Latency-Associated Sense Transcripts Are Expressed during \textit{in Vitro} Human Cytomegalovirus Productive Infection

Jennine M. Lunetta and Jean A. Wiedeman

University of California, Davis, Department of Pediatrics, Infectious Diseases, Davis, California 95616

Received June 23, 2000; returned to the author for revision July 26, 2000; accepted September 24, 2000; published online November 22, 2000

Published studies have identified novel sense transcripts in latent human cytomegalovirus (HCMV) infection. These cytomegalovirus latency-associated transcripts (CLTs) have start sites upstream from the MIE gene productive start site (PSS). We evaluated the expression of the sense CLTs in an \textit{in vitro} HCMV productive infection. Transcripts with initiation sites upstream from the PSS were detected in infected human fibroblasts using reverse transcription–polymerase chain reaction and 5' rapid amplification of cDNA ends (RACE) analysis. DNA sequencing of 5' RACE PCR products confirmed that the start sites were consistent with sense CLT expression. Furthermore, ribonuclease protection assay analysis showed that transcripts initiating at the latent start site-1 were most abundant at late times postinfection after transcription from the PSS had decreased. In addition, transcription consistent with sense CLT expression was identified in HCMV-infected dTHP-1 cells, monocyte-derived macrophages, and endothelial cells, as well as in clinical isolate-infected human fibroblasts. These findings clearly demonstrate the expression of sense CLTs during \textit{in vitro} HCMV infection.

\textbf{Key Words:} human cytomegalovirus; HCMV; latency transcripts; CLTs; reverse transcription–polymerase chain reaction; rapid amplification of cDNA ends; ribonuclease protection.

\section*{INTRODUCTION}

Human cytomegalovirus (HCMV) has the ability to establish a latent infection that persists for the life of the infected host. In individuals with immune system suppression, the latent virus can reactivate and cause severe disease. In fact, the majority of HCMV-related disease is due to reactivation of latent virus. Despite the significant role that latency plays in HCMV pathogenesis, little is known about the molecular mechanisms involved in establishing and maintaining this stage of the viral life cycle. The identification and characterization of latency-associated transcripts form an important initial step toward achieving a better understanding of the molecular events involved in HCMV latency.

Novel sense and antisense transcripts have been found to be expressed in experimentally infected granulocyte-macrophage progenitor cells, as well as in bone marrow cells from naturally infected individuals (Kondo et al., 1994, 1996; Kondo and Mocarski, 1995; Hahn et al., 1998; Slobedman and Mocarski, 1999). These HCMV latency-associated transcripts (CLTs) were mapped to the major immediate-early (MIE) region of the viral genome. The sense CLTs were found to have start sites (LSS-1 and LSS-2) in the MIE promoter-enhancer region 356 and 292 bp upstream from the MIE productive start site (PSS).

In this study, we investigated the possibility that the sense CLTs are expressed during \textit{in vitro} HCMV productive infection. A specific product was detected in HCMV-infected human fibroblasts using a reverse transcription–polymerase chain reaction (RT–PCR) method designed to evaluate transcription occurring upstream from the PSS. The RT–PCR product was detected over a wide range of times postinfection in human fibroblasts and was present in a number of different cell types and in clinical isolate-infected human fibroblasts. Transcripts with start sites consistent with that of the sense CLTs were detected in HCMV-infected human fibroblasts using 5' rapid amplification of cDNA ends (5' RACE). Furthermore, a ribonuclease protection assay (RPA) designed to detect transcripts initiating from the LSS-1, LSS-2, and PSS revealed that transcripts originating upstream from the PSS were most abundant at late times postinfection after the levels of the major lytic transcripts had reached a maximum. These findings support the conclusion that the sense CLTs are expressed during \textit{in vitro} productive infection and are most abundant late in infection.

\section*{RESULTS}

\subsection*{RT–PCR analysis}

An RT–PCR method was developed to evaluate whether transcription originating upstream from the MIE

0042-6822/00 $35.00

Copyright \textcopyright{} 2000 by Academic Press
All rights of reproduction in any form reserved.
gene PSS was occurring in \textit{in vitro} HCMV productive infection. First-strand cDNA was synthesized using an exon 2 primer and amplified by PCR using a nested exon 2 primer and an LSS-1-specific primer (Fig. 1). A 512-bp RT–PCR product was detected when RNA harvested from Toledo-infected primary human embryonic lung fibroblasts (HFs) at 5 days postinfection (d.p.i.) was reverse-transcribed using this method (Fig. 2A, lane 2). Southern blot analysis using probe LSS153 verified that the product was a specific amplification product. RNA was isolated from uninfected HFs in parallel with the other samples to control for cross-contamination (Fig. 2A, lane 1). Expected results were obtained for all of the controls (Fig. 2A, lanes 3–5).

To determine whether the transcription initiating upstream of the PSS was temporally regulated, RNA was isolated from Toledo-infected HFs at 2, 6, 18, and 48 h postinfection (h.p.i.) and analyzed by LSS-1-specific RT–PCR. A 512-bp RT–PCR product was detected at all of the time points evaluated (Fig. 2B, lanes 2–7). Southern blot analysis using probe LSS153 verified that the products were specific amplicons. Again, expected results were obtained for the uninfected sample and the controls (Fig. 2B, lanes 1 and 8–10).

RT–PCR analysis of various cell types infected with HCMV was performed to determine whether transcription occurring upstream of the MIE gene PSS was restricted to HFs. Several different cell types were chosen that have been shown to be either latently or persistently infected by HCMV during natural infection. RNA was isolated from Toledo-infected differentiated THP-1 cells and monocyte-derived macrophages (MDMs) at 7 d.p.i. RNA was isolated from AD169-infected aortic endothelial cells at 24 h.p.i. A 512-bp RT–PCR product was observed for each of the cell types examined (Fig. 2C, lanes 2–5). Southern blot analysis with probe LSS153 verified that the amplification products were specific.

The expression of transcripts initiating upstream of the MIE gene PSS was also evaluated in HFs infected with clinical isolates of HCMV using the LSS-1-specific RT–PCR method. RNA was isolated from clinical isolate-infected HFs at 7 d.p.i. A 512-bp RT–PCR product was obtained for each of the isolates examined (Fig. 2C, lanes 6 and 7). Southern blot analysis with probe LSS153 verified that the amplification products were specific.

**5’ RACE analysis**

To map the 5’ ends of the transcript(s) detected by RT–PCR, 5’ RACE methodology was used. Initial experiments were performed using cDNA synthesized with RNA from Toledo-infected HFs at 5 d.p.i. and random hexanucleotide primers. The cDNA was purified and tagged with cytose. The tagged cDNA was amplified with

---

**FIG. 1.** Locations and nucleotide sequences of the primers and probes used for RT–PCR, 5’ RACE, and RPA procedures. The first three exons of the MIE gene region are shown. The various start sites are indicated by PSS, LSS-1, and LSS-2.
an abridged anchor primer and an exon 2 primer (Fig. 1 and diagram in Fig. 3A). Ethidium bromide-stained agarose gels and corresponding Southern blots of the 5' RACE PCR products obtained from this amplification are shown in Fig. 3A. The anchored PCR products were loaded as replicate samples in lanes 1–3 of the two gels, and the membranes were hybridized with a probe specific for CMV sequence either downstream (4153) or upstream (LSS153) of the MIE gene PSS. Probe LSS153 was designed to detect transcripts originating upstream from the PSS, whereas 4153 was specific for transcripts initiating at all three start sites (PSS, LSS-1, and LSS-2). An intense band migrating just below the 220-bp marker was observed in the infected fibroblast sample (Fig. 3A, lane 1 of both gels) and corresponds to amplification of transcripts originating from the PSS (194 bp). A number of other amplification product bands were also visible in lane 1 of the gels migrating above the 220-bp marker. Southern blot analysis with probe 4153 yielded multiple probe positive products (Fig. 3A, lane 1), a very prominent band corresponding to the major lytic transcripts, and at least two other higher-molecular-weight bands. However, when the same PCR products were probed with LSS153, only the higher-molecular-weight species were visible, confirming that the products represented transcripts originating upstream from the PSS. One of the
probe-positive products exhibited a migration consistent with transcripts initiating at LSS-1 (548 bp). Although a product band was visible in both gels of the expected size (486 bp) for LSS-2 transcripts, a distinct corresponding band was not evident in the Southern blots. At least two additional specific amplification products (indicated by asterisks) were generated from this 5' RACE PCR. The structure of these products remains to be determined. However, the size of the products (∼260 and ∼400 bp), as well as the fact that the products were consistently detected by 5' RACE, suggests that additional MIE gene transcription start sites are present downstream from LSS-2 (between LSS-2 and the PSS). No amplification products were observed in the infected sample in the absence of terminal deoxynucleotidyl transferase (TdT) (Fig. 3A, lane 2), indicating specific amplification of cytosine-tailed cDNA in the sample containing TdT, or in the negative control (Fig. 3A, lane 3). This 5' RACE experiment verified that transcription originating upstream of the MIE gene PSS was occurring in Toledo-infected HFs. It was also evident from this experiment that transcripts initiating upstream of the PSS were present in relatively low abundance compared with the major lytic transcripts. To map the 5' ends of the transcripts initiating upstream of the PSS, another 5' RACE experiment was performed and the products TA cloned (Fig. 3B). In this experiment, a gene-specific PCR primer located upstream of the PSS was used for PCR to avoid amplification of transcripts originating from the PSS and therefore allow for selective cloning of PCR products representing only transcripts originating upstream of the PSS. The 5' RACE PCR products were separated on an ethidium bromide-stained agarose gel and subjected to Southern blot analysis with probe LSS153. A discrete product band migrating just above the 298-bp marker was visible in the gel for the
Toledo-infected HF sample (Fig. 3B, lane 1) and was consistent with transcripts initiating at LSS-1 (317 bp). Southern blot analysis with probe LSS153 demonstrated that the PCR product was specific. Although an amplification product corresponding to LSS-2 transcripts (255 bp) was not clearly visible in the gel or the blot, sequence data obtained from PCR product cloning did confirm transcription initiating at LSS-2 (see later). Another broad probe-positive band was visible on the Southern blot with a migration between 154 and 220 bp. This band represents amplification products that would correspond to transcripts initiating downstream from LSS-2 (data not shown). The two other clones exhibited 5′ ends that mapped proximate to the LSS-2, with the majority mapping slightly upstream (7–8 bp) of the LSS-1. This would place the transcription start site at −361, exactly 25 bp downstream from a putative TATA box. The cDNA sequenced from the “LSS-2-sized” clone had a 5′ end that mapped to the LSS-2 (−292). The two other clones exhibited 5′ ends that mapped to 320 and 430 bp upstream of the PSS. A cDNA clone that would correspond to the 5′ RACE PCR products indicative of transcripts initiating downstream from LSS-2 (below 292 bp) were not detected within the group of clones screened. The fact that only 10 white colonies were screened may account for the lack of detection, especially because the PCR products generated were small and the resulting inserts may not have interrupted the reading frame of the lacZ gene. RPA analysis of Toledo-infected HFs did yield a minor protected fragment after long exposure that would correspond to transcripts initiating downstream from LSS-2 (data not shown). The results obtained from the TA cloning of the 5′ RACE PCR products confirm that transcripts initiating proximate to the MIE gene LSS-1 and at the LSS-2 are expressed in productive infection. However, all 7 of the “LSS-1-sized” cDNA clones had 5′ ends that mapped to 320 and 430 bp upstream of the PSS. A cDNA clone that would correspond to the 5′ RACE PCR products indicative of transcripts initiating downstream from LSS-2 (below 292 bp) were not detected within the group of clones screened. The fact that only 10 white colonies were screened may account for the lack of detection, especially because the PCR products generated were small and the resulting inserts may not have interrupted the reading frame of the lacZ gene. RPA analysis of Toledo-infected HFs did yield a minor protected fragment after long exposure that would correspond to transcripts initiating downstream from LSS-2 (data not shown). The results obtained from the TA cloning of the 5′ RACE PCR products confirm that transcripts initiating proximate to the MIE gene LSS-1 and at the LSS-2 are expressed in Toledo-infected HFs.

**RPA analysis**

RPA analyses were performed to confirm the 5′ RACE results and to evaluate the relative abundance of the
various MIE region transcripts. A digoxigenin (DIG) or radiolabeled RNA probe was designed to detect transcripts initiating from the MIE region LSS-1, LSS-2, and PSS. After digestion with RNase, the RNA was precipitated and resuspended in loading buffer. The protected fragments were separated by electrophoresis through a denaturing polyacrylamide gel. (A) Northern blot of protected fragments generated from a nonradioactive RPA analysis using infected HF RNA at 5 d.p.i. and a DIG-labeled probe. (B) An autoradiogram of protected fragments generated from a radioactive RPA analysis using infected HF RNA at a range of times postinfection and a 32P-labeled probe. (A) Lanes 1 and 2, probe only (no RNase); 3, probe plus RNase; 4, uninfected HF RNA (50 μg); 5, infected HF RNA (50 μg) at 5 d.p.i. (B) Lane 1, probe only (no RNase); 2, probe plus RNase; 3, uninfected HF RNA (50 μg); 4–6, infected HF RNA (50 μg) at 2 and 48 h.p.i. and 5 d.p.i., respectively; 7–9, infected HF RNA at 12 (12 μg), 24 (11 μg), and 72 (20 μg) h.p.i., respectively; M, RNA Century Marker Plus molecular weight marker. The arrows indicate the positions of the protected fragments generated from transcripts originating at the LSS-1 (475 nt) and PSS (120 nt). The numbers to the left or right of the image indicate the size in nucleotides of the adjacent RNA marker.

FIG. 4. RPA analysis of Toledo-infected HFs. RNA from Toledo-infected HFs or uninfected HFs was incubated with a labeled RNA probe designed to detect transcripts initiating from the MIE region LSS-1, LSS-2, and PSS. After digestion with RNase, the RNA was precipitated and resuspended in loading buffer. The protected fragments were separated by electrophoresis through a denaturing polyacrylamide gel. (A) Northern blot of protected fragments generated from a nonradioactive RPA analysis using infected HF RNA at 5 d.p.i. and a DIG-labeled probe. (B) An autoradiogram of protected fragments generated from a radioactive RPA analysis using infected HF RNA at a range of times postinfection and a 32P-labeled probe. (A) Lanes 1 and 2, probe only (no RNase); 3, probe plus RNase; 4, uninfected HF RNA (50 μg); 5, infected HF RNA (50 μg) at 5 d.p.i. (B) Lane 1, probe only (no RNase); 2, probe plus RNase; 3, uninfected HF RNA (50 μg); 4–6, infected HF RNA (50 μg) at 2 and 48 h.p.i. and 5 d.p.i., respectively; 7–9, infected HF RNA at 12 (12 μg), 24 (11 μg), and 72 (20 μg) h.p.i., respectively; M, RNA Century Marker Plus molecular weight marker. The arrows indicate the positions of the protected fragments generated from transcripts originating at the LSS-1 (475 nt) and PSS (120 nt). The numbers to the left or right of the image indicate the size in nucleotides of the adjacent RNA marker.
ent until 7 d.p.i. Only 20 μg of RNA was assayed at 72 h.p.i. and could explain the lack of detection of the transcripts at this time point. Regardless, it was clear from both RPA analyses that the LSS-1 latency transcripts were most abundant late in infection (5 and 7 d.p.i.), after the levels of the major lytic transcripts had diminished. RPA analysis was also used to evaluate whether the LSS-1 latency transcripts were expressed in clinical isolate-infected HFs. An RPA was performed using RNA (25 μg) isolated from fetal clinical isolate-infected HFs at 7 d.p.i. A pattern of protected fragments identical to that observed with Toledo-infected HFs was generated when RNA from fetal clinical isolate-infected HFs at 7 d.p.i. and in primary MDMs. Monocytes/macrophages differentiated monocytic cell line infected with Toledo at 7 d.p.i. was analyzed (data not shown). This finding demonstrated that expression of transcripts initiating at the LSS-1 of the MIE gene was not limited to HCMV strains that have been subjected to laboratory passage.

**DISCUSSION**

A specific product was generated using RNA harvested from Toledo-infected HFs and an RT–PCR method designed to detect transcription initiating upstream from the MIE gene PSS. Additional RT–PCR analysis of infected HFs detected transcription initiating upstream from the PSS as early as 2 h.p.i. and as late as 7 d.p.i. In addition, a specific RT–PCR product was obtained from clinical isolate-infected HFs, indicating that the expression was not an adaptation induced by the laboratory passage of the virus. It was also determined that this expression was not limited to fibroblasts. Transcription was detected in three additional cell types after infection with HCMV. An RT–PCR signal was detected in a differentiated monocytic cell line infected with Toledo at 7 d.p.i. and in primary MDMs. Monocytes/macrophages are myeloid lineage cells that have been shown to be involved in latent viral carriage in healthy HCMV-seropositive individuals (Taylor-Wiedeman et al., 1991; Sinclair and Sissons, 1996; Bolovan-Fritts et al., 1999). RT–PCR signal was also detected in AD169-infected aortic endothelial cells 24 h after infection. Endothelial cells have been persistently infected with HCMV in vitro and may play a role in HCMV pathogenesis (Fish et al., 1998; Vossen et al., 1996). Therefore, by RT–PCR analysis it was determined that transcription upstream of the PSS was active in a variety of cell types infected with HCMV. Of course, a more detailed analysis would be necessary to identify different patterns of MIE region transcript expression among these cell types.

To confirm that the transcript detected by RT–PCR originated from the LSS-1, 5′ RACE methodology was used. Using a primer upstream from the PSS in 5′ RACE PCR, products of the approximate size for transcripts initiating at LSS-1 and LSS-2 were generated. DNA sequencing of the 5′ RACE PCR products revealed start sites similar to LSS-1 and identical to LSS-2. The 3′ ends of these transcripts have not been mapped in productive infection, so it is not known whether they share the same splicing patterns as that seen in latent infection.

An evaluation of the relative abundance of the MIE region transcripts initiating at LSS-1, LSS-2, and the PSS in HCMV-infected HFs was performed using RPA analysis. Protected fragments corresponding to transcripts originating at the PSS were detected at all time points postinfection and, as expected, were most abundant at immediate-early times postinfection. In contrast, the LSS-1 latency transcripts were most abundant at late times postinfection, after the level of the PSS transcripts had decreased. It is not known whether the levels of the LSS-1 latency transcripts at 5 and 7 d.p.i. represent an accumulation of the transcripts, because transcription upstream from the PSS was detected by RT–PCR as early as 2 h.p.i., or are due to an increase in transcription initiating at the latency start site. The low abundance of the LSS-1 latency transcripts in HFs at immediate-early times after HCMV infection may account for the inability of others to detect the transcripts by RPA analysis, because the assay was performed using RNA harvested from infected HFs at 2 h.p.i. (Kondo et al., 1996). The same laboratory has since detected the latency transcripts in productively infected HFs as early as 8 h.p.i. using RPA analysis (White et al., 2000). It is clear that transcripts initiating from LSS-1 are more abundant than LSS-2 in productive infection, because transcripts corresponding to LSS-1 use were easily detectable by 5′ RACE and RPA procedures, whereas LSS-2 transcripts were detected only after cloning of 5′ RACE PCR products. This finding is consistent with that found in the experimental system of latency where transcripts corresponding to LSS-1 use were predominant over those corresponding to LSS-2 use (Kondo et al., 1996).

The function of the latency-associated sense transcripts in productive or latent infection remains to be determined. A number of open reading frames can be predicted from their sequence. The largest predicted open reading frame encoded by LSS-1 or LSS-2 transcripts is ORF 94. Using an ORF 94 knockout virus (RC2710), it was recently shown that ORF 94 is not required for productive or latent infection in culture (White et al., 2000).

The fact that the sense CLTs are present during both latent and productive infection is consistent with that observed for LAT expression during other herpesvirus infections. For example, in herpes simplex virus type 1, the major 2.0-kb LAT is expressed in ganglia during acute infection in mice, although to a lesser extent than that observed during latent infection (Spivack and Fraser, 1987). In addition, the Epstein–Barr virus LAT, EBNA-1, is expressed during the lytic cycle, although it is under the control of a promoter distinct from those used during latency (Lear et al., 1992; Schaefer et al., 1995; Nonkwelo
et al., 1996). Transcription occurs from the porcine herpesvirus 1, or pseudorabies virus, LAT gene during productive infection, although the RNAs were found to be distinct from the large latency transcript (Cheung, 1990; Jin and Scherba, 1999).

In conclusion, sense transcripts with start sites in the promoter-enhancer region of the MIE gene were identified during in vitro HCMV productive infection. The initiation sites of these sense transcripts mapped to the latent start sites previously described for the sense CLTs. The LSS-1 transcripts were predominant over LSS-2 transcripts and were found to be most abundant at late times after infection. Additionally, RT–PCR analysis demonstrated that transcripts with start sites upstream from the PSS were expressed in HCMV-infected HFs over a wide range of times postinfection and were also present in a variety of different cell types.

Further studies designed to evaluate the role of the sense CLTs in in vitro HCMV productive infection are warranted, especially considering the inherent difficulties associated with the study of HCMV latency. Defining the role of the transcripts in productive infection may provide insight into their function during latent infection.

MATERIALS AND METHODS

Cell culture

Primary human embryonic lung fibroblasts (HFs) were cultured in Iscove's modified Eagle's medium (IMDM) containing 5–10% (v/v) fetal calf serum (FCS). Primary MDMs were prepared by adhering peripheral blood mononuclear cells obtained from seronegative individuals to plastic tissue culture dishes for 15 min in phosphate-buffered saline. Nonadherent cells were removed, and the remaining adherent cells were cultured for 6 d in IMDM containing 10% (v/v) FCS plus hydrocortisone (50 \( \mu \)M). The cells were incubated overnight with phorbol-12-myristate-13-acetate (PMA; 20 nM) the day before viral infection. Transformed human premonocytic (THP-1) cells (American Type Culture Collection, Rockville, MD) were differentiated with hydrocortisone and PMA as described here for MDMs. Aortic endothelial cells were cultured as described in the manual provided by the supplier (Clonetics Corporation, San Diego, CA).

Viruses

HCMV Toledo and AD169 strains were provided by Dr. E. S. Mocarski (Stanford University). HCMV AD169 was originally obtained from American Type Culture Collection, in 1986 (passage >200). HCMV Toledo was originally acquired from Dr. Stanley Plotkin and was passage 8 when received from Dr. Mocarski. The Toledo and AD169 viral strains were propagated and plaque-titered in HFs. All HFs and viral stocks were negative for mycoplasma, acholeplasma, and ureaplasma using a Myco-

plasma PCR Elisa kit (Roche Molecular Biochemicals, Welwyn Garden City, UK). The HCMV clinical isolates were derived from infected tissue obtained from a bronchoaveolar lavage of an adult male (adult clinical isolate) and lung from an abortus (fetal clinical isolate). HFs were infected with Toledo and the clinical isolates at a multiplicity of infection (m.o.i.) of 1. Differentiated THP-1 cells and MDMs were infected with Toledo at an m.o.i. of 1 and 2, respectively. Cultured aortic endothelial cells were infected with AD169 at an m.o.i. of 1.

RNA isolation

Total cellular RNA was isolated using an RNA extraction kit (RNAGents; Promega, Madison, WI) based on the method described by Chromczynski and Sacchi (1987). Contaminating genomic DNA was removed from RNA preparations by incubation with RQ1 RNase-free DNase (Promega) as described in the RNAGents kit technical bulletin. The RNA integrity and concentration of the samples were estimated by running a small aliquot on a 1% denaturing agarose gel. Known amounts of bacterial rRNA served as quantitation standards. The RNA content of the samples was also determined spectrophotometrically.

RT–PCR

First-strand cDNA synthesis was carried out using random hexanucleotide primers (Promega) or an exon 2 gene-specific primer and SuperScript II reverse transcriptase (GIBCO BRL, Grand Island, NY). An initial annealing reaction (25 \( \mu \)l) was performed with template RNA (10 ng for RT–PCR shown in Figs. 2A and 2B and 100 ng for Fig. 2C), primer [random hexanucleotide (500 ng) or gene-specific (5 pmol)], and rRNasin (10 U). The samples were incubated for 10 min at 68°C, followed by 45 s at 65, 60, 55, 50, 45, 40, 35, 30, and 25°C, and finally 10 min at 25°C. A reaction mix (25 \( \mu \)l) was then added to the samples to yield a 50-\( \mu \)l reaction volume containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl\(_2\), 10 \( \mu \)M dithiothreitol (GIBCO BRL), 500 \( \mu \)M concentration of each deoxynucleotide triphosphate (dNTP; Roche Molecular Biochemicals), and 200 U of SuperScript II reverse transcriptase (GIBCO BRL). The samples were then incubated at 42°C for 1 h, followed by 8 min at 95°C. The samples were cooled to 4°C and used immediately for cDNA amplification or stored at −70°C. cDNA (5 \( \mu \)l) was amplified in a 50-\( \mu \)l reaction mix containing 10 mM Tris–HCl, pH 8.5 at 25°C, 50 mM KCl, 1.5 mM MgCl\(_2\), 0.01% gelatin, 0.5% Tween 20, 0.1% Triton X-100, 1 \( \mu \)M concentration of each primer (GIBCO BRL), 200 \( \mu \)M concentration of each dNTP, and 1 U Taq DNA polymerase (Promega). A modified hot-start and an initial step-down annealing reaction were used. An initial denaturation at 94°C for 4 min, was followed by 10 cycles of 94°C for 20 s, 67–58°C (step-down 1°C each cycle) for 20 s, and
72°C for 1 min. Fifty cycles of the following were then performed: 94°C for 20 s, 55°C for 20 s, and 72°C for 1 min, followed by a final 7 min at 72°C. PCR products (5 µl) were separated by electrophoresis through a 1.5% agarose gel, and the bands were visualized by staining with ethidium bromide. All reactions were carried out in a GeneAmp PCR System 2400 thermal cycler Applied Biosystem (Foster City, CA). Positive and negative controls were included with each set of samples analyzed by RT–PCR. For the RT reaction and PCR negative controls, water was substituted for DNA template. Purified Toledo DNA (100 copies) served as DNA template for the PCR positive control. According to the known sequence of AD169, the expected size of the spliced product generated from this RT–PCR is 512 bp, whereas the DNA PCR product size is 1339 bp. The locations and nucleotide sequences of the primers and the probe used for RT–PCR are shown in Fig. 1.

RACE

A 5’ RACE kit (GIBCO BRL) was used to map the 5’ ends of the transcripts initiating upstream from the PSS. RNA was isolated from Toledo-infected HFs at 5 d.p.i. First-strand cDNA synthesis was carried out using 1 µg RNA and random hexanucleotide primers or an exon 2 gene-specific primer as described earlier. cDNA was purified and dC-tailed as described in the manual supplied with the kit. Tailed cDNA (5 µl) was amplified in a 50-µl reaction mix containing 10 mM Tris–HCl (pH 8.5 at 25°C), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.5% Tween 20, 0.1% Triton X-100, 500 nM concentration of each primer (GIBCO BRL), 200 µM of concentration of each dNTP, and 1 U Taq DNA polymerase (Promega). A modified hot start was performed, followed by an initial denaturation at 94°C for 4 min. Thirty-five cycles of the following were then performed: 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, followed by a final 7 min at 72°C. PCR products (25 µl) were separated by electrophoresis through an agarose gel (1.5%), and the bands were visualized by staining with ethidium bromide. The locations and nucleotide sequences of the primers and probes used for 5’ RACE are shown in Fig. 1. The expected sizes (CMV sequence plus 36-bp anchor primer) for transcripts initiating at the various MIE start sites were as follows for 5’ RACE PCR with primer downstream from PSS: 648, 486, and 194 bp for LSS-1, LSS-2, and PSS, respectively. For 5’ RACE PCR with a primer upstream of the PSS, the expected sizes of LSS-1 and LSS-2 transcripts were 317 and 255 bp, respectively.

TA cloning of 5’ RACE PCR products

RACE PCR products were TA cloned using an Original TA Cloning kit (Invitrogen Carlsbad, CA). An aliquot (2 µl) of the PCR sample was used in the ligation reaction. InvαF− cells were transformed as described in the manual provided by Invitrogen. White colonies (10) were picked and cultured overnight in 2 ml Luria-Bertani medium containing ampicillin (100 µg/ml). Plasmid DNA isolation was performed using a Quantum Prep Plasmid Mini Prep kit (BioRad, Hercules, CA). Plasmid DNA was quantified by electrophoresis through an agarose gel (0.8%) in the presence of DNA quantification standards (GIBCO BRL). To determine the size of the cloned insert, restriction enzyme digests of the plasmid DNA were performed using EcoRI. The DNA was sequenced at the UCD Division of Biological Sciences DNA Sequencing Lab.

Southern blot analysis

The specificity of PCR amplification products was evaluated by Southern blot analysis using the following methods. Agarose gels were blotted onto ZetaProbe membranes (BioRad) using an alkaline transfer method. Gels were transferred overnight in a 0.4 N NaOH solution. The membranes were rinsed briefly with 2× SSC and then air-dried. When a DIG-labeled molecular weight marker (Vi; Roche Molecular Biochemicals) was used for estimating the size of RACE PCR product bands, a traditional Southern blot transfer was performed. Hybridization was carried out using the 5× SSC procedure described in a DIG 3’ end-labeling kit (Roche Molecular Biochemicals) with the following exceptions. The membrane was incubated in prehybridization solution for 1 h at 37°C and then incubated overnight in hybridization solution containing ~25 pmol of a DIG-labeled oligonucleotide probe (4153 or LSS153; see Fig.1). The hybridized DIG probe was localized using an anti-DIG alkaline phosphatase/CDP-Star chemiluminescent detection system (Roche Molecular Biochemicals). End (3’)-labeled DIG-labeled probes were prepared with 100 pmol of oligonucleotide (GIBCO BRL) and terminal deoxynucleotidyl transferase (24 U, FPLC-pure; Amersham Pharmacia Biotech, Picataway, NJ) using a DIG oligonucleotide 3’ end-labeling kit (Roche Molecular Biochemicals).

Preparation of a labeled antisense RNA probe

A 592-bp PCR product was first generated using a primer set that spanned a region 115 bp upstream from the LSS-1 to 120 bp downstream from the PSS (see Fig. 1). Viral DNA prepared from Toledo-infected HFs was used as template. The PCR product (2 µl) was TA cloned as described earlier except that TOP10F− cells (Invitrogen) were used. The plasmid DNA was linearized with HindIII. An in vitro transcription reaction was performed using a Riboprobe kit (Promega) and [α-32P]CTP (NEN Life Science Products, Boston, MA) or DIG-11-UTP (Roche Molecular Biochemicals). The size of the antisense riboprobe generated from this transcription was 720 nt (592 nt of HCMV-specific sequence and 127 nt of vector sequence). A labeled RNA molecular weight
marker was also prepared by in vitro transcription of an RNA Century Marker Plus Template Set (Ambion, Austin, TX).

RPA

The relative abundance of the MIE region transcripts was evaluated using a Ribonuclease Protection kit (Roche Molecular Biochemicals) with the following minor modifications. RNA (60 μg, unless otherwise indicated) harvested from Toledo-infected HF or uninfected HF, and a radiolabeled (3 × 10^5 cpm) or DIG-labeled (35 ng) RNA probes were coprecipitated with sodium acetate and absolute ethanol. The RNA was resuspended in hybridization buffer and incubated at 95°C for 5 min, followed by an overnight incubation at 50°C. RNase digestion was performed at 30°C for 45 min. When a DIG-labeled probe was used, RNase A was excluded from the digestion reaction. The RPA products were separated by electrophoresis through a denaturing polyacrylamide gel (4.0% acrylamide/7 M urea). For the nonradioactive RPA, the hybridized DIG-labeled RNA probe was localized using an anti-DIG alkaline phosphatase/radioactive RPA, the hybridized DIG-labeled probe was used, RNase A was excluded digestion was performed at 30°C for 45 min. When a DIG-labeled probe was used, RNase A was excluded from the digestion reaction. The RPA products were separated by electrophoresis through a denaturing polyacrylamide gel (4.0% acrylamide/7 M urea). For the nonradioactive RPA, the hybridized DIG-labeled RNA probe was localized using an anti-DIG alkaline phosphatase/CDP-Star chemiluminescent detection system (Roche Molecular Biochemicals). For the radioactive RPA, a standard phosphor screen was exposed to the polyacrylamide gel, and the screen was scanned using a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The digitized image was analyzed using ImageQuant software (Molecular Dynamics). A labeled RNA molecular weight marker (RNA Century Marker Plus; Ambion) was used to estimate the sizes of the protected fragments.

ACKNOWLEDGMENTS

This work was supported by grants from the Children's Miracle Network and from the UCD School of Medicine Dean's Fund. The authors would like to thank Ms. Kirsten White, Dr. Edward Mocarski, Dr. Ravi Kaul, and Dr. Wanda Wenman for critical review of the manuscript. The authors would also like to thank Dr. Cynthia Bolovan-Fritts for endothelial cell RNA.

REFERENCES