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Discordant Expression of the Immediate-Early 1 and 2 Gene Regions of Human Cytomegalovirus at Early Times after Infection Involves Posttranscriptional Processing Events

THOMAS STAMMINGER,* ELKE PUCHTLER, AND BERNHARD FLECKENSTEIN

Institut für Klinische und Molekulare Virologie der Universität Erlangen-Nürnberg, Loschgestrasse 7, 8520 Erlangen, Federal Republic of Germany

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Expression of the immediate-early 1 and 2 (IE-1 and IE-2) gene region of human cytomegalovirus (HCMV) was studied during initial phases of the replicative cycle. Accumulation of RNA from IE-1 and -2 was found to be differential. Transcripts from IE-2 reached peak levels very early in infection between 3 and 5 h, whereas IE-1 RNA peak levels were detected later, between 6 and 8 h. A strong decrease in steady-state levels of a 2.2-kb IE-2 RNA was observed at a time when IE-1 transcripts showed a further increase in abundance. Northern (RNA) blot experiments revealed that expression of both the IE-1 RNA and the 2.2-kb IE-2 transcript is controlled by the IE-1 enhancer-promoter. Nuclear run-on experiments demonstrated equal rates of primary transcription for IE-1 and -2 at a time when different steady-state levels of RNA were observed. Concomitant with down-regulation of IE-2 RNAs, a decrease in the size of the IE-1 transcript was detected. At 2 to 5 h after infection the IE-1 transcript migrated at 1.95 kb, whereas later in the replicative cycle the RNA was found at 1.8 kb. RNase H blot analysis revealed that this size discrepancy is due to a shorter poly(A) tail of the IE-1 RNA at early times after infection. These experiments suggest that in addition to transcriptional regulation, specific posttranscriptional mechanisms are involved in controlling expression from the IE-1 and -2 gene region of HCMV.

Human cytomegalovirus (HCMV), which belongs to the beta subgroup of herpesviruses, is characterized by its narrow host range and prolonged replicative cycle in tissue culture systems. Gene expression of HCMV, which has been most extensively studied in primary human fibroblasts, occurs, as with other herpesviruses, in a temporally regulated manner (11, 20, 35, 36). At least three broad phases of viral gene expression have been described, which were termed immediate early (IE), early, and late. IE proteins are thought to exert important regulatory functions in the switch from restricted to extensive expression of the viral genome (18, 25, 26, 29). IE genes are transcribed primarily under control of host cell *trans*-acting factors, and their expression does not require de novo protein synthesis. Under experimental conditions, IE genes are defined as being transcribed in the presence of an inhibitor of protein synthesis such as cycloheximide or anisomycin. By this approach, an abundantly expressed IE gene region has been identified within HCMV which is located between map units 0.66 and 0.77 of the viral genome (11, 16, 20, 35, 37). Two transcription units within this region, termed IE-1 and IE-2, have been studied extensively (1, 31, 32). The IE-1 gene region codes for a 1.95-kb spliced mRNA which is translated into a phosphorylated protein of apparent molecular weight 72,000 (1, 3, 7, 13, 29, 31, 37). IE-2 codes for a series of mRNAs of 2.2, 1.7, and 1.5 kb (16, 28, 32, 33, 37). A strong transcriptional enhancer element is located immediately upstream of the IE-1 cap site (4, 34). Since it could be shown that the first exons of IE-1 are differentially spliced onto IE-2 exons (28, 32), this enhancer-promoter regulatory region drives expression of both IE-1 and IE-2 mRNAs. Transcription from IE-1 and -2 occurs throughout the HCMV replicative cycle, with the pattern of RNAs changing between early and late times (28). In particular, an abundant transcript of 1.5 kb that originates from within IE-2 could be detected at late times after infection (28).

It has been reported that IE-1 RNA steady-state levels undergo negative regulation during the time course of HCMV infection (16, 28, 30); however, neither the exact time point of negative regulation nor the responsible mechanism has been fully clarified. Since it could be shown that IE-2 gene products are able to negatively regulate gene expression from the IE-1 enhancer-promoter in transient expression assays (14, 25, 29), an autoregulatory mechanism of IE-1 and -2 gene expression has been proposed. Conflicting information is also available on the relative abundance of IE-1 and -2 gene transcripts during the first hours of HCMV infection. Stinski et al. (33) describe that IE-1 is the most abundant species of RNA on polyribosomes expressed during initial phases of infection, whereas Stenberg et al. (28) report about equal expression of IE-1 and -2 when analyzing cytoplasmic RNA. These discrepant findings were explained by the different RNA isolation procedures used (28).

In this study, we attempted to define the relative abundance of transcripts from IE-1 and -2 during the initial phases of HCMV infection. As most studies dealing with the splicing patterns of IE-1 and -2 transcripts were performed with RNA harvested in the presence of protein synthesis inhibitors, we were also interested in determining whether cycloheximide has an influence on expression of this gene region. We describe a biphasic negative regulation of transcripts originating from IE-1 and -2. Our data suggest that posttranscriptional mechanisms, occurring at the level of specific down-regulation of IE-2 RNAs and at the level of modification of the IE-1 RNA poly(A) tail length, are involved in regulation of IE-1 and -2 gene expression.

* Corresponding author.

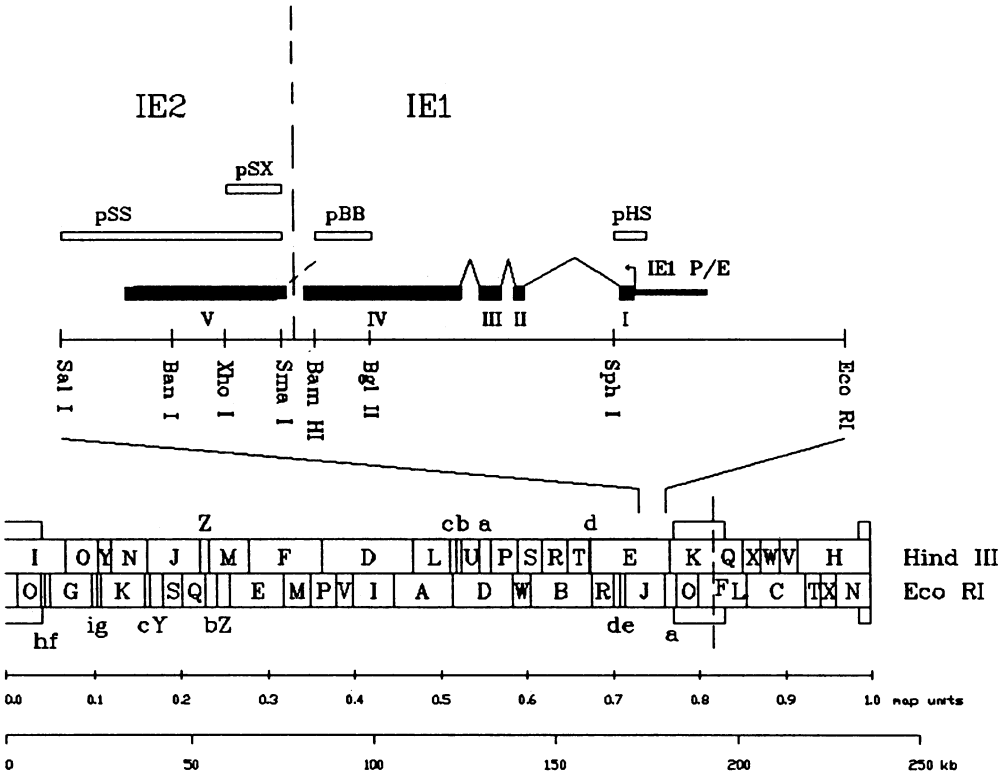

FIG. 1. Prototype arrangement of the HCMV AD169 genome and positions of plasmid clones used in this study. The prototype arrangement of the HCMV AD169 genome is shown in the lower half; restriction sites used for subcloning of DNA fragments that are specific for either IE-1 or IE-2 are indicated in the upper half. The splicing patterns of IE-1 and -2 (■; roman numerals refer to exons 1 to 5 of IE-1 and -2) and the position of the IE-1 enhancer-promoter region (——) are diagrammed. Sequences that were subcloned in plasmid vectors together with the nomenclature of the resulting plasmids (pSS, pSX, pBB, and pHS) are indicated by open bars at the top.

MATERIALS AND METHODS

Virus and cell culture. Primary human foreskin fibroblasts were prepared and cultured as previously described (16). The fibroblasts were infected with HCMV (AD169) at 60% confluence with 10 to 20 PFU per cell. Virus was allowed to adsorb to the cells for 1 h at 37°C. Cells were then washed two times with minimal essential medium supplemented with glutamine, gentamicin, and 5% fetal calf serum and were harvested at the indicated times after infection. For isolation of RNA in the presence of cycloheximide, the cells were infected and maintained in medium supplemented with 50 or 150 μg of cycloheximide per ml as indicated. Dactinomycin was added to the medium at a concentration of 5 μg/ml at the indicated times.

Plasmid cloning and DNA labeling. Cloning reactions were performed according to standard procedures. For construction of plasmids pBB, pSX, and pSS (Fig. 1), the respective fragments were isolated from plasmid pRR47 that contained a 6.7-kb *Eco*RI-*Sal*I insert of the *Eco*RI J fragment of HCMV strain AD169 (25a). After size fractionation in low-gelling agarose, DNA fragments were purified by chromatography on Elutip-d columns (Schleicher & Schuell, Dassel, Federal Republic of Germany) and ligated into the BlueScribe vector (Vector Cloning Systems, San Diego, Calif.). Plasmid pHS was created by inserting the *Hin*dIII-*Sph*I fragment of plasmid pRR38/8 (containing sequences from −92 to +174 of the IE-1 cap site; 25a) into the BlueScribe vector. DNA fragments used as probes in hybridization reactions were purified from vector sequences as described above and labeled by nick translation to a specific activity of 1×10^8 to 2×10^8 cpm/μg of DNA, using [α-^{32}P]dATP.

Isolation of RNA. Total cellular RNA was isolated according to Chomczynski and Sacchi (9). Briefly, cells of one 9-cm petri dish were lysed in 2 ml of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. Then 0.2 ml of 2 M sodium acetate (pH 4), 2 ml of water-saturated phenol, and 0.4 ml of chloroform-isoamyl alcohol (49:1) were added sequentially. The homogenate was shaken vigorously and incubated on ice for 15 min. After a centrifugation step of 20 min at 10,000 rpm in a Sorvall SS34 rotor, the aqueous phase was transferred to a new tube and precipitated twice, using an equal volume of isopropanol. The RNA pellet was washed several times with 75% ethanol, vacuum dried briefly, and resuspended in 200 μl of sterile water.

Cytoplasmic RNA was isolated by lysing the cells in a buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), and 0.6% Nonidet P-40. After a brief centrifugation step, the cytoplasmic supernatant was transferred to a tube containing 300 μl of buffer B (7 M urea, 1% sodium dodecyl sulfate [SDS], 0.35 M NaCl, 20 mM EDTA, 10 mM Tris HCl [pH 7.5]) and 600 μl of phenol-chloroform (1:1) and was shaken vigorously for at least 1 min. After centrifugation, the aqueous phase was transferred to a new tube and phenolization was repeated twice. The RNA was then precipitated by the addition of 2 volumes

ethanol and, after washing of the pellet with 80% ethanol, resuspended in sterile water.

RNase protection and Northern (RNA) blot analysis. RNase protection analyses were performed as described previously (27). Antisense transcripts were prepared by using plasmids pBB, pHS, and pSX, which contained various portions of the HCMV IE-1 and -2 gene region (Fig. 1) fused to T3 and T7 bacteriophage promoters. For in vitro transcription reactions, these plasmids were linearized with restriction enzymes, leaving sequences of the vectors' multiple cloning site attached to the insert. Therefore, the size of each probe changed after RNase digestion. RNA was hybridized with 250,000 cpm of the probe in a buffer containing 80% formamide, 40 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 400 mM NaCl, and 1 mM EDTA. Digestion conditions were as described previously (27).

For Northern blot analysis, RNA, denatured for 15 min at 65°C in the presence of formaldehyde, was separated on horizontal gels of 1 to 3% agarose containing 0.4 M formaldehyde, 20 mM morpholinepropanesulfonic acid electrophoresis buffer, 5 mM sodium acetate, and 1 mM EDTA at 200 V for 4 h. As molecular weight markers, an RNA ladder (GIBCO/BRL, Eggenstein, Federal Republic of Germany) ranging in size from 0.24 to 9.5 kb was run in parallel. RNA was visualized by ethidium bromide staining to control the amount of RNA loaded onto the gel. The RNA was transferred to nitrocellulose filters, fixed to the filters by heat (2 h, 80°C), and prehybridized for 12 h at 43°C in prehybridization solution containing 50% formamide, 5× SSC, 25 mM potassium phosphate (pH 7.4), 5× Denhardt's solution, and 50 μg of salmon sperm DNA per ml. As radioactive probes, DNA fragments corresponding to various portions of the IE-1 and 2 gene region were labeled by nick translation as described above. Hybridizations were performed for 12 h in prehybridization solution supplemented with 10% dextran sulfate (Sigma Chemical Co., Deisenhofen, Federal Republic of Germany) and the radioactive probe at 500,000 cpm/ml. The filters were washed several times after hybridization with buffer containing 20 mM sodium phosphate, 0.1% SDS, and decreasing concentrations of SSC. The dried filters were autoradiographed for 4 to 48 h at room temperature.

Nuclear run-on analysis. Nuclei of HCMV-infected fibroblasts were isolated by lysis of the cytoplasmic membrane of cells in a buffer containing 10 mM Tris HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (vol/vol) Nonidet P-40, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation for 5 min at 1,500 rpm (Beckman J6B centrifuge), the pellet containing the isolated nuclei was resuspended in 100 μl of 50 mM Tris HCl (pH 8.3)–40% (vol/vol) glycerol–5 mM $MgCl_2$–0.1 mM EDTA–0.1 mM PMSF. Preinitiated transcripts were elongated by incubation for 30 min at 30°C in the presence of 150 mM KCl, 5 mM $MgCl_2$, 20% glycerol, 25 mM Tris HCl (pH 8.0), 0.1 mM EDTA, 0.2 mM PMSF, 0.3 mg of heparin per ml, 2,500 U of RNAguard (Pharmacia, Freiburg, Federal Republic of Germany) per ml, 2.5 mM ATP, CTP, and GTP, and 100 μCi of [^{32}P]UTP. RNA was isolated from this reaction as described by Chomczynski and Sacchi (9). DNA fragments representing individual genomic regions of HCMV were purified from vector sequences and spotted onto nitrocellulose filters together with appropriate negative and positive control sequences. After fixation of the DNA by heat, filters were prehybridized as described for Northern blot analysis. Hybridizations were performed at 43°C for 72 h in prehybridization solution containing the radioactive RNA. After hybridization, filters were washed in 2× SSC at 65°C for 2 h and then incubated for 15 min in 2× SSC containing 8 μg of RNase A per ml.

PCR. Total cellular RNA (10 μg) was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) and oligo(dT)$_{15}$ as a primer. Oligonucleotides (30-mers) corresponding in sequence to the 5' and 3' ends of the IE-1 RNA were synthesized on a Cyclone DNA synthesizer (Milligene/Biosearch, Eschborn, Federal Republic of Germany). Amplification was performed in a buffer containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM deoxynucleoside triphosphates, 25 U of *Taq* polymerase (Perkin-Elmer Cetus) per ml, and 50 pmol of each primer. Polymerase chain reaction (PCR) cycling was accomplished with a DNA thermal cycler (Biotech, Greifenberg, Federal Republic of Germany). Thirty cycles of 40 s at 56°C for annealing, 3 min at 72°C for polymerization, and 40 s at 92°C for denaturation were used. PCR products were separated on 1% agarose gels.

RNase H blot analysis. RNase H blot analysis was performed essentially as described by Carrazana et al. (8). To separate the poly(A) tail from the body of the IE-1 RNA, a 30-mer oligonucleotide (H-oligo) complementary to a sequence 216 nucleotides (nt) upstream of the IE-1 3' end was synthesized. Then 5 to 10 μg of total RNA in 100 mM KCl–0.1 mM EDTA was mixed with 0.5 μg of H-oligo, denatured at 65°C for 5 min, and hybridized to the oligonucleotide at 22°C for 30 min. RNA was recovered by ethanol precipitation and digested with 40 U of RNase H (Boehringer, Mannheim, Federal Republic of Germany) per ml in 10 mM $MgCl_2$–80 mM KCl–1 mM dithiothreitol–0.5 mg of bovine serum albumin per ml–50 mM Tris HCl (pH 7.5). To destroy the poly(A) tail of RNA, 10 μg of total RNA was hybridized to 5 μg of oligo(dT)$_{15}$. After RNase H digestion, RNA was phenol-chloroform extracted and analyzed by Northern blotting as described above.

RESULTS

Biphasic negative regulation of transcripts from the IE-1 and -2 gene region during initial phases of infection. In an attempt to define the relative abundance of IE-1 and -2 transcripts, expression of RNA originating from this gene region of HCMV was studied during the immediate-early and early phases of infection. Since IE-1 and IE-2 show a complex splicing pattern, with the first exons of IE-1 being spliced onto exons of IE-2, it was necessary to construct probes that are specific either for IE-1 or for IE-2. As a probe specific for IE-1, a 474-nt *Bgl*II-*Bam*HI fragment from exon 4 of IE-1 was chosen. A 1,884-nt *Sma*I-*Sal*I fragment and a 469-nt *Sma*I-*Xho*I fragment were used as probes that are specific for IE-2 transcripts (Fig. 1). These fragments were subcloned into the BlueScribe vector to allow the generation of antisense transcripts for RNase protection experiments. Human foreskin fibroblasts were infected at a high multiplicity of infection to create one-step multiplication conditions. Total cellular RNA was isolated at 3, 6, 12, 24, and 72 h after infection and analyzed by RNase protection, using plasmids pBB and pSX (Fig. 1) to generate specific antisense transcripts (Fig. 2). Both IE-1- and -2-specific transcripts could be abundantly detected at 3 h after infection, with IE-2 RNA levels being higher than IE-1 RNA levels (Fig. 2, lanes 4 and 9). Surprisingly, steady-state levels of RNA from IE-1 showed a further increase in abundance at 6 h after infection, whereas a strong reduction in RNA from IE-2 was observed (Fig. 2, lanes 5 and 10). The levels of RNA from IE-2 at 6 h of infection were about the same as observed at early times,

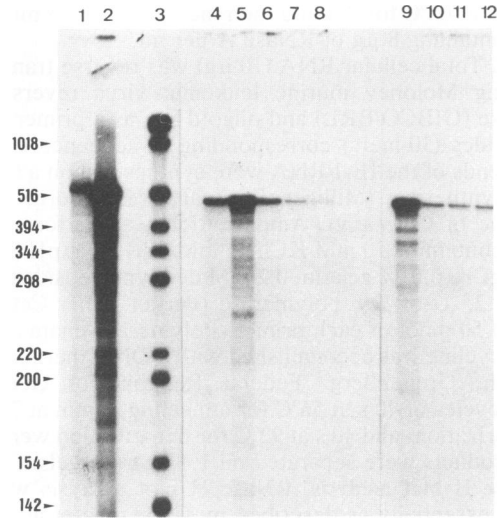

FIG. 2. RNase protection analysis with RNA isolated during the HCMV replicative cycle. Lanes: 1, antisense transcript derived from plasmid pBB; 2, antisense transcript derived from plasmid pSX; 3, molecular weight marker kilobase ladder; 4 to 8, antisense transcript derived from plasmid pBB used for hybridizations to detect IE-1-specific transcripts; 9 to 12, antisense transcript derived from plasmid pSX used for hybridizations to detect IE-2-specific transcripts; 4 and 9, 5 μg of RNA harvested at 3 h after infection; 5 and 10, 5 μg of RNA harvested at 6 h after infection; 6 and 11, 5 μg of RNA harvested at 12 h after infection; 7 and 12, 5 μg of RNA harvested at 24 h after infection; 8, 5 μg of RNA harvested at 72 h after infection. Sizes of molecular weight markers (in nucleotides) are indicated on the left.

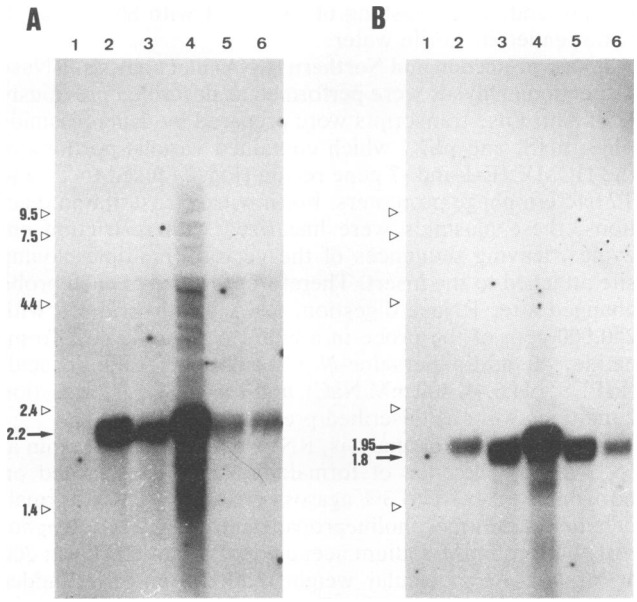

FIG. 3. Northern blot analysis with RNA purified during the initial phases of the HCMV replicative cycle to detect IE-1- and -2-specific transcripts. (A) The insert of plasmid pSS was used as probe to detect IE-2-specific transcripts; (B) the insert of plasmid pBB was used as probe to detect IE-1-specific transcripts. Lanes: 1 to 6, 10 μg of RNA harvested at 1, 3, 6, 6 h after HCMV infection in the presence of cycloheximide (50 μg/ml), 8 and 12 h after HCMV infection. Sizes (in kilobases) of major transcripts are indicated by arrows; positions and sizes of molecular weight markers (in kilobases) are indicated by triangles.

whereas IE-1 RNA decreased during early times, reaching minimum levels at late times (Fig. 2, lanes 6 to 8 and 10 to 12). This kinetics of IE-1 and -2 gene expression observed in RNase protection experiments was obtained with several independent RNA preparations using viral stocks of different passage as well as primary human fibroblasts of various individuals (data not shown).

Northern blot experiments were then performed to determine the sizes of transcripts expressed during the initial phases of infection from IE-1 and -2 (Fig. 3). Total cellular RNA, harvested at indicated times after infection, was separated on 1.2% formaldehyde agarose gels, transferred to nitrocellulose filters, and hybridized with the insert of plasmid pSS (Fig. 1) to detect IE-2-specific transcripts (Fig. 3A). At 3 h after infection, one major signal corresponding to a size of 2.2 kb was observed in Northern blot experiments (Fig. 3A, lane 2). At 6 h after infection and later on the replicative cycle, a decrease in steady-state levels of this 2.2-kb transcript was obvious (Fig. 3A, lanes 3, 5, and 6). When RNA was harvested 6 h after infection in the presence of cycloheximide (50 μg/ml), the 2.2-kb IE-2 RNA was abundantly present. In addition, signals of 1.7, 1.4, and 1.1 kb could be observed in RNA isolated under the experimental conditions of cycloheximide block (Fig. 3A, lane 4).

To assess the relative abundance of IE-1 transcripts in the same RNA preparation, the filter used for Fig. 3A was rehybridized with the insert of plasmid pBB as a probe to detect IE-1-specific transcripts. This probe detected an RNA of 1.95 kb at 3 h after infection (Fig. 3B, lane 2). Signals at 6 and 8 h after infection showed an increase in intensity relative to the signal at 3 h but corresponded now to a size of 1.8 kb, as could also be observed with RNA harvested at 12 h after infection (Fig. 3B, lanes 3, 5, and 6). In the presence of cycloheximide (50 μg/ml), the IE-1 RNA was at a size of 1.95 kb (Fig. 3B, lane 4). Thus, the kinetics of IE-1 and -2 gene expression observed in RNase protection experiments was confirmed by Northern blot studies. Accumulation of transcripts from IE-1 and -2 was found to be differential in whole cell RNA. Transcripts from IE-2 reached peak levels very early in infection, between 3 and 5 h after infection, and then rapidly declined, whereas IE-1 RNA peak levels could be observed later, between 6 and 8 h. To test whether the levels of IE-1 and -2 transcripts were the same in the cytoplasm, RNase protection experiments were performed with cytoplasmic RNA harvested 3, 6, 8, and 10 h after infection of fibroblasts with HCMV. Again, plasmids pBB and pSX were used to synthesize IE-1- or IE-2-specific riboprobes. The same differential pattern of RNA steady-state levels could be observed as with total cellular RNA (Fig. 4).

These experiments demonstrate a differential regulation of transcripts from IE-1 and IE-2 that could be detected both in total cellular and in cytoplasmic RNA. IE-2 transcripts were negatively regulated at a time when IE-1 expression showed a further increase. Negative regulation of IE-2 gene expression could not be observed if cycloheximide was used to prevent protein synthesis.

Posttranscriptional events negatively regulate IE-2 gene expression at early times. Northern blot experiments detected a 2.2-kb IE-2 transcript that was negatively regulated at early times after infection. As a previous study suggested the existence of two different 2.2-kb IE-2 transcripts, one of which shared exons with IE-1 (15), additional Northern blot

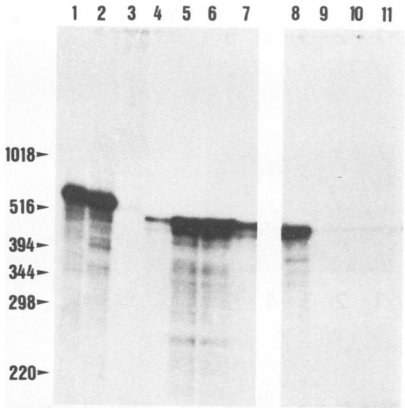

FIG. 4. RNase protection analysis with cytoplasmic RNA harvested at 3, 6, 8, and 10 h after HCMV infection of human fibroblasts. Lanes: 1, antisense transcript derived from plasmid pBB; 2, antisense transcript derived from plasmid pSX; lane 3, molecular weight marker kilobase ladder; 4 to 7, antisense transcript derived from plasmid pBB used for hybridizations to detect IE-1-specific transcripts; 8 to 11, antisense transcript derived from plasmid pSX used to detect IE-2-specific transcripts; 4 and 8, 5 μg of cytoplasmic RNA harvested at 3 h after infection; 5 and 9, 5 μg of cytoplasmic RNA harvested at 6 h after infection; 6 and 10, 5 μg of cytoplasmic RNA harvested at 8 h after infection; 7 and 11, 5 μg of RNA harvested at 10 h after infection. Sizes of molecular weight markers (in nucleotides) are indicated on the left.

experiments were performed. Total cellular RNA was harvested at 3, 6, and 6 h in the presence of cycloheximide (150 μg/ml); at 24, 48, and 72 h after infection, it was separated by electrophoresis, transferred to nitrocellulose filters, and hybridized to the insert of plasmid pSX. The same pattern of IE-2 RNA steady-state levels was observed as in previous hybridizations (Fig. 5A, lanes 1 to 4). Again, additional signals at 1.7, 1.4, and 1.1 kb were seen when RNA was harvested in the presence of cycloheximide (Fig. 5A, lane 3). These transcripts could not be detected with RNA harvested without the drug, even on long exposures of Northern blots (Fig. 5D, lanes 1 and 2).

To test whether the 2.2-kb IE-2 transcript contains exons of IE-1, we performed hybridizations using the insert of plasmid pHS (Fig. 1). This DNA fragment contains exon 1 of IE-1 and should detect the 1.95-kb IE-1 transcript as well as IE-2 transcripts containing the first exons of IE-1. In RNA isolated at 3 h after infection, the main signal with the exon 1-specific probe corresponded to a size of 2.2 kb. A second, fainter band was visible at 1.95 kb which was easily detectable on longer exposures (Fig. 5B, lane 1). At 6 h after infection, the probe hybridized to an RNA of 1.8 kb (Fig. 5B, lane 2). In the presence of cycloheximide, two prominent signals of 2.2 and 1.95 kb were visible which were identical in intensity (Fig. 5B, lane 3; Fig. 5D, lane 3). In addition, a ladder of discrete bands with sizes of 1.7 to 0.5 kb appeared with the exon 1 probe that was not observed during the natural time course of infection (Fig. 5B, lane 3; Fig. 5D, lane 3). The 1.8-kb signal observed at 6 h, which was also present later in the infectious cycle (Fig. 5B, lane 4; Fig. 5C, lanes 5 and 6), could not be detected in the presence of cycloheximide.

To distinguish between IE-1- and IE-2-specific transcripts, the filter used for Fig. 5B was rehybridized with the insert of plasmid pBB (Fig. 1), which should exclusively detect IE-1-specific transcripts. This probe detected a transcript of 1.95 kb at 3 h after infection and at 6 h after infection in the presence of cycloheximide (Fig. 5C, lanes 1 and 3; Fig. 5D, lane 4; Fig. 5E). At 6 h after infection without the drug and later in the replicative cycle, the signal was at 1.8 kb (Fig. 5C, lanes 2, 4, 5, and 6; Fig. 5E).

Since the exon 1-specific probe detected the 2.2-kb IE-2 RNA, this RNA shares exons with IE-1. Northern blots

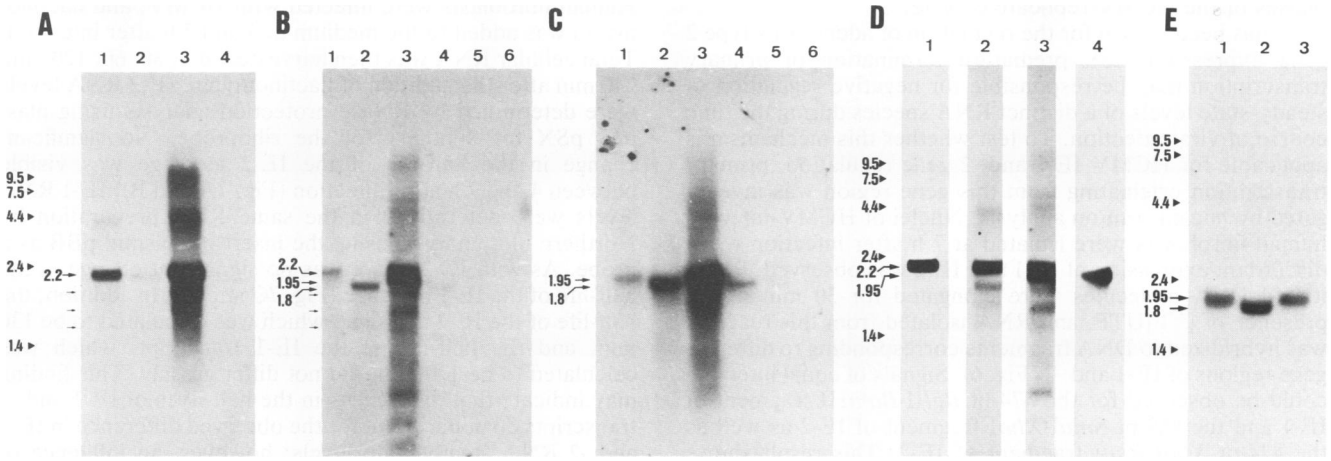

FIG. 5. Northern blot analysis with RNA purified at different times postinfection to detect IE-1- and -2-specific transcripts. (A) The insert of plasmid pSS was used as a probe to detect IE-2-specific transcripts; (B) the insert of plasmid pHS was used as a probe to detect transcripts containing exon 1 of IE-1; (C) the insert of plasmid pBB was used as a probe to detect IE-1-specific transcripts. Lane 3 contained 10 μg of RNA harvested at 3, 6, and 6 h after HCMV infection in the presence of cycloheximide (150 μg/ml). Lanes 4 to 6, at 24, 48, and 72 h, RNA was harvested without cyclohexamide. (D) Longer and shorter exposures of the lanes in panels A to C. Lanes: 1, long exposure of lane 1 in panel A; 2, short exposure of lane 3 in panel A; 3, short exposure of lane 3 in panel B; 4, short exposure of lane 3 in panel C. (E) The insert of plasmid pBB was used to detect IE-1-specific transcripts. Lanes: 1, 20 μg of RNA harvested at 3 h after infection; 2, 5 μg of RNA harvested at 6 h after infection; 3, 0.5 μg of RNA harvested at 6 h after infection in the presence of cycloheximide (150 μg/ml). Sizes (in kilobases) of major transcripts are marked large arrows; small arrows indicate transcripts of 1.7, 1.4, 1.1, and 0.5 kb in the presence of cycloheximide. Positions and sizes of molecular weight markers (in kilobases) are indicated by triangles.

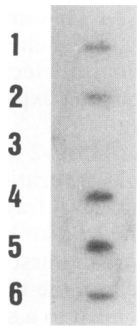

FIG. 6. Nuclear run-on analysis with HCMV-infected fibroblasts. Nuclei of HCMV-infected fibroblasts were harvested at 7 h after infection. Preinitiated primary transcripts were elongated in the presence of [^{32}P]UTP as the radioactive nucleotide. RNA was isolated and hybridized to different DNA fragments spotted onto a nitrocellulose filter. Bands: 1, 1 μg of glyceraldehyde 3-phosphate dehydrogenase DNA (1.4 kb) as a positive control; 2, 1 μg of actin DNA (2.2 kb) as a positive control; 3, 1 μg of pUC19 DNA as a negative control; 4, 1 μg of the *Bgl*II-*Bam*HI fragment of IE-1 (474 nt); 5, 1 μg of the *Sma*I-*Xho*I fragment of IE-2 (469 nt); 6, 1 μg of the *Xho*I-*Ban*I fragment of IE-2 (424 nt) (for locations of restriction enzyme sites, see Fig. 1).

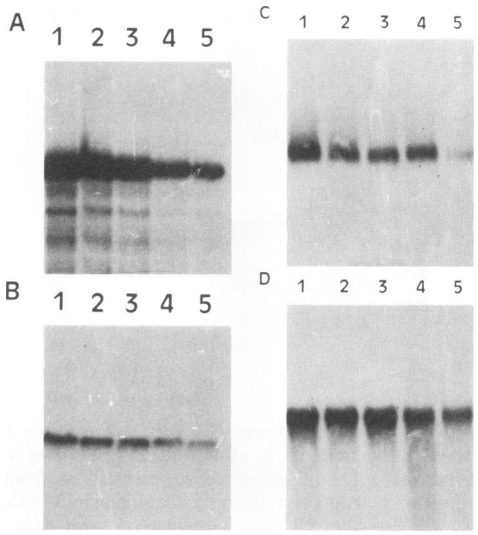

FIG. 7. Determination of IE-1 and -2 RNA levels in the presence of dactinomycin. Human fibroblasts were infected with HCMV, and dactinomycin was added at different times after infection. RNA was harvested at the indicated times after the addition of dactinomycin. (A and B) IE-2 RNA levels were determined in RNase protection experiments using a riboprobe derived from plasmid pSX; (C and D) IE-1 RNA levels were determined in Northern blot experiments using the insert of plasmid pBB as a probe; (A and C) dactinomycin was added at 4 h after infection; (B and D) dactinomycin D was added at 7 h after infection. Lanes 1 to 5 contained RNA that was harvested at 1, 30, 60, 120, and 240 min after the addition of dactinomycin.

using a probe that is specific for exon 3 of IE-1 detected also the 2.2-kb IE-2 RNA (data not shown). Thus, the 2.2-kb IE-2 transcript contains the first exons of IE-1 spliced to exon 5 of IE-2 (32). These experiments confirmed the kinetics of IE-1 and -2 gene expression that was observed in RNase protection experiments. At early times, negative regulation could be observed for a 2.2-kb IE-2 transcript when expression of IE-1 showed a further increase. Both transcripts are under control of the IE-1 enhancer-promoter regulatory region, since both transcripts hybridized with exon 1 of IE-1. We conclude from these data that regulation of promoter activities alone cannot account for the differing steady-state levels of IE-1 and -2 RNA that were observed during initial phases of the HCMV replicative cycle.

As has been shown for the regulation of adenovirus type 2 gene expression (23), premature termination of primary transcription may be responsible for negative regulation of steady-state levels of a distinct RNA species during the time course of viral infection. To test whether this mechanism is applicable for HCMV IE-1 and -2 gene regulation, primary transcription originating from this gene region was investigated by nuclear run-on analysis. Nuclei of HCMV-infected human fibroblasts were isolated at 7 h after infection when discordant expression of IE-1 and IE-2 was observed. Preinitiated RNA molecules were elongated for 30 min in the presence of [^{32}P]UTP, and RNA isolated from this reaction was hybridized to DNA fragments corresponding to different gene regions of IE-1 and -2 (Fig. 6). Signals of equal intensity could be observed for the 474-nt *Bgl*II-*Bam*HI fragment of IE-1 and the 469-nt *Sma*I-*Xho*I fragment of IE-2 as well as the 424-nt *Xho*I-*Ban*I fragment of IE-2. This result shows that there is still significant primary transcription at 7 h after infection as well as an equal distribution of primary transcription between IE-1 and IE-2. Given this result, we argue that premature transcriptional termination may not be the major factor accounting for down-regulation of IE-2 RNA steady-state levels during the early phase of HCMV gene expression. This experiment, together with the observation that both RNAs are transcribed under control of the same enhancer-promoter region, suggests that a posttranscriptional mechanism is responsible for down-regulation of IE-2 RNA steady-state levels.

To test whether changes in the half-life of IE-2 transcripts occur during the first hours of the replicative cycle, RNA levels were determined in the presence of dactinomycin. Human fibroblasts were infected with HCMV, and dactinomycin was added to the medium at 4 and 7 h after infection. Total cellular RNA was then harvested at 1, 30, 60, 120, and 240 min after the addition of dactinomycin. IE-2 RNA levels were determined by RNase protection analysis using plasmid pSX for synthesis of the riboprobe. No significant change in the half-life of the IE-2 message was visible between 4 and 7 h after infection (Fig. 7A and B). IE-1 RNA levels were determined in the same RNA preparation by Northern blot analysis using the insert of plasmid pBB as a probe. As with IE-2, there was no significant change in the half-life of the IE-1 message (Fig. 7C and D). In addition, the half-life of the IE-2 message, which was calculated to be 130 min, and the half-life of the IE-1 transcript, which was calculated to be 170 min, did not differ grossly. This finding may indicate that differences in the half-lives of IE-1 and -2 transcripts do not account for the observed difference in IE-1 and -2 RNA steady-state levels; however, an influence of dactinomycin treatment on mRNA half-lives cannot be excluded at present.

Poly(A) tracts of the IE-1 transcript are shortened during the time course of infection. Concomitant with negative regulation of IE-2 RNA steady-state levels, a qualitative change in IE-1 gene expression was observed. Northern blot hybridizations detected a 1.95-kb IE-1 transcript at 2 to 5 h after infection (Fig. 3B, lane 2; Fig. 5C and E, lanes 1; data not shown), whereas later in the infectious cycle a 1.8-kb

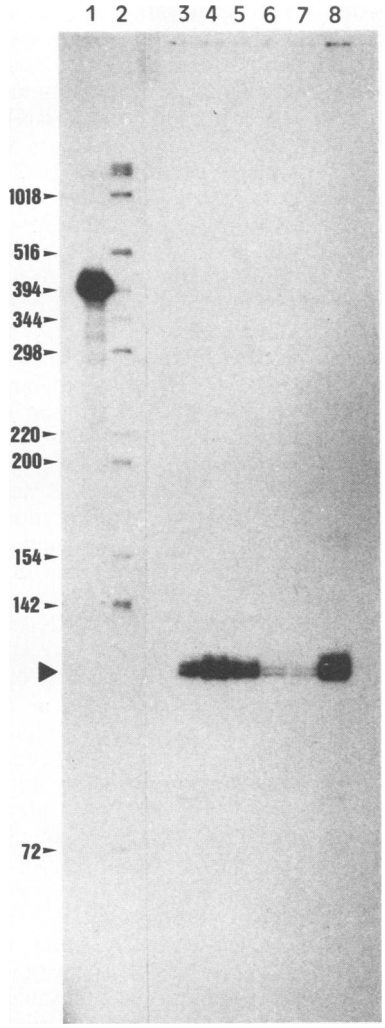

FIG. 8. Determination of the 5′ ends of IE-1 and -2 RNAs by RNase protection analysis using plasmid pHS and total cellular RNA harvested at different times postinfection. Lanes: 1, antisense transcript derived from plasmid pHS; 2, molecular weight marker kilobase ladder; 3 to 7, the riboprobe from plasmid pHS hybridized with 5 μg of RNA harvested at 3, 6, 10, 24, and 72 h after infection; 8, the riboprobe from plasmid pHS hybridized with 1 μg of RNA harvested at 6 h after infection in the presence of cycloheximide (150 μg/ml). The triangle indicates the position of protected fragments; sizes (in nucleotides) of molecular weight markers are shown on the left.

RNA originated from this region (Fig. 3B, lanes 3, 5, and 6; Fig. 5C and E, lanes 2, 4, 5, and 6). The change in IE-1 transcript size was prevented by cycloheximide treatment of cells (Fig. 3B, lane 4; Fig. 5C, lane 3; Fig. 5D, lane 4; Fig. 5E, lane 3). This size variation during the time course of infection could be due to alterations of the transcriptional start site, of the splicing pattern, or of the 3′ end of the IE-1 RNA. To distinguish among these possibilities, RNase protection analyses were performed with an antisense transcript derived from plasmid pHS (Fig. 1) as a probe (Fig. 8). RNA isolated at 3, 6, 10, 24, 72, and 6 h in the presence of cycloheximide was hybridized with the exon 1-specific probe. After digestion and polyacrylamide gel electrophoresis separation of protected fragments, signals migrating at the expected size of about 120 nt were visible. Thus, the transcriptional start site of IE-1 and -2 gene expression did not change during the time course of infection. An alternative splicing pattern could also account for a smaller IE-1 transcript. To test this hypothesis, RNA isolated at various times after infection was reverse transcribed, and the resulting DNA was amplified by PCR, using a primer specific for the 5′ and 3′ termini of IE-1 RNA. After size fractionation of the resulting amplification products by agarose gel electrophoresis, bands migrating at the expected size of 1.7 kb were present; however, no size variation could be observed (data not shown). This result argues against an alternative splicing pattern in IE-1 gene expression.

To test whether changes at the 3′ end of the transcript occur, we used the RNase H blot technique (8). This method makes use of the fact that RNase H selectively cleaves within a DNA-RNA hybrid. It allows the selective removal of the poly(A) tract of an RNA by RNase H-catalyzed cleavage after hybridization with oligo$(dT)_{15}$ or with an oligonucleotide binding within the 3′ region. RNA isolated at 6 h after infection and at 6 h after infection in the presence of cycloheximide, representing the two size classes of the IE-1 transcript, was hybridized to a 30-mer oligonucleotide (H-oligo) that binds to a sequence 216 nt upstream of the IE-1 3′ end (Fig. 9B). In parallel, the same RNAs were hybridized to an excess of oligo$(dT)_{15}$ (Fig. 9C). After digestion with RNase H, samples were separated on a 1.5% formaldehyde agarose gel, transferred to nitrocellulose filters, and hybridized with the insert of plasmid pBB (Fig. 9). Digestion in the presence of the H-oligo resulted in an incomplete cleavage of IE-1 transcripts (Fig. 9, lanes 3 and 4). The cleaved products, however, representing the body of the RNA had an identical mobility of 1.5 kb. The poly(A) tract of the 1.95-kb RNA could be detected at a size of approximately 0.4 kb (Fig. 9, lane 4). After removal of the poly(A) tracts by RNase H digestion in the presence of oligo$(dT)_{15}$, the IE-1 RNAs had the same size of 1.75 kb (Fig. 9, lanes 5 and 6). As the smaller poly(A) tract was not resolved on this gel system, RNA harvested at 6 h after infection was again hybridized with the H-oligo and, after RNase H digestion, separated on a 3% formaldehyde agarose gel. After hybridization with the insert of plasmid pBB and autoradiography, two signals were visible (Fig. 9, lane 7). The upper signal, corresponding to a size of 1.5 kb, represented the body of the IE-1 RNA, whereas the lower band, migrating at 0.24 kb, represented the 3′ end of the RNA, having the smaller poly(A) tract.

These data demonstrate that the reduction in size of IE-1 transcripts observed at 6 h of infection and later is due to a modulation of poly(A) tract length. The poly(A) tail of IE-1 had about 200 to 250 nt up to 5 h after infection, whereas later it was shortened to approximately 30 nt. This change in mRNA poly(A) tail length seems to be specific for the IE-1 transcript, since the same phenomenon could not be observed for RNAs originating from IE-2 (Fig. 3A and 5A).

DISCUSSION

Expression of the major IE gene region of HCMV shows a complex regulation during the viral replicative cycle which involves both transcriptional and posttranscriptional mechanisms (1, 4, 12, 14, 25, 26, 28–30). This study describes a differential regulation of IE-1 and -2 transcripts that was detected during our studies of the relative abundance of expression from this genomic region during HCMV replication. At 2 to 5 h after infection, IE-2 transcripts reached peak levels and IE-1 was also abundantly expressed. This finding is in agreement with a study by Stenberg et al. (28), who

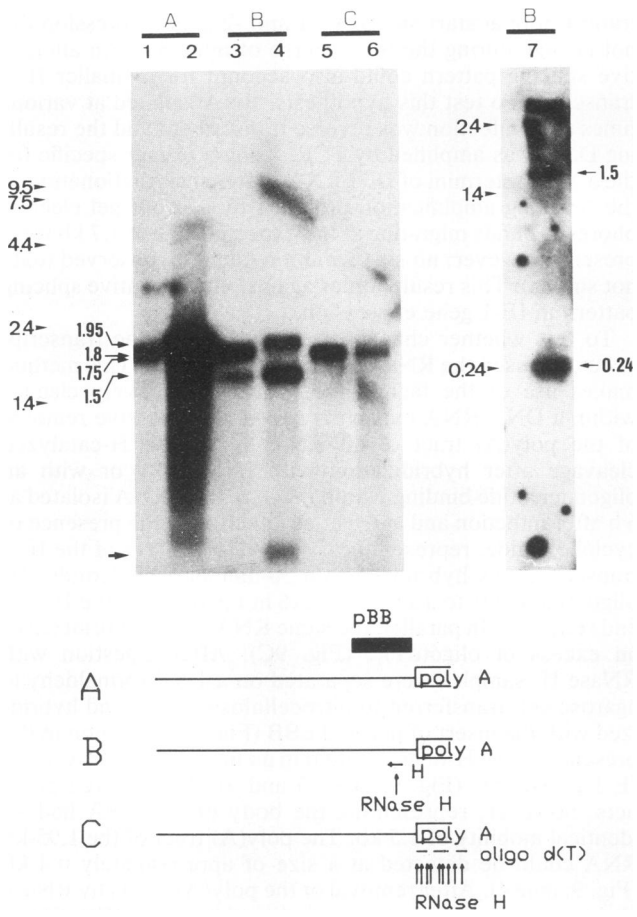

FIG. 9. RNase H blot analysis of IE-1-specific transcripts. RNA representing the different size classes of the IE-1 transcript was untreated (A) or was hybridized with the H-oligo and digested with RNase H (B) or hybridized with oligo(dT)$_{15}$ and digested with RNase H (C). The insert of plasmid pBB was used to detect IE-1-specific transcripts. Lanes: 1, 10 μg of RNA harvested at 8 h after infection; 2, 5 μg of RNA harvested at 8 h after infection in the presence of cycloheximide; 3, 5, and 7, 10 μg of RNA harvested at 6 h after infection; 4 and 6, 5 μg of RNA harvested at 6 h after infection in the presence of cycloheximide. RNA in lanes 1 to 6 was separated on a 1.5% formaldehyde agarose gel; RNA in lane 7 was separated on a 3% formaldehyde agarose gel. Sizes (in kilobases) of the transcripts are indicated by small arrows; the large arrow at the bottom indicates the poly(A) tail of the 1.95-kb IE-1 transcript. Sizes and positions of molecular weight markers are indicated by arrowheads.

performed their experiments with total cytoplasmic RNA. At 6 h after infection a strong decrease in steady-state levels of IE-2 RNA was observed, whereas IE-1 expression showed a further increase. IE-2 gene products have been proposed to negatively regulate gene expression from the IE-1 enhancer-promoter in transient expression assays (14, 25, 29). Thus, the observed decrease in expression from IE-2 may account for concomitant up-regulation of transcription rates from the IE-1 enhancer-promoter. The 2.2-kb IE-2 RNA that was found to be down-regulated hybridized with exon 1 of IE-1. It most probably corresponds to the IE-2 message that contains the first three exons of IE-1 spliced to exon 5 of IE-2 (32). Therefore, transcription of this RNA is driven by the IE-1 enhancer-promoter regulatory region. RNase protection experiments with RNA isolated at various times after infection did not reveal any alternative transcriptional start sites that could account for discordant regulation of IE-1 and -2 mRNAs. Another transcriptional mechanism that could be responsible for differential RNA expression would be premature termination of primary transcription. This has been described for adenovirus type 2 gene expression during its replicative cycle (23). Nuclear run-on analyses detected primary transcription at approximately equal ratios for both IE-1 and IE-2 at a time when negative regulation of IE-2 mRNA steady-state levels was observed. This finding argues against transcriptional regulation being involved in the described event; it suggests a posttranscriptional mechanism. However, we cannot exclude the possibility that attenuation of transcription is sensitive to the manipulations associated with nuclear run-on assays even if this method has been successfully used to demonstrate a decline of transcriptional activities in other systems (19). Since the decrease of IE-2 mRNAs was seen when we analyzed total cellular RNA, specific retention or segregation of the transcript within a cellular compartment could also not be responsible for down-regulation. In addition, the same kinetics were observed when cytoplasmic RNA was analyzed. Specific destabilization of IE-2 RNAs seems not to occur, since the half-life of the IE-2 message in the presence of dactinomycin did not change significantly during the first hours of the replicative cycle. Possibly, as has been described in several systems (6, 10, 17, 21), regulation occurs at the level of poly(A) site cleavage or splice site selection. Further experiments will be required to differentiate between these possibilities.

It was observed that down-regulation of the IE-1 RNA occurs also during the early phase of the replicative cycle after 8 h of infection. The mechanism responsible for that effect remains unclear; however, since IE-2 transcripts are down-regulated earlier, IE-2 proteins may not play the dominant role in this event. Possibly, posttranscriptional as well as transcriptional regulation mechanisms are involved, as efforts to observe repression of transcription from the IE-1 enhancer-promoter during the HCMV replicative cycle in a transient expression assay were not successful (26a).

When RNA was isolated at 6 h after infection in the presence of cycloheximide, a strong increase of IE-2 RNA steady-state levels was observed relative to RNA isolated at the same time without cycloheximide. This effect could be due to a selective strong stabilization of the 2.2-kb IE-2 RNA in the presence of the drug. However, as IE-1 and IE-2 mRNA levels are the same in the presence of cycloheximide, it seems more reasonable to assume that the synthesis of a protein involved in IE-2 negative regulation is prevented. We hypothesize that either a cellular function induced by the virus or a virus-encoded function is involved in posttranscriptional regulation of IE-2 mRNA levels at early times after HCMV infection.

Cycloheximide treatment of cells also induced qualitative changes in the pattern of RNAs observed. This was most remarkable when hybridizations were performed with exon 1 of IE-1; a ladder of distinct bands appeared that was never observed in RNA without the drug. In particular, a spliced IE-2 RNA of 1.7 kb which has been described in several studies (28, 32, 33) could not be detected in Northern blot experiments in the absence of protein synthesis inhibitors. We cannot exclude the possibility that this RNA would be detectable with use of more sensitive methods like RNase protection analysis; however, the use of cycloheximide seems to have an influence on the proportion of spliced to

unspliced RNAs originating from this genomic region of HCMV. This view is in agreement with data on the expression of the putative 55-kDa gene product of this RNA, which could not be detected during initial phases of the HCMV replicative cycle without the use of protein synthesis inhibitors (28).

Another event that was influenced by cycloheximide was the appearance of a 1.8-kb IE-1 RNA. During the first hours after infection and in the presence of cycloheximide, a 1.95-kb IE-1 transcript was present, whereas at 6 h and later in the replicative cycle the IE-1-specific probe detected a 1.8-kb RNA. RNase H blot analysis showed that this change in IE-1 transcript size is due to the fact that the mRNA poly(A) tail decreases from 200 to 250 nt at immediate-early times to a size of 30 nt at early and late times. This change appears to be specific for the IE-1 message, since no size variation could be observed for the 2.2-kb IE-2 transcript or for glyceraldehyde 3-phosphate dehydrogenase mRNA during the replicative cycle (data not shown). At present, the function of the poly(A) tail of eukaryotic mRNA is incompletely defined (5). Postulated functions include mRNA stabilization (40) as well as determination of translational efficiency (24). Poly(A) shortening has been shown to precede degradation of the mRNA body (38, 39). However, other studies suggest that a poly(A) tail length of about 20 nt is sufficient for binding of the poly(A)-binding protein which is required to stabilize a RNA (2). In addition, no change in the half-life of the IE-1 message could be detected during the first hours of the replicative cycle when poly(A) tracts were found to be shortened. A recent report by Munroe and Jacobson (22) proposes that an mRNA poly(A) tail functions as a 3′ enhancer of translational initiation. They describe a correlation between the length of poly(A) tails and the effectiveness with which such mRNAs are translated. This finding suggests that IE-1 mRNAs at immediate-early times, having a longer poly(A) tail, are more efficiently translated into protein than later in the replicative cycle. In addition, a long poly(A) tail may be an advantage in competing for the translational machinery of the cell. This view may correlate with the finding of Stinski et al. (33) and Stenberg et al. (28) that the IE-1 mRNA is preferentially associated with polysomes at immediate-early times of infection. In summary, this study demonstrates a differential accumulation of transcripts from IE-1 and -2 during initial phases of the HCMV replicative cycle. Our experiments suggest that in addition to transcriptional control mechanisms, posttranscriptional events like modification of poly(A) tail length contribute to the complexity of HCMV IE gene expression.

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REFERENCES

1. **Akrigg, A., G. W. G. Wilkinson, and J. D. Oram.** 1985. The structure of the major immediate early gene of human cytomegalovirus. Virus Res. **2**:107–121.
2. **Bernstein, P., S. W. Peltz, and J. Ross.** 1989. The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability in vitro. Mol. Cell. Biol. **9**:659–670.
3. **Blanton, R. A., and M. J. Tevethia.** 1981. Immunoprecipitation of virus-specific immediate-early and early polypeptides from cells lytically infected with human cytomegalovirus strain AD169. Virology **112**:262–273.
4. **Boshart, M., F. Weber, G. Jahn, K. Dorsch-Häsler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41**:521–530.
5. **Brawerman, G.** 1987. Determinants of messenger RNA stability. Cell **48**:5–6.
6. **Breitbart, R. E., H. T. Nguyen, R. M. Medford, A. T. Destree, V. Mahdavi, and B. Nadal-Ginard.** 1985. Intricate combinatorial patterns of exon splicing generate regulated troponin T isoforms from a single gene. Cell **41**:67–82.
7. **Cameron, J. M., and C. M. Preston.** 1981. Comparison of the immediate early polypeptides of human cytomegalovirus isolates. J. Gen. Virol. **54**:421–424.
8. **Carrazana, E. J., K. B. Pasieka, and J. A. Majzoub.** 1988. The vasopressin mRNA poly(A) tract is unusually long and increases during stimulation of vasopressin gene expression in vivo. Mol. Cell. Biol. **8**:2267–2274.
9. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
10. **Danner, D., and P. Leder.** 1985. Role of an RNA cleavage/poly(A) addition site in the production of membrane-bound and secreted IgM mRNA. Proc. Natl. Acad. Sci. USA **82**:8658–8662.
11. **DeMarchi, J. M.** 1981. Human cytomegalovirus DNA: restriction enzyme cleavage maps and map locations for immediate-early, early, and late RNAs. Virology **114**:23–38.
12. **DeMarchi, J. M.** 1983. Post-transcriptional control of human cytomegalovirus gene expression. Virology **124**:390–402.
13. **Gibson, W.** 1981. Immediate-early proteins of human cytomegalovirus strains Ad169, Davis and Towne differ in electrophoretic mobility. Virology **112**:350–354.
14. **Hermiston, T. W., C. L. Malone, and M. F. Stinski.** 1990. Human cytomegalovirus immediate-early two protein region involved in negative regulation of the major immediate-early promoter. J. Virol. **64**:3532–3536.
15. **Hermiston, T. W., C. L. Malone, P. R. Witte, and M. F. Stinski.** Identification and characterization of the human cytomegalovirus immediate-early region 2 that stimulates gene expression from an inducible promoter. J. Virol. **61**:3214–3221.
16. **Jahn, G., E. Knust, H. Schmolla, T. Sarre, J. A. Nelson, J. K. McDougall, and B. Fleckenstein.** 1984. Predominant immediate-early transcripts of human cytomegalovirus AD169. J. Virol. **49**:363–370.
17. **Laski, F. A., D. C. Rio, and G. M. Rubin.** 1986. Tissue specificity of Drosophila P element transposition is regulated at the level of mRNA splicing. Cell **44**:7–19.
18. **Malone, C. L., D. H. Vesole, and M. F. Stinski.** 1990. Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins. J. Virol. **64**:1498–1506.
19. **Mather, E. L., K. J. Nelson, J. Haimovich, and R. P. Perry.** 1984. Mode of regulation of immunoglobulin μ- and δ-chain expression varies during B-lymphocyte maturation. Cell **36**:329–338.
20. **McDonough, S., and D. Spector.** 1983. Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD 169. Virology **125**:31–46.
21. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1984. Control of adenovirus E1B mRNA synthesis by a shift in the activities of RNA splice sites. Mol. Cell. Biol. **4**:966–97.
22. **Munroe, D., and A. Jacobson.** 1990. mRNA poly(A) tail, a 3′ enhancer of translational initiation. Mol. Cell. Biol. **10**:3441–3455.
23. **Nevins, J. R., and M. C. Wilson.** 1981. Regulation of adenovirus-2 gene expression at the level of transcriptional termination and RNA processing. Nature (London) **290**:113–118.
24. **Palatnik, C. M., S. Wilkins, and A. Jacobson.** 1984. Translational control during early dictyostelium development: possible involvement of poly(A) sequences. Cell **36**:1017–1025.
25. **Pizzorno, M. C., P. O'Hare, L. Sha, R. L. LaFemina, and G. S.**

Hayward. 1988. *trans*-activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. J. Virol. **62**:1167–1179.

25a. **Rüger, R.** Unpublished data.

26. **Spaete, R. R., and E. S. Mocarski.** 1985. Regulation of cytomegalovirus gene expression: α and β promoters are *trans* activated by viral functions in permissive human fibroblasts. J. Virol. **56**:135–143.

26a. **Stamminger, T.** Unpublished data.

27. **Stamminger, T., H. Fickenscher, and B. Fleckenstein.** 1990. Cell type-specific induction of the major immediate early enhancer of human cytomegalovirus by cyclic AMP. J. Gen. Virol. **71**:105–113.

28. **Stenberg, R. M., A. S. Depto, J. Fortney, and J. A. Nelson.** 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. J. Virol. **63**:2699–2708.

29. **Stenberg, R. M., J. Fortney, S. W. Barlow, B. P. Magrane, J. A. Nelson, and P. Ghazal.** 1990. Promoter-specific *trans* activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. J. Virol. **64**:1556–1565.

30. **Stenberg, R. M., and M. F. Stinski.** 1985. Autoregulation of the human cytomegalovirus major immediate-early gene. J. Virol. **56**:676–682.

31. **Stenberg, R. M., D. R. Thomsen, and M. F. Stinski.** 1984. Structural analysis of the major immediate-early gene of human cytomegalovirus. J. Virol. **49**:190–199.

32. **Stenberg, R. M., P. R. Witte, and M. F. Stinski.** 1985. Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. J. Virol. **56**:665–675.

33. **Stinski, M. F., D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein.** 1983. Organization and expression of the immediate-early genes of human cytomegalovirus. J. Virol. **46**:1–14.

34. **Thomsen, D. R., R. M. Stenberg, W. F. Goins, and M. F. Stinski.** 1984. Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **81**:659–663.

35. **Wathen, M. W., and M. R. Stinski.** 1982. Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection. J. Virol. **41**:462–477.

36. **Wathen, M. W., D. R. Thomsen, and M. F. Stinski.** 1981. Temporal regulation of human cytomegalovirus transcription at immediate early and early times after infection. J. Virol. **38**:446–459.

37. **Wilkinson, G. W. G., A. Akrigg, and P. J. Greenaway.** 1984. Transcription of the immediate early genes of human cytomegalovirus strain AD169. Virus Res. **1**:101–116.

38. **Wilson, M. C., S. G. Sawicki, P. A. White, and J. E. Darnell.** 1978. A correlation between the rate of poly(A) shortening and half-life of messenger RNA in adenovirus transformed cells. J. Mol. Biol. **126**:23–26.

39. **Wilson, T., and R. Treisman.** 1988. Removal of poly(A) and consequent degradation of c-fos mRNA facilitated by 3′ AU-rich sequences. Nature **336**:396–399.

40. **Zeevi, M., J. R. Nevins, and J. E. Darnell, Jr.** 1982. Newly formed mRNA lacking polyadenylic acid enters the cytoplasm and the polyribosomes but has a shorter half-life in the absence of polyadenylic acid. Mol. Cell. Biol. **2**:517–525.