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Expression of a Human Cytomegalovirus Late Gene Is Posttranscriptionally Regulated by a 3′-End-Processing Event Occurring Exclusively Late after Infection

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A phenomenon of posttranscriptional regulation has been previously identified in cytomegalovirus-infected human fibroblast cells (Wathen and Stinski, J. Virol. 41:462, 1982). A region typifying this phenomenon has been located within the large unique component of the viral genome (map units 0.408 to 0.423). Even though this transcriptional unit was highly transcribed at early times after infection, mRNAs from this region were only detectable on the polyribosomes after viral DNA replication. Thus, this region is believed to code for a late gene. Single-strand-specific nuclease mapping experiments of viral transcripts established that the transcriptional initiation sites and the 5′ ends of a downstream exon were identical at early and late times. However, the late transcripts differed from the early transcripts by the processing of the 3′ end of the viral RNAs. This involved either the removal of a distinct region of the transcript by the selection of an upstream cleavage and polyadenylation site or the differential splicing of the RNA molecule. The upstream cleavage and polyadenylation site was identified by nuclease mapping analyses and DNA sequencing. The 3′-end processing of these transcripts is necessary for the detection of these viral RNAs within the cytoplasm of the infected cell. We propose that human cytomegalovirus either codes for a factor(s) that is involved in the 3′-end-processing event at late times after infection or stimulates the synthesis of a host cell factor(s) involved in this complex regulatory event. This level of regulation may have an influence on the types of cells that permit productive cytomegalovirus replication.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is the etiologic agent of a variety of human disorders, including HCMV mononucleosis syndrome, interstitial pneumonia, and cytomegalic inclusion disease. The association of HCMV with Kaposi's sarcoma (6, 35) and the ability of defined regions of the genome to transform cells in culture (6, 8, 36) demonstrate other interactions that may occur between the virus and the host cell.

The genome of HCMV, a linear double-stranded DNA molecule of approximately 240 kilobase pairs (kb) (14), is composed of a long unique (U_L) and a short unique (U_S) region, with both regions being bound by inverted repeats (26). HCMV, like other herpesviruses, exhibits a sequential pattern of gene expression (12, 15, 22, 31, 63). The immediate-early genes are expressed in the absence of preceding viral protein synthesis. The immediate-early transcripts arise from a few distinct regions of the genome. Within the region of highest transcriptional activity, there exist three transcription units whose RNA and protein products have been investigated in detail (12, 24, 31, 47, 48, 51, 53, 62). After the immediate-early proteins have been synthesized, there is a switch from restrictive to extensive transcription of the genome (15, 31, 62, 63). The transcripts synthesized at this time represent both early and late genes. Synthesis of early-gene mRNAs and the appearance of their protein products occur prior to viral DNA replication. In contrast, expression of the late viral genes in terms of mRNA in the cytoplasm and the translation of the viral protein are dependent on replication of the viral genome (49, 62).

Expression of many of the HCMV transcripts appears to be regulated by a posttranscriptional mechanism(s) (13, 18, 62; Martinez and St. Jeor, personal communication). Even though the HCMV genome is extensively transcribed at early times after infection (13, 15, 62), only a minimal number of these transcripts are present on the polyribosomes or in the cytoplasm (13, 18, 62). In contrast, at late times transcripts from all regions of the genome are present on the polysomes or in the cytoplasm in varying abundance (13, 62). Therefore, we hypothesize that a posttranscriptional mechanism(s) is affecting either the transport of numerous viral transcripts synthesized at early times after infection or the steady-state levels of these mRNAs in the cytoplasm.

This paper describes a posttranscriptional phenomenon in HCMV-infected cells related to the nuclear retention or cytoplasmic stability of viral transcripts at early times after infection, i.e., prior to viral DNA replication. A region typifying this pattern of regulation was localized to the large unique component of the genome within the *Xba*I-B restriction fragment between 0.408 and 0.423 map units. The transcripts originating within the large repeat sequence of the viral genome, within the *Bam*HI-S restriction fragment between 0.805 to 0.809 and 0.814 to 0.823 map units, were selected as controls. Transcripts from this reiterated region of the viral genome were not retained in the nucleus and consequently were present on the polysomes at both early and late times after infection (13, 62).

Northern blot hybridizations and single-strand-specific nuclease mapping analyses were used to define the structure of the transcripts originating within *Xba*I-B at map units 0.408 to 0.423 at both early and late times postinfection (p.i.). We detected posttranscriptional processing events that occurred only after viral DNA replication. These 3′-end-processing events correlated with the detection of these mRNA species within the cytoplasm of the infected cell. Posttranscriptional regulation at the mRNA processing level

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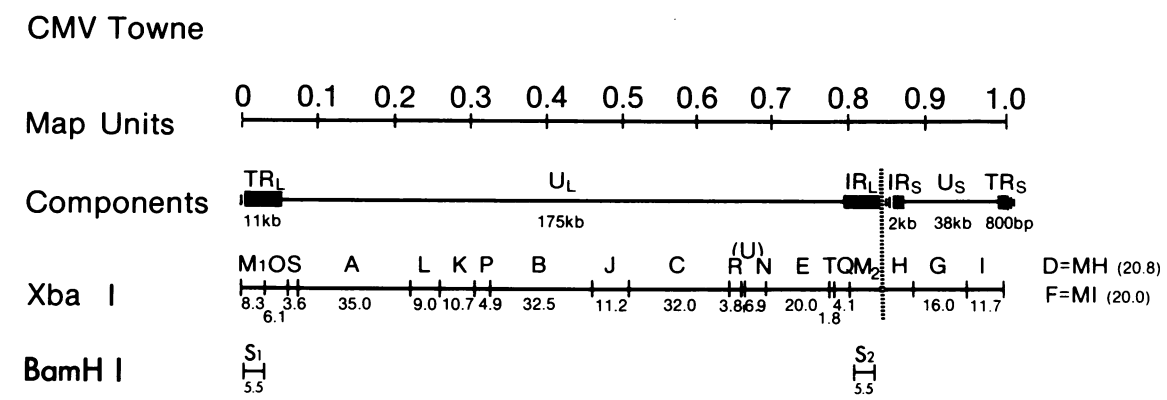


FIG. 1. Physical map of the HCMV (Towne) genome according to the prototype arrangement. The HCMV genome comprises a long unique (U_L) and a short unique (U_S) component bounded by inverted (IR) and terminal (TR) repeats. The total genomic length is 240 kb. The sizes (in kilobases) for each section of the genome are designated. The *Bam*HI-S restriction fragment is located within the *Xba*I-D restriction fragment and is part of the large repeat sequence of the genome.

and its relationship to productive HCMV replication are discussed.

MATERIALS AND METHODS

Virus and tissue culture. The growth of human foreskin fibroblast cells and the propagation of HCMV (Towne) have been described previously (50).

Recombinant plasmid DNA and physical map of *Xba*I-B. The cloning and purification of HCMV recombinant plasmids have been described previously (58). Recombinant plasmid pCB9, containing the *Bam*HI-S restriction fragment, was a gift from R. LaFemina and G. Hayward. The physical map of *Xba*I-D was previously determined by LaFemina and Hayward (26). The *Xba*I-B restriction fragment was mapped by the partial digestion method of Smith and Birnstiel (44), in combination with double restriction enzyme digestion. Restriction enzymes, as well as other enzymes used in this study, were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), New England Biolabs (Beverly, Mass.), and Boehringer Mannheim Biochemicals (Indianapolis, Ind.). The conditions for enzyme use were those recommended by the supplier. The *Xba*I-B restriction fragment was subcloned as *Eco*RI or *Sal*I restriction fragments as described previously (58).

Viral infection and RNA pulse-labeling. Human foreskin fibroblasts were infected with HCMV at a multiplicity of 10 to 20 PFU/cell. To label early RNA, cells were treated with 200 μg of phosphonoacetic acid (PAA) per ml at 2 h p.i. Cells were pulse-labeled from 12 to 24 h p.i. with 1 mCi of $^{32}P_i$ (Amersham Corp., Arlington Heights, Ill.) per ml in phosphate-free Eagle medium containing 2% dialyzed fetal calf serum. To label late RNA, cells were pulse-labeled as above, except that the labeling period was from 60 to 72 h p.i. in the absence of PAA.

Isolation of RNA. All reagents, plasticware, and glassware were treated with 0.1% diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.) and autoclaved before use. Polysome-associated RNA was isolated by the magnesium precipitation method of Palmiter (37, 62). Whole-cell RNA was isolated by the guanidine hydrochloride-CsCl gradient procedure as described previously (63). Nuclear and cytoplasmic RNAs were isolated by a modification of the procedure of Craig and Raskas (10, 62). Polyadenylated [poly(A)$^+$] RNA was selected from total polysome-associated or total cell RNA by oligodeoxythymidylic acid-cellulose chromatography (Collaborative Research, Inc., Waltham, Mass.) as described previously (63).

Southern blot hybridization. *Xba*I-B DNA was digested with various restriction enzymes (*Bam*HI, *Eco*RI, *Pst*I, and *Xho*I) plus *Xba*I, and *Xba*I-D was digested with either *Bam*HI or *Eco*RI plus *Xba*I, and the resulting fragments were fractionated on 1.0% agarose gels by the procedure of Bachi and Arber (2). The DNA was then transferred to nitrocellulose paper by the procedure of Southern (46). Each blot was pretreated at 42°C for 24 h in prehybridization buffer containing 50% formamide, 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.08% Denhardt reagent (0.08% each bovine serum albumin, Ficoll, and polyvinylpyrrolidine), and 200 μg each of denatured calf thymus DNA and *Saccharomyces cerevisiae* soluble RNA per ml. For the early and late whole-cell blots, 50×10^6 cpm of ^{32}P-labeled early or late whole-cell RNA was heated to 100°C for 2 min and then added to the hybridization buffer as prepared above, except that the concentration of Denhardt reagent was 0.04%. Hybridization was performed at 42°C for 48 h. For the hybridization-competition, the blots were hybridized with 43 μg of unlabeled early whole-cell RNA as described above. After 48 h of hybridization, the blots were hybridized for an additional 48 h with 50×10^6 cpm of ^{32}P-labeled late whole-cell RNA. All blots were washed as previously described (62). Hybridization of virus-specific RNA to the blots was detected by autoradiography with X-Omat XAR-5 film (Eastman Kodak Co., Rochester, N.Y.). The amount of ^{32}P-labeled virus-specific RNA that hybridized to the blotted DNA of *Xba*I-B and *Xba*I-D was determined by scanning with a Beckman DU-8 spectrophotometer with the DU-8 gel-scanning Compuset module.

Preparation of radioactive probes for Northern and dot blot analyses. Recombinant plasmid DNA fragments were labeled with [α-^{32}P]dCTP by nick translation by the procedure of Rigby et al. (40). A radioactively labeled cDNA probe was prepared from cellular polysome-associated poly(A)$^+$ RNA primed with calf thymus DNA and oligo(dT) by reverse transcriptase (avian myeloblastosis virus) as described previously (53).

Dot blot hybridizations. Total nuclear or polysome-associated poly(A)$^+$ RNA, isolated from cells at various times p.i., was serially diluted from 500 to 8 ng and immobilized in duplicate onto nitrocellulose paper by the procedure of White and Bancroft (64). The filters were pretreated as described above and hybridized with ^{32}P-labeled nick-

translated probes or ^{32}P-labeled cDNA synthesized from uninfected cell poly(A)$^+$ RNA. The hybridization conditions were the same as above, except that 5× SSPE was used in place of 4× SSC. The blots were washed four times for 5-min periods with 2× SSC–0.1% sodium dodecyl sulfate (SDS) at room temperature and three times for 15-min periods with 0.1× SSC–0.1% SDS at 50°C. Detection of the amount of hybridization and scanning of the autoradiograms were performed as described above. Duplicate dots were averaged, and the amount of hybridization was quantitated.

Northern blot hybridization. Total whole-cell, poly(A)$^+$ whole-cell, or cytoplasmic RNA was fractionated in a 1.5% denaturing agarose gel containing 2.2 M formaldehyde by the method of Lehrach et al. (27). *Escherichia coli* 23S (3.3 kb) and 16S (1.7 kb) rRNAs (P-L Biochemicals, Inc., Milwaukee, Wis.) and 28S (5.3 kb) and 18S (2.0 kb) human fibroblast rRNAs were included as molecular weight standards. The RNAs were transferred to nitrocellulose paper (57) as described previously (48, 63). A ^{32}P-labeled nick-translated probe consisting of the 2.9-kb *Bgl*II fragment was hybridized to the blot as described previously (63).

Preparation of ^{32}P-end-labeled probes. The dephosphorylated 5′ termini of DNA fragments were labeled by the forward reaction technique (29) with [γ-^{32}P]ATP (Amersham Corp.) and T4 polynucleotide kinase (P-L Biochemicals). The 3′ termini of DNA fragments were labeled by the fill-in reaction (33) with the appropriate [α-^{32}P]deoxynucleotide triphosphate (Amersham Corp.) and the large fragment (Klenow) of *E. coli* DNA polymerase I (Boehringer Mannheim) (48). To generate uniquely end-labeled probes, the ^{32}P-labeled DNA fragments were digested with a second restriction enzyme and gel purified.

Structural analysis of RNA. The structure of the virus-specific transcripts was determined by the nuclease mapping technique of Berk and Sharp (4) with mung bean nuclease (P-L Biochemicals) and exonuclease VII (Bethesda Research Laboratories) as described previously (47, 48). ^{32}P-end-labeled probes were hybridized to 10 to 50 μg of early or late whole-cell or late polysomal RNA in 80% formamide–0.4 M NaCl–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4]–1 mM EDTA at 55°C for 4 h. Nuclease-resistant hybrids were fractionated on either 1.5% denaturing agarose (4) or 6% urea–acrylamide gels (29). The denaturing agarose gels were dried, and the specific hybrids were detected by autoradiography. Standard size markers for denaturing agarose gels were prepared from a recombinant plasmid, pSV325 (a gift from L. Villarreal), that was digested with restriction endonuclease *Hin*dIII and labeled with [α-^{32}P]dATP (Amersham Corp.) and Klenow polymerase (Boehringer Mannheim) as described above. Standard size markers for 6% urea–acrylamide gels were prepared from recombinant plasmid pBR322 that was digested with restriction endonucleases *Hin*fI and *Eco*RI and labeled with [α-^{32}P]dATP (Amersham Corp.) and Klenow polymerase (Boehringer Mannheim) as described above.

DNA sequencing. DNA sequence analysis was accomplished by the chemical cleavage method of Maxam and Gilbert (29) as described previously (48). The secondary structure of the RNA molecule was predicted with the P-C Fold computer program (65).

RESULTS

Location of the transcriptional units within *Xba*I-B and *Xba*I-D. A physical map of the HCMV genome showing the location of the two regions of interest in this study, *Xba*I-B within the large unique component and *Xba*I-D, which contains the *Bam*HI-S fragment within the long repeat sequence of the genome, is depicted in Fig. 1. A detailed map of both regions is displayed underneath the autoradiograms in Fig. 2.

The transcriptional units within *Xba*I-B and *Xba*I-D, which contains the *Bam*HI-S region, were mapped by hybridizing ^{32}P-labeled early or late whole-cell RNA to Southern (46) blots of cloned DNA from the two regions digested with various restriction endonucleases. Throughout this manuscript, early RNA is defined as RNA isolated from infected cells prior to DNA replication (i.e., at 12 h p.i.) or infected cells treated with PAA. In contrast, late RNA is defined as the RNA isolated from infected cells at 24 to 72 h p.i. in the absence of PAA. One region within *Xba*I-B hybridized extensively to both early and late ^{32}P-labeled whole-cell RNA (Fig. 2A). This region was contained within the large *Bam*HI-A, *Eco*RI-A, and *Pst*I-A fragments of *Xba*I-B. This transcriptional unit was further localized to two adjacent *Xho*I fragments (*Xho*I-F_2 and *Xho*I-I) and was designated *Xba*I-B transcriptional region I. Other DNA fragments that hybridized to ^{32}P-labeled early and late RNA, but at a lower intensity, constituted transcription region II. All fragments that hybridized to early whole-cell RNA also hybridized to late RNA. Within *Xba*I-D, intense hybridization of early and late ^{32}P-labeled whole-cell RNA to the *Bam*HI-S and *Eco*RI-O_2 fragments delineated a region of high transcriptional activity, designated *Xba*I-D transcriptional region I (Fig. 2B). An additional transcriptional unit (*Xba*I-D region II) was defined by hybridization of ^{32}P-labeled late whole-cell RNA to the *Eco*RI-H fragments. As with the *Xba*I-B blots, all DNA fragments that demonstrated hybridization to early whole-cell RNA also showed hybridization to late RNA. Thus, transcriptional units within *Xba*I-B and *Xba*I-D are highly transcribed prior to and after viral DNA synthesis, in agreement with the results of Wathen and Stinski (62).

To prove that the regions actively transcribed at early times after infection were the same regions transcribed at late times, a competition analysis was performed. In this analysis, "cold" early whole-cell RNA was first hybridized for 48 h to the blots of *Xba*I-B and *Xba*I-D. The blots were hybridized to an equivalent amount (in micrograms) of ^{32}P-labeled late whole-cell RNA and analyzed as described in Materials and Methods. A reduction in the amount of ^{32}P-labeled late whole-cell RNA that hybridized to the respective DNA fragments was detected (Fig. 2). This reduction was not the result of elution of the immobilized DNA since the early and late hybridizations pretreated in a similar manner but with nonspecific yeast soluble RNA instead of "cold" early whole-cell RNA did not show this reduction in the extent of hybridization. The autoradiograms shown in Fig. 2 were all exposed for the same period of time (2 h). Densitometry scans for the late RNA or competition blots demonstrated two peaks, designated *Xho*I-F_2 and *Xho*I-I, from *Xba*I-B (Fig. 2A). The lone peak detected in the *Bam*HI digestion scans corresponded to the *Bam*HI-S fragment generated from *Xba*I-D (Fig. 2B). The relative amount of ^{32}P-labeled late RNA that hybridized to the *Xba*I-B or *Xba*I-D region was reduced four- to fivefold when an equivalent microgram amount of cold early RNA was used for competition hybridization. Since the amount of virus-specific RNA present within the infected cell is greater at late times p.i. (7), increased levels of unlabeled early RNA would be required for complete competition of the hybridization of labeled late RNA to the blots. This result indicated

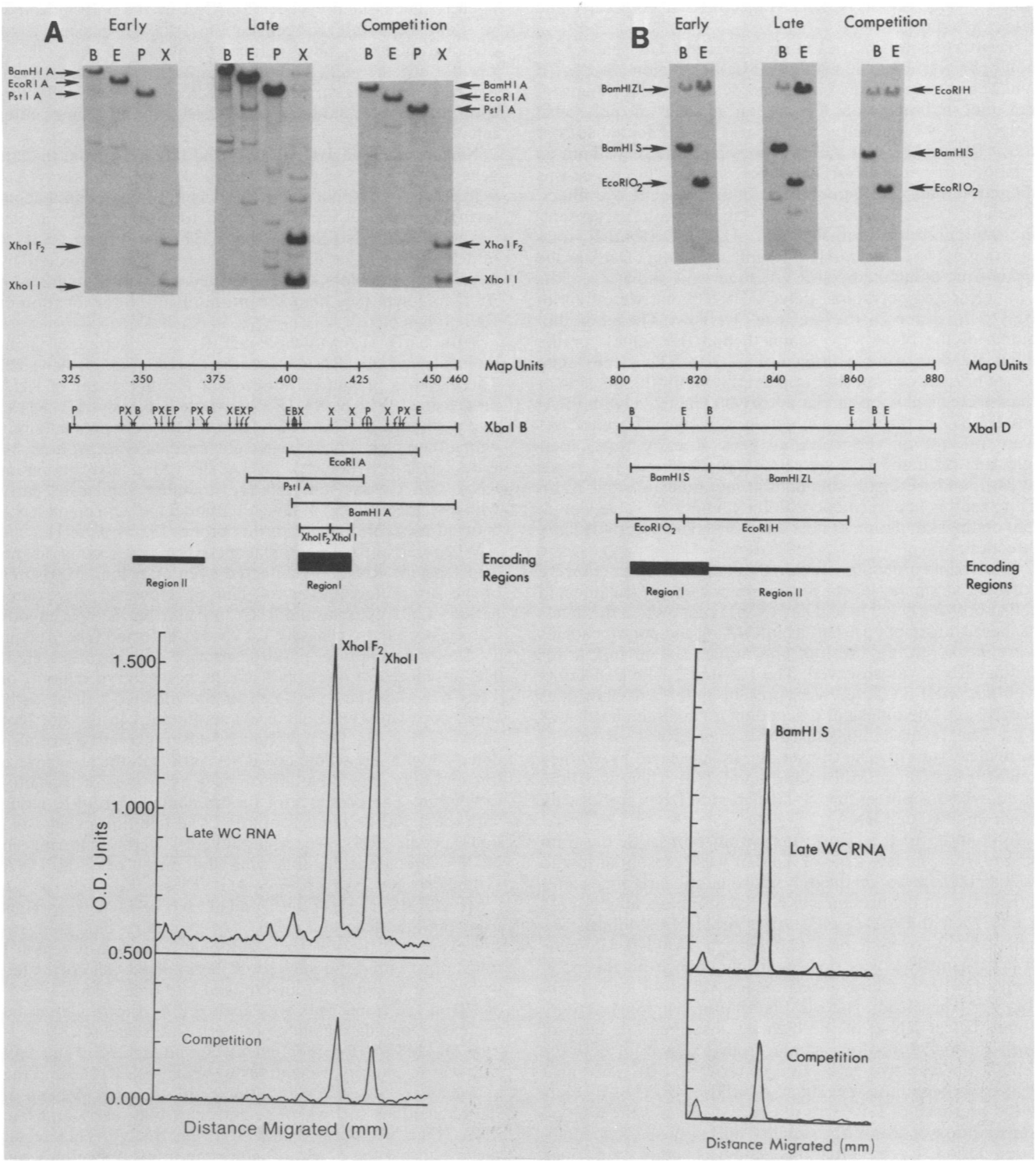


FIG. 2. Southern blots of ^{32}P-labeled early or late whole-cell RNA hybridized to *Xba*I-B DNA (A) and *Xba*I-D DNA (B) cleaved with various restriction enzymes. Digested DNA (B, *Bam*HI; E, *Eco*RI; P, *Pst*I; X, *Xho*I) from the two regions was fractionated by gel electrophoresis and subsequently immobilized onto nitrocellulose as described in the text. A total of 50×10^6 cpm of ^{32}P-labeled RNA was used in the hybridizations. The competition blots were performed by prehybridizing the blots with an equivalent amount of unlabeled early whole-cell (WC) RNA and then probing the blots with 50×10^6 cpm of late ^{32}P-labeled whole-cell RNA. Restriction maps of the two fragments, including map units and regions of transcription, are shown. The thickness of the bars corresponds to the abundance of transcript originating within the various regions. The region of highest transcription was designated region I. Densitometry scans of the *Xho*I restriction endonuclease digestion lane of *Xba*I-B late RNA or early RNA competition blots and the *Bam*HI restriction endonuclease digestion lane of *Xba*I-D late RNA or early RNA competition blots are shown.

that the same region is being transcribed at both early and late times p.i.

Transcriptional analyses of an early and a late gene by dot blot hybridization. The highly transcribed regions of *Xba*I-B and *Xba*I-D were studied in further detail by the technique of dot blot hybridization (64). Total nuclear or polysomal poly(A)$^+$ RNA was harvested from HCMV-infected cells or mock-infected cells at various times p.i. with or without an inhibitor of viral DNA synthesis, PAA. The RNA was diluted serially and immobilized onto nitrocellulose filters. The filters were hybridized to ^{32}P-labeled probes representing the highly transcribed region of *Xba*I-B (*Xho*I-F_2 fragment) and *Xba*I-D (*Bam*HI-S fragment) or to a cDNA probe made from uninfected-cell RNA. Figure 3A shows dot blots of nuclear and polysomal poly(A)$^+$ RNA that were hybridized to the probe for the late gene (*Xho*I-F_2). The autoradiograms of the blots were scanned, and the values for the duplicate dots were averaged (Fig. 3B). The steady-state levels of host cell-specific transcripts within the nucleus of the infected cell were similar at early (12 h or 72 h with PAA) and late (72 h) times after infection. Cell-specific transcripts were present on the polyribosomes at early times after infection (12 h and 72 h with PAA), reached maximal levels at 24 h, and were still abundant at late times (48 and 72 h). This result is in agreement with the findings of Tanaka et al. (55) on the stimulation of host cell RNA synthesis by HCMV infection.

Transcripts originating within the *Bam*HI-S fragment were detected at similar levels within the nucleus of the infected cell at both early (12 h) and late (72 h) times. When the levels of these transcripts present as mRNA on the polyribosomes were measured, a pattern of appearance similar to that for cellular RNA was observed. The 1.2- and 2.7-kb transcripts within *Bam*HI-S (32, 62) were present on the polyribosomes at early and late times but reached their maximum level at 24 h p.i. This result demonstrated that these two RNA species were clearly early gene transcripts, as suggested by Wathen and Stinski (62) and McDonough et al. (32). There was little or no hybridization of the ^{32}P-labeled viral DNA probes with mock-infected cell RNA.

In contrast to the transcripts within *Bam*HI-S, the transcripts from the late gene of *Xba*I-B were present at low to undetectable levels on the polyribosomes at early times after infection or in the presence of a DNA synthesis inhibitor (12 h and 72 h with PAA) (Fig. 3). After viral DNA synthesis was initiated (12 to 16 h p.i.) (23, 50), the *Xba*I-B late-gene (*Xho*I-F_2) mRNA was present in abundance on the polysomes (24 h), reaching a maximum level at 48 to 72 h p.i. (Fig. 3). The steady-state levels of transcriptional activity (total nuclear RNA) of the highly transcribed late gene of *Xba*I-B showed a pattern similar to that shown for both the cellular and the two early viral genes (Fig. 3B). The slight decrease visible in the cellular, *Bam*HI-S, or *Xho*I-F_2 blots in the presence of PAA was due to the effect of the drug on transcription or transcript stability during the 72-h accumulation period. Even though this gene was highly transcribed at early times, the presence of mRNA on the polyribosomes was linked to viral DNA replication. Total cytoplasmic RNA demonstrated a similar pattern (data not shown). These results suggested that a posttranscriptional regulatory mechanism functioning in HCMV-infected cells was affecting transport of the late-gene viral RNAs at early times after infection.

Northern blot analysis of early and late RNA from the late gene of *Xba*I-B. Early and late total whole-cell, poly(A)$^+$ whole-cell, or cytoplasmic RNA was fractionated in a denaturing agarose gel and transferred to nitrocellulose as described in Materials and Methods. The various size classes of RNA present at early or late times were detected by hybridizing the blots to a ^{32}P-labeled nick-translated 2.9-kb *Bgl*II fragment (see map at bottom of Fig. 6 for location of *Bgl*II fragment).

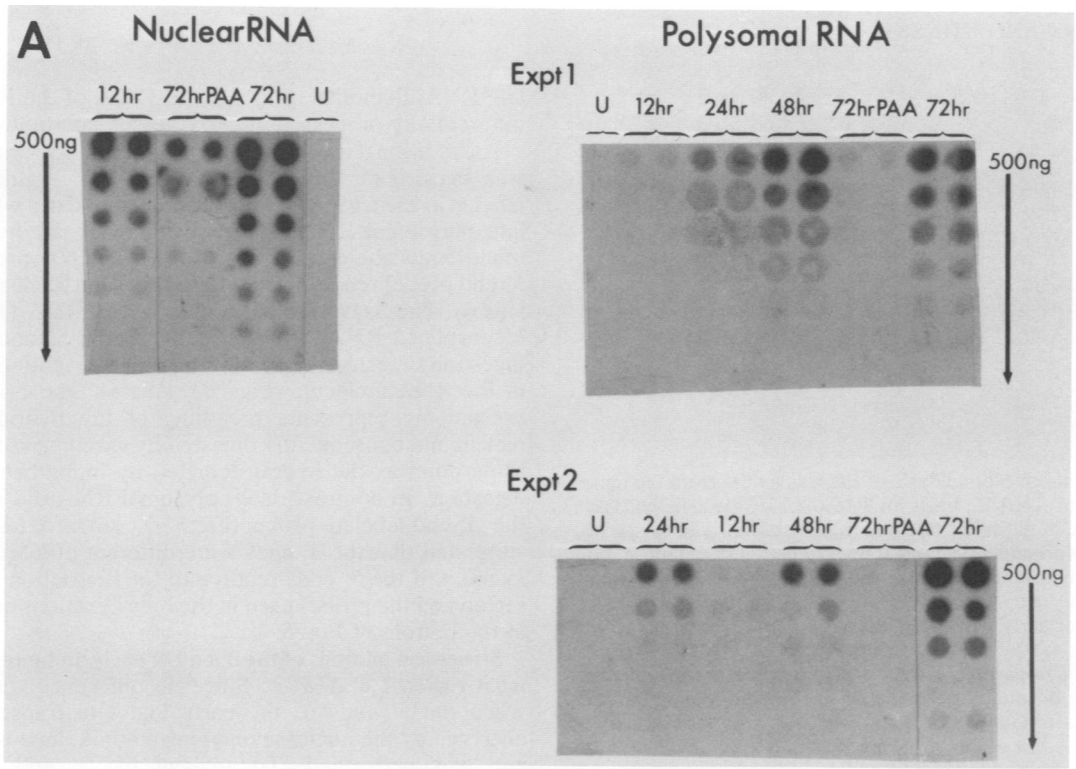
With early (PAA) whole-cell RNA, a series of high-molecular-weight RNAs were detected (Fig. 4, lane 2). These RNAs presumably represent various stages of RNA processing. With early (PAA) whole-cell poly(A)$^+$ RNA, two predominant RNAs of 7.4 and 6.4 kb were present along with other high-molecular-weight species (Fig. 4, lane 3). Late whole-cell poly(A)$^+$ RNA showed a distribution of RNA size classes and relative amounts similar to early whole-cell RNA (Fig. 4, lane 4). Uninfected whole-cell RNA showed no hybridization to this probe (Fig. 4, lane 1).

When early (PAA) cytoplasmic RNA was examined, no virus-specific RNA was detected even after long exposures of the autoradiogram (Fig. 4, lane 6). However, by examination of late cytoplasmic RNA, a major but heterogeneous size class of approximately 1.9 kb was detected (Fig. 4, lane 7). Lower levels of a 2.5-kb transcript were also detected. The absence of transcripts from the late gene of region I of *Xba*I-B within the cytoplasm of the infected cell at early times correlated with our previous findings in the dot blot analyses (Fig. 3). The 1.9- and 2.5-kb transcripts were also detected with late whole-cell RNA, although the levels were low and only detectable after longer exposures of the autoradiogram. The presence of the predominant 1.9-kb, along with the 2.5-kb, size class of mRNA at late times suggested that specific RNA-processing events were occurring in HCMV-infected cells following the onset of viral DNA synthesis.

Structural analysis of the 5′ end of early and late transcripts from the late gene of *Xba*I-B. The Northern blot analyses suggested that the posttranscriptional retention of region I transcripts within the nucleus of the infected cell correlated with processing of the transcripts at late times. Thus, the 5′ end(s) of the transcripts was analyzed by the single-strand nuclease mapping technique of Berk and Sharp (4).

A 2.0-kb *Sal*I-*Bst*EII fragment, 5′-end labeled at the *Sal*I site, was used in hybridizations to whole-cell RNA, and the hybrids were digested with mung bean nuclease. A band of 900 nucleotides (nt) was detected with both early and late RNA (Fig. 5A, lanes 2 and 3, respectively). This result suggested that the 5′ end of either the transcript or an exon

FIG. 3. Dot blot analyses, showing the temporal appearance of transcripts of an early and a late HCMV gene during infection of human fibroblast cells. Total nuclear or polysome-associated polysomal poly(A)$^+$ RNA was harvested at various times p.i. with and without PAA, an inhibitor of viral DNA synthesis. Serial dilutions of the RNA samples were blotted in duplicate onto nitrocellulose as described in the text. The ^{32}P-labeled probes used in the hybridizations were cDNA to polysomal poly(A)$^+$ RNA from uninfected human fibroblast cells, the *Bam*HI-S fragment, and the *Xho*I-F_2 fragment from *Xba*I-B. (A) Sample blots of total nuclear and polysomal poly(A)$^+$ RNA hybridized with the *Xho*I-F_2 probe. The samples for 62.5- and 31.25-ng dots were inadvertently reversed on dot blot experiment 2. (B) The total nuclear and the polysomal poly(A)$^+$ (expt 1) blots from panel A as well as dot blots of the early RNAs from *Bam*HI-S or cellular RNA were scanned, and the average of the duplicate dots was plotted as scanner units versus nanograms of RNA blotted. Symbols: △, 12 h; ●, 24 h; ○, 48 h; ■, 72 h; □, 72 h with PAA.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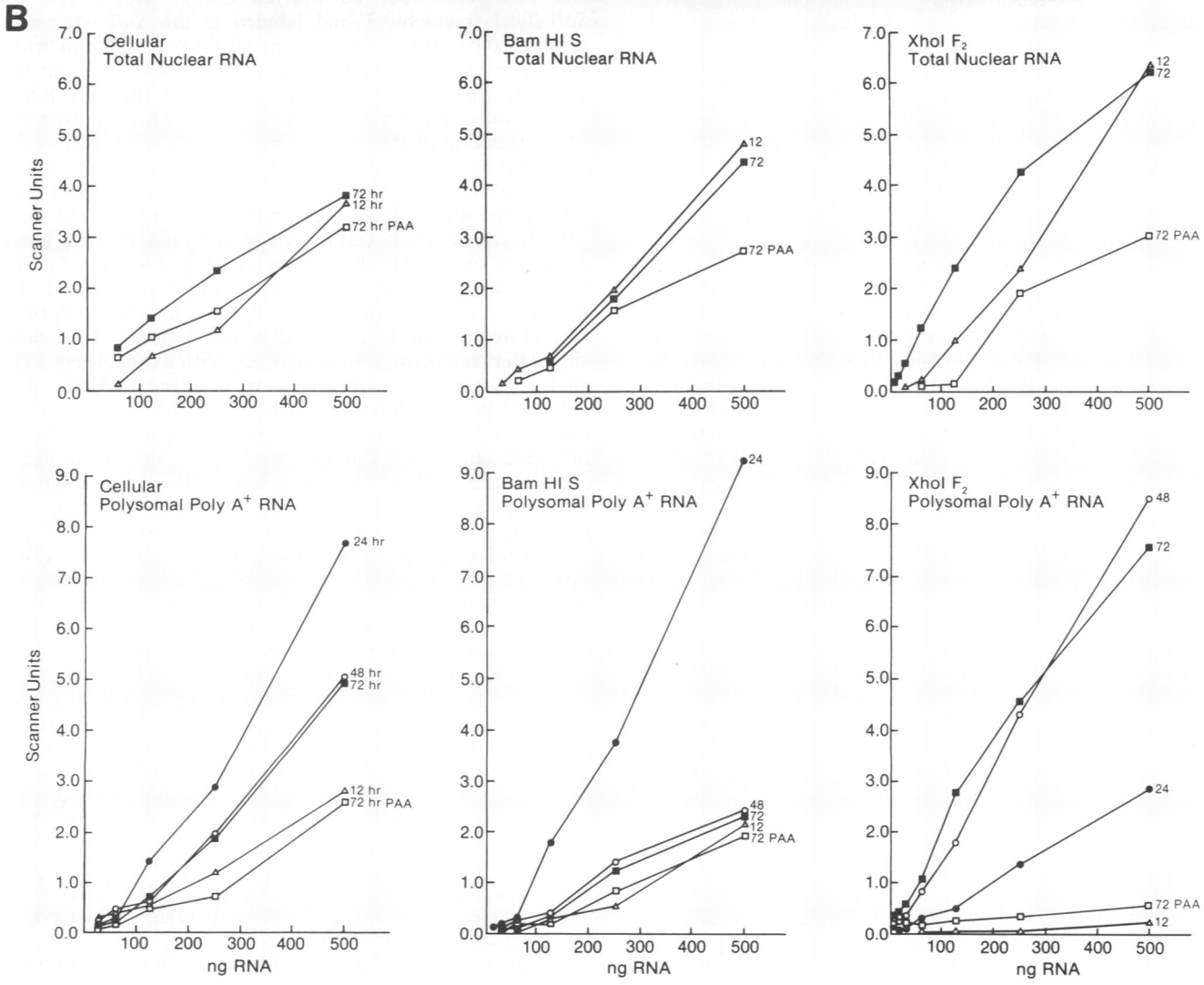
B
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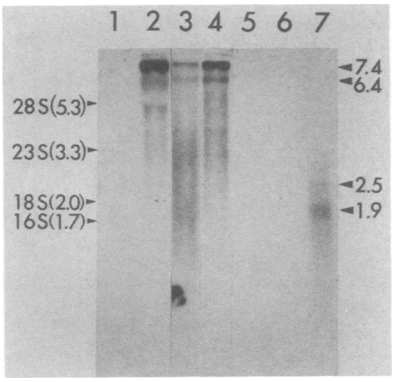

FIG. 4. Northern blot analysis of the transcripts from the major late gene within *Xba*I-B. Early or late total whole-cell, poly(A)$^+$ whole-cell, or cytoplasmic RNA was fractionated in a 1.5% agarose gel containing formaldehyde and transferred to nitrocellulose, and the filter was hybridized to a nick-translated 2.9-kb *Bgl*II probe as described in the text. Lanes: 1, 5 μg of mock-infected whole-cell RNA; 2, 5 μg of early whole-cell RNA; 3, 5 μg of early whole-cell poly(A)$^+$ RNA; 4, 5 μg of late whole-cell poly(A)$^+$ RNA; 5, 10 μg of mock-infected cytoplasmic RNA; 6, 10 μg of early cytoplasmic RNA; 7, 10 μg of late cytoplasmic RNA. The autoradiogram was exposed for 8 h for lanes 1 and 2 and 5 to 7, 24 h for lane 3, and 2 h for lane 4. The sizes of the virus-specific transcripts are shown (in kilobases). The molecular weight standards described in the text are shown to the left.

of the transcript existed 900 nt upstream of the *Sal*I site. This also established the direction of transcription, left to right according to the prototype arrangement of the HCMV (Towne) genome. Probes from the *Sal*I site failed to detect RNA transcription in the opposite direction.

The 5′ end was analyzed further by using a 2.40-kb *Xho*I fragment (*Xho*I-F$_2$) that was labeled at the 5′ end. For this experiment, hybrids were treated with mung bean nuclease or exonuclease VII. Mung bean analysis on early whole-cell RNA detected a band of 450 nt (Fig. 5B, lane 4). This result mapped the 5′ end to the same spot that the *Sal*I-*Bst*EII probe did. However, after digestion of the hybrids with exonuclease VII, the 450-nt band was no longer detectable, and a new band of 1,600 nt was detected by using early whole-cell RNA (Fig. 5B, lane 5). The minor bands between 1,600 and 450 nt were also present in the mock-infected cell RNA control (lanes 2 and 3) and were more easily observed after longer exposure of the autoradiogram. The artifactual bands were detected in both the mung bean nuclease and exonuclease VII lanes. The bands did not shift between mung bean nuclease and exonuclease VII treatment. It is possible that some of the minor bands were also the result of digestion of the DNA probe due to breathing of the DNA-RNA hybrid. These results showed that the 5′ end of the transcripts at early times mapped to a site 1,600 nt upstream from the *Xho*I site. To confirm the location of the 5′ end, a 1.85-kb *Xho*I-*Sal*I fragment, 5′-end labeled at the *Xho*I site, was hybridized to early whole-cell or late polysomal RNA. A 450-nt band was detected with both RNAs after digestion with mung bean nuclease (Fig. 5C, lanes 3 and 5, respectively). The light 1,600-nt bands (Fig. 5C, lanes 3 and 5) represent the RNA-DNA hybrids formed with unspliced RNA. After digestion with exonuclease VII, the 450-nt band disappeared and a prominent band of 1,600 nt was detected with both RNAs (Fig. 5C, lanes 4 and 6, respectively). Therefore, the 5′ end of the early and late transcripts is located between the *Sal*I and *Bst*EII sites (Fig. 5). In addition, an intron(s) can be removed from both early and late RNA, demonstrating that the 5′ end of the RNAs is not differentially processed at early versus late times.

An additional analysis of the 5′ end of early and late RNA was performed. A 3.85-kb *Cla*I-*Bam*HI fragment, 5′-end labeled at the *Cla*I site, was hybridized to early whole-cell or late polysomal RNA. After digestion of the hybrids with mung bean nuclease, early whole-cell RNA protected the 5′-end probe, rendering bands of 1,450 and 2,600 nt (Fig. 5D, lane 3). The 2,600-nt band represents an RNA-DNA hybrid of unspliced RNA with the DNA probe. Exonuclease VII digestion detected the 2,600-nt band representing the 5′ end of the RNA molecule (Fig. 5D, lane 4). The 1,450-nt band presumably represents breathing of the hybrid that was nicked and consequently digested by exonuclease VII to the same point as the hybrid detected by mung bean nuclease digestion. In contrast, late polysomal RNA did not protect the 5′-end-labeled probe (Fig. 5D, lanes 5 and 6). This suggested that the 3′ ends were different at late times. The location of the 5′ ends relative to the restriction map of the region and the probes used in the hybridizations are depicted at the bottom of Fig. 5.

Structural analysis of the 3′ end of early and late transcripts from region I of *Xba*I-B. Since no difference was detected when the 5′ ends of the early and late transcripts were analyzed by the nuclease mapping method, analysis of the 3′ end was performed. To analyze the 3′ end, a 1.95-kb *Sal*I-*Bgl*II fragment, 3′-end labeled at the *Sal*I site, was hybridized to early or late whole-cell RNA, and the hybrids were digested with either mung bean nuclease or exonuclease VII. Mung bean digestion of hybrids with early RNA gave a protected band of about 1,200 nt (Fig. 6A, lane 2). After digestion of the same hybrids with exonuclease VII, the same band of 1,200 nt was observed (Fig. 6A, lane 3). This result showed that the 3′ end of the RNA molecule at early times after infection extended 1,200 nt past the *Sal*I site. When late whole-cell RNA was analyzed by mung bean digestion, two bands were detected (Fig. 6A, lane 4). As seen previously, a minor 1,200-nt band was detected. In contrast to the early transcripts, the late hybrids showed a second major band mapping 270 nt downstream of the *Sal*I site. After treatment of these hybrids with exonuclease VII, analogous bands were detected (Fig. 6A, lane 5). This result indicated that a different and unique 3′ end was present at late times. The 3′ end of the transcripts was studied in further detail by performing a time course analysis to demonstrate the differential processing of the 3′ end during infection. Cytoplasmic RNA from an equal number of uninfected and infected cells was hybridized to a 1.60-kb *Xho*I-*Xho*I fragment, 3′-end labeled at the *Xho*I site (Fig. 6B). The resulting hybrids were treated with mung bean nuclease and analyzed on 6% urea–acrylamide gels (Fig. 6B). A total of three bands were detected as early as 24 h p.i. (Fig. 6B, lane 5). These bands increased in intensity when RNA isolated at 48 and 72 h p.i. was analyzed (Fig. 6B, lanes 6 and 7). The size of the protected hybrids were 380, 430, and 710 nt. The 380- and 430-nt bands mapped to positions containing putative splice donor sites (11). The 710-nt band mapped to the 3′ end detected when the *Sal*I-*Bgl*II probe (Fig. 6A) was used to analyze late transcripts. This 3′ end was analyzed in further detail and is discussed below. The location of the 3′ ends of the exons relative to the *Xho*I-*Xho*I probe and the restriction map of the region are detailed at the bottom of Fig. 6. These results indicated that differential processing of the 3′ end of the *Xba*I-B region I transcripts occurred late after viral infection.

Sequence analysis of the 3′ end located 270 nt downstream

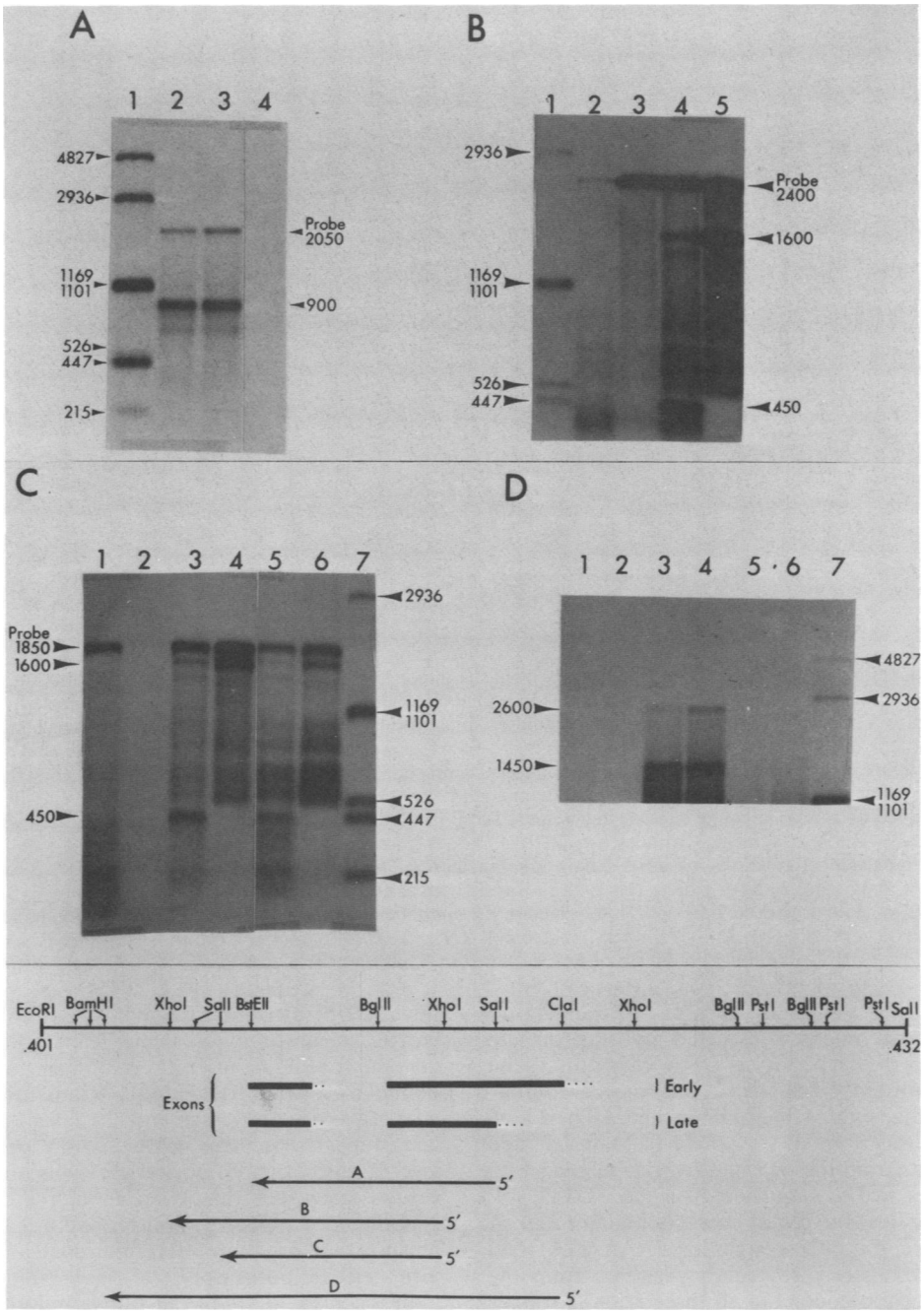

FIG. 5. Nuclease mapping of the 5′ end of the transcripts from the major late gene within *Xba*I-B at early and late times after infection. Early or late whole-cell or late polysomal RNA was hybridized to ^{32}P-end-labeled DNA probes and digested with either mung bean nuclease or exonuclease VII, and the hybrids were fractionated on denaturing 1.5% agarose gels as described in the text. The location of the probes used in the hybridizations relative to the early and late exons, as well as the restriction endonuclease map of the region, is shown at the bottom of the figure. Dotted lines denoting exon boundaries represent 5′ or 3′ ends of exons not mapped by this experiment. The map units of the region are also shown. The sizes of the probes and the protected hybrids are indicated (in nucleotides). (A) 5′-end analysis with a *Sal*I-*Bst*EII probe end-labeled with [γ-^{32}P]ATP at the *Sal*I site. Lanes: 1, ^{32}P-labeled molecular weight standard; 2 through 4, early whole-cell RNA, late whole-cell RNA, and mock-infected cell RNA, respectively, treated with mung bean nuclease. (B) 5′-end analysis with a *Xho*I-*Xho*I fragment end-labeled with [γ-^{32}P]ATP. Lanes: 1, ^{32}P-labeled molecular weight standard; 2, mock-infected whole-cell RNA (mung bean nuclease); 3, mock-infected whole-cell RNA (exonuclease VII); 4, early whole-cell RNA (mung bean nuclease); 5, early whole-cell RNA (exonuclease VII). (C) 5′-end analysis with a *Xho*I-*Sal*I probe end-labeled with [γ-^{32}P]ATP at the *Xho*I site. Lanes: 1, mock-infected whole-cell RNA (mung bean nuclease); 2, mock-infected whole-cell RNA (exonuclease VII); 3, early whole-cell RNA (mung bean nuclease); 4, early whole-cell RNA (exonuclease VII); 5, late polysomal RNA (mung bean nuclease); 6, late polysomal RNA (exonuclease VII); 7, ^{32}P-labeled molecular weight standard. (D) 5′-end analysis with a *Cla*I-*Bam*HI probe end-labeled with [γ-^{32}P]ATP at the *Cla*I site. Lanes: 1, mock-infected whole-cell RNA (mung bean nuclease); 2, mock-infected whole-cell RNA (exonuclease VII); 3, early whole-cell RNA (mung bean nuclease); 4, early whole-cell RNA (exonuclease VII); 5, late polysomal RNA (mung bean nuclease); 6, late polysomal RNA (exonuclease VII); 7, ^{32}P-labeled molecular weight standards.

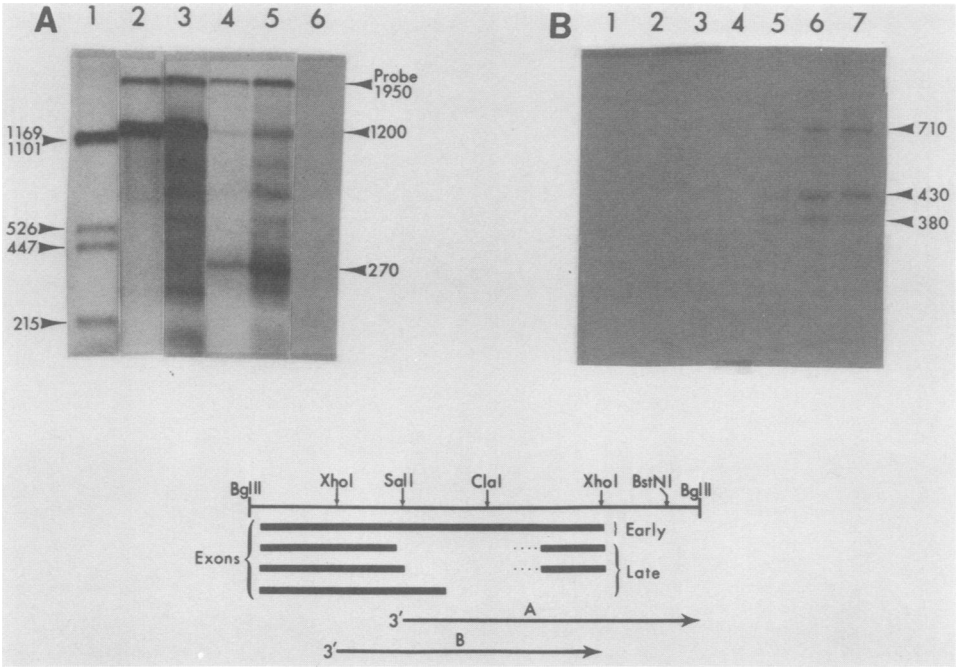

FIG. 6. Nuclease mapping of the 3′ end of the transcripts from the major late gene within *Xba*I-B at early and late times after infection. Early or late whole-cell, polysomal, or cytoplasmic RNA was hybridized to ^{32}P-end-labeled DNA probes and digested with either mung bean nuclease or exonuclease VII, and the hybrids were fractionated on denaturing 1.5% agarose gels or 6% urea–acrylamide gels. The location of the probes used in the hybridizations relative to the early and late exons, as well as the restriction endonuclease map of the region, is shown at the bottom of the figure. Dotted lines denoting exon boundaries represent the 5′ ends of exons mapped in this study. The sizes of the protected hybrids are shown in nucleotides. (A) 3′-end analysis with a *Sal*I-*Bgl*II probe end-labeled with [α-^{32}P]TTP at the *Sal*I site. The hybrids were fractionated on a 1.5% alkaline agarose gel. Lanes: 1, ^{32}P-labeled molecular weight standards; 2, early whole-cell RNA (mung bean nuclease); 3, early whole-cell RNA (exonuclease VII); 4, late whole-cell RNA (mung bean nuclease); 5, late whole-cell RNA (exonuclease VII); 6, mock-infected whole-cell RNA (mung bean nuclease). The autoradiogram was exposed for 2 h for lane 1, 4 h for lanes 2 and 3, 7 h for lanes 4 and 5, and 24 h for lane 6. (B) 3′-end analysis with a *Xho*I-*Xho*I probe end-labeled with [α-^{32}P]TTP at the XhoI site. Cytoplasmic RNA was isolated at various times p.i., hybridized to the end-labeled probe, digested with mung bean nuclease, and fractionated on a 6% urea–acrylamide gel. Lanes: 1, 35 μg of mock-infected cell RNA; 2, 35 μg of RNA, 2 h p.i.; 3, 45 μg of RNA, 5 h p.i.; 4, 55 μg of RNA, 10 h p.i.; 5, 65 μg of RNA, 24 h p.i.; 6, 70 μg of RNA, 48 h p.i.; 7, 85 μg of RNA, 72 h p.i.

of the *Sal*I site that is detectable only at late times after infection. Mung bean nuclease-digested hybrids of late polysomal RNA to a 0.5-kb *Sal*I-*Cla*I DNA fragment, 3′-end labeled at the *Sal*I site, were analyzed on 12% urea–acrylamide gels adjacent to the Maxam and Gilbert (29) chemical cleavage reactions as described in Materials and Methods. The bands from the digested hybrids aligned with the two C residues in the sequence 5′-GAGCTCTTTT-3′ (Fig. 7A), assuming the 1- to 1.5-nt correction factor (20). A consensus sequence for polyadenylation (AATAAA) (39) was detected approximately 180 nt downstream from the *Sal*I site (Fig. 7B). Stop codons in all three reading frames were found both upstream and downstream of the polyadenylation and cleavage sites (Fig. 7B). The theoretical secondary structure of the RNA molecule in this region was predicted from the DNA sequence by the PC-Fold computer program (65). A very stable secondary structure (−71.2 kcal) was predicted that positioned the polyadenylation signal adjacent to the cleavage sites (Fig. 7C). A cluster of T residues was detected immediately downstream of the cleavage sites.

DISCUSSION

Previous work by DeMarchi (13) and Wathen and Stinski (62) demonstrated that even though the HCMV genome showed extensive transcription at early times after infection, few viral transcripts were present on the polyribosomes. One region typifying this phenomenon was located within the *Xba*I-B restriction fragment (32.5 kb). Hybridization of early nuclear or cytoplasmic RNA to this region of the genome revealed a 6:1 ratio of nuclear to cytoplasmic RNA bound to *Xba*I-B DNA (62). When the genes within the *Xba*I-D restriction fragment (20.8 kb), located within the large repeat sequences of the HCMV genome, were examined, a ratio of approximately 1:1 was observed (62). These results suggested that even though these regions of the genome are extensively transcribed at early times, a mechanism exists for the preferential nuclear retention of some virus-specific transcripts.

In the present study the transcription units within these two regions were mapped in further detail, and the regions displaying the highest level of transcriptional activity were examined by dot blot analysis. The results of this series of experiments indicated that even though both regions (region I of *Xba*I-B and region I of *Xba*I-D) were highly transcribed at early and late times after infection, only transcripts from the region within the large repeat sequences were present on the polyribosomes at early times. The appearance of transcripts from region I of *Xba*I-B in the cytoplasm did not take place when viral DNA replication was inhibited.

The above results further characterized the general phenomenon of posttranscriptional regulation in HCMV-infected cells, but gave no insight into the possible mechanism for the retention of certain viral transcripts within the nucleus of the cell at early times after infection. The study of the size and abundance of transcript species from region I of

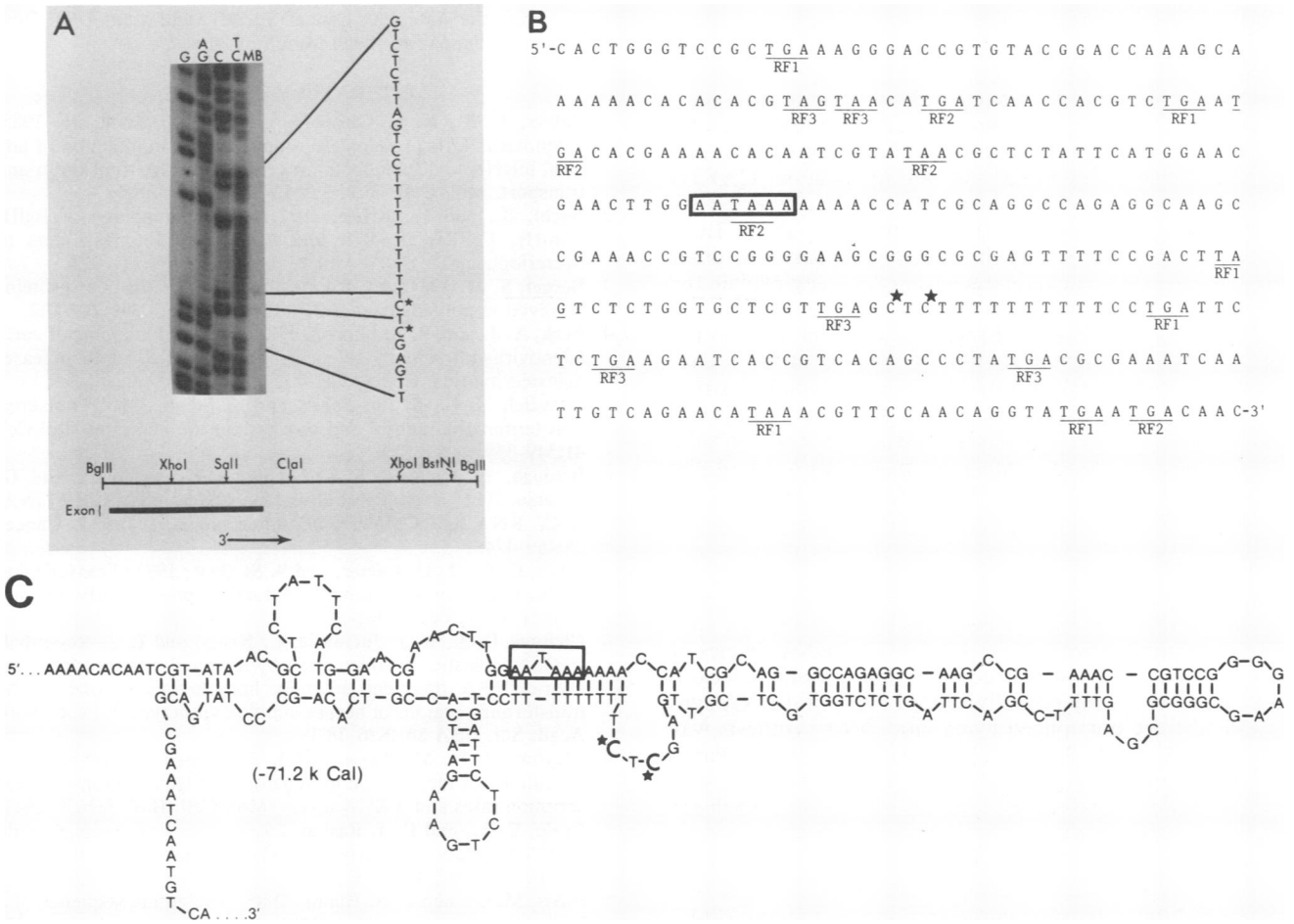

FIG. 7. Sequence analysis of the 3′ end located 270 nt downstream of the *SalI* site that is detectable only at late times after infection. (A) Late polysomal RNA was hybridized to a *SalI-ClaI* probe end-labeled with [α-^{32}P]TTP at the *SalI* site. The hybrid was digested with mung bean nuclease (MB) and fractionated on a 12% urea–acrylamide gel in a lane adjacent to the Maxam-Gilbert chemical cleavage reactions of the *SalI-ClaI* fragment end-labeled with [γ-^{32}P]ATP at the *SalI* site. The sequence of the region is shown adjacent to the gel and in greater detail in panel B. The stars denote the nucleotides at which the RNA molecule is cleaved. (B) Nucleotide sequence of the region containing the cleavage and polyadenylation site. Important features within the region, such as the polyadenylation signal (AATAAA, boxed), the sites of cleavage (stars) and the stop codons present within all three reading frames (RF) are indicated. (C) Theoretical secondary structure of the RNA molecule within this region as predicted from the DNA sequence by the PC-Fold computer program (65). The stability of the postulated secondary structure is shown in kilocalories.

*Xba*I-B present at early and late times was made possible by Northern blot and nuclease mapping analyses. Even though numerous high-molecular-weight virus-specific RNA species were detected within the nucleus at early times, little or no virus-specific transcripts originating within *Xba*I-B region I were present within the cytoplasm of the infected cell. In contrast, transcripts originating from region I of *Xba*I-B were found in the cytoplasm when viral DNA synthesis was not inhibited. The late RNA species were detectable within the cytoplasm at 24 h p.i. and increased in relative amounts at 48 and 72 h p.i. Nuclease mapping analyses demonstrated that a preferential processing event at the 3′ ends of the RNA molecules occurred late in infection, concurrent with viral DNA synthesis. These 3′-end-processing events correlated with the transport of the RNA molecules to the cytoplasm. Alternatively, the 3′-end-processing events stabilized these RNAs while in the cytoplasm. The unprocessed RNAs could have been transported but rapidly degraded. Nuclease mapping experiments on early and late RNA revealed that the mechanism of posttranscriptional regulation was at the level of differential processing of the 3′ ends of the viral transcripts either by alternative 3′-terminal cleavage and polyadenylation or by differential splicing.

Posttranscriptional regulatory phenomena have been observed in various systems, including cellular (9, 16, 17, 28, 56) and viral (1, 18, 25, 38, 60, 61) genes. Many possible mechanisms have been suggested: differential RNA stability, methylation of specific transcripts, differential splicing patterns, 3′-end cleavage and polyadenylation, and differential affinity for ribonuclear protein complexes.

Proper cleavage and polyadenylation seem to involve many factors. Originally, the AAUAAA consensus sequence was believed to be the factor required for 3′-end processing (39). It is now thought that many factors may be required to achieve this processing event. A preferential site of cleavage, located somewhere within 20 nt downstream of the AAUAAA consensus sequence, has been established. Cleavage usually occurs at a C(A) dinucleotide that precedes a G/T cluster (5). The HCMV 1.9-kb RNA of *Xba*I-B region I, which is processed at an upstream polyadenylation signal, is cleaved at either of two C(T) dinucleotides. The C(T) dinucleotides are considerably further downstream from the

AAUAAA consensus sequence (approximately 90 nt) than previously detected in other systems (5). The characteristic G/T cluster (YGTGTTYY) (5, 21, 34, 41), present within the 30 nt following the C(A) dinucleotide of various eucaryotic genes, shows marginal sequence similarity to the stretch of T residues following the cleavage sites. Another sequence that may play a role in cleavage and polyadenylation, CAYTG (3), shows similarity to a sequence, CATCG, detected six bases downstream from the polyadenylation signal. Even though this HCMV 3′ end shows some similarity to other eucaryotic 3′ ends, the differences detected may explain the differential recognition of this site.

The HCMV 1.9-kb *Xba*I-B region I transcript displays the ability to form a potential stem-loop structure at the 3′ end. Formation of the RNA into this theoretical secondary structure allows for the C(T) dinucleotides to be placed adjacent to the AAUAAA polyadenylation signal, even though the cleavage site is approximately 90 nt downstream of the AAUAAA signal. A similar RNA stem and loop structure has been hypothesized to form in RNA from human T-cell leukemia virus type I, in which the cleavage site is located 276 bases downstream from the polyadenylation signal (42).

In many systems in which the splicing process has been perturbed, unspliced transcripts accumulate within the nucleus of the cell (43, 60). The HCMV 1.9-kb RNA species of region I of *Xba*I-B, which contains more than two exon sequences, were preferentially spliced after viral DNA synthesis and were not spliced when viral DNA synthesis was inhibited with PAA. The unspliced precursors accumulated within the nucleus of the cell at both early and late times after infection. Thus, removal of the intron closest to the 3′ end of the RNA molecule coincides with transport of the RNA species to the cytoplasm.

A factor may be responsible for the accurate cleavage, polyadenylation, and splicing of the HCMV late transcripts. This factor is present within the infected cell following viral DNA synthesis and may be a virus-encoded protein or RNA, or it may be a cellular factor that is stimulated by infection of the cell with HCMV (54). A study characterizing the expression of α_1-acid glycoprotein, a plasma protein synthesized in response to diverse stimuli, demonstrated that glucocorticoids permitted the expression of this gene by inducing an RNA processing factor involved in maturation of the α_1-acid glycoprotein primary transcript (59). In HCMV-infected cells a similar factor may allow the removal of the sequences constituting a second intron of the *Xba*I-B region I late gene either by processing of the transcript at the earliest 3′ cleavage and polyadenylation site or by differential splicing.

Adenoviruses produce a factor that facilitates the accumulation of late viral transcripts and disturbs the transport of host cell transcripts. Through the use of viral mutants, the E1B 55-kilodalton protein has been shown to be the factor that plays the crucial role in transport or stabilization of the late viral transcripts (1, 38). HCMV may code for a similar factor involved in selective transport of late viral transcripts.

In summary, we have shown that posttranscriptional regulation of the RNA from a late gene of HCMV is linked to 3′-end-processing events involving either the selection of an upstream cleavage and polyadenylation site or the removal of a region of the primary transcript by alternative splicing.

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