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Identification and Characterization of the Human Cytomegalovirus Immediate-Early Region 2 Gene That Stimulates Gene Expression from an Inducible Promoter

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The human cytomegalovirus (HCMV) *Xba*I E cloned DNA fragment of approximately 20 kilobases can complement an adenovirus mutant (*dl*312) defective in the E1a viral gene product (D. J. Spector and M. J. Tevethia, Virology 151:329–338, 1986). This viral DNA fragment contains three immediate-early (IE) genes between 0.709 and 0.751 map units (M. F. Stinski, D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein, J. Virol. 46:1–14, 1983). Two of the IE genes, IE1 and IE2, were isolated and tested for a role in regulating viral gene expression. Since HCMV early and late promoters require additional characterization, the chloramphenicol acetyl transferase (*cat*) gene, driven by the adenovirus E2 promoter, was used as an indicator of gene expression. *cat* expression from this heterologous viral promoter was shown to be stimulated by HCMV at early times after infection. The IE1 gene product did not function independently in activating this promoter. The IE2 gene products could independently stimulate the expression of a plasmid when the *cat* gene was placed downstream of the inducible E2 promoter (E2CAT). Five proteins of different sizes have been predicted to originate from IE2, depending on mRNA splicing. The protein products specified by the IE2 gene were characterized with an antibody to a synthetic peptide according to the open reading frame of exon 2. Three of the five proteins are encoded by exon 2. Three viral proteins of 82, 54, and 28 kilodaltons (kDa) were detected. The exons contained in the region designated as IE2a have open reading frames that could code for two of the smaller proteins of 27 and 30 kDa. This region, when driven by the HCMV enhancer, could independently stimulate gene expression from E2CAT to a high level. A plasmid with the HCMV enhancer upstream of exons, that could code for the HCMV IE2 proteins of 48 and 51 kDa, as well as 27- and 30-kDa proteins, also stimulated E2CAT expression but at a lower level. The activity of this plasmid was augmented by the IE1 gene product, despite the fact that the latter gene product alone was inactive. It is proposed that the HCMV IE region 2 gene products are involved in the regulation of viral or host cell promoters either independently or in combination with other HCMV IE proteins.

Human cytomegalovirus (HCMV), a herpesvirus, can cause congenital malformation in the newborn and pneumonitis in the immunocompromised adult, as well as a subclinical infection in the normal healthy individual. The HCMV double-stranded DNA genome of 240 kilobases (kb) can establish latency in certain cell types of the host or undergo a sequential, regulated expression of the viral genes, ultimately leading to the production of infectious virus. Sequential gene expression is broadly categorized in three phases, immediate-early (IE), early, and late. The IE genes are the first genes expressed after infection, and their viral RNAs arise from a few distinct regions of the genome (3, 19, 37). Within the region of highest IE transcriptional activity, there exist three transcription units whose RNA structures have been investigated in detail (14, 32, 33, 36). After the IE proteins have been synthesized, there is a switch from restrictive to extensive transcription of the genome (3, 19, 38). Therefore, one or more of the IE proteins are likely to mediate this switch and thus, regulate transcription of other viral genes.

Viral regulatory proteins have been described in other herpesviruses. Pseudorabies virus (6), varicella-zoster virus (4), and murine cytomegalovirus (15) each encode a large viral protein (180, 140, and 89 kilodaltons [kDa], respectively) believed to be involved in regulation of viral gene expression. A similar viral protein specified by Epstein-Barr virus has recently been identified, but this phosphorylated viral protein is considerably smaller, 47 kDa, than the other herpesvirus *trans*-acting proteins (17, 41). Herpes simplex virus (HSV) codes for two IE proteins that directly or indirectly regulate transcription of early promoters (5, 8, 18, 22–26). These viral proteins have been designated ICP4 (IE3, Vmw175) and ICPO (IE1, Vmw110). Although ICP4 activates inducible HSV promoters, it can suppress promoters containing *cis*-acting enhancer elements. ICPO (Vmw110) generally has a stimulatory effect on nonenhancer- and enhancer-containing promoters. The synergistic interaction between these two viral proteins may regulate transcription of HSV genes during the course of infection. In general, herpesvirus *trans*-acting proteins can stimulate expression from heterologous promoters, as well as homologous promoters (4, 6, 15, 17, 41).

The region of the HCMV genome which reportedly activates heterologous promoters in transient assays is *Hin*dIII E for strain Ad169 and *Xba*I E for strain Towne (4). The same region can also rescue an adenovirus deletion mutant, *dl*312, which is defective in its *trans*-acting gene product, E1a (29). This region of the HCMV genome has at least four transcription units (30, 36) within approximately 20 kb. Three of these transcription units are positioned downstream of the major IE promoter regulatory region which contains *cis*-acting enhancer elements (2, 35). These HCMV genes have been designated IE1, IE2, and IE3 (36). The transcripts from these regions are complicated because of alternate

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splicing patterns that result in a variety of sizes of mRNAs which could code for several different viral proteins (33). Two of these regions, IE1 and IE2, have been assayed for the ability to stimulate expression from a viral promoter.

To date, no HCMV genes or gene products have been identified that regulate cellular or viral transcription. Several reports either proposed or implied that the first HCMV IE gene product (IE1) was independently involved in the stimulation of viral gene transcription (4, 15, 29, 36). This was a logical choice because IE1 is the first gene downstream of the enhancer and by analogy, papovaviruses, adenoviruses, and several of the herpesviruses have a gene that encodes a *trans*-acting protein immediately downstream of an enhancer. However, our results indicate no independent regulation by the IE1 gene product. Rather, this report describes independent *trans*-acting regulatory HCMV gene products specified by IE2. The IE2 gene in the Towne strain codes for at least five proteins of different sizes due to differential splicing of the mRNAs. We show here that the HCMV IE2 exons that encode one or perhaps both of the smallest proteins (27 and 30 kDa) can act in *trans* to stimulate *cat* expression from an early viral promoter, the adenovirus E2 promoter. Further investigation is necessary to characterize HCMV early or late promoters and the IE gene products necessary for their stimulation. The HCMV IE2 genes and gene products described in this report are also capable of complementing adenovirus mutants defective in E1a (M. J. Tevethia, D. J. Spector, K. M. Leisure, and M. F. Stinski, submitted for publication).

MATERIALS AND METHODS

Virus and cell culture. The growth of human foreskin fibroblast cells and the propagation of HCMV (Towne strain) were as described previously (34). Virus infectivity was determined by plaque assay (40) or tissue culture infective doses (34).

Plasmid constructions. For the construction of recombinant plasmids, restriction endonucleases (Bethesda Research Laboratories, Inc., Gaithersburg, Md.; New England BioLabs, Inc., Beverly, Mass.), calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), Klenow DNA polymerase (Boehringer Mannheim), and T4 RNA and DNA ligases (New England BioLabs) were used by the specifications of the manufacturers. Plasmid DNAs were extracted and purified as described previously (37).

Plasmid pCC contains the IE1 promoter-regulatory and gene-coding regions. The preparation of this plasmid and expression of this viral gene product in transfected cells have been described previously (31). Construction of pCS was as follows. A *Bam*HI-to-*Sal*I fragment containing the IE2a promoter and coding region was isolated from plasmid pCB42 (36) and cloned into plasmid pCC. This construction placed the major IE promoter regulatory region upstream of the IE1 and IE2a coding sequences in a structure identical to that found normally in the viral genome (Fig. 1).

Several deletion clones were derived from plasmid pCS. Plasmid pCSdlCla contains a *Cla*I-to-*Cla*I 288-base-pair deletion which excised the first translation initiation codon (AUG) in the coding sequence of IE2a but retained the open reading frame (ORF) (Fig. 1). Plasmid pCSdlAcc was constructed by deleting an *Acc*I-to-*Acc*I 1,414-base-pair fragment. This deletion removed most of exon 4, except for 71 base pairs of the 3′ end in IE1, and left the first three exons. pCSdlAcc still retained the ATG and the Proudfoot box sequence 5′ AATAAA 3′ of IE1. The Proudfoot sequence of IE1 also served as the CAAT box in the sequence 5′ CAATAAA 3′ upstream of the TATA box for IE region 2a (Fig. 1). Plasmid pCC, pCS, pCSdlAcc, and pCSdlCla were all placed in a vector which contains the simian virus 40 origin of replication.

Plasmid pSB was constructed by isolating the *Bam*HI-to-*Sal*I fragment from pCS (Fig. 1). Plasmid pSBenh was constructed by inserting the above *Bam*HI-to-*Sal*I fragment into vector DNA at a site approximately 350 base pairs downstream of the strong IE promoter regulatory region. This clone retains the 3′ 89 base pairs of IE1, which includes the promoter of IE2a. The plasmid pLink760 contains the strong IE promoter regulatory region from +7 to approximately −760 cloned into bacterial plasmid pAT153 as described previously (35). Bacterial plasmid pSVOd was described previously (31). Indicator plasmid pE2CAT was a gift from J. Nevins (Rockefeller University, New York) and was constructed as described previously (13).

Transfections. All transfections were done on duplicate 100-mm plates on HFF cells. All transfections were done in permissive human fibroblast cells, rather than nonpermissive cells, so that the transfected cells could also be infected with HCMV. However, COS-1 cells were transfected as described previously (31) to evaluate transcription of the IE1 or IE2 genes. All DNA concentrations were checked by comparisons between the intensities of ethidium bromide-stained DNA fragments generated by digestion with various restriction endonucleases and fractionated in polyacrylamide gels. Therefore, the amount of the various plasmid DNAs used had approximately the same molar ratio, even though the plasmids differed in size. Transfection was carried out by the method of Graham and Van der Eb (10) with the following modifications. Human foreskin fibroblast cells were grown for 3 to 4 days, and the medium was replaced 24 h before transfection. The plasmid DNA (5 μg) containing the *cat* construct was combined with nonspecific pSVOd DNA or pLink760 DNA or with HCMV-specific plasmid constructs (5 μg unless noted). Sonicated salmon sperm DNA was added (20 μg), as carrier DNA, with H_2O to a final volume of 100 μl. This solution was added to 500 μl of 2× HEPES-buffered saline (HBS; 35 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM glucose, pH 7.4) and 250 mM $CaCl_2$, pH 6.8, to give a final concentration of 100 mM $CaCl_2$. A precipitate was allowed to form for 30 min at room temperature. After removing the medium, the precipitate was added dropwise to the monolayer and allowed to incubate at room temperature for 10 min. Fresh medium was then added, and the plates were incubated at 37°C for 4 h. After removal of the medium, the cells were shocked with 30% dimethyl sulfoxide in 1× HBS, pH 7.4, for 2 min. Cells were then washed and incubated in fresh medium at 37°C for 48 h.

The amount of pE2CAT DNA in each transfected plate was evaluated by Southern blot hybridization (28). Low-molecular-weight DNA was isolated by the method of Hirt (12). Transfections were done multiple times with different plasmid preparations to control variations in transfection efficiencies.

CAT assays. Cell harvesting and CAT assays were performed as described by Gorman et al. (9). Chromatography was described previously (35). All quantitative comparisons were made by measuring the Cm-3-Ac products by using enzyme levels which were shown to be on the linear part of the curve of product formation versus enzyme concentration and time. The percentage conversion to Cm-3-Ac products

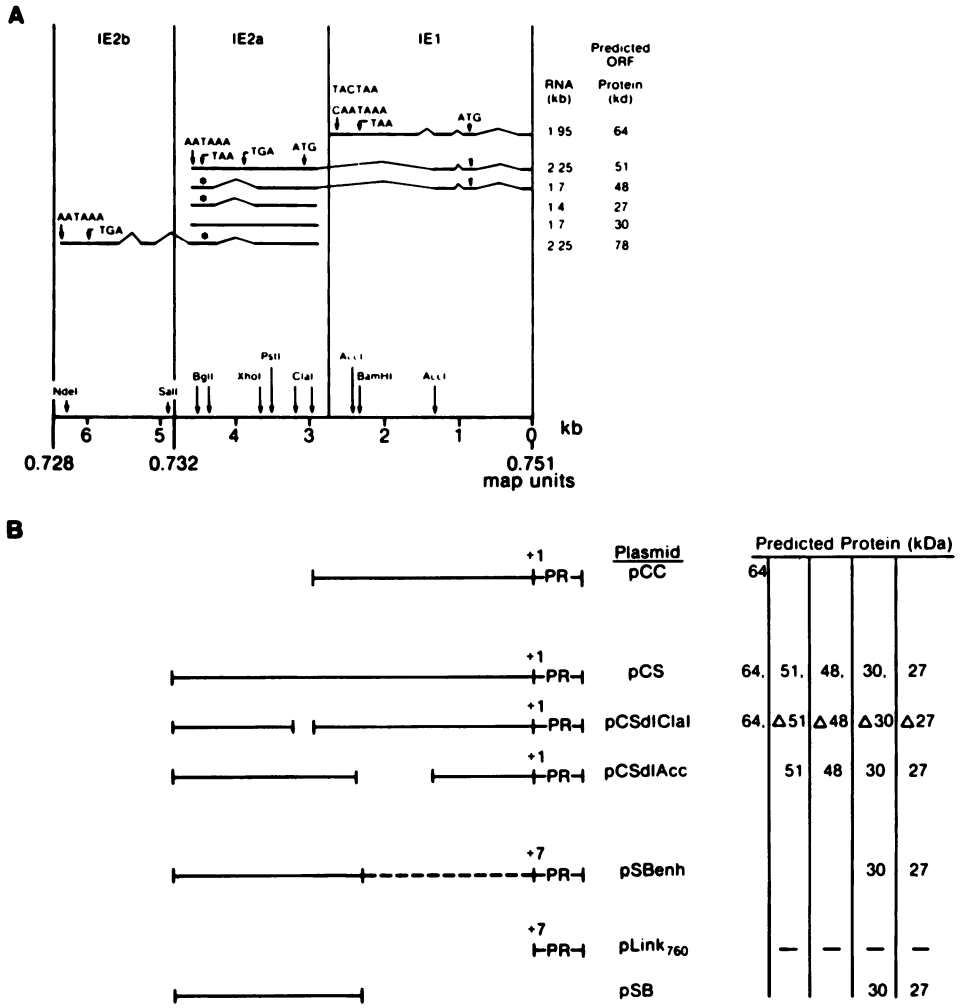

FIG. 1. Organization of HCMV IE genes and plasmid constructions. (A) The mRNA structures, sizes, and predicted ORFs for HCMV genes IE1 and IE2 are shown. Bold and thin lines represent mRNA exons and their introns, respectively. Transcription signals, such as the CAAT and TATA boxes for IE2 and the AATAAA boxes for IE1 and IE2, are shown. The strong promoter-regulatory region upstream of IE1 and the cap site have been described previously (32, 35). The location of restriction endonuclease sites, the distance (in kilobases), and the map units on the HCMV genome (in kilobases) are indicated. An asterisk indicates the location of the sequence used for preparation of the synthetic peptide. kd, Kilodalton. (B) Construction of the various plasmids is described in the text. The location of the strong major IE promoter regulatory regions upstream of the IE genes and the junctions with the transcriptional unit relative to the cap sites are indicated. Deletions in IE1 or IE2 are shown as a gap. For plasmid pSBenh, the dash represents 350 bp of vector DNA. All plasmids are identified on the right, as is the predicted gene product in kilodaltons (kDa). Δ indicates a deletion.

was always measured relative to either the same number of cells or milligrams of protein. Only the Cm-3-Ac product was analyzed because this product provides valid estimates of CAT activity under these conditions (27). Known amounts of purified CAT enzyme (P-L Biochemicals, Inc., Milwaukee, Wis.) were used to quantitate the microunits of CAT activity. At 37°C and pH 7.8, 1 U of CAT catalyzes the acetylation of 1 nmol of chloramphenicol per min. The relative expression levels from the adenovirus E2 *cat* construct in combination with the above plasmid DNAs were consistent. However, the absolute enzyme activity varied between experiments, presumably because of differences in cell passage or plating efficiency.

Immunoprecipitation. Cells were infected with 10 PFU of HCMV per ml in the presence of cycloheximide as described previously (34). After release of the cycloheximide block, proteins were radioactively labeled with either [^{35}S]methionine (20 μCi/ml) or $^{32}P_i$ (40 μCi/ml) as described previously (34). Immunoprecipitations were performed by a modification of the method of Grose et al. (11). Monoclonal antibody E-3 was obtained from Genetic Systems (Seattle, Wash.). Polyclonal antibody 1218 was prepared to a synthetic 17-amino-acid peptide, Gln-Phe-Glu-Gln-Pro-Thr-Glu-Thr-Pro-Pro-Glu-Asp-Leu-Asp-Thr-Leu-Ser, according to the ORF in exon 2 of IE region 2a (33) and in collaboration with M. Oldstone and J. Nelson (La Jolla, Calif.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polypeptides were fractionated in sodium dodecyl sulfate-polyacrylamide gels as described previously (34). Molecular weight standards containing ^{14}C-labeled proteins ranging in molecular weight from 200,000 to 14,300 were obtained from Bethesda Research Laboratories.

RESULTS

Viral proteins specified by the IE1 and IE2 genes. The organization of HCMV IE1 and IE2 has been described

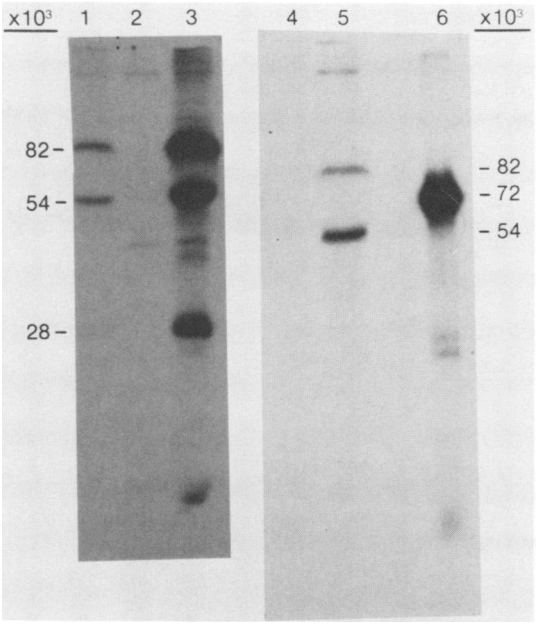

FIG. 2. Proteins encoded by the HCMV IE1 and IE2 genes. Mock-infected or infected cells were treated with cycloheximide for 16 h, washed, and then pulse-labeled with either [^{35}S]methionine or $^{32}P_i$. Viral proteins were immunoprecipitated with either monoclonal antibody E-3 to IE1 antigen or polyclonal antibody 1218 to IE2 antigens. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent autoradiography as described in the text. Lanes: 1, ^{32}P-labeled infected-cell lysates plus 1218 antiserum; 2, ^{35}S-labeled infected-cell lysates plus pre-1218 antiserum; 3, ^{35}S-labeled infected-cell lysate plus 1218 antiserum; 4, ^{32}P-labeled mock-infected cell lysates plus 1218 antiserum; 5, ^{32}P-labeled infected-cell lysate plus 1218 antiserum; 6, ^{32}P-labeled infected-cell lysate plus E-3 monoclonal antibody. The molecular masses of the proteins are given at the side of the gels.

previously (33, 36). Figure 1A summarizes the mRNA structures derived from regions IE1 and IE2 for the Towne strain of HCMV. The IE1 gene is immediately downstream of the strong IE promoter regulatory region that contains *cis*-acting enhancer elements (2, 35). In infected cells, the IE1 mRNA and protein products are expressed at levels 10- to 20-fold higher than the IE2 genes are at early times after infection (36). Expression of the IE2 RNAs occurs by (i) linkage to the IE1 upstream exons by differential RNA processing, rendering two mRNAs of 2.25 and 1.7 kb that have predicted ORFs for proteins of 51 and 48 kDa, respectively (33) or (ii) transcription from its own promoter coding for three mRNAs of 2.25, 1.7, and 1.4 kb, with predicted ORFs for proteins of 78, 30, and 27 kDa, respectively (33). The RNAs in the region designated IE2 contain two different polyadenylation and cleavage sites, with a splice site just upstream of the first polyadenylation signal, and consequently, IE2 has been subdivided into two regions designated IE2a and IE2b. Splicing in approximately the middle of IE2a and removing a stop codon before the first 5′ AATAAA 3′ signal in IE2a results in the synthesis of a 2.25-kb RNA from regions IE2a and IE2b, with an ORF for a predicted protein of 78 kDa.

The viral protein specified by IE1, as well as the entire amino acid sequence and predicted secondary structure, has been described (1, 32). The proteins specified by IE2 were predicted but not confirmed to exist in the infected cell. A polyclonal antibody prepared to a synthetic peptide of exon 2 of IE2a should detect three proteins predicted to have sizes of 78, 48, and 27 kDa. Because of a stop codon, the 51- and 30-kDa proteins should not be detected (Fig. 1). When cells were labeled with [^{35}S]methionine, three proteins of 82, 54, and 28 kDa were immunoprecipitated (Fig. 2, lane 3). When cells were labeled with $^{32}P_i$, only the 82- and 54-kDa proteins were found to be phosphorylated (Fig. 2, lanes 1 and 5). The migration of these IE2-encoded proteins (Fig. 2, lanes 1, 3, and 5) relative to the IE1-encoded protein (Fig. 2, lane 6) was compared. These data support the mRNA structures and predicted ORFs for IE2 (33) and substantiate that these IE2 proteins are present in the infected cell at early times after infection and at similar relative concentrations. The molecular weights determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were slightly higher than the molecular weights predicted from the ORFs. According to this interpretation, the 82-kDa protein would be encoded entirely by IE regions 2a and 2b. The 54-kDa protein would be encoded by a mRNA with exons 2 and 3 of IE1 and exons 1 and 2 of IE2a. The structure of this viral mRNA has been described previously (33). This protein would have 84 amino acids in common with the IE1 protein. The 28-kDa protein would be encoded totally within IE2a, and it is a nonphosphorylated protein. This viral protein encoded by IE2a was detected as early as 6 h after infection, as well as at 72 h after infection (data not shown).

***trans*-Activation of a heterologous early viral promoter.** The adenovirus E2 promoter is an inducible promoter responsive to the adenovirus E1a product (13), as well as herpesvirus regulatory proteins (6). To test if HCMV made a *trans*-acting protein(s) that could stimulate the heterologous E2 promoter, cells were transfected with a plasmid containing the adenovirus E2 promoter upstream of the *cat* gene, pE2CAT, and either mock infected or infected with 5 PFU of HCMV per ml at 24 h after transfection. Cell lysates were assayed for CAT activity as described in Materials and Methods. The amount of plasmid DNA associated with various samples, as determined by Southern blot hybridization, did not differ more than 1.0- to 1.5-fold, indicating that approximately equal levels of indicator pE2CAT DNA were present in each sample. Figure 3A demonstrates that infection with HCMV greatly stimulated (>100-fold) expression of the *cat* gene downstream of the adenovirus E2 promoter and that this stimulation occurred at early times after infection. Previous studies with HCMV, as well as other herpesviruses, have shown that expression of CAT enzyme activity from chimeric gene constructs provides a valid and sensitive assay for promoter activity and that increased levels of CAT activity correlate well with increased levels of cytoplasmic RNA (4, 5, 17, 22–24, 41). Thus, HCMV could code for a *trans*-acting protein(s) which stimulates heterologous, as well as homologous, promoters.

To determine whether HCMV IE genes are involved in stimulation of the adenovirus promoter, cells were cotransfected with pE2CAT DNA and various combinations of HCMV IE genes and the level of *cat* expression was assayed. Cotransfection of nonspecific SVOd DNA with pE2CAT DNA had no effect (Fig. 3B, lane 1). However, cotransfection of pCS DNA, which has both the IE1 and IE2 genes, with pE2CAT DNA caused a significant stimulation (24-fold) in *cat* expression above that of the control, pE2CAT plus pSVOd (Fig. 3B, lane 2). If the pCS DNA was linearized by digestion with restriction endonuclease *Sal*I, which cleaves downstream of IE2a at a single site in the plasmid (Fig. 1), there was some reduction in CAT activity, but there was still significant stimulation (17-fold) of *cat*

expression (Fig. 3B, lane 3). This reduction may be due to the fact that supercoiled DNAs, especially those containing enhancers, yield higher levels of expression than linearized DNAs do (39). Digestion of pCS DNA with restriction endonuclease *Cla*I linearized the plasmid at a site upstream of the first AUG of IE region 2. This digestion eliminated any synthesis of IE2 proteins made with the IE2 promoter and truncated IE2 proteins containing the amino acids from IE1 (Fig. 1) but allowed the synthesis of the IE1 protein. This digestion eliminated any stimulation in *cat* expression (Fig. 3B, lane 4), suggesting that the IE1 protein could not induce the adenovirus E2 promoter without the IE2 gene products.

To test the role of the IE2 gene products in the stimulation of the adenovirus promoter, a deletion in IE2 between the *Cla*I site in IE2a was made. This perturbed the amino-terminal end in IE2a but maintained the ORF in the deleted 2.25- and 1.7-kb mRNAs predicted to code for the 51- and 48-kDa proteins (Fig. 1). In addition, there was an AUG downstream of the second or 3′ *Cla*I site in the correct ORF that could serve for the synthesis of truncated forms of the 78-, 30-, and 27-kDa proteins. This deletion did not abolish CAT activity, but it reduced the original activity by approximately 75% (Fig. 3B, lane 5). These results again suggested that the critical region involved in stimulation of the adenovirus E2 promoter is located in IE2.

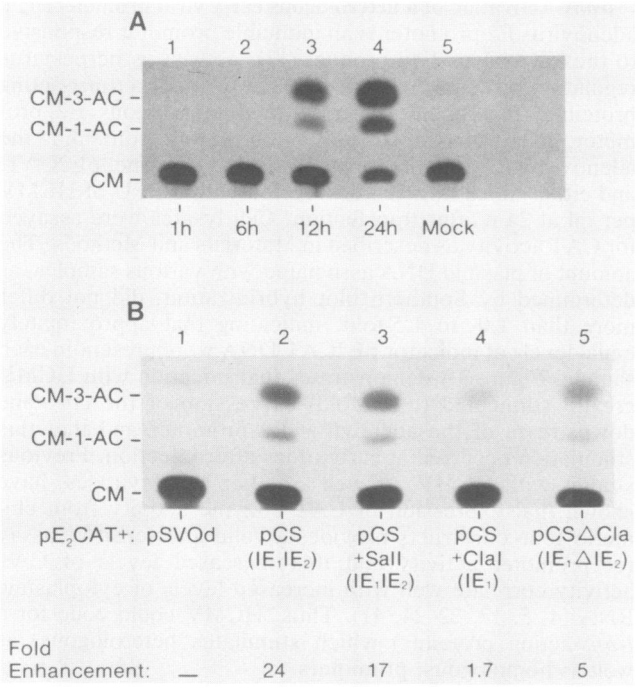

FIG. 3. Stimulation of *cat* expression driven by the adenovirus E2 promoter in permissive human fibroblast cells. (A) Cells were transfected with 5 μg of the indicator plasmid DNA pE2CAT. After 24 h, the cells were either mock infected or infected with 5 PFU of HCMV per ml. Cells were harvested at various times after infection and assayed for CAT activity as described in Materials and Methods. The hours after infection are shown under each lane. (B) Cells were transfected with 5 μg of pE2CAT DNA plus 5 μg of test plasmid DNA that was either supercoiled, linearized by restriction endonuclease digestion, or deleted as described in the text. The plasmid DNAs used are designated under each lane. Cell lysates were tested for CAT activity at 48 h after transfection. Plasmids capable of expressing the ORFs in regions IE1 or IE2 are shown under each lane. Δ indicates a deletion. The fold enhancement of E2CAT expression is shown at the bottom of the gel.

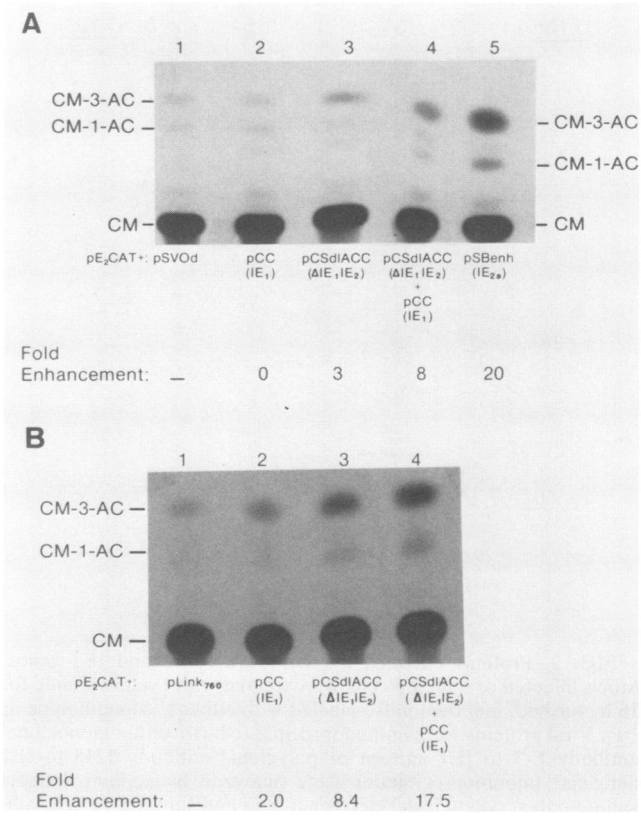

FIG. 4. The role of HCMV genes IE1 and IE2 in stimulation of *cat* expression driven by an early viral promoter. Cells were transfected with 5 μg of indicator plasmid DNA pE2CAT plus 1 to 5 μg of test plasmid DNA. Enzyme activities were determined as described in the text. (A) One microgram of pCC (IE1) DNA was used when cells were cotransfected with pE2CAT plus pCSdlAcc (ΔIE1 IE2). (B) Five micrograms of pCC (IE1) DNA was used when cells were cotransfected with pE2CAT plus pCSdlAcc (ΔIE1 IE2). The plasmid DNAs used are shown under each lane. Plasmids capable of expressing the ORFs in region IE1 or IE2 are shown under each lane. Δ indicates a deletion. The fold enhancement of E2CAT expression is shown at the bottom of the gel.

Separation of region IE2a. To determine whether both IE1 and IE2 are required for stimulation of pE2CAT expression, the genes were cloned on separate plasmids. The plasmid pCC expresses only IE1. Two additional clones were constructed to express region IE2. The clone pCSdlAcc has exon 4 of region IE1 deleted but retains the potential for splicing exons 1, 2, and 3 onto region IE2a. This plasmid should be able to code for the 48- and 51-kDa proteins and the 27- and 30-kDa proteins predicted from the IE2 ORFs (Fig. 1). The clone pSBenh has the strong IE promoter-regulatory region upstream of region IE2a. This plasmid should be able to code for only the 27- and 30-kDa proteins (Fig. 1). The levels of the 27- and 30-kDa proteins produced from plasmid pSBenh would be predicted to be considerably higher than those from pCSdlAcc, because the enhancer elements were directly upstream of IE2a in this construction (Fig. 1). Figure 4A (lanes 1 and 2) demonstrates that cotransfection of pE2CAT with either nonspecific pSVOd DNA or region IE1 DNA (pCC) had no significant effect on *cat* expression in the permissive human fibroblast cell. Cotransfection with pCSdlAcc DNA (ΔIE1 IE2) caused a small increase (threefold) in *cat* expression relative to that of the control, pE2CAT plus pSVOd (Fig. 4, lane 3). Expression of

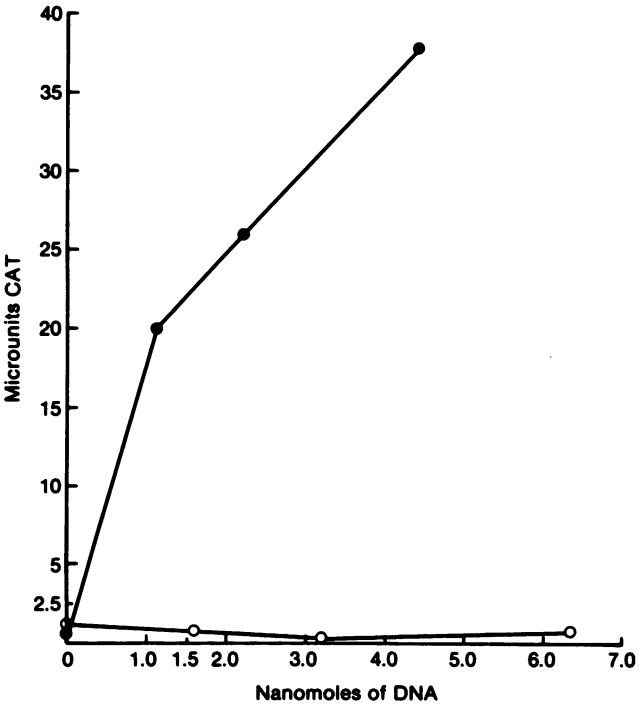

FIG. 5. Effect of increasing concentrations of the HCMV IE2a gene product on stimulation of E2CAT expression from an early viral promoter. Cells were cotransfected with 5 μg of indicator plasmid DNA pE2CAT and various concentrations of pSBenh (IE2a) DNA (●) or with just promoter-regulatory region pLink760 DNA (○). Enzyme activities were determined as described in the text.

both the IE1 and IE2 genes with the above plasmid was confirmed by transfection of COS-1 cells, followed by single-strand-specific nuclease protection experiments as described previously (31). In contrast, cotransfection with the IE2a gene placed downstream of the strong IE1 promoter regulatory region (pSBenh DNA) greatly stimulated *cat* expression (20-fold) (Fig. 4A, lane 5). However, a plasmid DNA without the enhancer elements upstream of IE2a, pSB (Fig. 1), had little to no effect on pE2CAT expression, suggesting that the level of IE2a expression is important (data not shown). When cells were cotransfected with pE2CAT plus 5-μg equivalents of pCSdlAcc (ΔIE1 IE2) and 1-μg equivalent of pCC (IE1), *cat* expression was stimulated eightfold (Fig. 4A, lane 4). When this experiment was repeated, pCSdlAcc (ΔIE1 IE2) again caused a slight increase (8.4-fold) in E2CAT expression (Fig. 4B, lane 3). When cells were cotransfected with 5-μg equivalents of both pCSdlAcc (ΔIE1 IE2) and pCC (IE1), E2CAT expression was stimulated 17.5-fold (Fig. 4B, lane 4). These data suggest that the IE2a viral proteins of 27 or 30 kDa can stimulate the E2CAT expression in *trans*. The lower level of stimulation of *cat* expression by pCSdlAcc (ΔIE1 IE2) may be due to the lower relative amount of IE2 transcription caused partly by the positional effect of the enhancer. Plasmid pCSdlAcc contains the simian virus 40 origin which can suppress the expression of genes on the same plasmid. In addition, pCSdlAcc (ΔIE1 IE2) could produce four proteins while pSBenh (IE2a) could produce only two (Fig. 1). How the IE1 gene product influences the activity of the pCSdlAcc plasmid and their gene products is not understood. Although quantitative comparisons of stimulation of *cat* expression are difficult, it is clear that the proteins made entirely within IE2a can stimulate *cat* expression from the E2 promoter. The 48- and 51-kDa viral proteins that can be coded for by plasmid pCSdlAcc (ΔIE1 IE2) may also have activity. The effect of IE1 on IE2 expression in plasmid pCSdlAcc or on the gene products requires further investigation.

Dose response to IE2a gene products. To establish a relationship between the level of region IE2a expression from the plasmid pSBenh and the degree of *cat* expression from the E2 promoter, cells were cotransfected with pE2CAT DNA and different concentrations of plasmid pSBenh or plasmid pLink760 that contains only the IE1 promoter regulatory region (Fig. 1). The latter plasmid DNA was used to eliminate the possibility that increased *cat* expression is due to a recombination between the IE1 promoter-regulatory region and the *cat* gene. Figure 5 demonstrates a linear relationship between the microunits of CAT activity and the nanomoles of plasmid pSBenh. These data substantiate the independent activity of IE2a which can encode for the 27- and 30-kDa proteins and establish a linear relationship between the amount of IE2a DNA and the stimulation of *cat* expression. Since no stimulation of CAT activity was detected in the presence of high levels of pLink760 DNA, stimulation of pE2CAT expression is not related to recombination of the *cat* gene with the HCMV IE promoter-regulatory region.

DISCUSSION

The adenovirus E1a protein acts in *trans* to provide a number of functions (20). Among these functions is the ability to stimulate the transcription of a variety of genes. The cloned *Xba*I E DNA of the HCMV genome has been shown to rescue the adenovirus E1a mutant, *dl*312 (29). This region of the HCMV genome contains a strong enhancer region upstream of three IE genes designated IE1, IE2, and IE3 (2, 35, 36). Thus, it was postulated that one or a combination of these genes may function to regulate HCMV by stimulating transcription from viral promoters. The HCMV early and late promoters detected in our laboratory require additional investigation. To study the ability of the IE gene and gene products to regulate viral promoters, it was necessary to use a system which was known to be responsive to *trans*-acting viral regulatory proteins, as well as to HCMV stimulation. The well-characterized adenovirus E2 promoter appears to mimic the HCMV early promoters. The pattern of E2CAT expression in response to HCMV infection is analogous to *cat* expression driven by an early HCMV promoter (E1.7) after HCMV infection (T. W. Hermiston, C. L. Malone, and M. F. Stinski, unpublished data).

Previous studies have proposed that IE1 may be responsible for this function (4, 15, 29, 36). However, this viral gene is expressed at a level far higher than that necessary for most *trans*-acting regulatory proteins. Since IE1 expression reaches maximum levels at 5 to 6 h after infection, much sooner than extensive transcription of the genome begins, it seemed unlikely that this area was responsible for expressing a function which rescues adenovirus *dl*312. When tested in permissive human fibroblast cells, the IE1 gene product, as predicted, demonstrated little to no stimulatory effect on the adenovirus E2-inducible promoter. Similar results were obtained in the nonpermissive monkey cells (M. J. Tevethia and D. J. Spector, personal communication). On the other hand, IE2 is made at levels more consistent with its role as a regulatory protein and reaches a maximum level of transcription at 48 to 72 h postinfection, when viral gene expres-

sion is at its highest level (38). As predicted, the HCMV IE2 gene was found to express a regulatory factor that significantly stimulated the *cat* expression from the adenovirus E2 promoter.

HCMV may code for other possible regulatory functions. The function of gene products from IE3 is also under investigation. The level of expression from this region and the time of appearance of the products are very similar to IE2 (Witte et al., unpublished data). This implies that IE3 may also have some regulatory function.

How these *trans*-acting viral proteins function is presently not known. It has been suggested that these viral proteins stimulate transcription nonspecifically by either inactivating an inhibitory cellular function (21), promoting assembly of stable transcription complexes (7), or altering the level of activity of a cellular transcription factor (16). It is possible that the HCMV early or late promoters interact poorly with the unmodified host cell transcriptional machinery and that the presence to the IE2 proteins, as well as other IE proteins of HCMV, facilitates the promoter recognition.

Although the IE1 gene product alone appears to have no activity in the E2CAT assay system, as well as other heterologous and homologous promoters tested to date (M. F. Stinski, C. L. Malone, and T. W. Hermiston, unpublished data), it may still interact positively, either directly or indirectly, in transcriptional stimulation. It is known that the IE1 protein negatively autoregulates its own expression (31). Since IE1 and IE2 exist in tandem and the IE1 protein product is highly expressed immediately after infection, the IE1 protein may positively regulate the relative abundance of the IE2 gene products. The possibility that the IE2 gene products act to stimulate their own promoter or interact to further suppress the IE1 promoter regulatory region requires further investigation. There is also the possibility that two or more gene products form a complex that favors their activity. Alternate splicing patterns of the mRNAs from IE1 and IE2 predicted a number of gene products of different sizes (33). Predicted ORFs for the IE2 proteins of 48 and 51 kDa contain 84 amino acids in common with the IE1 protein. These common amino-terminal amino acids may facilitate an interaction between IE1 and IE2 proteins.

Although there are protein products of various sizes associated with IE2 and these gene products are detected in infected cells at early times after infection, we were able to demonstrate that the IE2a gene, which can code for proteins of 27 and 30 kDa, can independently stimulate *cat* expression from the adenovirus E2 promoter in permissive human fibroblast cells. One of the IE2a gene products was detected in HCMV-infected cells as a nonphosphorylated protein of 28 kDa. It is interesting that phosphorylation may not be necessary for *trans*-acting regulatory activity.

Recently, M. J. Tevethia, D. J. Spector, K. M. Leisure, and M. F. Stinski (manuscript in preparation) have been able to complement adenovirus mutants deleted in the E1a gene, *dl*312, and *dl*1500, with plasmids pCS (IE1 IE2) and with pCC (IE1) plus pCSdlAcc (ΔIE1 IE2) DNA. Although pCSdlAcc (ΔIE1 IE2) alone also rescued these adenovirus deletion mutants, the relative amount of rescue was lower. Plasmid pCC (IE1) alone was unable to rescue adenovirus deletion mutants. These observations imply that all the adenovirus early promoters can be regulated in *trans* by the HCMV IE2 gene products or a combination of IE1 and IE2 but not by IE1 alone.

Although herpesviruses can complement an adenovirus mutant defective in E1a (6), there are no reports that adenovirus is able to complement a HSV defective in IE regulatory proteins. Thus, the adenovirus system seems to be less stringent than HSV is in its requirement for *trans*-acting regulatory proteins. Although the adenovirus E2 promoter requires only the proteins contained within the IE2a region, the HCMV system may require either the entire IE2 repertoire of proteins or the proteins of the other IE genes, independently or in combination, to obtain the full level of activation of promoters seen in the sequential regulated viral infection. Several HCMV early and late promoters are currently being isolated and characterized for their response to a variety of the HCMV IE gene products. Multiple *trans*-acting proteins of HCMV may be required to regulate differentially the numerous early and late viral promoters, as well as cellular promoters.

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