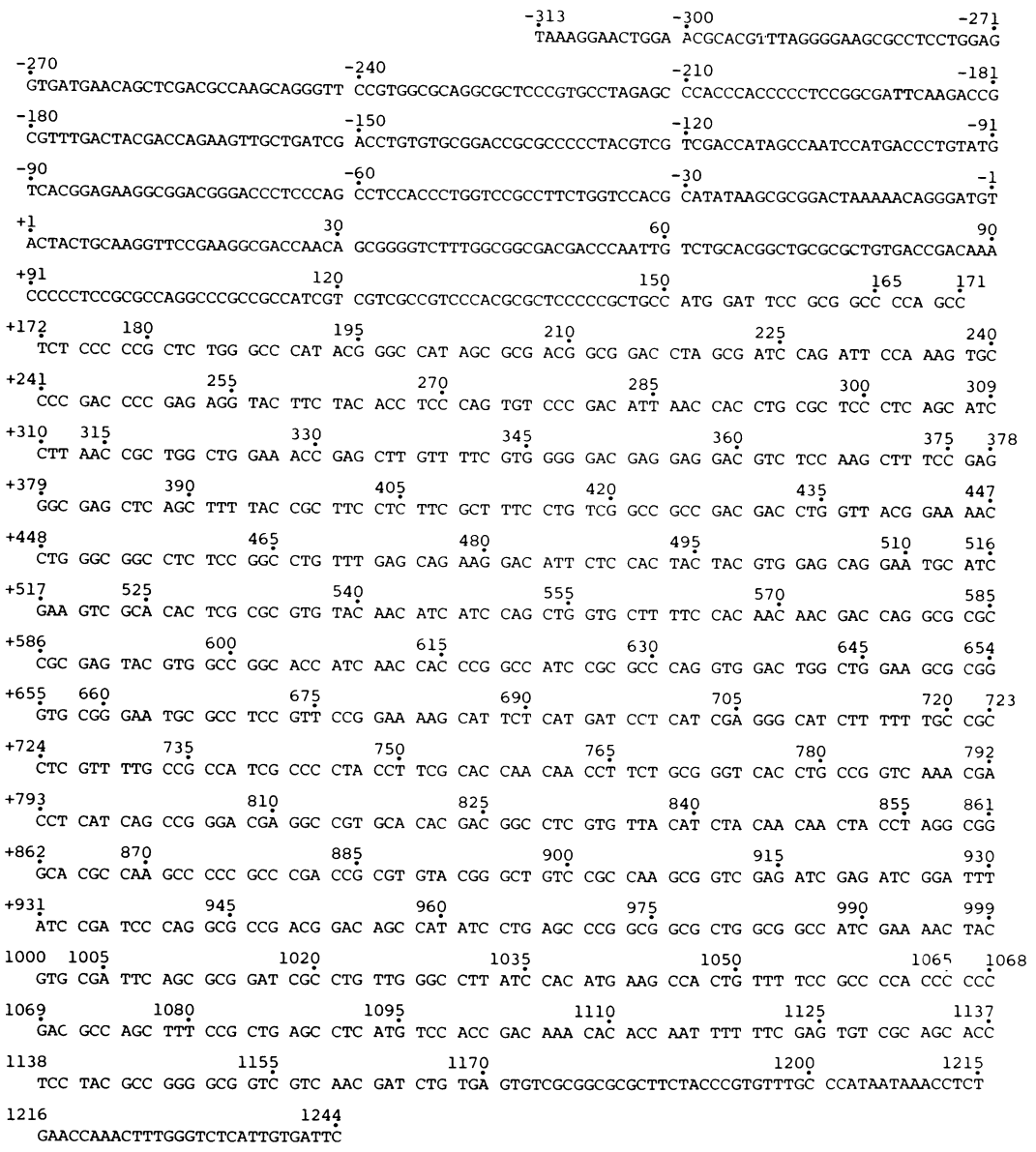

FIG. 3. Nucleotide sequence of the noncoding strand of HSV-1 DNA encoding the 1,200-base β mRNA and its 5′ and 3′ flanking regions. DNA sequence analysis was performed by the procedure of Maxam and Gilbert (15). Cloned DNA was end labeled with [^{32}P]ATP, and isolated strands of DNA were then sequenced, using gels (30 by 80 cm by 0.5 mm). All sequences were done at least in duplicate, and both DNA strands were sequenced. The sequence from 313 bases upstream of the 5′ end of the mRNA to 1,244 bases downstream is shown for the mRNA sense strand. As discussed in the text, the putative CAT box signal is at position −90, the AC-rich region is at −112 through −104, and the TATA box is between −29 and −24. The translation termination codon TAA is seen at −313 and defines reading frame 1. Other terminator sequences in this frame are seen at positions 201, 285, 312, 471, 693, and 855. Translation termination codons in frame 2 are seen at positions −290, −216, −156, −102, and −15. The frame is opened with the AUG (ATG) codon at position 151 and closed again at positions 1168, 1204, and 1207. This is the reading frame for the encoded polypeptide whose molecular weight is 37,970, based on its calculated amino acid composition. The third potential reading frame has translation terminators at positions −269, −266, −176, −113, −26, +80, +218, +965, +1040, and +1085. The restriction sites indicated in Fig. 1B are as follows: *Hin*fI sites (GANTC) are at positions −192, +154, +228, +1004, and +1240; the *Sal*I site (GTCGAC) is at −121, the *Ava*I site (CPyCGPuG) is at position +247; the *Hin*dIII site (AAGCTT) is at position +367; the *Pvu*II site (CAGCTG) is at position +550; and *Dde*I sites (CTNAG) are at positions +964 and +1084.

temporal control may be operating in the two infectious cycles.

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