Identification of Sequence Requirements and *trans*-Acting Functions Necessary for Regulated Expression of a Human Cytomegalovirus Early Gene

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We analyzed the regulation of expression of a human cytomegalovirus (HCMV) early transcription unit encoded by *Eco*RI fragments R and d (map units, 0.682 to 0.713), located within the long unique segment of the genome. This region specified a 2.2-kilobase class of spliced transcripts which encode several related proteins. To define important upstream regulatory elements of this gene, we generated hybrid plasmids in which 5'-promoter sequences were fused to the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene and tested these hybrid genes in transient expression assays in human fibroblast cells. The stimulation of CAT activity in HCMV-infected cells was found to reflect an induction of correctly initiated hybrid mRNA, which was dependent on the *de novo* synthesis of some virally induced factor(s). A time course experiment showed the correct early kinetics of CAT expression. Analysis of a series of 5'-promoter deletion plasmids, ending between -323 and -7 base pairs relative to the transcription start site, showed a stepwise reduction in inducible CAT activity, suggesting that this HCMV early promoter consists of multiple elements. One of these elements resembles the binding site of a previously identified cellular "transcription" factor. We also examined the role of specific virus-encoded factors in the transactivation of this promoter. Cotransfection of human fibroblasts with the 2.2-kilobase RNA promoter-CAT construct and plasmids containing different immediate-early genes showed that expression of CAT from this promoter was stimulated by the region of the HCMV genome encoding the immediate-early 1 and 2 gene products.

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus capable of causing a variety of disease conditions in newborns and immunosuppressed individuals (for a review, see reference 38). Few of the viral functions involved in HCMV infection of its host have been identified, although studies indicate that HCMV, like other herpesviruses, undergoes a sequential order of gene expression following infection of permissive cells in tissue culture (9, 32, 64, 65).

The first genes expressed after infection are the immediate-early (IE) genes, which can be transcribed in the absence of *de novo* protein synthesis (9, 23, 24, 32, 57, 64-66). All strains of HCMV studied to date have one major region of IE transcription. The major site of IE transcription for HCMV strain AD169 is located in the long unique segment corresponding to *Hind*III fragment E and *Eco*RI fragments J, e, d, and R (23, 24, 32, 66; see Fig. 1). This site is located in a similar position on both the Davis and Towne strains of HCMV (9, 57) and encodes at least three transcription units designated IE1, IE2, and IE3 (21, 54, 55, 64, 65). A fourth IE transcription unit initiating in this region specifies a 5.0-kilobase (kb) RNA (10, 23, 24, 64). In addition, a second region of IE gene transcription is found (9, 32, 64, 66) which in strain AD169 is contained within *Bgl*II fragment O (32, 66; see Fig. 1).

The best characterized of the IE genes, IE1, encodes a 72-kilodalton nuclear phosphoprotein (18, 54, 57, 60) and is efficiently expressed following either infection or transfection of the isolated gene into tissue culture cells. This expression is mediated via a strong enhancer element, which

consists of different subsets of repeats that can independently substitute for enhancer activity (4, 56). An HCMV component further enhances IE gene expression in *trans* via these repeat sequences (50, 56). Immediately downstream of IE1 is IE2, which is composed of two subregions (IE2a and -b) determined by the presence of two sets of termination and polyadenylation sites (21). Transcription of regions IE1 and IE2 is very complex, with different transcripts being produced as a result of alternate splicing. The predominant transcript that originates from the IE1 promoter consists of all four exons of IE1, but there are also several additional transcripts which consist of the first three exons of IE1 linked to region IE2a (21). As the infection proceeds, transcription from the IE1 promoter is down-regulated, and transcription begins at the IE2 promoter (21).

HCMV early genes are transcribed before the onset of viral DNA replication, and their transcription appears to require the prior expression of one or more IE genes. However, the mechanism of this activation and the important *cis*-acting elements of the HCMV early genes responsible for their temporal regulation have yet to be elucidated. In one report, it was shown that a hybrid gene consisting of 3.2 kilobase pairs (kbp) of promoter-proximal and 135 base pairs (bp) of transcribed sequences of a major HCMV early gene fused to an indicator gene is expressed in a temporally authentic manner in transfected cells that have been subsequently infected with HCMV, but the domains of this upstream regulatory element were not defined further (50).

In this study, we have analyzed the regulation of the expression of an early transcription unit encoded by *Eco*RI fragments R and d (map units, 0.682 to 0.713), located at the right end of the long unique segment of the HCMV strain AD169 genome (Fig. 1B and C). This site is near the region

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FIG. 1. (A) Map of the 729-CAT plasmid containing 694 bp of upstream sequences and 35 bp of leader sequences of the 2.2-kb early RNA fused to the reported gene CAT (see Materials and Methods). (B) Map of the 2.2-kb RNA transcription unit encoded by *EcoRI* fragments R and d (53). The positions of the exons are indicated by the black bars below, with sizes given in nucleotides. (C) Diagram of the HCMV AD169 genome structure, the *EcoRI* restriction endonuclease sites, and the location of the IE genes (52).

of major IE transcription (9, 23, 24, 33, 57, 64–66). It also is adjacent to the transforming region described by Nelson et al. (39, 40), overlaps the second transforming region described by El Beik et al. (13) and Clanton et al. (6), and contains cell-related sequences (42, 44). In a previous report, we described the kinetics of appearance and complex spliced structure of a 2.2-kb class of transcripts encoded by this region (53). Fine mapping of the 5' end of this RNA and analysis of upstream sequences allowed the identification of putative early promoter-regulatory sequences, as well as a 5'-coding exon containing the cell-related sequences. In a separate study (67), we have characterized four phosphoproteins encoded by this transcription unit.

Here, we report our efforts to define functionally important regulatory elements of this early gene by generation of hybrid plasmids in which 5'-promoter sequences were fused to the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene. These hybrid genes were tested in transient expression assays in human fibroblast cells and were found to respond to HCMV infection with the correct kinetics of stimulation and transcription initiation. Furthermore, the regulation of this hybrid gene appeared to be at the transcriptional level. A series of 5'-promoter deletion mutants was used to identify important *cis*-regulatory regions upstream of this early gene.

We also have analyzed the *trans*-activation of this HCMV early gene promoter by specific subgenomic viral fragments. On the basis of temporal expression and the requirement for prior protein synthesis, it has been assumed that one or more HCMV IE gene products would play an important role in the regulation of early gene expression. In this regard, several previous studies have shown that HCMV IE genes could activate other promoters, but these promoters were not of HCMV origin. Using transient expression assays, Everett (15) first demonstrated that regions of the HCMV genome encoding the IE1, IE2, and IE3 genes can stimulate transcription from a herpes simplex virus early gene promoter. Other studies have shown that the IE2a region (exon 5) is essential for *trans*-activation of a variety of heterologous promoters. Hermiston et al. (21) have found that IE2a, when linked to a strong promoter, is sufficient for *trans*-activation. In contrast, Pizzorno et al. (41) and Davis et al. (8) have shown that *trans*-activation only occurs when IE2a is physically linked to the first three exons of IE1. Because different promoters and cell lines were used in these studies, a direct comparison of the results is difficult. In this report, we present the first evidence for the *trans*-activation of a bona fide HCMV early promoter by HCMV IE genes and show that the region of the HCMV genome encoding the IE1 and IE2 gene products can *trans*-activate this early promoter in transient assays.

MATERIALS AND METHODS

Recombinant DNA techniques. Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc., and Boehringer Mannheim Biochemicals and were used in the buffers recommended by the suppliers. Cloned viral DNA fragments were prepared, cleaved from vector plasmid, and isolated by agarose gel electrophoresis in Tris-borate-EDTA buffer (31), followed by electrophoresis onto and high-salt elution from DE-81 filter paper (Whatman, Inc.). Ligation reactions were carried out with T4 DNA ligase (Bethesda Research Laboratories) as described previously (59). Transformation of recombinant plasmids into competent host *E. coli* HB101 or DH5 cells (Bethesda Research Laboratories), was accomplished by the method recommended by the supplier.

Two similar vector plasmids, differing only in available restriction endonuclease sites, were used in the construction of the HCMV-CAT fusion plasmids used in this study. Vector pPCL (obtained from M. G. Rosenfeld), a derivative of pSV2ACAT (25), differed from the latter by the absence of the *BamHI* site 3' of the CAT gene. pPCL also contained exogenous promoter sequences (in place of the original simian virus 40 regulatory sequences), upstream of the CAT gene, which were excised by *BamHI*-*HindIII* cleavage. Vector pSV2AXho (11) was a derivative of pSV2ACAT in which the *BamHI*-*HindIII* fragment containing the simian virus regulatory sequences had been removed and replaced by an *XhoI* site. This vector was further modified by cleavage with *XhoI* and treatment with the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories) and deoxyribonucleoside triphosphates (31) to produce blunt ends, followed by treatment with calf intestinal phosphatase (Boehringer Mannheim Biochemicals). The modified pSV2AXho vector was ligated to a gel-purified polylinker sequence from vector pSP64 (Promega Biotec, Inc.), which had been excised with *HindIII* and *EcoRI* and treated with the Klenow fragment of DNA polymerase I and deoxyribonucleoside triphosphates to produce blunt ends. Acrylamide

gel electrophoresis was used to identify a recombinant clone which contained the *Hind*III site at the 5' end of the polylinker (relative to the CAT gene) and was designated pSV01.

The strategy used to clone 729-CAT, a recombinant clone containing 694 bp of sequences upstream of the initiation site of the 2.2-kb RNA and 35 bp of leader sequences (53) fused to the *E. coli* CAT gene, was as follows. First, the 1.5-kbp *Bgl*II-*Bam*HI fragment of *Eco*RI fragment R (53) containing sequences 5' of the initiation site and part of the first exon of the 2.2-kb RNA was subcloned (in both orientations) into the *Bam*HI site of vector pUC9 (61). This subclone was cleaved at the unique *Xho*II site (generated by the ligation of the *Bam*HI vector end to the *Bgl*II insert end) and a *Hae*III site located 35 bp downstream of the 2.2-kb RNA transcription initiation site. This gave rise to a 0.73-kbp "promoter" fragment which was larger than the remaining *Hae*III-generated fragments and thus could be gel purified. This fragment was ligated to pPCL (the pSV2CAT vector described above) which had been first cleaved with *Hind*III, treated with T4 DNA polymerase to produce blunt ends, and finally cleaved with *Bam*HI. Ampicillin-resistant colonies were screened by restriction enzyme analysis.

Two approaches were used to clone 5'-end deletions of the HCMV promoter-regulatory sequences. In the first series of experiments, we used pRd, which consists of *Eco*RI fragment R fused to the adjacent 1.5-kbp *Eco*RI-*Bam*HI subfragment of d and cloned into the *Bam*HI-*Eco*RI site of pUC9 (53). pRd was linearized with *Bgl*II 0.7 kbp upstream of the transcription start site of the 2.2-kb RNA, and Bal31 exonuclease (Bethesda Research Laboratories) was used to produce deletions through the upstream sequences. The resulting fragments were treated with T4 DNA polymerase to produce blunt ends and cleaved 3' of the R-d sequences at a *Hind*III site within the pUC9 polylinker. Deletion fragments were isolated by gel purification and ligated to *Hinc*II-*Hind*III-cleaved pUC13. Recombinant clones were analyzed by acrylamide gel electrophoresis, and clones found to terminate 531 and 113 bp upstream of the transcription start site were used to generate two promoter deletion-fusion plasmids, 566- and 148-CAT, respectively. This was achieved by cleaving the deletion clones at an *Xba*I site located 5' of the HCMV sequences in the pUC13 polylinker and at a *Hae*III site located 35 bp downstream of the transcription start site. Fragments prepared in this manner were ligated to *Xba*I-*Sma*I-cleaved vector pSV01, and ampicillin-resistant colonies were screened for the presence of the *Xba*I-*Hae*III promoter-specific fragments.

In the second approach, plasmids 566- and 148-CAT were used to generate additional 5' deletions of the HCMV regulatory sequences. Both plasmids were first cleaved at the *Xba*I site 5' of the HCMV sequences, digested with Bal31 exonuclease, treated with T4 DNA polymerase to produce blunt ends, and finally cleaved with *Pvu*I (Fig. 1A) within the vector sequences. The promoter-CAT fusion fragments were isolated by gel purification and ligated to the complementary gel-purified 2.0-kbp *Pvu*I-*Sma*I subfragment of pSV01, regenerating promoter-CAT fusion plasmids. This approach resulted in 358-, 259-, 93-, and 65-CAT constructs which contained 323, 224, 58, and 30 bp, respectively, upstream of the mRNA start site.

DNA sequence analysis. The precise endpoint of each promoter deletion plasmid was determined by the dideoxy-chain termination method of DNA sequencing (43). The GemSeq K/RT Sequencing System (Promega Biotec) was used in conjunction with an oligonucleotide primer (synthe-

sized by the University of California, San Diego, Oligonucleotide Synthesis Facility) corresponding to the polylinker sequence upstream of the cloned inserts. [α - 35 S]dATP (1,000 Ci/mmol) was purchased from Amersham Corp.

Virus and cells. HCMV strain AD169 was obtained from the American Type Culture Collection. Human foreskin fibroblasts were a gift from Stephen Spector. Methods for cell culture and viral infections have been described before (58).

Suspension transfection procedure. Human foreskin fibroblasts were transfected with plasmid DNAs by a modification of the technique described by Shen et al. (45). HEPES (2 $\times$; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline consisted of the following (in grams per liter): HEPES, 10; NaCl, 16; KCl, 0.74; Na₂HPO₄, 0.2; dextrose, 2. This buffer was adjusted to pH 7.05 with NaOH, filter sterilized, and stored at -20°C in 25-ml samples. CaCl₂, 2 M, was stored in 5-ml samples at -20°C. Prior to preparation of the calcium phosphate-DNA coprecipitate, the 2 $\times$ HEPES-buffered saline, 2 M CaCl₂ and DNA solutions were warmed to room temperature. For each 75-cm² flask (Corning Glass Works) corresponding to a total of 1 $\times$ 10⁶ to 2 $\times$ 10⁶ cells, 0.5 ml of 2 $\times$ HEPES-buffered saline, 10 μ g of plasmid DNA solution, and water to give a final volume of 0.95 ml were mixed. We then added 50 μ l of 2 M CaCl₂ as a drop on the side of the test tube and mixed this drop with the HEPES-DNA solution by rapid vortexing. The transfection cocktail remained at room temperature for 20 min while the cells were prepared.

Almost confluent cell monolayers were transfected in suspension essentially as described by Shen et al. (45), except that Dulbecco modified Eagle medium (DME; Irvine Scientific) containing 10% fetal bovine serum (FBS) was used and the polyethylene glycol-sucrose shock was omitted. Cells were trypsinized with 0.25% trypsin and then pelleted in a clinical centrifuge (700 $\times$ g). The cell pellet was suspended in approximately 0.1 ml of overlaying medium, followed by the addition of 0.9 ml of the transfection cocktail and incubation at room temperature for 20 min. To this, 9 ml of DME containing 10% FBS was added, and the suspension was plated into one 75-cm² flask and incubated at 37°C for 8 to 12 h before the medium was changed. In cases in which cells were infected with HCMV, virus was introduced to the cells at a multiplicity of infection of 5 at 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.

Monolayer transfection. Human foreskin fibroblast monolayers were transfected with plasmid DNAs by a modification of the DEAE-dextran technique described previously (50). Confluent monolayers in 75-cm² flasks were washed twice with phosphate-buffered saline (pH 7.4) and subsequently layered with 4 ml of plasmid DNA (5 to 48 μ g) in DME (without FBS)-50 mM Tris hydrochloride (pH 7.4)-400 μ g of DEAE-dextran (Sigma Chemical Co.) per ml. After incubation for 2 h at 37°C with occasional agitation, monolayers were washed twice with phosphate-buffered saline and once with DME containing 10% FBS and maintained in DME-10% FBS. Cells were harvested 48 h posttransfection. Duplicate flasks were transfected for each experimental condition.

Transient expression assays. At given times following infection, cells were harvested. In experiments in which more

than one plasmid was being analyzed, the contents of each flask were split into two parts: one for analysis of CAT activity; the other for preparation of nuclear DNA and assessment of transfection efficiency (1).

Soluble extracts were prepared and assayed for CAT activity essentially as described by Gorman et al. (20), except that the acetyl coenzyme A concentration was increased to 4 mM for longer reaction times (1 to 7 h) (63).

Transfection efficiency was determined by isolation of transfected cell nuclear DNA and determination of relative amounts of plasmid DNA sequences present (1). Trypsinized cells were washed free of media and lysed in 0.03 M Tris hydrochloride (pH 7.6)–0.15 M NaCl–0.2% Nonidet P-40–0.0015 M MgCl₂ for 5 min at 0°C. Nuclei were pelleted by centrifugation, suspended in 0.01 M Tris hydrochloride (pH 8.0)–0.1 M NaCl–0.01 M EDTA–1% sodium dodecyl sulfate–500 µg of pronase per ml, and incubated at 37°C for 1 to 2 h. The nucleic acid was then extracted with phenol-chloroform-isoamyl alcohol (50:48:2) and chloroform-isoamyl alcohol (96:4) and precipitated with ethanol. Isolated nuclear DNA was digested with *Eco*RI, and equivalent amounts of DNA, determined by ethidium bromide staining intensities, were analyzed by agarose gel electrophoresis, transfer to nitrocellulose filters, and hybridization with ³²P-labeled vector (pSV01) sequences (59). The relative hybridization signals detected by autoradiography were used to assess the relative copy number of plasmid DNAs in different samples.

RNA analysis. RNA was prepared 28 h after infection of human foreskin fibroblasts that had been transfected with the 729-CAT plasmid. Control transfected, uninfected cells were also harvested at the same time. For preparation of transfected cell RNA made in the presence of cycloheximide, 24 h after transfection, cells were pretreated with 100 µg of cycloheximide per ml for 1 h followed by HCMV infection in the presence of 200 µg of cycloheximide per ml. Whole-cell RNA was isolated by a modification of the method of Shields and Blobel (46), in which cells were lysed in a solution containing 50 mM Tris hydrochloride (pH 8.3), 100 mM NaCl, 5 mM EDTA, 1% sodium dodecyl sulfate, and 500 µg of proteinase K per ml, and the DNA was sheared by passage four times through an 18-gauge needle (2). S1 nuclease reactions were performed by modifications of the method described by Berk and Sharp (3, 53). Samples were denatured in buffer containing 80% formamide at 77°C for 10 min and hybridized for 10 h at 51°C before digestion with S1 nuclease.

RESULTS

Identification of 2.2-kb RNA promoter sequences. Previous mapping studies localized the transcription initiation site of the 2.2-kb early RNA within *Eco*RI-R, 340 bp to the left of the *Pst*I site (Fig. 1). Fine mapping allowed positioning of the 5' end of the RNA with respect to the nucleotide sequence and indicated that it mapped 27 bp downstream of a consensus TATA box and appeared to encode for a 76-nucleotide leader sequence preceding the first methionine codon. The upstream sequences included AC- and GC-rich regions conserved in many herpes simplex virus (HSV) early promoters (62). Having thus identified the putative promoter-regulatory sequences of the 2.2-kb RNA, we were interested in functionally defining the promoter region through the use of a marker gene. To do this, we fused a 729-bp fragment of 2.2-kb RNA upstream sequences, including 35 bp of putative leader sequences, to the *E. coli* CAT gene.

The resultant fusion plasmid, 729-CAT (Fig. 1A), was first tested for its ability to direct transient expression of the CAT

FIG. 2. CAT assay of the 729-CAT plasmid and the equivalent plasmid lacking any HCMV sequences (pSV01) in uninfected (uninf.) and infected (inf.) cells at 28 h p.i. Duplicate plates of cells were transfected by the calcium phosphate suspension method and infected, and extracts from samples representing about 2×10^5 cells were incubated for 1 h as described in Materials and Methods. The percent conversion of chloramphenicol (CM) to the acetylated forms (AC-CM), a measure of CAT enzyme activity, is indicated at the top.

gene in human fibroblast cells, in both the absence and the presence of HCMV infection. In the first experiment, two recombinant plasmids, pSV01 and 729-CAT, were compared. pSV01 contains the same vector sequences present in 729-CAT, but lacks the HCMV promoter sequences. Each plasmid was transfected into human fibroblasts by the calcium phosphate suspension procedure (see Materials and Methods), and 24 h later, one-half of the cells transfected with each plasmid were infected by HCMV. At 28 h postinfection (p.i.), duplicate plates of cells were harvested. One-half the contents of each plate were used to make extracts to assay for CAT activity, and the other half was used to isolate nuclear DNA for quantitation of the relative efficiency of transfection. The results of the CAT assay are shown in Fig. 2. pSV01 generated no detectable levels of CAT activity. 729-CAT generated a low basal level of CAT activity, represented by 1.7% conversion of chloramphenicol to the acetylated form. This basal level of activity was significantly induced in HCMV-infected cells, represented by 76% conversion. Analysis of nuclear DNA from the transfected cells (data not shown) indicated that the plasmids had been transfected with equal efficiencies. Thus, the 729-CAT plasmid appeared to contain sequences capable of directing expression of a fused marker gene; furthermore, these sequences appeared to be responsive to factors in the viral infection.

S1 nuclease analysis of RNA made in the transient assay. The studies described above suggested that the HCMV sequences identified in the 729-CAT plasmid were capable of acting like a transcriptional promoter, as reflected by the induction of the fused marker gene or CAT activity following HCMV infection. To determine whether the induction of CAT activity reflected an induction of 729-CAT mRNA and whether the authentic viral genomic transcription initiation site was being used for the generation of this hybrid mRNA, we carried out a quantitative S1 nuclease analysis of the RNA. Cells were transfected by the calcium phosphate suspension procedure with the 729-CAT plasmid; 24 h later, one-half of the cells were infected with HCMV, followed by extraction of whole-cell RNA at 28 h p.i. The 5'-end-labeled DNA probe used in this experiment was the 729-CAT

FIG. 3. Expression of 729-CAT RNA in the transient assay. (A) Quantitative S1 nuclease analysis of 729-CAT RNA in uninfected and HCMV-infected cells. The 729-CAT probe, a 0.88-kbp fragment (panel C), was hybridized alone (probe) or to a 50- μ g sample of transfected/uninfected-cell RNA (uninf.) or transfected/infected-cell RNA (inf.). In these experiments, transfection was by the calcium phosphate suspension method. Samples were treated with S1 nuclease, and portions of the reaction mixtures were electrophoresed on a denaturing 4% acrylamide gel with phage ϕ X174 DNA cleaved with *Hinf*I as the size markers (lane M). Sizes are in nucleotides. The 0.88-kbp probe would run at the top of the gel and therefore is not visible in the control lane labeled "probe." (B) Effect of cycloheximide on 729-CAT RNA expression. The 729-CAT probe was hybridized to 25 μ g of transfected/infected-cell RNA from cycloheximide-treated (+CH) or drug-free (-CH) cells. Samples were treated as in panel A.

plasmid, digested with *Pvu*II, which cleaves once within the CAT gene coding sequences, 150 bp downstream of the HCMV-CAT leader sequence junction (Fig. 3C). The 729-CAT probe was hybridized to transfected/uninfected-cell RNA or to transfected/infected-cell RNA and subjected to S1 nuclease digestion. Figure 3A shows the specific protection of a 185-nucleotide DNA fragment with transfected/infected-cell RNA. This is the exact size expected if the previously mapped 2.2-kb RNA transcription initiation site was used (53). Furthermore, the RNA represented by this

assay appeared to be lacking in transfected/uninfected cells. Thus, it appeared that the induction of 729-CAT enzyme activity at 28 h p.i. was occurring at the level of mRNA accumulation.

We were also interested in determining whether the sequences identified in the 729-CAT plasmid were a faithful representation of an early promoter, as defined by their dependence on de novo protein synthesis for expression. To address this, we set up another transfection/infection experiment in which we analyzed the effects of cycloheximide treatment on HCMV induction of 729-CAT RNA. Cells were transfected by the calcium phosphate suspension procedure with the 729-CAT plasmid; 24 h later, one-half of the cells were pretreated with 100 μ g of cycloheximide per ml for 1 h, followed by HCMV infection of the pretreated cells in the presence of 200 μ g of cycloheximide per ml. Transfected, drug-free cells were also infected at the same time. At 10 h p.i., whole-cell RNA was extracted and subjected to S1 nuclease analysis as described above. Figure 3B shows the specific protection of the 185-nucleotide DNA fragment representative of 729-CAT RNA, in the transfected/infected drug-free cells. This band was missing in the cycloheximide-treated cells, demonstrating that the promoter present in the 729-CAT plasmid needed the de novo synthesis of some virally induced factor(s) in order to respond to the viral infection. Thus, it appeared that these sequences were behaving like a true early promoter.

Time course of 729-CAT induction. The above experiments demonstrated that, when assayed for CAT activity at 28 h p.i., the 729-CAT construct expressed elevated levels of CAT mRNA and enzyme activity relative to that seen in uninfected cells. To determine the kinetics of 729-CAT expression in response to HCMV infection, we next examined this induction as a function of time. Cells were transfected by the calcium phosphate suspension procedure with the 729-CAT plasmid; 24 h later, one-half of the cells were infected with HCMV. At 2, 6, 10, and 28 h p.i., duplicate plates of infected cells as well as uninfected controls were harvested and assayed for CAT activity. Figure 4 shows the fold induction of 729-CAT activity in infected cells, over that measured in uninfected cells, as a function of time. For this experiment, a longer reaction time (5 h) was used to show minimal levels of CAT activity at early times. Consequently, the measured CAT activity in uninfected cells at later times appears deceptively high, and, for the infected cell, a 28-h time point is no longer within the linear range. Therefore, the fold induction in infected versus uninfected cells at 28 h is actually underestimated. From this and two similar experiments (data not shown), it was clear that the 729-CAT activity was induced as early as 6 h and clearly by 10 h p.i., consistent with the expectation for an early promoter. Thus, the hybrid gene showed the same temporal regulation with regard to induction by HCMV infection as the 2.2-kb class of HCMV RNA transcripts (53).

Analysis of 2.2-kb RNA promoter deletions. Having determined that the 729-CAT plasmid contained sequences responsive to the viral infection in a temporally authentic fashion, we next constructed a set of recombinant vectors to further sublocalize the *cis*-acting sequences responsible for this induction. To do this, we generated a series of 5'-promoter deletion-CAT fusion plasmids to be tested in the transient expression assay. The approach taken for the generation of these recombinant clones (see Materials and Methods) necessitated that we derive them from vectors other than the original 729-CAT plasmid. Thus, plasmids 566-CAT and 148-CAT, containing 566 and 148 bp of pro-

FIG. 4. Kinetics of induction of 729-CAT expression in response to viral infection. Cells were transfected with the 729-CAT plasmid by the calcium phosphate suspension method; 24 h later, one-half of the samples were infected with HCMV. Duplicate plates of uninfected and infected cells were harvested at different times postinfection, and extracts from 3×10^6 cells were assayed for CAT activity (A). (B) Kinetics of the 729-CAT expression is represented as fold induction of 729-CAT activity in infected relative to uninfected cells. The uninfected 6-h time point was extrapolated by plotting the other uninfected time points.

moter and leader sequences, differed from 729-CAT such that the former contained an additional 16 bp of polylinker sequences between the HCMV and CAT gene leader sequences at the point of fusion. Additional deletion clones were generated from 566- and 148-CAT, in a manner which did not alter the HCMV-CAT leader sequence fusions, giving rise to recombinant constructions 358-, 259-, 93-, 65-, and 42-CAT. To ascertain that the introduction of polylinker sequences was not deleterious to CAT expression, we verified that 566-CAT demonstrated the same level of CAT expression upon HCMV infection as did the 729-CAT plasmid (see Table 1 and Fig. 5), indicating that the introduction of these additional sequences was not affecting expression from these plasmids.

We tested these promoter deletion plasmids in a series of transient expression assays in which four plasmids at a time were compared with the 729-CAT construct. Transfection and HCMV infection of cells were carried out as before, and cells were harvested to make CAT extracts at 28 h p.i. The results of these experiments are summarized in Fig. 5 and Table 1. Figure 5A shows the deletion endpoint of each construct, as determined by the dideoxy-chain termination method of sequencing (43), using an oligonucleotide primer corresponding to the polylinker sequence upstream of the cloned inserts. Table 1 shows the inducible CAT activity of each plasmid, presented as a percentage of the activity expressed by the 729-CAT plasmid.

From these results, we determined that the 358-CAT plasmid retained virtually the same level of CAT activity as

FIG. 5. Nucleotide sequence of the 2.2-kb RNA promoter-regulatory region used in construction of HCMV-CAT fusion plasmids and a summary of their activities. (A) The HCMV fragment cloned into the 729-CAT plasmid is enclosed in brackets (nucleotide sequence from Kouzarides et al. [24]). Nucleotide numbers relative to the transcription start site are indicated in the left-hand margin. The nucleotide to which the 5' end of the 2.2-kb RNA was previously mapped (53) is indicated by the number 1 directly above it, and the TATA box is underlined. Arrows labeled DR1 and DR2 show the positions of direct repeat sequences. The endpoints of deletions used in this study are indicated by vertical arrows. (B) Schematic representation of the pertinent promoter deletion plasmids and their observed CAT activities, relative to that of the 729-CAT plasmid.

did the original 729-CAT plasmid, indicating that 323 bp of upstream sequences was sufficient for full induction by HCMV infection. With further successive deletions, we observed reductions in this inducible CAT activity. Thus, plasmids containing 224, 113, and 58 bp of 5' sequences resulted in 2- to 3-fold, 4- to 6-fold, and 25- to 50-fold reductions, respectively, of CAT activity. A further deletion beyond -58 down to -30 relative to the start site of transcription did not appear to lower the inducible CAT activity significantly, although it is possible that the measured conversion (1 to 5%) in these experiments was too low to detect significant differences. Thus, a construct containing only the TATA box and 3 additional bp upstream appeared capable of directing a minimal level of promoter activity.

TABLE 1. CAT activity of promoter deletion mutants

Expt ^a	Plasmid ^b	Normalized % conversion ^c	% 729-CAT activity
1	729-CAT	20.4	100
	566-CAT	24.3	119
	358-CAT	15.6	76.5
	65-CAT	0.2	1.0
	42-CAT	0	0
2	729-CAT	10.8	100
	358-CAT	12.4	115
	259-CAT	5.0	46.3
	148-CAT	1.9	17.6
	93-CAT	0.5	4.6
3	729-CAT	38.6	100
	259-CAT	11.7	30.3
	148-CAT	10.7	27.7
	93-CAT	0.6	1.6
	65-CAT	1.0	2.6

^a In experiment 1, 10 or 30 µg of extract was incubated for 7 h. In experiment 2, 46 µg of extract was incubated for 4 h. In experiment 3, 15 µg of extract was incubated for 5 h.

^b The deletion endpoint of each plasmid is shown in Fig. 5B, except for 566-CAT, which ends at position -531.

^c Percent conversion of substrate chloramphenicol reflects the average of duplicate samples. Within an experiment, numbers are corrected for the micrograms of extract assayed and the transfection efficiency as determined by an analysis of nuclear plasmid DNA (see Materials and Methods). The 42-CAT plasmid gave no detectable levels of CAT activity.

Finally, a plasmid which lacked the TATA box and which contained only 7 bp upstream of the mRNA start site resulted in total loss of CAT activity.

With this stepwise reduction of inducible promoter activity in mind, we analyzed the minimal 323 bp of promoter sequences required for full induction for potential regulatory sequences. This was done by visual inspection and computer-assisted homology searches against a number of viral early gene promoters and known cellular transcription factor binding sites. The sequence was also analyzed for the presence of repeat and inverted repeat sequences. The most outstanding homology detected in this analysis registered as a 9-bp direct sequence repeat (DR1) which formed part of an adenovirus early promoter-regulatory element. The positions of the DR1s are indicated in Fig. 5. A sequence including the DR1 which lies closest to the transcription start site (-71 to -61) could be aligned with an 11-bp region of the adenovirus E2 promoter (TGACGTAGTTT), matching 10 of 11 bp. The adenovirus sequence, which lies in a similar position (-76 to -66), constitutes part of an important promoter-regulatory element (22, 37) involved in binding of a cellular factor (48, 49). For the 2.2-kb early RNA promoter, deletion of the DR1 sequences correlated with the largest reductions in CAT activity. Another 9-bp direct repeat (designated DR2 in Fig. 5) was detected at positions -244 and -217, and again its deletion correlated with reductions in CAT activity. The potential significance of these sequence homologies is addressed in the Discussion.

trans-Activation of the 2.2-kb RNA promoter. Since with other herpesviruses it has been demonstrated that IE genes are involved in the activation of early genes, we expected that one or more of the IE regions of HCMV would be responsible for activating the 2.2-kb RNA promoter. To identify the gene(s) encoding the *trans*-activating factor(s), human foreskin fibroblasts were transfected by the monolayer transfection procedure and DEAE-dextran (see Mate-

TABLE 2. *trans*-Activation of the 2.2-kb RNA promoter

Transfection ^a	Relative CAT activity ^b
729-CAT	1
729-CAT + <i>Hind</i> III fragment E	118
729-CAT + <i>Eco</i> RI fragment J	125
729-CAT + <i>Bgl</i> II fragment O	1
729-CAT + pGEM-1	3
729-CAT + HCMV	273

^a Human foreskin fibroblast monolayers were transfected with plasmid DNAs by the DEAE-dextran technique. Cells were infected at 24 h posttransfection (i.e., 729-CAT and HCMV), and all monolayers were harvested at 48 h posttransfection.

^b CAT assays were performed with 100 µg of extract or less, incubated at 37°C for 10 h. The relative CAT activity was determined by comparing the corrected percent conversion of substrate chloramphenicol for cells transfected with 729-CAT alone and cells transfected with 729-CAT and either cotransfected with another plasmid DNA or superinfected with HCMV. Numbers are corrected for micrograms of extract assayed and the transfection efficiency as determined by an analysis of nuclear plasmid DNA (see Materials and Methods).

rials and Methods), with the 729-CAT construct either singly or in combination with a plasmid vector containing a fragment encoding one of the major IE regions (Fig. 1). The 729-CAT construct alone exhibited a low basal level of CAT expression (Table 2). However, when the fibroblasts were cotransfected with a construct containing *Hind*III fragment E, which encodes IE regions 1, 2, and 3 and the 5' end of the 5.0-kb RNA, the CAT activity increased by 118-fold. Similar results were observed with *Eco*RI fragment J, which encodes only IE regions 1 and 2, suggesting that IE regions 1 and 2 are sufficient for activation of the 2.2-kb RNA gene. No *trans*-activation was observed when plasmid alone (pGEM-1) or the DNA fragment representing another region of IE gene expression (*Bgl*II-O) was used in the cotransfection experiments. For comparison, we also show that, under similar conditions of transfection, HCMV superinfection resulted in a 273-fold increase in CAT expression over basal levels.

DISCUSSION

In this study, we identified and characterized the functional promoter-regulatory sequences of an HCMV early transcription unit encoded by *Eco*RI fragments R and d in the long unique segment of the genome (map units, 0.682 to 0.713). These sequences specify a 2.2-kb class of RNAs which are first detected at early times (8 h) postinfection and have a complex spliced structure which includes a 5'-coding exon containing cell-related sequences (42, 44, 53, 67). In previous analyses of HCMV transcription in permissively infected cells, it has been determined that HCMV, like other herpesviruses, has a temporal program of gene expression (9, 32, 64, 65). Furthermore, genes induced with similar kinetics can display different patterns of expression as the infection progresses. For instance, the 2.7- and 1.2-kb RNAs encoded by the long repeat of the AD169 genome are both detected at 8 h p.i., but show marked amplification by 28 and 72 h p.i., respectively (33, 53). These patterns are in contrast to the 2.2-kb RNAs analyzed in this study, whose steady-state levels remain about the same after 8 h p.i., characteristic of true beta RNAs (51).

The mechanisms by which early gene expression is regulated during HCMV infection still remain elusive. The best studied early genes, those of the HSV, appear to require relatively short (about 100-bp) stretches of promoter-prox-

imal sequences for their maximal induction in transient expression assays (14, 34). For the HSV thymidine kinase gene, three important control regions have been defined: a TATA box, a first distal signal (−79 to −47) containing the transcription factor SP1 binding site, and a second distal signal (−105 to −86) containing SP1 and CCAAT transcription factor binding sites (7, 35). To date, promoter mutation analyses of HSV beta genes have not detected early gene-specific inducing/enhancing elements which can be separated from sequences required for basal promoter activity (12, 15). To better understand early gene regulation in HCMV, we have analyzed the 5' regulatory-promoter sequences of the 2.2-kb RNAs encoded by *EcoRI*-R and -d.

Our previous studies localized the transcription initiation site of the 2.2-kb RNAs within *EcoRI*-R and allowed for the identification of putative promoter-regulatory sequences including AC- and GC-rich regions and a TATA box, but no "CCAAT" box (53). It should be mentioned that the transcription start site of the 2.2-kb RNAs is of the opposite polarity and about 1.2 kb away from a 5.0-kb IE RNA characterized by Jahn et al. (24). Using this information, we constructed a promoter-CAT fusion plasmid (729-CAT), in which 694 bp of upstream and 35 bp of leader sequences were fused to the *E. coli* CAT gene (Fig. 1A and B). When introduced into human fibroblast cells by transfection, this plasmid was able to direct a low level of CAT expression which was markedly induced by HCMV infection (Fig. 2). It should be noted that, in the experiment shown in Fig. 2, 76% acetylation of the substrate chloramphenicol is probably no longer within the linear range of the assay and therefore represents an underestimate of the fold induction (45×) of 729-CAT activity in infected cells over that seen in uninfected cells. In subsequent experiments within the linear range of the assay (data not shown), we routinely detected 100- to 200-fold induction of 729-CAT activity in infected cells. These experiments indicated that the 729-CAT plasmid contained sequences capable of directing expression of a fused marker gene and that these sequences were responsive to factors present in HCMV-infected cells.

By quantitative S1 nuclease analysis, we were able to show that the induction of 729-CAT activity in HCMV-infected cells reflected an induction of 729-CAT mRNA correctly initiated at the previously mapped 2.2-kb RNA start site (Fig. 3A) (53). This was an important observation, considering that certain HCMV genes may be posttranscriptionally regulated (16, 17, 19). Using this same assay, we further determined that the 729-CAT sequences were behaving like a true early promoter, in that they were unable to respond to HCMV infection in the presence of cycloheximide (Fig. 3B). Thus, the promoter in the 729-CAT plasmid requires *de novo* synthesis of some important transcription-regulatory factors for induction by HCMV.

A time course experiment further supported the notion that the 729-CAT promoter sequences showed the correct temporal induction. From Fig. 4 and two similar experiments (data not shown), we concluded that 729-CAT activity was induced as early as 6 h and clearly by 10 h p.i. This is consistent with the expectation for an early promoter and is similar to the pattern of expression of the 2.2-kb RNAs and their gene products (53, 67).

Having determined that the cloned promoter-regulatory sequences behaved in an authentic fashion, we further sublocalized the *cis*-acting sequences responsible for this induction. This was done by analyzing a series of 5'-promoter deletion plasmids in the transient expression assay. The results presented in Fig. 5 and Table 1 are only for

induced (infected) levels of fusion plasmid CAT activity. The basal (uninfected) levels of CAT activity generated by these plasmids were too low (0.5 to 5%) to generate meaningful comparisons. However, all plasmids tested for basal activity (729-, 566-, 148-, 93-, and 65-CAT) showed similar low levels of activity.

From the summarized results in Fig. 5 and Table 1, all of which have been corrected for transfection efficiency by analysis of nuclear plasmid DNAs, we concluded that 323 bp of the 2.2-kb RNA upstream sequences was sufficient for full induction by HCMV infection. A deletion to −224 bp upstream of the transcription start site reproducibly gave a two to threefold reduction in this activity. These deletions define a somewhat longer upstream sequence requirement than that observed for the HSV early genes, thymidine kinase and glycoprotein D, which require 109 and 83 bp, respectively, for their maximal induction in similar assays (14, 34). Our deletion analyses showed a stepwise reduction in inducible CAT activity, suggesting that this HCMV early promoter consists of multiple elements, the sum total of which results in full inducibility. The most important of these elements appears to be defined by the deletions to −113 and −58 bp relative to the start site, which resulted in 4- to 6- and 25- to 50-fold reductions, respectively, in CAT activity. Inspection of these sequences reveals the presence of a 9-bp direct repeat element (DR1) at positions −193 and −69. Furthermore, as shown below, the DR1 at −69 can be aligned with 10 bp of an 11-bp region of the adenovirus E2 promoter which constitutes an important promoter element:

The position of the adenovirus element at −76 relative to the transcription start site is similar to that of the HCMV sequences. This homology merits discussion, as this sequence constitutes part of a larger sequence which has been identified as an important adenovirus regulatory element which apparently binds two cellular factors. SivaRaman et al. (48), in an analysis of linker-scanning mutants by DNA-binding assays and methylation interference experiments, showed that a HeLa cell factor (designated E2A-EF) bound to a sequence between positions −82 and −66. The binding activity of this factor remained constant in both infected and uninfected cells. At a site which is overlapping and downstream of this adenovirus sequence (−68 to −33), Kovessdi et al. (27, 28) observed binding of another HeLa cell factor (E2F) which was induced by adenovirus infection. The adenovirus E2 promoter, an early promoter, is stimulated by an E1A gene product, and it appears that the E2F promoter binding factor mediates this transcriptional stimulation. To resolve the ambiguities concerning these two factors, SivaRaman and Thimmappaya (49) have used different competitors in gel shift assays and have demonstrated that E2A-EF and E2F are distinct cellular factors that recognize adjacent promoter sequences. The HCMV 2.2-kb early RNA promoter only contains the former of these two sequences. To verify that the HCMV DR1 sequence is important for promoter activity, it will be necessary to demonstrate specific factor binding to the detected sequence homology, as well as to precisely sublocalize the *cis*-acting sequences necessary for induction; these experiments are in progress.

Recent comparative studies suggest that the cellular transcription factor, E2A-EF, likely corresponds to factors E4F1 and E3F2 which interact with the E1A-inducible adenovirus promoters E4 and E3, respectively (30). This factor has now been designated ATF. The consensus sequence for binding of ATF, A/TCGTCA, is functional in either orientation and has been detected in a variety of viral and cellular E1A-dependent promoters (29, 30). The presence of the ATF sequence in an essential region of the HCMV 2.2-kb RNA promoter (−66 to −71) suggests that ATF may play a role in regulating this gene. It is also interesting to note that the ATF consensus sequence is located within the cyclic AMP responsive element sequence (36, 47), implying a possible interplay between these transcription factors.

The minimal promoter sequences required for expression are defined by the −30 deletion in the 65-CAT plasmid. It should be noted that this plasmid lacks the nearly perfect SP1 consensus sequence (5), or GC-rich region, yet does not appear to differ significantly in activity from the 93-CAT construct which contains this sequence. Thus, it is not likely that this GC-rich stretch is important for induction of the 2.2-kb early RNA.

From a comparison of the 2.2-kb early RNA promoter with that of other CMV or HSV early promoters, it appears that relatively little sequence homology is shared by these seemingly analogous regulatory units. This sequence diversity invokes the possibility that viral gene regulation is mediated through a variety of *cis*-acting regulatory elements and *trans*-acting viral factors and cellular factors which may be induced or modified by viral IE genes. The presence or absence of such cellular factors may also determine whether a particular cell type is permissive for HCMV infection.

In this study, we have demonstrated that the HCMV major IE region can *trans*-activate the 2.2-kb early RNA promoter (i.e., 729-CAT). *Eco*RI fragment J, which encodes the major IE protein gene, IE1, as well as the IE2 gene products, is the smallest sequence tested which exhibits *trans*-activation. A recent report by Hermiston et al. (21) also supports the notion that the major IE region encodes a regulatory factor(s) for gene expression. In their studies, it was shown by cotransfection of the HCMV IE genes and a construct containing the adenovirus E2 early gene promoter linked to CAT that IE2, particularly IE2a, could independently stimulate expression from the E2 promoter. Although IE1 alone did not activate the construct, maximum activity was observed when both IE1 and IE2 were present. Other studies have demonstrated the importance of IE2a for *trans*-activation of heterologous promoters, but stimulation was observed only when IE2a was linked to the first three exons of IE1 (8, 41). In drawing conclusions from the above studies, however, it must be kept in mind that all of the experiments for *trans*-activation involve transient expression assays with hybrid plasmid constructs containing promoters linked to a heterologous indicator gene. Further studies will be needed to determine whether additional factors, either viral or cellular, are involved in the regulation of the HCMV 2.2-kb early RNA during the normal course of the infection and how these factor requirements compare with those of other HCMV early genes.

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