Sequences in the Human Cytomegalovirus 2.7-Kilobase RNA Promoter Which Mediate Its Regulation as an Early Gene

KEVIN M. KLUCHER, DOUGLAS K. RABERT, AND DEBORAH H. SPECTOR*

Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093

Received 1 May 1989/Accepted 2 August 1989

We have studied the regulation of expression of a major human cytomegalovirus (HCMV) early transcription unit located within the long repeat of the strain AD169 genome. This region specified a 2.7-kilobase RNA which underwent its largest increase in abundance between 8 and 14 h postinfection. To study the regulation of this gene, its promoter was cloned 5' of the gene for chloramphenicol acetyltransferase (CAT) for use in transient expression assays. A construct containing 651 base pairs of upstream sequence and 54 base pairs of leader sequence was transfected into human fibroblast cells, followed by HCMV infection. Analysis of the steady-state levels of RNA expressed from this hybrid gene indicated that it accumulated with the same kinetics as the authentic viral transcript early in the infection. Cotransfection of human fibroblasts with the 2.7-kilobase RNA promoter-CAT construct and plasmids containing different HCMV immediate-early (IE) genes showed that the region of the HCMV genome encoding the transcription units corresponding to IE1 and 2 and the 5' end of IE3 is capable of stimulating promoter activity but not to the same extent as HCMV infection. To define important *cis*-acting regulatory elements in the promoter, a series of 5' deletion mutants was constructed. Transient expression analysis showed a stepwise reduction in inducible CAT activity, suggesting the presence of multiple regulatory sites. To further characterize the nature of these sites, we used gel mobility shift assays to study DNA-protein interactions occurring within this promoter sequence. With nuclear extracts prepared from HeLa cells as well as from infected and uninfected human foreskin fibroblasts, we found specific binding of a cellular factor to a region of the promoter important in HCMV inducible activity. This region contains a palindromic octamer with homology to the binding site of the cellular factor USF/MLTF.

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is an important pathogen implicated in a variety of diseases in newborn and immunocompromised individuals (for a review, see reference 30). Like other members of the herpesvirus family, studies indicate that HCMV genes undergo a temporal order of expression following infection of permissive cells (9, 27, 46, 47).

The first HCMV genes expressed after infection are the immediate-early (IE) genes, characterized as being able to be transcribed in the absence of de novo protein synthesis (9, 21, 22, 27, 42, 43, 46-48). The major site of IE gene transcription in HCMV strain AD169 is located in the long unique segment of the genome (corresponding to the *Hind*III E fragment and *Eco*RI fragments, J, e, d, and R) (21, 22, 27, 48). At least three transcription units, designated IE1, IE2, and IE3, are encoded in this region as well as in a similar position in the Davis and Towne strains of HCMV (9, 19, 21, 40, 41, 43, 46-48). In addition, a fourth IE transcript, 5.0 kilobases (kb) in length, initiates in this region (10, 21, 22, 32, 46, 48).

Early genes are the next temporal class of genes to be transcribed. They are transcribed prior to viral DNA replication and presumably require the expression of one or more HCMV IE gene products. Within this class, one can identify at least three differential patterns of expression as the infection progresses. One group, exemplified by a family of transcripts encoded by HCMV AD169 *Eco*RI fragments R and d (originally designated the 2.2-kb RNAs), is detected early postinfection (p.i.), with its steady-state levels remaining approximately the same throughout the infection (39). A second group of RNAs, represented by a 2.7-kb transcript

from the long repeat of the genome (corresponding to *Eco*RI fragments O and W in strain AD169), continues to increase in abundance until the midpoint of the infection (after the onset of viral DNA replication) (28). Finally, members of the third group, including a 1.2-kb early transcript also originating from the long repeat of the genome, demonstrate a major increase in abundance from the midpoint until late in the infection (28).

We have been studying the sequence requirements and *trans*-acting functions necessary for the regulated expression of the HCMV early class of transcripts. In a previous study, we presented evidence that the promoter of the family of HCMV early transcripts encoded by *Eco*RI fragments R and d consists of multiple elements, one of which resembles the binding site of a previously identified cellular transcription factor (38). We also showed in transient expression assays that this promoter was maximally stimulated by the region of the HCMV genome encoding the IE1 and IE2 gene products. Other studies have demonstrated that the gene products specified by this IE region can activate other HCMV early gene promoters (6, 11) as well as a number of heterologous viral promoters, including herpes simplex virus early gene promoters (13, 31), the adenovirus E2 early promoter (19), and the human immunodeficiency virus long terminal repeat (8).

The promoters which can be activated by the gene products specified by the HCMV IE1 and IE2 region show little sequence homology other than the TATA sequence. This diversity suggests that the HCMV IE gene products may not interact directly with early viral promoters but, rather, may exert their influence indirectly through the activation or modification of cellular factors. A similar mechanism has been proposed for both the adenovirus E1A protein and the

* Corresponding author.

pseudorabies virus IE protein, which seem to act through cellular factors to initiate transcription of adenovirus early and late genes (1, 26).

This paper focuses on the regulation of expression of the 2.7-kb transcript, a member of the second group of early transcripts described above. Previous work by Spaete and Mocarski (36) showed that the regulatory region of this gene was contained within 1,000 base pairs (bp) upstream of the start site of transcription and 135 bp of transcribed sequence. Further work (16) demonstrated the presence of a *cis*-acting element within the 5' leader sequence of the RNA which appeared to be responsible for posttranscriptional control of gene expression. This control was mediated by one or two upstream AUG codons which were able to reduce the level of translation, thus giving the appearance of altered kinetics of gene expression (15). We have analyzed the promoter region of this gene by using transient expression assays for which the HCMV promoter was fused to the gene encoding the bacterial enzyme chloramphenicol acetyltransferase (CAT). These hybrid constructs did not contain the upstream AUG codons responsible for translational control and therefore could be used for studies focusing on transcriptional regulatory signals. The hybrid CAT gene was found to respond to viral factors in a manner temporally consistent with the viral gene at the RNA level. Promoter deletions were constructed and analyzed for determination of important *cis*-acting elements. We also studied the *trans* activation of this HCMV early promoter by viral immediate-early genes and found that, analogous to the results previously reported for the promoter directing the synthesis of the 2.2-kb RNAs (38), the region of the HCMV genome encoding the IE1 and IE2 gene products can also *trans* activate the 2.7-kb RNA promoter in transient expression assays. However, the data also indicate that other factors appear to be important for full activation of this promoter during the permissive infection. Finally, we have shown that a cellular factor binds to a region of this promoter important for HCMV-inducible activity.

MATERIALS AND METHODS

Cells and virus. Human foreskin fibroblasts were a gift from Stephen Spector (University of California, San Diego). Cells were maintained in Dulbecco modified Eagle medium (Irvine Scientific) containing 10% fetal bovine serum. HCMV strain AD169 was obtained from the American Type Culture Collection. Methods for cell culture and viral infection have been described (44). HeLa cell suspension cultures were a gift from M. Karin (University of California, San Diego).

Molecular cloning. Restriction enzymes used were obtained from Bethesda Research Laboratories, Inc., or Boehringer Mannheim Biochemicals and used as recommended by the manufacturers. Competent *Escherichia coli* DH5 cells (Bethesda Research Laboratories) were used as recommended by the suppliers for transformation of recombinant plasmids.

The promoter-CAT fusion plasmids used in the transient expression assays were constructed from the previously described vector plasmid, pSV01 (38), which is a derivative of pSV2ACAT (23).

Plasmids containing 5'-end deletions of the 2.7-kb RNA promoter were constructed in two ways. p705WOCAT, a recombinant clone containing 651 bp of sequences upstream of the initiation site of the 2.7-kb RNA (18) and 54 bp of leader sequence fused to the *E. coli* CAT gene, was con-

structed by digesting the HCMV *Eco*RI O fragment with *Sma*I, followed by Bal 31 nuclease. This deletion fragment was treated with the Klenow fragment of DNA polymerase I to produce blunt ends, digested with *Eco*RI, and ligated to pUC13 cleaved with *Sma*I and *Eco*RI. This clone was digested with *Hind*III (a site within the polylinker of pUC13) and *Hae*III, and the fragment containing the 2.7-kb promoter was ligated to pSV01 cleaved with *Hind*III and *Sma*I. p269WOCAT and p170WOCAT were constructed in a similar manner, with the exception that the HCMV deletion clones were digested with *Hind*III and *Nae*I (a restriction site overlapping the *Hae*III site) yielding plasmids containing 213 bp upstream of the 2.7-kb RNA initiation site in p269WOCAT and 114 bp upstream of the initiation site in p170WOCAT. Both plasmids contained 56 bp of leader sequence.

p150WOCAT, p135WOCAT, p109WOCAT, p94WOCAT, p89WOCAT, and p69WOCAT, containing 94, 79, 53, 38, 33, and 13 bp of sequences upstream of the RNA initiation site, respectively, were constructed by cleaving p170WOCAT with *Hind*III and digesting with Bal 31 nuclease. The resulting deletions were treated with the Klenow fragment of DNA polymerase I to produce blunt ends and cleaved with *Pvu*I within the vector sequence. The promoter-CAT fusion fragments were isolated by gel purification and ligated to the complementary 2-kb *Pvu*I-*Sma*I fragments of pSV01, regenerating the promoter-CAT fusion plasmids.

p729GEMCAT was constructed by digesting the previously described 729CAT (38), an HCMV promoter-CAT fusion plasmid containing the promoter of the family of RNAs encoded by HCMV *Eco*RI fragments R and d, with *Xho*II and *Eco*RI. The 980-bp fragment containing this RNA promoter-CAT fusion was ligated to pGEM1 (Promega Biotech, Inc.) which had been cleaved with *Bam*HI and *Eco*RI.

p2.7GEM was generated by digesting the HCMV strain AD169 *Eco*RI fragment H with *Bam*HI and *Eco*RI. A 1.8-kb fragment containing the 3' end of the 2.7-kb RNA coding sequence was ligated to pGEM1 which had been digested with *Bam*HI and *Eco*RI (28).

DNA sequence analysis. The endpoint of each promoter deletion plasmid was determined by the dideoxy-chain termination method of DNA sequencing (33). The GemSeq K/RT Sequencing System (Promega Biotech, Inc.) was used in conjunction with an oligonucleotide primer (synthesized by the University of California, San Diego, Oligonucleotide Synthesis Facility) corresponding to the polylinker sequence upstream of the cloned inserts. [α -³⁵S]dATP (1,000 Ci/mmol) was purchased from Amersham Corp.

Cell transfection. Two methods were used for cell transfection. First, calcium phosphate suspension transfections were used for transient expression analysis comparing promoter deletion mutants. In this method, human foreskin fibroblasts were transfected with plasmid DNAs by using the suspension method first described by Shen et al. (35) and modified by Staprans et al. (38). In cases where cells were infected with HCMV, virus was introduced to the cells at a multiplicity of infection (MOI) of 5 approximately 24 h after transfection. The suspension transfection approach allowed for larger numbers of cells to be pooled and transfected with the same plasmid DNA in a single test tube, thus avoiding flask-to-flask differences in transfection efficiency. In all experiments, duplicate flasks of transfected cells were plated for each condition under study.

Second, DEAE-dextran monolayer transfection was used for RNA analysis of hybrid CAT RNA and cotransfection studies as well as promoter deletion studies. Human foreskin

fibroblast monolayers were transfected with plasmid DNAs by using the DEAE-dextran technique previously described by Staprans et al. (38). In cases where cells were to be infected with HCMV, virus was introduced to the cells at an MOI of 5 approximately 24 h posttransfection.

Transient expression assays. Approximately 24 h p.i., cells were harvested. In experiments where more than one plasmid was being analyzed, the contents of each flask were split into two parts: one for the analysis of CAT activity and the other for the preparation of nuclear DNA and assessment of transfection efficiency. Soluble extracts were prepared and assayed for CAT activity essentially as described by Gorman et al. (17) except that the acetyl coenzyme A concentration was increased to 4 mM for longer reaction times (10 h). Transfection efficiency was determined by isolation of transfected cell nuclear DNA and determination of relative amounts of plasmid DNA sequences present as described by Staprans et al. (38). Briefly, isolated nuclear DNA was digested with *EcoRI* and equivalent amounts of DNA, determined by ethidium bromide staining intensities, were electrophoresed on agarose gels followed by transfer to nitrocellulose filters. The filters were prehybridized for 6 to 8 h at 37°C in a buffer containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM sodium phosphate (pH 6.5), 5× Denhardt solution (1× Denhardt solution is 0.02% each polyvinylpyrrolidone, Ficoll, and bovine serum albumin), 250 µg of denatured salmon sperm DNA per ml, 4 mM EDTA, and 0.2% sodium dodecyl sulfate (SDS), followed by hybridization with ³²P-labeled vector (pSV01) sequences for 16 to 24 h at 37°C in a buffer containing 50% formamide, 5× SSC, 50 mM sodium phosphate (pH 6.5), 1× Denhardt solution, 100 µg of denatured salmon sperm DNA per ml, 1 mM EDTA, 0.05% SDS, and 10% dextran sulfate. After the desired time, the filters were washed four times for 5 min each at room temperature in 2× SSC, followed by two washes for 30 min each at 50°C in 0.1× SSC–0.1% SDS. The filters were quickly rinsed in 0.1× SSC at room temperature, air dried, and subjected to autoradiography. The relative hybridization signals detected by autoradiography were used to assess the relative copy number of plasmid DNAs in different samples.

RNA analysis. Human foreskin fibroblasts were transfected with p705WOCAT, followed 24 h later by mock or HCMV infection at an MOI of 5. Whole-cell RNA was purified at various times p.i. by lysis in a buffer containing 0.01 M Tris hydrochloride (pH 8), 0.1 M NaCl, 0.01 M EDTA, 1% SDS, and 200 µg of proteinase K per ml. The dishes were scraped with a rubber policeman, and the solution was incubated at 37°C for 30 min, followed by two phenol:chloroform:isoamyl alcohol (50:48:2) extractions and two chloroform:isoamyl alcohol (96:4) extractions. After precipitation, the nucleic acid was treated with 100 µg of RNase-free DNase per ml. Mock-transfected cell RNA was prepared in the same manner. In some experiments, RNA was harvested from cells infected and maintained in the presence of 300 µg of phosphonoacetic acid (PAA) per ml to inhibit viral DNA replication.

RNA probes were generated by using SP6 or T7 RNA polymerase and [α -³²P]UTP as described by Melton et al. (29) and as modified by the manufacturer (Promega Biotec, Inc.). RNase mapping studies were performed by using a T7 RNA probe generated from p729GEMCAT. Whole-cell RNA (40 µg) was dissolved with probe in 30 µl of a buffer containing 80% formamide, 40 mM piperazine-*N,N'*-bis[2-ethanesulfonic acid] (PIPES) (pH 6.7), 0.4 M NaCl, and 1 mM EDTA, denatured at 85°C for 5 min, and hybridized for

17 h at 45°C. Following hybridization, 300 µl of a buffer containing 10 mM Tris hydrochloride (pH 7.5), 5 mM EDTA, 300 mM NaCl, 40 µg of RNase A per ml, and 2 µg of RNase T1 per ml was added, and the mixture was incubated for 1 h at 30°C. The RNase digestion reaction was terminated by 20 µl of 10% SDS and 50 µg of proteinase K with further incubation at 37°C for 15 min followed by phenol:chloroform:isoamyl alcohol (50:48:2) extraction and ethanol precipitation. The precipitate was suspended in a buffer containing 90% deionized formamide, 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, 0.02% (wt/vol) bromophenol blue, and 0.02% (wt/vol) xylene cyanol and was subjected to electrophoresis through a 4% acrylamide–8 M urea denaturing gel. The gel was dried, and the resulting autoradiographs were scanned by a laser densitometer for quantitation.

S1 nuclease reactions were performed by a modification of the method described by Berk and Sharp (3). Samples were denatured in buffer containing 80% formamide at 77°C for 10 min, followed by hybridization for 10 h at 51°C before digestion with S1 nuclease. The S1 probe was generated by cleaving p705WOCAT with *EcoRI*, isolating the 3.8-kb fragment containing the HCMV promoter, dephosphorylating the 5' ends, and 5' end labeling with [γ -³²P]ATP and T4 polynucleotide kinase.

RNA slot blot analysis of whole-cell RNA was performed by using a Schleicher & Schuell Minifold II manifold as recommended by the manufacturer. Briefly, purified RNA was suspended in a buffer containing 6× SSC and 7.4% (wt/wt) formaldehyde and heated at 60°C for 15 min. Samples were applied to filtration manifold in 10× SSC. RNA samples immobilized on nitrocellulose filters were prehybridized at 55°C for 4 to 6 h in a buffer containing 50% formamide, 5× SSC, 50 mM sodium phosphate (pH 6.5), 5× Denhardt solution, 250 µg of denatured salmon sperm DNA per ml, 4 mM EDTA, 0.2% SDS, 100 µg of polyadenylic acid per ml, and 100 µg of transfer RNA per ml. The filters were then hybridized at 55°C for 16 h with a ³²P-labeled SP6 RNA probe generated from p2.7GEM to detect the 2.7-kb viral transcript in a buffer containing 50% formamide, 5× SSC, 50 mM sodium phosphate (pH 6.5), 1× Denhardt solution, 100 µg of denatured salmon sperm DNA per ml, 1 mM EDTA, 0.05% SDS, 100 µg of polyadenylic acid per ml, 100 µg of transfer RNA per ml, and 10% dextran sulfate. A parallel filter was also hybridized with a nick-translated ribosomal DNA probe at 45°C for 16 h to normalize for the amount of RNA in each sample. Blots were washed as described earlier in Materials and Methods. Densitometric scanning was used on the resulting autoradiographs for quantitation.

Cell extracts. Human foreskin fibroblasts were grown in roller bottles, trypsinized, infected with HCMV in suspension at an MOI of 3, and plated onto 100-mm tissue culture plates at a density of approximately 10⁷ cells per plate. At 20 to 24 h p.i., the cells were scraped from the plates, and nuclear extracts were prepared essentially as described by Dignam et al. (12) with the following modifications. Human foreskin fibroblasts were lysed with a Dounce homogenizer with an A-type pestle. Nuclei were suspended in a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9), 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA [ethylene glycol-bis(aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 2 mM dithiothreitol, 25% glycerol, and 0.1 volume of 4 M ammonium sulfate. After 30 min of rotation at 4°C on a Lab Quake, the nuclear suspension was centrifuged at 100,000 × *g* for 1 h. Protein in the supernatant was precipitated with the addition of 0.33 g of solid ammonium sulfate per ml and

dialyzed as described by Dignam et al. (12). Nuclear extracts from mock-infected foreskin fibroblasts and HeLa cells were prepared as described above. Protein concentrations were determined as described by Bradford (4).

Gel retardation assay. Probes for gel retardation assays were prepared by gel purifying DNA fragments containing sequences from -213 to +56 relative to the 2.7-kb RNA start site and 3' end labeling with [α - 32 P]dCTP and the Klenow fragment of DNA polymerase I. Probe (0.5 ng) was mixed with 20 μ g of poly(dA-dT) (Sigma Chemical Co.) and 50 ng of a 200-bp pGEM1 *Hpa*II fragment in a buffer described by Andrisani et al. (2). Appropriate specific DNA competitors were added in 100-fold molar excess over probe. Approximately 5 to 10 μ g of crude nuclear extracts was added to the probe mix and incubated at room temperature for 30 min. After binding, 3 μ l of a solution containing 50% glycerol, 0.1% (wt/vol) bromophenol blue, and 0.1% (wt/vol) xylene cyanol was added, and the mix was electrophoresed on a 4% acrylamide gel as described by Speck and Baltimore (37). The gel was dried and subjected to autoradiography.

RESULTS

Promoter-CAT plasmids. Studies by Greenaway and Wilkinson (18) previously mapped a potential start site for the 2.7-kb transcript to a position 606 bp upstream of the *Eco*RI site between *Eco*RI fragments O and W. This was confirmed in our laboratory by using S1 nuclease analysis (data not shown). Analysis of the region upstream of the potential RNA start site showed a TATA box (5) as well as alternating purine-pyrimidine tracts and G-C-rich regions conserved in many herpes simplex virus RNA promoters (45). With these data as background, we proceeded to functionally define the promoter for the 2.7-kb RNA species. p705WOCAT was generated by fusing 705 bp of HCMV *Eco*RI fragment O to the gene encoding the bacterial enzyme CAT (Fig. 1A). This plasmid contains 651 bp of sequence upstream of the RNA initiation site and 54 bp of putative leader sequence. To determine whether this promoter-CAT fusion gene was responsive to HCMV infection, p705WOCAT was transfected by the calcium phosphate suspension method into human foreskin fibroblasts, cells fully permissive for HCMV infection. Analysis of CAT activity, as measured by the acetylation of [14 C]chloramphenicol, in uninfected cells showed a low basal level of activity which was increased in this experiment 155- to 167-fold by HCMV infection (Fig. 1B).

To verify that induction of CAT activity was due to an increase of p705WOCAT mRNA and to determine whether the authentic viral transcription initiation site was being used for the synthesis of this mRNA, we performed an S1 nuclease analysis of the RNA. Human fibroblast cells were transfected with p705WOCAT by the DEAE-dextran transfection method, followed 24 h later by mock infection or HCMV infection (MOI of 5). At 27 h p.i., RNA was harvested as described in Materials and Methods. A 5'-end-labeled probe generated from the 3.8-kb *Eco*RI fragment of p705WOCAT (Fig. 2B) was hybridized with the RNA followed by S1 nuclease digestion and denaturing gel electrophoresis. Specific protection of a 329-nucleotide DNA fragment (infected lane) was seen (Fig. 2A). This was the expected size for an RNA containing 54 bp of leader sequence fused to 275 bp of CAT and linker sequences. Thus, this fragment defines the start site of transcription of the hybrid CAT RNA at the same site previously reported for the viral transcript (18). No hybrid CAT RNA could be

FIG. 1. (A) Map of p705WOCAT, an HCMV promoter-CAT fusion plasmid containing 651 bp of upstream sequence and 54 bp of leader sequence of the 2.7-kb early RNA. (B) CAT assay of p705WOCAT in uninfected (UNINF.) and infected (INF.) cells at 24 h p.i. Duplicate flasks of human fibroblast cells were transfected by the calcium phosphate suspension method and mock or HCMV infected 24 h later. Cells were harvested and assayed for CAT activity as described in Materials and Methods. Twice as much protein extract was used in the second and fourth lanes (20 μ g) as in the first and third lanes (10 μ g), respectively. The percent conversion of chloramphenicol (CM) to the acetylated forms (AC-CM) is indicated at the top.

detected in transfected cells which had not been infected with HCMV, indicating that the induction of the CAT enzyme activity was occurring at the level of mRNA accumulation.

Time course of accumulation of the hybrid RNA. The experiments described above indicated that the HCMV promoter of p705WOCAT was indeed directing the synthesis of a transcript in response to viral factors. To further determine whether these sequences were responding faith-

FIG. 2. (A) Quantitative S1 nuclease analysis of RNA in transient assays. Whole-cell RNA (40 μ g) from p705WOCAT transfected and uninfected (UNINF.) or transfected and infected (INF.) cells was hybridized with the 5'-end-labeled *Eco*RI fragment of p705WOCAT containing the HCMV promoter (see panel B). p705WOCAT was transfected by the DEAE-dextran method. Samples were treated with S1 nuclease and electrophoresed on a denaturing 4% acrylamide gel. ϕ X174 DNA cleaved with *Hinf*I was used as the size markers (M). (B) Probe used for S1 nuclease analysis. A 3.8-kb *Eco*RI fragment of p705WOCAT was 5' end labeled with T4 polynucleotide kinase and [γ - 32 P]ATP. $\square$, HCMV DNA sequences. nt, Nucleotide.

fully as an HCMV promoter on this template, we compared the temporal accumulation of the hybrid CAT transcript with that of the authentic viral 2.7-kb transcript. Human foreskin fibroblasts were transfected with p705WOCAT by the DEAE-dextran method followed by mock or HCMV infection. Cells were also infected in the absence or presence of PAA, an inhibitor of HCMV DNA synthesis, to study the effect of viral DNA replication on viral gene expression. At various times p.i., whole-cell RNA was harvested and assayed for both the viral 2.7-kb RNA and the hybrid CAT RNA. For the detection of the viral RNA, we used slot blot hybridization with a probe specific for the 2.7-kb RNA (Fig. 3A). The hybrid CAT RNA was assayed by hybridization to a probe designed to detect only the CAT sequence followed by RNase digestion and denaturing gel electrophoresis (Fig. 3B). The amount of RNA used in these experiments was normalized by hybridization to a ribosomal DNA probe (data

not shown). In the absence of PAA, the viral 2.7-kb transcript was detected by 8 h p.i. with a 10- to 12-fold increase in viral RNA abundance occurring between 8 and 14 h after infection. A 2- to 3-fold increase in viral RNA accumulation was seen between 20 and 26 h p.i. as well as a further 1.5-fold increase between 26 and 48 h after infection. During the first 26 h of infection in the absence of PAA, the temporal accumulation of the hybrid CAT RNA was similar to that seen for the viral transcript. The hybrid CAT RNA was detected by 8 h p.i. with the largest increase in accumulation (10-fold) also occurring between 8 and 14 h. A further two- to threefold increase in hybrid CAT RNA was seen between 20 and 26 h after infection. However, in contrast to the viral transcript, a threefold decrease in hybrid CAT RNA abundance was seen between 26 and 48 h p.i.

In the presence of PAA, the temporal accumulation of the viral and hybrid CAT RNA was similar throughout the infection. The viral RNA was again detected by 8 h p.i., with a fivefold increase in viral RNA detected between 8 and 14 h p.i. A further twofold increase in viral RNA abundance was detected between 14 and 20 h p.i. and remained at that level throughout the rest of the infection (20 to 48 h p.i.). The hybrid CAT RNA was also detected by 8 h after infection and increased in abundance between 8 and 14 h (6-fold), 14 and 20 h (2-fold), and 20 to 26 h (1.5-fold) p.i. It should be noted that in Fig. 3B the amount of hybrid CAT RNA sample loaded for the 14-h time point in the presence of PAA was threefold lower than that loaded for the other time points. The hybrid CAT RNA remained at the same level between 26 and 48 h p.i., as was seen for the viral transcript. No hybrid CAT RNA was detected under any conditions in transfected cells which had not been infected with HCMV or mock-transfected cells.

Deletion analysis. To define important 5' regulatory regions, a series of 5' promoter deletion mutants was generated for use in transient expression assays. Figure 4 shows the deletion mutants generated and their 5' endpoints as determined by dideoxy sequence analysis. These deletion plasmids were transfected into human foreskin fibroblasts by calcium phosphate or DEAE-dextran transfection methods, followed 24 h later by HCMV infection. Cells were harvested at 24 h p.i., and extracts were analyzed for CAT activity. We determined from these experiments that p269WOCAT had essentially the same level of CAT activity as p705WOCAT, indicating that 213 bp of upstream sequence was sufficient for full inducibility by HCMV infection. Further successive deletions reduced this inducible activity. Deletion plasmids p170WOCAT, p150WOCAT, p109WOCAT, and p94WOCAT (containing 114, 94, 53, and 38 bp of upstream sequence, respectively) had reductions in activity of 3- to 5-fold, 7- to 10-fold, 20- to 30-fold, and 50- to 100-fold relative to p269WOCAT. Finally, a plasmid with the TATA box removed (p69WOCAT) showed a complete loss of inducible CAT activity.

trans-Activation analysis. In transient expression studies analogous to those previously reported for the promoter of the family of HCMV early transcripts encoded by strain AD169 *Eco*RI fragments R and d (38), we examined specific IE gene *trans* activation of the 2.7-kb RNA gene promoter. Foreskin fibroblasts were cotransfected by the DEAE-dextran method with the promoter-CAT hybrid and a construct encoding the major HCMV IE gene products (data not shown). From this analysis, it was determined that *Eco*RI fragment J, which contains IE regions 1 and 2 and the 5' end of IE region 3, encodes a factor(s) capable of stimulating the 2.7-kb RNA gene promoter. This IE region increased CAT

FIG. 3. Temporal analysis of viral and hybrid CAT RNA. (A) Slot blot analysis of viral 2.7-kb RNA. Whole-cell RNA from p705WOCAT-transfected cells was harvested at various time p.i. in the absence (–) or presence (+) of PAA, a viral DNA synthesis inhibitor, slot blotted, and hybridized with an RNA probe (p2.7GEM) specific for the 2.7-kb viral transcript. Tenfold dilutions of RNA (1,000, 100, and 10 ng) were slotted. (B) RNase mapping study of hybrid-CAT transcript. RNA samples (40 µg) were hybridized with a ³²P-labeled RNA probe (p729GEMCAT) to detect CAT RNA. Samples were treated with RNase A and T1 and electrophoresed on a denaturing 4% acrylamide gel. Detection of a specific 259-nucleotide (nt) protected fragment indicated CAT RNA. The time of harvest (p.i.) is indicated at the top of the lane. Mock-transfected (MOCK) RNA is also indicated. The top and bottom panels represent different exposures of the same gel. Threefold-less RNA was loaded in the 14-h +PAA lane.

activity by 37-fold over that of basal level. However, in these experiments, the level of activity induced by the IE genes was significantly less when compared with induction of the promoter by HCMV infection (4,974-fold over that of basal level).

Interaction of the 2.7-kb RNA promoter with a cellular factor. To define further the nature of the *cis*-acting sites detected by deletion analysis and potential *trans*-acting factors interacting with these sites, we used gel retardation assays (14). Nuclear extracts were prepared from mock-infected human foreskin fibroblasts, HCMV-infected human foreskin fibroblasts harvested at 20 or 24 h p.i., and uninfected HeLa cells. A probe containing sequences from –213 to +56 relative to the RNA start site of the 2.7-kb RNA was 3' end labeled and then incubated with protein from the various nuclear extracts. All three nuclear extracts showed formation of the same major specific complex (Fig. 5, band a). However, the uninfected fibroblast extract also showed the presence of three faster-migrating complexes (bands b, c, and d) which were barely detectable in the infected fibroblast extracts and not detectable at all in the HeLa extract. Complex formation with all three extracts was greatly reduced by the addition of a 100-fold excess of DNA containing the 5' sequences corresponding to nucleotides –213 to +56 (lanes 3, 7, 11, and 15) and –114 to +56 (lanes 4, 8, 12, and 16). However, no competition was seen with a 100-fold excess of DNA containing the 5' sequences –94 to +56

(lanes 5, 9, 13, and 17). Therefore, it would appear that a specific protein-DNA interaction is occurring between a normal cellular protein and a segment of DNA spanning nucleotides –114 to –95.

DISCUSSION

To better understand the regulation of HCMV early genes, we have studied the promoter of the 2.7-kb early RNA and have compared the results of these studies with our previous analysis of the promoter of the 2.2-kb class of HCMV early RNAs (38). Although both groups of RNA are first detected at early times, they display differential patterns of expression as the infection progresses. Previous work has shown that the 2.7-kb RNA can be detected throughout the infection, undergoing a large increase in accumulation between 8 and 27 h after infection (28). In contrast, the 2.2-kb transcripts encoded by *Eco*RI fragments R and d in the long unique region of the genome are also detected at early times (8 h) after infection but remain at approximately the same level of abundance throughout the infection (39).

A

```

      -651
- 657 CAGGGTGAA ATCTTACCAC AGTTGATGAT AGAAGACGGT TTTCATCGG GTGGGAACA
      |
- 597 TGGGATAACG GTGGTGACTA ATAATGCTAC AACGGTCGTC AATACAACAG CCGTGTGTTT
      |
- 537 AAGTTGTTGG CATACGTGCG TTGTGCTTTG CAATATGAAG CAGCAGACTG ATTGGTTGTA
      |
- 477 CGGAGTGGGT CATCGGTTGA ATGAAGGAAG AGATGGTGAA CTGTGGAGAG TTTCGGTTTC
      |
- 417 TTAATAATCC CATACGACAT GTGTTCATTT ATATCTAATT TTAGGATGAT GACTATAGTA
      |
- 357 TAACCTGGG GAACAAATAT CATACGTAA TCACCTTAAG TTACGCGGTT AGGAAGAAGA
      |
- 297 AATCAGTCAG AATGAACAT AGTCAGCGCA ATGATACAGC AATAGCTTGT TTACAACGTG
      |
- 237 TTCTTTTTTA CATTATGAAC GTGGCTTGCT TTTTATACAC ACATGGAGAC AGAGGTCOCT
      |
- 177 CAGGCTTGT CAGCAACAT CCGTTTCTCT AAACGGTATG TGCTCCAAAC CGTATCTCCT
      |
- 117 CATGCTCAGG TGAATACCA TGGGACCCCT TTTCGTCACA CAGCTCTTTC CGCTTACCCA
      |
- 57 ACGGCTCAGC CCGGCTCGG CAGAGCTACC ATATAAAAC GCAGGGGTTT AGCAGCTTTC
      |
+ 4 CCAGATGCTT GCTGCCCCGG CGTTCTCCAG AAGCCCCGGC GGGGGAATCG GGGGCTGCTT
      |
      Hae III
      Nae I

```

B

FIG. 4. 5' Promoter deletion analysis. (A) Nucleotide sequence of HCMV DNA used in p705WOCAT (sequence data from Greenaway and Wilkinson [18]). The nucleotide numbering relative to the RNA start site is shown at the left margin. The 5' endpoints of the deletion mutants are shown above the sequence by vertical arrows. (B) CAT activity of promoter deletion plasmids relative to p705WOCAT (100%). Promoter-CAT plasmids were transfected into human fibroblasts by calcium phosphate or DEAE-dextran methods (each set of experiments used one or the other method). The data shown represents the range of two to five individual experiments.

Sequence analysis of the upstream region for the 2.7-kb RNA showed a number of promoterlike features. From this analysis, we isolated a region containing likely regulatory elements and cloned it 5' to the CAT gene. p705WOCAT, containing 651 bp of DNA sequence upstream of the RNA initiation site and 54 bp of putative leader sequence, was found to be responsive to HCMV infection when transfected into human fibroblast cells. It should be noted that the fold increase seen was dependent on both the type of transfection method and the amount of protein extract used, as the basal level of activity in uninfected cells was too low to be accurately measured. Quantitative S1 nuclease analysis of p705WOCAT-transfected cell RNA (Fig. 2A) showed that the induction of CAT activity was occurring at the level of

correctly initiated mRNA accumulation. However, since this assay only determines the steady-state level of RNA, we cannot determine from this experiment whether the mRNA accumulation is due to an increase in the rate of transcription of the hybrid CAT gene or an increase in the stability of its transcript.

An RNase protection assay was used to analyze the temporal accumulation of the hybrid transcript. The results suggest that the 705 bp of HCMV sequence found in p705WOCAT is sufficient for the time-dependent transcriptional regulation of this HCMV early gene. The presence of PAA allowed us to compare the hybrid CAT transcript with viral RNA produced from a nonreplicating template. Under these conditions, the steady-state levels of RNA expressed from the hybrid CAT gene as the infection progressed closely paralleled that seen with the viral 2.7-kb RNA, with the major increase in abundance occurring between 8 and 14 h p.i. When PAA was absent and viral DNA replication proceeded normally, the temporal accumulation of the hybrid CAT RNA paralleled that of the viral transcript up to 26 h p.i. but decreased 3-fold between 26 and 48 h after infection. This was in contrast to the viral transcript which increased 1.5-fold during the same time period. This difference, which occurs at the time when there is marked amplification of the viral template, may be due to a decreased transcription rate of the hybrid CAT RNA as a result of a limiting amount of factors necessary for transcription. Alternatively, at this time in the infection, the presence of a negative regulatory factor may down-regulate transcription from the 2.7-kb RNA promoter. Although the viral 2.7-kb RNA increases slightly between 26 and 48 h p.i., negative regulation may still be occurring, as the template amplification during this time period is 20 to 40 times larger than the RNA increase seen. This negative regulatory factor would necessarily be dependent on viral DNA replication, as the RNA decrease is not seen in the presence of PAA. In addition, we cannot formally rule out the possibility, although unlikely, that PAA specifically increases the stability of the promoter-CAT plasmid or the hybrid CAT transcript.

Spaete and Mocarski (36) studied the regulation of expression of the gene encoding the 2.7-kb RNA by fusing approximately 1,000 bp of promoter-proximal sequence and 135 bp of 5' leader sequence to the *E. coli lacZ* gene for use in transient assays. They found a large induction in β -gal activity following infection with the Towne strain of HCMV at an MOI of 20 beginning at 12 h p.i. and continuing through 24 h p.i. Geballe et al. (16) further identified a *cis*-acting signal contained within the 5' leader sequence (nucleotides +62 to +142) which acted at the translational level to regulate expression of the gene encoding the 2.7-kb RNA. Subsequently, it was determined that this translational control was mediated by one or two upstream AUG codons located within the leader sequence (15). The two AUG codons are part of short open reading frames (7 and 35 codons in length) preceding a longer 170-codon open reading frame. The presence of either of the two AUG codons was sufficient to reduce the levels and delay the overall accumulation of the *lacZ* gene. The constructs we have employed in this study do not contain either of the two upstream AUG codons. Therefore, they are not subject to this translational control mechanism, but rather are under the transcriptional control mediated by the *cis*-acting sites contained within the 651 bp upstream of the RNA start site and 54 bp of leader sequence. Our results strongly suggest that these sequences are sufficient for the transcriptional control of the gene encoding the 2.7-kb RNA.

FIG. 5. Mobility shift assay. A 3'-end-labeled probe containing sequences from -213 to +56 relative to the 2.7-kb RNA start site was incubated with 20 μ g of poly(dA-dT) and 50 ng of a 200-bp pGEM *Hpa*II fragment for nonspecific binding competition and a 100-fold excess of appropriate specific competitor where indicated. Additions were made of 6.2 μ g of uninfected fibroblast nuclear extract (lanes 2 to 5), 5 μ g of 24-h.p.i.-infected fibroblast nuclear extract (lanes 6 to 9), 7 μ g of 20-h.p.i.-infected fibroblast nuclear extract (lanes 10 to 13), and 7.5 μ g of uninfected HeLa cell nuclear extract (lanes 14 to 17), and the assay was performed as described in Materials and Methods. Lane 1, Probe alone; lanes 2, 6, 10, and 14, extract and probe with no specific competitor; lanes 3, 7, 11, and 15, extract and probe with an excess of a DNA fragment containing sequences -213 to +56 as specific competitor; lanes 4, 8, 12, and 16, extract and probe with an excess of a DNA fragment containing sequences -114 to +56 as specific competitor; lanes 5, 9, 13, and 17, extract and probe with an excess of a DNA fragment containing sequences -94 to +56 as specific competitor. a, b, c, and d are specific complexes (see text). F, Uncomplexed probe.

5' Promoter deletion mutants were constructed to further define important upstream regulatory elements (Fig. 4). In transient expression assays, a plasmid with 213 bp of sequence upstream of the RNA start site showed the same level of induction as p705WOCAT. Further deletions resulted in a stepwise reduction in inducible CAT activity with a complete loss of inducible activity occurring in a plasmid lacking the TATA box. These results were similar to what we found for another HCMV early gene promoter directing synthesis of a family of RNAs within *Eco*RI fragments R and d (38). This stepwise reduction in inducible activity suggests that each of these early promoters has multiple regulatory elements; however, with the exception of the TATA box, little sequence homology is seen between the two promoters. Although further fine deletion analysis is necessary to clearly define these elements, nevertheless it would appear that the TATA box alone is sufficient for viral induction of transcription. This is similar to what has been proposed for the adenovirus E1a induction of adenovirus promoters through the E1a-dependent increased activity of the TATA box transcription factor, TFIID (26), as well as for the pseudorabies virus immediate early protein activation of the adenovirus major late promoter through the facilitation of TFIID: promoter interactions (1). The upstream elements necessary for temporal regulation have yet to be determined.

The complex transcriptional pattern of the 2.7-kb RNA gene promoter suggests that a variety of *trans*-acting factors, viral and cellular, are required for full regulation of this

promoter. From the cotransfection analysis, it appears that one (or more) of the IE gene products encoded within *Eco*RI fragment J participates in the induction of the promoter-CAT construct. However, there is a marked difference in the level of induction seen between *Eco*RI J transfection studies and HCMV infection. One explanation for this differential effect is that the level of HCMV IE gene expression following transfection is too low for maximal activation of the 2.7-kb RNA promoter. A more likely possibility is that gene products other than those specified by the major HCMV IE region are necessary for full induction of this promoter.

Gel retardation assays were used to study DNA-protein interactions (Fig. 5). Nuclear extracts from mock-infected human foreskin fibroblasts, HCMV-infected human foreskin fibroblasts harvested at 20 or 24 h p.i., and uninfected HeLa cells showed formation of the same major specific complex (band a). In addition, with the nuclear extract from mock-infected and infected fibroblasts, three other retarded complexes (bands b, c, and d), which migrated faster in the gel than the major complex, were detected. Although all of the retarded complexes appear in both uninfected and infected fibroblast extracts, there is a quantitative difference in the proportion of material in the different complexes between uninfected and infected fibroblast extracts. Studies are currently in progress to determine the nature of these differences. However, it should be noted that although this factor is present in uninfected HeLa cells, p705WOCAT-transfected HeLa cells showed no CAT activity (data not shown).

Therefore, this factor itself cannot be sufficient for full HCMV-inducible activity.

Competition studies localized the binding of this cellular factor from all three extracts to a region corresponding to nucleotides -114 and -95 relative to the RNA start site of the 2.7-kb RNA. Deletion of this region was shown to cause an approximately two- to threefold decrease in HCMV-inducible activity (Fig. 4). A palindromic octamer, TCACGTGA, is found within the site defined by competition studies between nucleotides -112 to -105. This is similar to the consensus binding site of MLTF/USF (CCACGTGA) which consists of an imperfect palindrome located in the promoter of the adenovirus major late gene (7, 34). Also, within this region is the sequence CGTCA (-114 to -110), which corresponds to the core consensus site for binding of the ATF/CREB transcription factor. ATF is a cellular factor important in promoter activity of the adenovirus early genes E2, E3, and E4 (20, 24, 25). However, this pentamer is repeated from nucleotides -84 to -80 but does not compete for formation of the complex at -114 to -95 in competition studies, nor does deletion of this sequence cause a reduction in inducible activity. Therefore, it is unlikely that this latter sequence itself is sufficient for binding of the cellular factor. The specificity of the binding activity to this region has been further investigated by DNase footprinting analysis and competition binding studies with defined oligonucleotides. Although preliminary, the results provide additional support for the binding of MLTF/USF to this site (manuscript in preparation). A careful temporal study of complex formation along with further mutational analysis of the region encompassed within nucleotides -114 to -95 will be necessary to determine the role of this region in the viral induction of transcription.

It is intriguing that deletion analysis shows the presence of at least five regions important in promoter activity (Fig. 4), but gel retardation assays show only one of these *cis*-acting sites binding a protein. In our studies, we used crude nuclear extracts which may prevent the detection of transcription factors which are either less abundant, are less stable, or have a lower affinity of binding than the protein(s) we detected in our studies. If this is the case, then purification of crude extracts will be necessary to define what role these other *cis*-acting sequences play in the regulation of this gene.

ACKNOWLEDGMENTS

We thank D. Jaquish for excellent technical assistance; V. Koval, R. Spencer, E. Wade, and D. Wright for helpful discussions; and D. David for preparation of the manuscript.

This investigation was supported by Public Health Service grant no. 34729 awarded by the National Cancer Institute to D. H. Spector and by Public Health Service training grant GM07240 from the National Institutes of Health.

LITERATURE CITED

- Abmayr, S. M., J. L. Workman, and R. G. Roeder. 1988. The pseudorabies immediate early protein stimulates *in vitro* transcription by facilitating TFIID:promoter interactions. *Genes & Dev.* 2:542-553.
- Andrisani, O. M., D. A. Pot, Z. Zhu, and J. E. Dixon. 1988. Three sequence-specific DNA-protein complexes are formed with the same promoter element essential for expression of the rat somatostatin gene. *Mol. Cell. Biol.* 8:1947-1956.
- Berk, A. J., and P. A. Sharp. 1978. Spliced early mRNA's of simian virus 40. *Proc. Natl. Acad. Sci. USA* 75:1274-1278.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Breathnach, R., and P. Chambon. 1981. Organization and expression of eukaryotic split genes coding for proteins. *Annu. Rev. Biochem.* 50:349-383.
- Chang, C.-P., C. L. Malone, and M. F. Stinski. 1989. A human cytomegalovirus early gene has three inducible promoters that are regulated differentially at various times after infection. *J. Virol.* 63:281-290.
- Chodosh, L. A., R. W. Carthew, and P. A. Sharp. 1986. A single polypeptide possesses the binding and transcription activities of the adenovirus late transcription factor. *Mol. Cell. Biol.* 6:4723-4733.
- Davis, M. G., S. C. Kenney, J. Kamine, J. S. Pagano, and E. S. Huang. 1987. Immediate-early gene region of human cytomegalovirus trans-activates the promoter of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 84:8642-8646.
- Demarchi, J. M. 1981. Human cytomegalovirus DNA: restriction enzyme cleavage and map locations for immediate-early, early and late RNAs. *Virology* 114:23-38.
- Demarchi, J. M. 1983. Post-transcriptional control of human cytomegalovirus gene expression. *Virology* 124:390-402.
- Depto, A. S., and R. M. Stenberg. 1989. Regulated expression of the human cytomegalovirus pp65 gene: octamer sequence in the promoter is required for activation by viral gene products. *J. Virol.* 63:1232-1238.
- Dignam, J. D., R. M. Lebowitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11:1475-1489.
- Everett, R. D. 1984. A detailed analysis of an HSV-1 early promoter: sequences involved in trans-activation by viral immediate-early gene products are not early-gene specific. *Nucleic Acids Res.* 12:3037-3056.
- Fried, M., and D. M. Crothers. 1981. Equilibria and kinetics of lac repressor operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* 9:6505-6525.
- Geballe, A. P., and E. S. Mocarski. 1988. Translational control of cytomegalovirus gene expression is mediated by upstream AUG codons. *J. Virol.* 62:3334-3340.
- Geballe, A. P., R. R. Spaete, and E. S. Mocarski. 1986. A *cis*-acting element within the 5' leader of a cytomegalovirus β transcript determines kinetic class. *Cell* 46:865-872.
- Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044-1051.
- Greenaway, P. J., and G. W. G. Wilkinson. 1987. Nucleotide sequence of the most abundantly transcribed early gene of human cytomegalovirus strain AD169. *Virus Res.* 7:17-31.
- Hermiston, T. W., C. L. Malone, P. R. Witte, and M. F. Stinski. 1987. Identification and characterization of the human cytomegalovirus immediate-early region 2 gene that stimulates gene expression from an inducible promoter. *J. Virol.* 61:3214-3232.
- Hurst, H. C., and N. C. Jones. 1987. Identification of factors that interact with the E1A-inducible adenovirus E3 promoter. *Genes & Dev.* 1:1132-1146.
- Jahn, G., E. Knust, H. Schmulla, T. Jarre, J. A. Nelson, J. K. McDougall, and B. Fleckenstein. 1984. Predominant immediate early transcripts of human cytomegalovirus AD169. *J. Virol.* 41:462-477.
- Jahn, G., J. A. Nelson, B. Plachter, J. K. McDougall, and B. Fleckenstein. 1984. Transcription of a human cytomegalovirus DNA region which is capable of transforming rodent cells. *UCLA Symp. Mol. Cell. Biol. New Ser.* 21:455-463.
- Kadesch, T., and P. Berg. 1986. Effects of the position of the simian virus 40 enhancer on expression of multiple transcription units in a single plasmid. *Mol. Cell. Biol.* 6:2593-2601.
- Lee, K. A. W., and M. R. Green. 1987. A cellular transcription factor E4F1 interacts with an E1A-inducible enhancer and mediates constitutive enhancer function *in vitro*. *EMBO J.* 6:1345-1353.
- Lee, K. A. W., T. Y. Hai, L. SivaRamen, B. Thimmappaya, H. C. Hurst, N. C. Jones, and M. R. Green. 1987. A cellular protein activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters. *Proc. Natl.*

- Acad. Sci. USA **84**:8355–8359.
26. Leong, K., L. Brunet, and A. J. Berk. 1988. Factors responsible for the higher transcriptional activity of extracts of adenovirus-infected cells fractionate with the TATA box transcription factor. *Mol. Cell. Biol.* **8**:1765–1774.
 27. McDonough, S. H., and D. H. Spector. 1983. Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD169. *Virology* **125**:31–46.
 28. McDonough, S. H., S. I. Staprans, and D. H. Spector. 1985. Analysis of the major transcripts encoded by the long repeat of human cytomegalovirus strain AD169. *J. Virol.* **53**:711–718.
 29. Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12**:7035–7056.
 30. Nankervis, G. A., and M. L. Kumar. 1978. Diseases produced by cytomegaloviruses. *Med. Clin. North Am.* **62**:1021–1035.
 31. Pizzorno, M. C., P. O'Hare, L. Sha, R. L. LaFemina, and G. S. Hayward. 1988. *trans*-Activation and autoregulation of gene expression by immediate-early region 2 gene products of human cytomegalovirus. *J. Virol.* **62**:1167–1179.
 32. Plachter, B., B. Traupe, J. Albrecht, and G. Jahn. 1988. Abundant 5 kb RNA of human cytomegalovirus without a major translational reading frame. *J. Gen. Virol.* **69**:2251–2266.
 33. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 34. Sawadogo, M., and R. G. Roeder. 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. *Cell* **43**:165–175.
 35. Shen, Y., R. R. Hirshorn, W. E. Mercer, E. Surmacz, Y. Tsutsui, K. J. Soprano, and R. Baserga. 1982. Gene transfer: DNA microinjection compared with DNA transfection with a very high efficiency. *Mol. Cell. Biol.* **2**:1145–1154.
 36. 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 fibroblasts. *J. Virol.* **56**:135–143.
 37. Speck, N. A., and D. Baltimore. 1987. Six distinct nuclear factors interact with the 75-base-pair repeat of the Moloney murine leukemia viral enhancer. *Mol. Cell. Biol.* **7**:1101–1110.
 38. Staprans, S. I., D. K. Rabert, and D. H. Spector. 1988. Identification of sequence requirements and trans-acting functions necessary for regulated expression of a human cytomegalovirus early gene. *J. Virol.* **62**:3463–3473.
 39. Staprans, S. I., and D. H. Spector. 1986. 2.2-kilobase class of early transcripts encoded by cell-related sequences in human cytomegalovirus strain AD169. *J. Virol.* **57**:591–602.
 40. 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.
 41. 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.* **55**:665–675.
 42. Stinski, M. F., and T. J. Roehr. 1985. Activation of the major immediate-early gene of human cytomegalovirus by *cis*-acting elements in the promoter-regulatory sequence and by virus-specific *trans*-acting components. *J. Virol.* **55**:431–441.
 43. 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.
 44. Tamashiro, J. C., L. J. Hock, and D. H. Spector. 1982. Construction of a cloned library of the *EcoRI* fragments from the human cytomegalovirus strain AD169 DNA. *J. Virol.* **52**:541–548.
 45. Wagner, E. K. 1985. Individual HSV transcripts, p. 45–104. *In* B. Roizman (ed.), *The herpesviruses*. Plenum Publishing Corp., New York.
 46. Wathen, M. W., and M. F. 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.
 47. 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.
 48. 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.