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In Vivo and In Vitro Analysis of Transcriptional Activation Mediated by the Human Cytomegalovirus Major Immediate-Early Proteins

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To define mechanistically how the human cytomegalovirus (HCMV) major immediate-early (IE) proteins induce early-gene transcription, the IE1 72-kDa protein, the IE2 55-kDa protein, and the IE2 86-kDa protein were analyzed for their ability to activate transcription from an HCMV early promoter in vivo and in vitro. In transient-expression assays in U373MG astrocytoma/glioblastoma and HeLa cells, only the IE2 86-kDa protein was able to activate the HCMV early promoter to high levels. In HeLa cells, the IE1 72-kDa protein was able to activate the promoter to a low but detectable level, and the level of promoter activity observed in response to the IE2 86-kDa protein was increased synergistically following cotransfection of the constructs expressing both IE proteins. To examine the interaction of the HCMV IE proteins with the RNA polymerase II transcription machinery, we assayed the ability of *Escherichia coli*-synthesized proteins to activate the HCMV early promoter in nuclear extracts prepared from U373MG cells, HeLa cells, and *Drosophila* embryos. The results of the in vitro experiments correlated well with those obtained in vivo. The basal activity of the promoter was minimal in both the HeLa and U373MG extracts but was stimulated 6- to 10-fold by the IE2 86-kDa protein. With a histone H1-deficient extract from *Drosophila* embryos, the HCMV early promoter was quite active and was stimulated two- to fourfold by the IE2 86-kDa protein. Addition of histone H1 at 1 molecule per 40 to 50 bp of DNA template significantly repressed basal transcription from this promoter. However, the IE2 86-kDa protein, but none of the other IE proteins, was able to counteract the H1-mediated repression and stimulate transcription at least 10- to 20-fold. The promoter specificity of the activation was demonstrated by the inability of the IE2 86-kDa protein to activate the *Drosophila Krüppel* promoter in either the presence or absence of histone H1. These results suggest that one mechanism of transcription activation by the IE2 86-kDa protein involves antirepression.

In the examination of virus-host interactions, much attention has been given to the role of viral gene products, especially those functioning at early times, in transcriptional regulation. These studies have been important not only for advancing our understanding of how the viral life cycle is regulated, but also for helping elucidate the general mechanisms that operate to control gene expression. Although the mechanisms by which these viral proteins affect transcription have not yet been defined fully, the fact that many of these proteins (particularly the immediate-early [IE] proteins of DNA viruses) are functionally interchangeable suggests that there may be common themes (21, 31, 74, 84, 85).

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 reviews, see references 2 and 59). Like other members of the herpesvirus family, the genome of HCMV is temporally expressed during the viral life cycle (16, 56, 81, 88, 89). The IE gene products are synthesized immediately after viral infection and rely primarily on host factors for their expression. Early genes are transcribed prior to viral DNA replication, and their expression requires one or more viral IE gene products. Finally, late genes, which constitute a majority of the viral genome, are transcribed in abundance only after viral DNA replication.

The major site of IE gene transcription is located in the long unique segment of the genome and includes two genetic units, designated IE1 (0.739 to 0.755 map units) and IE2 (0.732 to 0.739 map units) (16, 17, 29, 33, 34, 56, 64, 79, 80, 82, 88, 89, 91). At least three IE RNAs have been found to be transcribed from these regions (Fig. 1B). A 1.95-kb RNA, transcribed from the IE1 region (79), encodes a nuclear phosphoprotein approximately 72 kDa in size. IE region 2 encodes two IE RNAs of 2.25 and 1.7 kb that, through differential splicing mechanisms, contain the three 5′ exons of IE1 fused to the IE2 region (80). These RNAs encode proteins of approximately 86 and 55 kDa, respectively. Additional, less-defined RNAs and proteins that appear to initiate within IE2 at late times in the infectious cycle have also been reported (62, 66, 77, 80).

In previous studies, we and others have used transient-expression assays to demonstrate that HCMV early promoters as well as heterologous viral and cellular promoters can be activated by the region of the genome specifying the IE1 and IE2 gene products (4, 5, 9, 12, 15, 18, 20, 23, 26, 29, 39, 53, 60, 63, 67, 75, 85, 87). Recent studies suggest that the IE2 86-kDa protein plays a major role in activating HCMV early promoters as well as in repressing its own promoter (the major IE promoter), while the IE1 72-kDa protein acts to enhance the activity of the major IE promoter and may augment the stimulatory effect of the IE2 86-kDa protein (10–12, 26, 28, 50, 53, 61–63, 69, 78).

Any consideration of the mechanism by which the HCMV IE1 and IE2 proteins affect transcription must take into account the fact that multiple viral and cellular promoters

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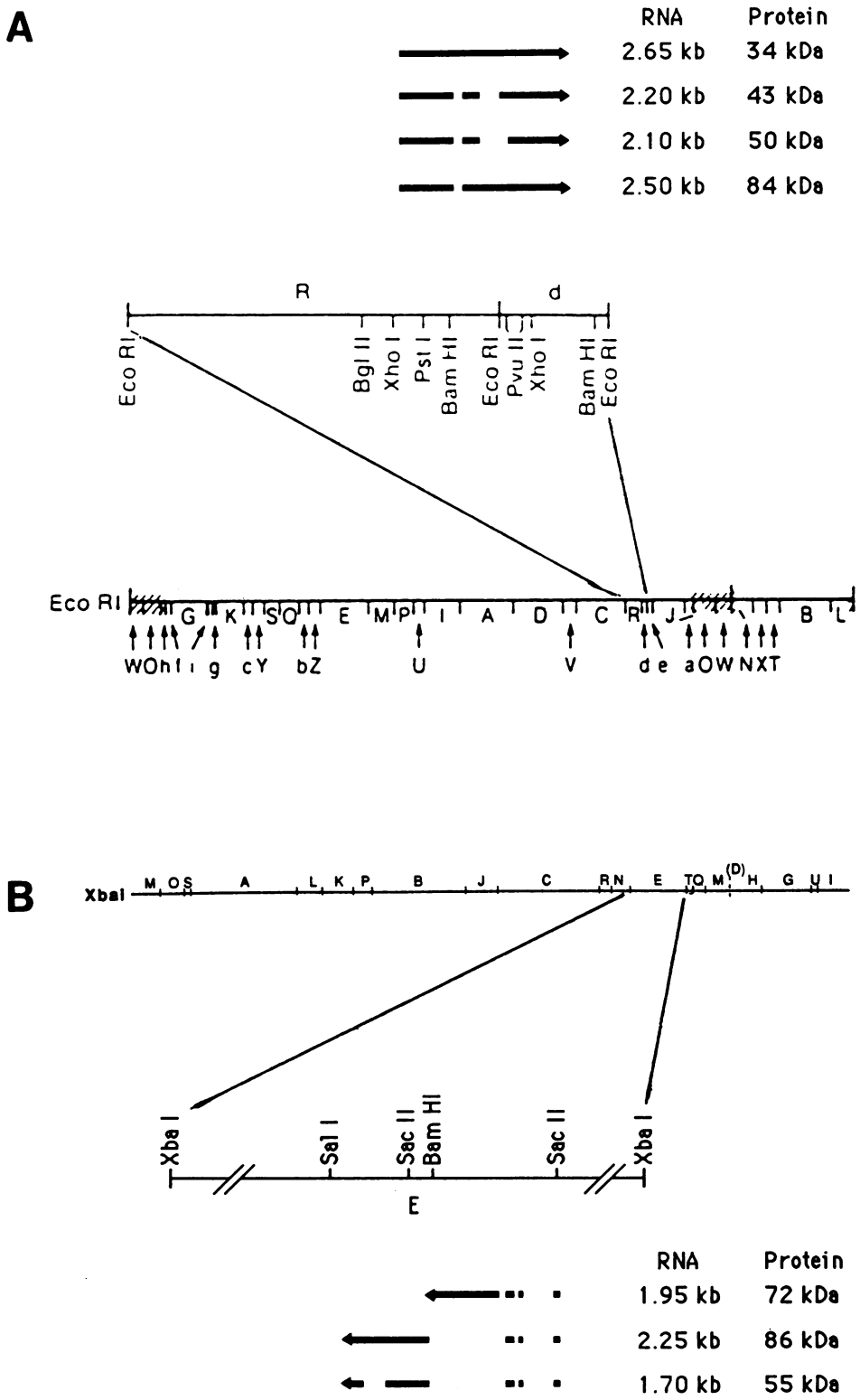

FIG. 1. Map locations of early RNA and major IE regions of HCMV. (A) Diagram of the HCMV strain AD169 genome structure, with *Eco*RI restriction endonuclease sites shown (73). *Eco*RI fragments R and d, encoding a family of RNAs that are derived through alternative splicing (76, 96, 97), are shown (top). The sizes of the early RNAs and corresponding proteins are indicated. (B) Diagram of the HCMV strain Towne genome structure, with *Xba*I endonuclease restriction sites shown (44). An expanded map of *Xba*I fragment E (containing IE regions 1 and 2) and the positions of the three major IE RNAs and corresponding proteins encoded by these regions are indicated at the bottom.

which seemingly lack any common element other than a TATA box are responsive to these proteins. Furthermore, these proteins have no known sequence-specific DNA-binding ability (40). This implies that they either interact with multiple targets or act through the general transcription factors. Alternatively, since any DNA in the nucleus, whether it be of viral or cellular origin, likely forms some higher-order protein-DNA complex, the IE proteins may function through a factor common to these structures. Because the conversion of genes from a repressed to an active state in vivo is often accompanied by a change in chromatin structure (for a review, see references 22 and 90), one mechanism used by cells to regulate transcriptional activity may involve nucleosomes or higher-order structures. In this regard, there are a number of reports demonstrating that transcription by RNA polymerase II in vitro is repressed upon reconstitution of chromatin structure (41, 52, 54, 93). Furthermore, it has been found that sequence-specific DNA-binding factors can prevent the repression mediated by chromatin formation if these factors are assembled on the template prior to chromatin reconstitution (92, 95). A similar series of experiments have also been performed with the pseudorabies virus IE protein, which functions as a transcriptional activator but lacks defined sequence specificity (1). In these studies, it was shown that in the absence of the pseudorabies virus IE protein, nonspecific DNA-binding proteins in HeLa nuclear extracts competed with TFIID for binding to the promoter. At low template concentrations, the pseudorabies virus IE protein appeared to facilitate the TFIID-promoter interaction and stimulate transcription by a mechanism that could be mimicked either by preincubating the DNA template with TFIID or by titrating the nonspecific DNA-binding proteins with non-promoter-containing DNA. Subsequently, Croston et al. (14) identified histone H1 as a major transcriptional repressor in nuclear extracts made by standard procedures. By using purified histone H1 and extracts specifically depleted of the inhibitory nonspecific DNA-binding proteins, they were able to demonstrate that the H1-mediated transcriptional repression could be relieved by prior assembly of sequence-specific DNA-binding factors on the template. Furthermore, it appeared that some site-specific transcription factors function only as antirepressors, whereas others seem to act as both true activators and antirepressors. Recently, these studies have been extended further to document that the relief of H1-mediated inhibition by sequence-specific transcription factors can also occur on DNA assembled into nucleosomes (45, 46).

In this study, we have used a combination of in vivo and in vitro assays to examine the functional properties of the HCMV IE proteins. As a target, we used the well-characterized HCMV early promoter for the 2.2-kb class of RNAs (open reading frames UL 112-113), which encode four nuclear phosphoproteins (Fig. 1A) (75, 76, 96, 97). For the in vivo analysis, cDNA constructs specifying individual gene products from the major IE region of the HCMV genome were used in transient-expression assays to identify the role that each protein plays in HCMV early-promoter activation. We also expressed the individual IE proteins in *Escherichia coli*, and with the purified proteins, we were able to reproduce in vitro the HCMV early-promoter activation observed in vivo. We find that the IE2 86-kDa protein is the major transactivator and that, in vitro, this protein can relieve histone H1-mediated repression of HCMV early-gene transcription.

MATERIALS AND METHODS

Cells. HeLa cell suspension cultures were a gift from M. Karin (University of California, San Diego) and were maintained in Joklik's modified minimum essential medium containing 5% calf serum. Human U373MG astrocytoma/glioblastoma cells were a gift from R. LaFemina (Merck, Sharpe and Dohme) and were maintained in Dulbecco's modified Eagle's medium (Irvine Scientific) supplemented with high glucose and containing 5% fetal bovine serum and Mito Plus serum extender (Collaborative Research).

Molecular cloning. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., or Boehringer Mannheim Biochemicals and used as recommended by the manufacturers. Competent *E. coli* DH5α cells (Bethesda Research Laboratories) were transformed with recombinant plasmids as recommended by the suppliers.

The construction of p729CAT, an HCMV early promoter-CAT fusion plasmid containing the promoter for the family of RNAs encoded within *Eco*RI fragments R and d, was described previously (75). The plasmid containing the *Drosophila Krüppel* promoter includes sequences between −861 and +426, relative to the major upstream start site, cloned into pUC119 (37, 65).

Plasmids used for the expression of the IE gene products in the analysis of IE gene-mediated early-promoter activation were constructed as follows. pSGIE72, pSGIE86, and pSGIE55 were constructed by first cleaving with *Hin*dIII the plasmids pIE72, pIE86, and pIE55 (obtained from R. Stenberg); these clones contained the individual cDNAs corresponding to the IE1 72-, IE2 86-, and IE2 55-kDa proteins, respectively (78). pIE72 was then cleaved to completion and pIE86 and pIE55 were partially cleaved with *Sac*II. The appropriate fragments were isolated, blunt ended with the Klenow fragment of DNA polymerase I, and attached to *Bam*HI linkers. After digestion with *Bam*HI, the resulting DNA fragments were isolated and cloned into pSG5 (Stratagene) that had been cleaved with *Bam*HI and dephosphorylated. Restriction enzyme analysis was used to identify clones containing inserts in the correct orientation.

Bacterial expression plasmids for the IE gene products were cloned as follows. pSGIE72, pSGIE86, and pSGIE55 were digested with *Bam*HI, and the IE coding sequences were isolated and ligated into pRMHa1 (7) that had been digested with *Bam*HI and dephosphorylated. These intermediate plasmids were designated pRMHa72, pRMHa86, and pRMHa55, respectively. pRMHa72, pRMHa86, and pRMHa55 were digested with *Sal*I and then either completely digested (pRMHa72) or partially digested (pRMHa86 and pRMHa55) with *Ava*II. DNA fragments containing all of the coding sequences of the IE genes except the 5′-terminal nucleotides were isolated. These fragments were ligated with an oligonucleotide that contained the missing 5′ nucleotides as well as an *Eco*RI restriction site at its 5′ end (synthesized by the University of California, San Diego, Oligonucleotide Synthesis Facility) and inserted into the glutathione-*S*-transferase (GST) fusion vector pGEX-KG (25), which had been cleaved with *Eco*RI and *Sal*I. The resulting clones (designated pGEX72, pGEX86, and pGEX55) were analyzed on a 10% acrylamide–sodium dodecyl sulfate (SDS) gel for production of fusion proteins of the expected sizes. The deletion plasmid of the 86-kDa IE fusion protein, pGEX86-StuI, which would express a protein missing 37 amino acids from the carboxy terminus, was constructed by cleaving pGEX86 with *Hin*dIII (a site within the polylinker 3′ of the IE gene) and *Stu*I (a site within the IE2 coding sequences), blunt-

ending the termini with the Klenow fragment of DNA polymerase I, and religating the ends.

Cell transfection. Human U373MG glial cells or HeLa cell monolayers were transfected with plasmid DNAs by the DEAE-dextran technique previously described by Staprans et al. (75). Briefly, 2×10^6 cells were washed twice with phosphate-buffered saline (PBS) prior to the addition of 5 ml of a solution containing plasmid DNAs and 400 μg of DEAE-dextran per ml in medium buffered with 50 mM Tris-HCl (pH 7.4) in the absence of serum. After 2 h, this solution was removed, and the cells were washed twice with PBS and once with medium plus serum. Ten milliliters of medium plus serum was added, and the cells were placed at 37°C. In all experiments, duplicate flasks were transfected.

Transient-expression assays. At approximately 48 h post-transfection, cells were harvested. When more than one plasmid was being analyzed, the contents of each flask were split into two parts: one for the analysis of chloramphenicol acetyltransferase (CAT) activity, and the other for the isolation of nuclear DNA and assessment of transfection efficiency. Soluble extracts were prepared and assayed for CAT activity essentially as described by Gorman et al. (24) except that the acetyl coenzyme A concentration was increased to 4 mM for longer reaction times (5 to 10 h). All CAT assays were performed so that the reactions were in the linear range. Transfection efficiency was determined by purification of transfected-cell nuclear DNA and determination of the relative amounts of plasmid DNA sequences present, as described by Staprans et al. (75).

Nuclear extracts for in vitro transcription assays. Nuclear extracts were prepared from uninfected U373MG glial cell monolayers and HeLa cell suspension cultures as described by Dignam et al. (19) with modifications that reportedly increase the efficiency of the nuclear extracts (72). Briefly, cells 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 0.4 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 0.33 g of solid ammonium sulfate per ml and dialyzed as described by Dignam et al. (19). The soluble nuclear fraction from *Drosophila* embryos, which is deficient in histone H1, was prepared by the procedures outlined by Kamakaka et al. (36) except that each gram of nuclei was suspended in 0.5 ml of HEMG (25 mM HEPES K^+ [pH 7.6], 12.5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 1.5 mM dithiothreitol) plus 0.1 M KCl prior to centrifugation in a Beckman SW28 rotor at 24,000 rpm for 1 h. *Drosophila* histone H1, purified as previously described (14), was a generous gift from Glenn Croston and Paul Laybourn. The concentrations of all proteins except histone H1 were determined as described by Bradford (6). The histone H1 concentration was determined with the Micro BCA assay (Pierce Chemical Co.).

Bacterial overexpression and purification of proteins. Overnight bacterial cultures containing the GST-IE fusion plasmids were diluted 1:10 in LB containing 50 μg of ampicillin per ml and grown for 2 h at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and cultures were harvested 3 h later. Cultures were centrifuged at 4,000 × *g* for 10 min, and the bacterial pellets were frozen at −70°C for 5 to 16 h. The bacterial cells were suspended in buffer (20 ml/g of wet pellet) containing 50 mM Tris-HCl (pH 8), 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin per ml, 1 mM sodium metabisulfite, and 1 mg of lysozyme per ml and incubated on ice for 30 min, after which Triton X-100 was added to 0.1%, and the cells were incubated on ice for a further 30 min. KCl was added to 200 mM, and the solution was centrifuged at 15,000 × *g* for 30 min. The supernatant was removed (the pellet was discarded), and Triton X-100 was added to a 1% final concentration. Then 2.5 μl of 50% (vol/vol) glutathione agarose was added for every milliliter of bacterial culture, and the mixture was rocked for 5 to 10 min at room temperature and centrifuged at 1,000 × *g* for 2 min. The supernatant was removed, and the pellet was washed three times with PBS plus 1% Triton X-100. Fusion proteins were eluted with 50 mM Tris-HCl (pH 8)–5 mM reduced glutathione–20% glycerol–1 mM phenylmethylsulfonyl fluoride–2 μg of aprotinin per ml by incubation at room temperature for 10 min. The supernatant was removed, frozen in liquid nitrogen, and stored at −70°C or in liquid nitrogen.

SDS-PAGE. Proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) essentially as described by Laemmli (43). SDS-PAGE gels consisted of a 5% acrylamide–0.13% bisacrylamide stacking gel and a 10% acrylamide–0.13% bisacrylamide resolving gel. Prior to electrophoresis, protein samples were suspended in SDS-PAGE sample buffer (2% SDS, 5 mM Na_2HPO_4 [pH 7], 10% glycerol, 5% 2-mercaptoethanol, 0.1 mM dithiothreitol, 0.01% bromophenol blue) and heated at 100°C for 5 min. Following electrophoresis, gels were stained with Coomassie brilliant blue. Molecular weights were estimated by comparison with molecular weight standards (Bio-Rad).

Western immunoblot analysis. Protein samples were subjected to SDS-PAGE as described above. After transfer of the proteins to nitrocellulose, the blots were blocked for 1 h at room temperature in Tris-buffered saline (TBS; 20 mM Tris [pH 7.6], 137 mM NaCl), 5% Nonidet P-40 (NP-40), and 5% (wt/vol) dried milk. Blots were rinsed twice in TBS plus 5% NP-40 and then washed once for 15 min and twice for 5 min each in TBS plus 5% NP-40. The blots were then incubated with a 1:200 dilution of monoclonal antibody CH-160 (specific for exon 3 of the major IE proteins; a gift from L. Pereira, University of California, San Francisco) for 45 min at room temperature in TBS plus 5% NP-40. The blots were washed as described above and then incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-mouse immunoglobulin secondary antibody for 45 min at room temperature in TBS plus 5% NP-40. After being washed as described above, the blots were treated with chemiluminescent detection reagents and exposed to film as described by the manufacturer (ECL kit; Amersham).

In vitro transcription and primer extension. Reactions were carried out essentially as described by Kadonaga (35). Standard in vitro transcription reaction mixes with human cell extracts contained 32.5 mM HEPES K^+ (pH 7.9), 6 mM Tris-HCl, 50 mM KCl, 6.25 mM $MgCl_2$, 0.04 mM EDTA, 0.04 mM EGTA, 0.4 mM dithiothreitol, 0.6 mM glutathione, 10% glycerol, 18 U of RNasin (Promega), 8 μg of supercoiled DNA template per ml, 1 to 2 mg of nuclear extract per ml, and various amounts of IE fusion proteins in a final volume of 25 μl. After 30 min at 30°C, all four ribonucleoside triphosphates (rNTPs) were added to a final concentration of 0.5 mM each, and the mix was further incubated at 30°C for 30 min. For the in vitro transcription assays with the *Drosophila* extracts, standard reaction mixes contained 32.5 mM HEPES (pH 7.6), 6 mM Tris-HCl, 50 mM KCl, 6.25 mM

$MgCl_2$, 0.5 mM dithiothreitol, 0.05 mM EDTA, 0.6 mM glutathione 5% glycerol, 1% polyvinyl alcohol, 1% polyethylene glycol, 0.0036% NP-40, 0.8 U of Inhibit-ACE (5 Prime → 3 Prime, Inc.), 0.5 mM each of the four rNTPs, 2.7 to 4 μg of supercoiled DNA template per ml, 1.2 to 1.7 mg of *Drosophila* embryo extract per ml, and various amounts of IE fusion proteins and *Drosophila* histone H1 in a final volume of 25 to 37 μl. In some experiments, DNA was preincubated with the IE proteins and extract prior to addition of the rNTPs; the precise conditions are described in each figure legend. Following addition of the rNTPs, the complete reaction mixes were incubated at 21°C for 30 min.

Transcription reactions were stopped by adding 100 μl of 20 mM EDTA (pH 8.0)–0.2 M NaCl–1% SDS–0.25 mg of tRNA or glycogen per ml as the carrier. Proteinase K (2.5 mg/ml, 5 μl) was added, and the reaction mix was incubated at room temperature for 5 min, after which 300 μl of 0.3 M sodium acetate was added. Reaction mixes were extracted once with phenol-chloroform (1:1, vol/vol) and once with chloroform-isoamyl alcohol (24:1, vol/vol). The aqueous layer was removed, 0.06 pmol of 5′-end-labeled primer was added, and ethanol precipitation was performed. The primer for reverse transcription analysis of the CAT RNA was 5′-TTTAGCTTCCTTAGCTCCTG, which hybridizes to the CAT transcript from p729CAT at positions +49 to +68 relative to the major RNA start site. The primer for reverse transcription analysis of the *Krüppel* RNA was 5′-TATTAC TCGCGGTTGTGTGTGGCACAAC, which hybridizes to the *Krüppel* transcript at positions +45 to +72 relative to the major upstream start site (37). The pellet was dissolved in 0.3 M sodium acetate (200 μl) and reprecipitated with ethanol. Pellets were washed in 75% ethanol, dried in a SpeedVac concentrator, and dissolved in 2 mM Tris-HCl (pH 7.8)–0.25 M KCl–0.2 mM EDTA (10 μl). This mixture was heated at 70°C for 1.5 min and then at 58°C for 1 h to allow hybridization of the primer to the RNA.

To the products of the in vitro transcription reactions with the human cell extracts was added 25 μl of a solution containing 20 mM Tris-HCl (pH 8.8), 10 mM $MgCl_2$, 5 mM dithiothreitol, 125 μg of dactinomycin per ml, 0.33 mM each of the four deoxynucleoside triphosphates (dNTPs), and 10 U of avian myeloblastosis virus reverse transcriptase (Promega). For the products resulting from the in vitro transcription reactions with the *Drosophila* extracts, 40 μl of a solution containing 62.5 mM Tris-HCl (pH 8.3), 1.25 mM $MnCl_2$, 125 μg of dactinomycin per ml, 0.33 mM each of the four dNTPs, and 0.5 μg of *E. coli*-synthesized Moloney murine leukemia virus reverse transcriptase (a gift from Michael Bulger) was added. The reaction mixes were incubated at 37°C for 1 h, and reactions were terminated by adding 300 μl of ethanol. Following centrifugation, the pellet was washed in 75% ethanol, dried in a SpeedVac concentrator, and dissolved in loading dye (formamide loading dye–0.1 M NaOH, 2:1). Samples were heated to 100°C for 3 min before being loaded on a 6 or 8% acrylamide–8 M urea sequencing gel. In some experiments, the primer extension products of in vitro transcription reactions were subjected to gel electrophoresis adjacent to the products of dideoxy sequencing reactions (70) prepared by using p729CAT as the template and the CAT oligonucleotide as the primer. The gels were dried and subjected to autoradiography at −70°C. Quantitation of the radioactivity in the primer extension products was done with a Phosphorimager (Molecular Dynamics).

RESULTS

Activation of an HCMV early RNA promoter by IE cDNA constructs in permissive U373MG astrocytoma/glioblastoma cells. Previously, we reported that a plasmid expressing both IE1 and IE2 in a genomic configuration was able to activate the early promoter for the family of RNAs encoded within *Eco*RI fragments R and d in transient-expression assays (75). In order to define clearly which IE gene or genes were responsible for this activation, individual cDNAs for each of the major IE proteins were cloned into eukaryotic expression vectors under the control of the simian virus 40 (SV40) early promoter. This eliminated the potential complication of negative autoregulation of the IE promoter by the 86-kDa protein from the IE2 region (10, 50, 61, 78). These constructs were cotransfected into permissive human U373MG astrocytoma/glioblastoma cells with the vector containing the HCMV early promoter (designated p729CAT) for transient-expression analysis (Fig. 2A). A cDNA construct expressing the IE2 86-kDa protein significantly stimulated the HCMV early promoter (62-fold over the background level). In contrast, a cDNA construct expressing the IE1 72-kDa protein was unable to activate transcription from this promoter. When both the 72- and 86-kDa cDNA constructs were transfected together, a small (1.4-fold) increase in activation of the HCMV early promoter was seen relative to that with the IE2 86-kDa cDNA construct alone.

Because the cDNA construct expressing the IE2 86-kDa protein still contained the splice sites to produce the IE2 55-kDa protein, we used a construct expressing a cDNA coding for the IE2 55-kDa protein to determine what role, if any, this protein played in early-promoter activation. By itself, the cDNA construct expressing the IE2 55-kDa protein was unable to activate transcription from the HCMV early promoter. However, when both the 55-kDa and 86-kDa cDNA constructs were transfected together with the reporter gene, the level of early promoter activation was reduced fourfold relative to the level obtained with the 86-kDa cDNA construct alone.

Activation of an HCMV early RNA promoter by IE cDNA constructs in nonpermissive HeLa cells. Because our long-range goal is to elucidate the biochemical mechanisms involved in IE-mediated early-gene activation, we were interested in developing an in vitro system to study this process (see below). Ideally, we would like to use extracts prepared from cells that are fully permissive for HCMV replication. Unfortunately, the two types of cells available, primary human fibroblasts and the human astrocytoma/glioblastoma cell line U373MG, do not grow in suspension and thus are extremely difficult to use for large-scale protein preparation. In contrast, HeLa cells can grow in suspension, and these cells have been used extensively as a source of factors for in vitro transcription systems. Although HeLa cells are nonpermissive for HCMV infection and do not express the IE genes when the virus is introduced into the cell by infection, IE genes are expressed when transfected into these cells, indicating that the block in the infection most likely occurs at the initial entry phase (86). Before HeLa cells could be used, however, it was necessary to compare the pattern of IE-mediated early-gene activation in these cells with that observed in the permissive U373MG cells.

As described above, IE cDNA constructs were cotransfected into HeLa cells with the HCMV early RNA promoter. In general, the pattern of gene activation was similar but not identical to that obtained in U373MG cells (Fig. 2B). The cDNA construct expressing the IE2 86-kDa protein was the

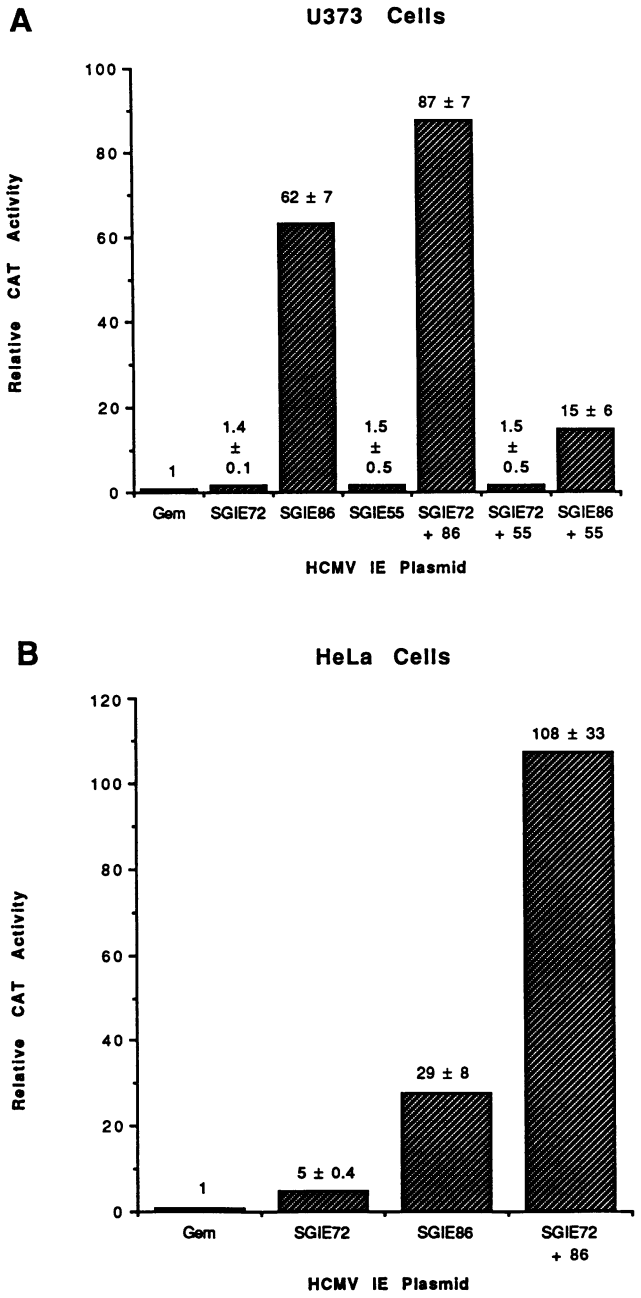

FIG. 2. Transactivation of an HCMV early promoter by IE cDNA constructs in permissive and nonpermissive cells. Duplicate flasks of permissive U373MG astrocytoma/glioblastoma cells (A) and nonpermissive HeLa cells (B) were cotransfected by the DEAE-dextran method with the HCMV early promoter construct p729CAT and with combinations of the following cDNA constructs driven by the SV40 early promoter: pSGIE72 (cDNA encoding the IE1 72-kDa protein), pSGIE86 (cDNA encoding the IE2 86-kDa protein), and pSGIE55 (cDNA encoding the IE2 55-kDa protein). pGem-1 (Gem) was transfected as a negative control. Cells were harvested at 48 h posttransfection and assayed for CAT activity as described in Materials and Methods. CAT activity relative to that with pGem-1 is shown above each bar. The values represent the averages of two to six independent assays, with the standard error of the mean given.

major transactivator in these cells (29-fold-increased activation). However, in the HeLa cells, the cDNA construct expressing the IE1 72-kDa protein was also able to activate this promoter, albeit to a low level (approximately fivefold). In addition, in HeLa cells, cotransfection of the cDNA constructs expressing both the IE1 72- and IE2 86-kDa proteins resulted in much greater stimulation of this promoter than did transfection by either cDNA construct alone. These results were in contrast to those with U373MG cells, in which the IE1 72-kDa protein did not significantly activate the early promoter (1.4-fold over background) and only minor increases in activity (1.4-fold) were seen upon cotransfection of cDNA constructs expressing both the IE1 72- and the IE2 86-kDa proteins relative to that after transfection of the cDNA construct expressing the IE2 86-kDa protein alone.

Activation of an HCMV early promoter in vitro in human cell extracts. Having established the nature of the activation of the HCMV early promoter by the HCMV IE genes in vivo, we next proceeded to analyze their activity in vitro. In order to provide large quantities of the IE gene products, the cDNA constructs expressing the IE1 72-, IE2 86-, and IE2 55-kDa proteins used in the above studies were cloned into vectors allowing their expression in *E. coli* as fusion proteins containing GST at the amino terminus. Full-length fusion proteins were detected in protein extracts from induced bacteria by Western blot analysis by using an antibody to exon 3 of the IE1 region (data not shown). Partially purified fusion proteins or the GST protein alone was then used in conjunction with crude nuclear protein extracts from both U373MG glial cells and HeLa cells for in vitro transcription studies.

Figure 3 shows the results of transcription assays with U373MG cell extracts. In reaction mixes containing 200 ng of template per assay, the basal level of transcription was barely detectable. However, a 6- to 10-fold increase in transcription was observed when the IE2 86-kDa fusion protein was present. In these reaction mixes, a band of approximately 70 bp appeared; the size compares favorably with that of the expected primer extension product. With 400 ng of template, the basal level was significantly increased, but only a twofold increase in transcription was observed with the IE2 86-kDa fusion protein relative to that with GST alone (data not shown). The IE1 72-kDa fusion protein either was unable to activate or activated transcription only slightly over the levels obtained with GST alone, and the presence of both the IE2 86- and IE1 72-kDa fusion proteins gave no further increase in transcription relative to that with the IE2 86-kDa fusion protein alone. This experiment also demonstrated that the IE2 86-kDa fusion protein was not present in excess, because using twice as much protein gave approximately twice as much RNA.

The results of in vitro transcription assays with crude nuclear extracts from HeLa cells were similar to those obtained with nuclear extracts from permissive U373MG cells (Fig. 4). Again, the IE2 86-kDa fusion protein markedly activated transcription from the HCMV early promoter, while the IE1 72-kDa fusion protein was unable to activate transcription. Addition of both the IE1 72- and IE2 86-kDa fusion proteins also resulted in no significant increase (less than twofold) in transcription relative to that with the IE2 86-kDa protein alone. We also tested a 37-amino-acid carboxy-terminal deletion mutant of the IE2 86-kDa fusion protein (protein 86d) for its ability to activate transcription from the HCMV early RNA promoter. When a similar construct was used by Pizzorno et al. (62, 63) in transient-

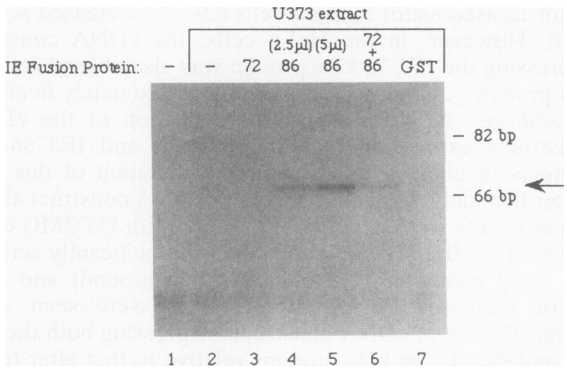

FIG. 3. In vitro transcription from an HCMV early promoter with permissive U373MG cell nuclear extracts. In vitro transcription was assayed by incubating 200 ng of p729CAT with 25 μg of U373MG cell nuclear extract, 125 ng of IE2 86-kDa fusion protein (lane 4), 250 ng of IE2 86-kDa fusion protein (lane 5), 400 ng of 72-kDa IE fusion protein (lane 3), or 125 ng of IE2 86-kDa fusion protein plus 400 ng of IE1 72-kDa fusion protein (lane 6) for 30 min at 30°C, after which rNTPs were added. After 30 min at 30°C, the nucleic acids were isolated and subjected to primer extension. As controls, 1,500 ng of GST (lane 7) or buffer (lane 2) was used to replace the IE fusion protein. Lane 1 did not contain the U373MG cell extract or IE fusion proteins. The IE fusion proteins used are indicated at the top. The arrow identifies the specific primer extension product of 68 bp. The amounts of IE fusion proteins and GST are based on the amount of full-length protein seen after Coomassie blue staining of acrylamide gels. The amount of total bacterial protein used in the assays was: GEX86, 790 ng (lane 4) and 1,580 ng (lane 5); GEX72, 400 ng; GEX72 plus GEX86, 1,190 ng; GST, 2,300 ng.

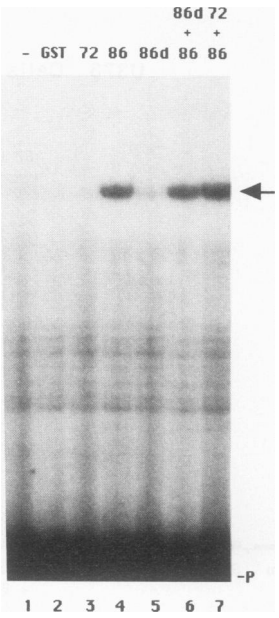

FIG. 4. In vitro transcription from an HCMV early promoter with nonpermissive HeLa extracts. In vitro transcription was assayed by incubating 200 ng of the HCMV early promoter construct p729CAT with 55 μg of HeLa nuclear extract and 200 ng of IE1 72-kDa fusion protein (lane 3), 88 ng of IE2 86-kDa fusion protein (lane 4), 100 ng of 86-kDa carboxy-terminal deletion IE fusion protein 86d (lane 5), 88 ng of IE2 86-kDa fusion protein plus 100 ng of 86d (lane 6), or 200 ng of IE1 72-kDa fusion protein and 88 ng of IE2 86-kDa fusion protein (lane 7) for 30 min at 30°C, after which rNTPs were added. After 30 min at 30°C, the nucleic acids were isolated and subjected to primer extension. As controls, 705 ng of GST (lane 2) or buffer (lane 1) was used to replace the IE fusion protein. The IE fusion proteins used are indicated at the top. The arrow identifies the specific primer extension product. P indicates free primer. The amounts of IE fusion proteins and GST are based on the amount of full-length protein seen after Coomassie blue staining of acrylamide gels. The amount of total bacterial protein used in the assays was: GEX72, 220 ng; GEX86, 375 ng; GEX86d, 125 ng; GEX86d plus GEX86, 500 ng; GEX72 plus GEX86, 595 ng; GST, 705 ng.

expression assays, it was unable to activate a herpes simplex virus early promoter. As shown in Fig. 4, this carboxy-terminal deletion protein also did not activate the HCMV early promoter over background levels in in vitro transcription assays. In addition, when the full-length and deleted 86-kDa fusion proteins were added together, there was no change in the amount of RNA produced relative to the levels obtained when only the full-length protein was used.

The IE2 86-kDa protein can specifically activate an HCMV early promoter in *Drosophila* embryo nuclear extracts. Nuclear extracts prepared from *Drosophila* embryos are highly active and have been shown to be capable of accurate RNA polymerase II-directed transcription from a variety of eukaryotic promoters, including those of mammalian origin (27, 36, 45). The fact that large amounts of extract and specific factors can be prepared at a relatively low cost, coupled with the high performance level of this system, makes it especially attractive. Recently, Kamakaka et al. (36) have demonstrated that even more efficient RNA polymerase II transcription can be obtained with a soluble nuclear fraction from *Drosophila* embryos. At least part of its increased activity appears to be due to its negligible content of nonspecific DNA-binding factors, especially histone H1, that can inhibit transcription (14). In fact, the nonlinear increase in basal activity and the decrease in the level of activation of the HCMV early promoter by the IE2 86-kDa fusion protein in the U373MG extracts as the DNA template concentration was raised suggested that the extracts contained nonspecific DNA-binding factors that might inhibit transcription.

To assess whether this soluble nuclear fraction from *Drosophila* embryos could be used to study transcription directed by the HCMV early promoter, we examined the activity of various amounts of the template DNA (p729CAT) in the presence and absence of the IE2 86-kDa fusion protein. As a control, we also assayed the levels of transcription obtained with various amounts of the *Drosophila Krüppel* promoter, which has been shown to be highly active in these extracts (14, 36). Figure 5 shows that, as the concentration of p729CAT DNA was increased from 50 to 200 ng per reaction mix, the total amount of transcription increased in a linear manner. When 25 ng of template was used, the levels of transcription were low but could be detected with longer exposures of the autoradiograms. Synthesis of RNA was also inhibited by the presence of 4 μg of α-amanitin per ml, which indicates that transcription was being directed by RNA polymerase II. From the pattern of extension products obtained, it appeared that several start sites were being utilized, but the major band was identical in size to that obtained with the human extracts. This is shown more directly in the experiment described below and shown in Fig. 6. As expected, the *Krüppel* promoter was also active in these extracts and yielded the expected pattern of extension products. When the IE2 86-kDa fusion protein was included

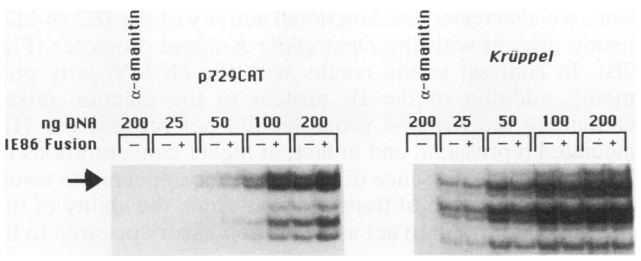

FIG. 5. In vitro transcription with *Drosophila* embryo nuclear extracts. In vitro transcription was assayed by incubating various amounts of the HCMV early-promoter construct p729CAT or the construct containing the *Drosophila Krüppel* promoter with 44 μg of *Drosophila* embryo nuclear extract in the presence or absence of 150 ng of IE2 86-kDa fusion protein in a total volume of 22.5 μl for 30 min at 21°C, after which 2.5 μl of rNTPs was added. One reaction mix for each promoter contained 4 μg of α-amanitin per ml. After 30 min at 21°C, the nucleic acids were isolated and subjected to primer extension. The arrow indicates the position of the major primer extension product for reaction mixes containing p729CAT. The amount of IE fusion protein was based on the amount of full-length protein seen after Coomassie blue staining of acrylamide gels; total bacterial protein was 950 ng.

in the reaction mixes, there was a small increase (approximately two- to threefold) in the amount of RNA synthesized from the HCMV early promoter. In contrast, addition of the IE2 86-kDa fusion protein to reaction mixes containing the *Krüppel* DNA either had no effect or resulted in a slightly lower level of transcription. These results suggested that the ability of the IE2 86-kDa protein to activate the HCMV early promoter was promoter specific and was not simply due to a general stimulation of basal transcription.

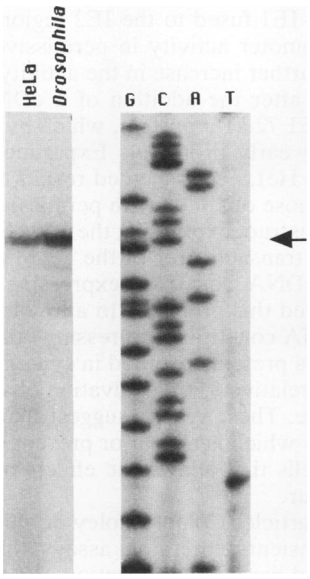

FIG. 6. Determination of the start site for transcription from the HCMV early promoter in nuclear extracts from HeLa and *Drosophila* embryo cells. Primer extension analysis was performed with the nucleic acids resulting from in vitro transcription reactions with the HCMV early-promoter construct p729CAT as the template and extracts from either HeLa cells or *Drosophila* embryo cells in the presence of the IE2 86-kDa fusion protein (similar to the experiments shown in Fig. 4 and 5). The extension products were then subjected to denaturing gel electrophoresis adjacent to the products of dideoxy sequencing reactions prepared by using p729CAT as the template and the CAT oligonucleotide as the primer. The arrow marks the position of the major RNA start site.

To determine precisely the start site of transcription directed by the HCMV early promoter and to document that the same initiation site was being utilized in the extracts from human and *Drosophila* cells, we subjected the primer extension products resulting from the in vitro transcription reactions to denaturing gel electrophoresis adjacent to the products of dideoxy sequencing reactions. In Fig. 6, it can be seen that in the presence of the IE2 86-kDa fusion protein, transcription initiates at the same position in extracts prepared from HeLa and *Drosophila* cells. The in vitro site corresponds to a position two nucleotides downstream from the previously reported initiation site used in vivo (see Fig. 6 in reference 76). This minor disparity is likely due to some compression in the earlier gels as well as to the different procedures used for the mapping; i.e., for the in vivo studies, the products of S1 nuclease reactions were electrophoresed alongside a sequencing ladder prepared by the method of Maxam and Gilbert (55). Nevertheless, the results showed that both the human and *Drosophila* extracts were capable of accurate initiation of transcription on this HCMV early promoter.

The IE2 86-kDa protein can act as an antirepressor of histone H1-mediated inhibition of basal RNA polymerase II transcription. Abmayr et al. (1) first presented evidence from in vitro transcription assays that the pseudorabies virus IE protein might function to counter the block in transcription initiation mediated by nonspecific DNA-binding proteins. Subsequently, Croston et al. (14) identified a major transcriptional repressor present in nuclear extracts as histone H1 and demonstrated that several sequence-specific transcription factors could counteract the H1-mediated repression. To determine whether the HCMV IE proteins, which lack known sequence-specific DNA-binding ability (40), might also function in a similar way, we tested their activity in the presence of added histone H1. In the experiment shown in Fig. 7A, we used the soluble nuclear fraction from *Drosophila* embryos described above, which is deficient in histone H1, and the DNA containing the HCMV early promoter (p729CAT) as the template. When histone H1 was not present in the reaction mixes, the IE2 86-kDa fusion protein activated the HCMV promoter approximately threefold over the levels seen in the control reactions with GST protein alone. The level of transcription in the presence of the IE1 72-kDa fusion protein was the same as that observed with the GST protein alone, while the level of transcription in the presence of the carboxy-terminal deletion mutant of the IE2 86-kDa protein was reduced approximately twofold. The IE2 55-kDa protein also was unable to activate transcription above the basal level. When 0.6 or 0.8 U of histone H1 was added to the extracts (1 U corresponds to approximately 1 molecule of histone H1 per 30 to 45 bp of template DNA), the level of transcription was reduced by greater than 90% in all reaction mixes except those that contained the full-length IE2 86-kDa fusion protein. Thus, the IE2 86-kDa fusion protein was able to counteract the histone H1-mediated repression and effect a net 10- to 20-fold increase in the level of transcription. Addition of the IE1 72-kDa fusion protein or the IE2 55-kDa fusion protein to extracts containing the IE2 86-kDa fusion protein in either the presence or absence of histone H1 gave no further increase in transcription relative to that seen with the IE2 86-kDa fusion protein alone.

To document the specificity of the observed antirepres-

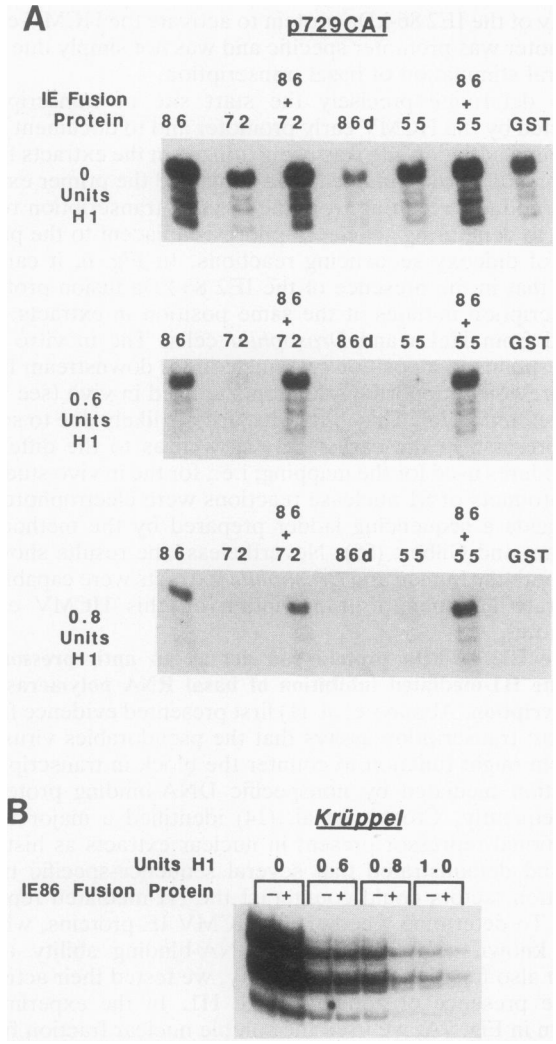

FIG. 7. The IE2 86-kDa protein but not the other IE1 or IE2 proteins can function as an antirepressor of histone H1-mediated inhibition of transcription from the HCMV early promoter. (A) In vitro transcription was assayed by preincubating 100 ng of the HCMV early promoter p729CAT with one of the following combinations of IE fusion proteins at 4°C for 30 min: 90 ng of the IE2 86-kDa fusion protein; 400 ng of the IE1 72-kDa fusion protein; 90 ng of the IE2 86-kDa fusion protein plus 400 ng of the IE1 72-kDa fusion protein; 360 ng of the IE2 86-kDa carboxy-terminal deletion fusion protein 86d; 105 ng of the IE2 55-kDa fusion protein; 90 ng of the IE2 86-kDa fusion protein plus 90 ng of the IE2 55-kDa fusion protein; or, as a control, 430 ng of GST. This DNA-protein mix was then added to reaction mixes containing 44 μg of *Drosophila* embryo nuclear extract in the presence of various amounts of *Drosophila* histone H1, after which rNTPs were added; the total volume of each reaction mix was 37 μl. One unit of histone H1 corresponds to 140 ng. After 30 min at 21°C, the nucleic acids were isolated and subjected to primer extension. The amount of IE fusion protein was based on the amount of full-length protein seen after Coomassie blue staining of acrylamide gels. The amount of total bacterial protein in each assay was as follows: GEX86, 432 ng; GEX72, 440 ng; GEX86 plus GEX72, 872 ng; GEX86d, 414 ng; GEX55, 455 ng; GEX86 plus GEX55, 822 ng; GST, 440 ng. (B) In vitro transcription was assayed by preincubating 100 ng of the construct containing the *Drosophila Krüppel* promoter with 150 ng of IE2 86-kDa fusion protein or buffer alone at 4°C for 30 min. This DNA-protein mix was then added to reaction mixes containing 35 μg of *Drosophila* embryo nuclear extract in the presence of various amounts of *Drosophila* histone H1, after which rNTPs were added; the total volume of each reaction mix was 25 μl. After 30 min at 21°C, the nucleic acids were isolated and subjected to primer extension. The amount of IE fusion protein was based on the amount of full-length protein seen after Coomassie blue staining of acrylamide gels; total bacterial protein was 950 ng.

sion, we also tested the functional activity of the IE2 86-kDa fusion protein with the *Drosophila Krüppel* promoter (Fig. 7B). In contrast to the results with the HCMV early promoter, addition of the IE protein to the reaction mixes containing the *Krüppel* promoter did not prevent the H1-mediated repression, and in fact, at higher concentrations of histone H1, the presence of the IE protein appeared to result in further inhibition of transcription. Thus, the ability of the IE2 86-kDa protein to act as an antirepressor appeared to be promoter specific.

DISCUSSION

To progress through their life cycle, most DNA viruses require coordinated activation and repression of multiple regions of the genome. This progression appears to be critically dependent on the initial expression of one or more IE gene products. As we learn more about the properties of these various IE gene products, we begin to see that they form a unique group of regulatory proteins that appear to function in a similar manner. Complete understanding of their mode of action, however, requires in vivo studies to elucidate physiological properties and in vitro analyses to dissect the molecular mechanisms governing their function. In this study, we have used this combined approach to analyze the functional properties of the major IE proteins specified by HCMV.

In vivo role of the HCMV IE1 and IE2 proteins in early-gene regulation. In the studies presented here, we used transient-expression assays to demonstrate that the IE2 region of the major IE gene was the key region involved in the activation of an HCMV early gene. In particular, a cDNA construct expressing the IE2 86-kDa protein, which contains the first three exons in IE1 fused to the IE2 region, strongly stimulated early-promoter activity in permissive U373MG cells. Only a small further increase in the activity of this promoter was observed after the addition of a cDNA construct expressing the IE1 72-kDa protein, which by itself was unable to activate the early promoter. Experiments conducted in nonpermissive HeLa cells yielded results that differed only slightly from those obtained with permissive U373MG cells. The cDNA construct expressing the IE2 86-kDa protein was still the major transactivator of the HCMV early promoter. However, a cDNA construct expressing the IE1 72-kDa protein activated this promoter to a low level, and cotransfection of cDNA constructs expressing both the IE1 72-kDa and IE2 86-kDa proteins resulted in synergistic activation of this promoter relative to the activation observed with either construct alone. These results suggest that HeLa cells contain an activity which is absent or present only at low levels in U373MG cells that allows the effects of the IE1 72-kDa protein to occur.

In a recent article, Colberg-Poley et al. (12) reported the results of transient-expression assays that are similar to those presented here. Cotransfection of a truncated version of the promoter used in our studies (which contained 323 bp rather than 694 bp of upstream sequence) and a genomic construct that expressed the IE2 gene products under the control of the major IE promoter resulted in a high level of

promoter activity in both permissive human fibroblasts and HeLa cells. In their experiments, the IE1 gene product by itself did not activate this HCMV early promoter in either fibroblasts or HeLa cells, but cotransfection of both the IE1 and IE2 genomic constructs resulted in a 2- to 2.5-fold-higher level of promoter activity than was observed with the IE2 genomic construct alone.

Previously, Chang et al. (9) reported that a genomic construct containing the IE2 region fused to the first three exons of IE1 could stimulate transcription from another HCMV early promoter, but the levels of activation were augmented significantly by the presence of the complete IE1 region. In contrast, Stenberg et al. (78) detected almost no activation of the HCMV early promoter for the DNA polymerase gene by a genomic construct expressing the first three exons of IE1 fused to IE2 or a cDNA construct expressing the IE2 86-kDa protein. However, this promoter was activated when constructs expressing both the IE1 72-kDa and IE2 86-kDa proteins were cotransfected. Taken together, these results indicate that the requirement for the IE1 72-kDa protein for HCMV early gene expression may be both cell type and promoter specific. However, another explanation for the variable results may be related to the specific construct used. In this regard, it has been reported that the IE2 86-kDa protein can downregulate expression from its own promoter and that the IE1 72-kDa protein can alleviate this repression (50, 53, 63, 78). Thus, the synergistic effect seen by some (9, 12, 78) after addition of both the IE1 and IE2 regions may be due, at least in part, to this autoregulation, since the IE constructs used in those studies were under the control of the IE promoter. The constructs used in our studies had the SV40 early promoter directing expression of the IE genes. This promoter does not contain the site involved in the autorepression and therefore should not be downregulated.

The function of the IE2 55-kDa protein remains to be clarified. This protein has the same amino and carboxy termini as the IE2 86-kDa protein but is missing an internal region of 155 amino acids (Fig. 1B). Pizzorno et al. (62) reported that this protein is present in infected cells only after protein synthesis inhibitors are removed, and thus it is possible that it does not play a physiological role in the viral infection but is an artifact of the experimental conditions. Although there are two reports that this protein can activate the HCMV major IE promoter, the human immunodeficiency virus long terminal repeat, and the SV40 promoter to a low level (3, 23), in our transient-expression experiments and in vitro assays, we detected no role for the IE2 55-kDa protein by itself in early-gene activation. Stenberg et al. (78) also reported that a cDNA construct expressing the IE2 55-kDa protein did not activate the HCMV DNA polymerase early promoter even in the presence of the complete IE1 region. However, we did find that a cDNA construct expressing the IE2 55-kDa protein repressed the levels of HCMV early-promoter activity that had been induced by a cDNA construct expressing the IE2 86-kDa protein. It is possible that the IE2 55-kDa protein interferes with the ability of the IE2 86-kDa protein to activate transcription of early genes, perhaps by binding and sequestering a factor required for the activation mediated by the IE2 86-kDa protein.

HCMV IE2 86-kDa protein can activate early-gene transcription in vitro. By using crude protein extracts from nuclei of permissive U373MG astrocytoma/glioblastoma cells and partially purified IE fusion proteins expressed in *E. coli*, we were able to reproduce early-gene activation in vitro. The IE2 86-kDa fusion protein alone was able to activate the HCMV early promoter, as we had seen in transient-expression assays. Consistent with the in vivo results, we also found that addition of the IE1 72-kDa fusion protein to the IE2 86-kDa protein gave no further activation of this promoter.

As shown in Fig. 4, in vitro transcription experiments with nuclear extracts from HeLa cells also demonstrated that the HCMV early promoter was activated significantly by the IE2 86-kDa fusion protein. However, in contrast to our results in transient-expression assays, the IE1 72-kDa fusion protein did not activate this promoter in vitro. There are several possible explanations for this result. The IE1 72-kDa fusion protein produced in the bacterial cells may be inactive, or a posttranslational modification that occurs only in eukaryotic cells may be required for its proper functioning. Alternatively, during the preparation of the HeLa cell extract, the auxiliary factor(s) required for the IE1 72-kDa protein-mediated early-gene activation detected in transient-expression assays may have been lost or inactivated. However, the results of our experiments do suggest that extracts from HeLa cells should be sufficient for purifying the factors required for activation by the IE2 86-kDa protein and for studying the mechanisms used by this protein to activate early-gene transcription.

Crude *Drosophila* embryo extracts with and without histone H1 have also been used for in vitro transcription assays with a variety of eukaryotic promoters (27, 36, 45). In our experiments with *Drosophila* extracts in the absence of histone H1, the basal activity of the HCMV early promoter was much higher than that seen in the U373MG or HeLa cell nuclear extracts. This may be due to the faster rate of transcription initiation in *Drosophila* embryos than in mammalian cells (35) or, more likely, to the presence of contaminating histone H1 in the human nuclear extracts. Our experiments indicated that the IE2 86-kDa fusion protein was able to activate transcription from the HCMV early promoter two- to threefold in the absence of histones. The addition of histone H1 to the reaction mix after preincubation of the DNA template with GST or buffer alone markedly reduced basal-level activity. However, when the IE2 86-kDa fusion protein was added to the template prior to histone H1 addition, the histone H1-mediated repression of basal activity was prevented, resulting in a net 10- to 20-fold increase in the level of transcription. Thus, the IE2 86-kDa protein can function, at least in vitro, as an antirepressor. This function, however, appears to be promoter specific and not simply the result of global stimulation of general transcription or neutralization of histone H1.

In determining whether or not the biochemical experiments reflect the mechanisms that occur in vivo, it is important to consider how closely the in vitro assays simulate the in vivo conditions. When HCMV enters the cell, the early genes are transcriptionally repressed, and they are not activated until after the IE gene proteins begin to accumulate. Although the state of the HCMV genome in the nucleus during the course of the infection has yet to be characterized, it is improbable that it exists as naked DNA. Furthermore, it is well established that plasmid DNA transfected into mammalian cells by the DEAE-dextran method is rapidly assembled into minichromosomes with appropriately spaced nucleosomes (8, 68). Because transcription factors normally interact with chromatin in vivo, it has been argued that in vitro assays which utilize naked DNA might not allow the full range of functional properties of the specific factors to be observed. In this regard, several studies have shown

that the relative magnitude of transcriptional activation effected by a number of specific factors in vitro more closely approximates that seen in vivo when the DNA templates are assembled into chromatin or histone H1-DNA complexes (1, 14, 42, 45, 46, 92–95). One advantage of the histone H1-DNA complexes is that they are easier to prepare than chromatin templates, and the identification of histone H1 as the transcriptional repressor is unambiguous. This fact, coupled with the finding that the in vitro transcriptional properties of both types of templates are comparable (14, 45), has made H1-DNA complexes a useful, simple model system for transcriptionally repressed DNA templates.

Possible mechanisms of action. The IE protein-mediated activation of early-gene transcription does not appear to act through high-affinity DNA-protein interactions. In DNase I footprinting and gel shift analyses, we have not detected any binding of the IE2 86-kDa protein to the HCMV early promoter (40). Thus, this HCMV IE protein is similar to the adenovirus E1A protein, which is able to stimulate early-gene transcription but does not appear to possess specific DNA-binding ability. Other viral IE proteins, including herpes simplex virus IE proteins (57, 58) and the pseudorabies virus IE protein (13), have been found to interact with sites on viral promoters, although consensus binding sites have been hard to define. It remains to be determined whether the HCMV IE proteins have low-affinity but specific DNA-binding ability or can interact nonspecifically with double- or single-stranded DNA. However, the IE2 86-kDa protein does appear to have some promoter specificity, because it did not stimulate transcription from the *Drosophila Krüppel* promoter in nuclear extracts in either the presence or the absence of histone H1. Preliminary experiments also indicate that a minimal HCMV early promoter containing only 58 bp of upstream sequence, including the TATA box, has high basal activity in these extracts but does not respond to the IE2 86-kDa protein (71).

An alternative mechanism for IE-mediated transcriptional activation involves only protein-protein interactions. Both E1A and the herpes simplex virus acidic activator VP16 have been reported to interact with general transcription factors: E1A with TFIID (30, 47) and VP16 with TFIIB and TFIID (32, 48, 49, 83). In addition, it has been proposed that E1A also interacts with the cellular transcription factor ATF, which can bind to many sites in adenovirus early promoters (51). In preliminary experiments, we have found that the HCMV IE2 86-kDa protein but not the IE2 72-kDa protein is able to interact with both TFIIB and TFIID (38). Hagemeier et al. (26) recently reported similar results with respect to binding of these IE proteins to TFIID. However, as discussed above, the fact that the *Krüppel* promoter and a minimal HCMV early promoter are both not responsive to the IE2 protein indicates that sequences other than the TATA box are involved in the activation. Furthermore, the results of experiments with partially purified basal transcription factors suggest that the activation properties of the IE2 86-kDa protein in both the presence and absence of histone H1 require interactions with an ancillary factor that does not copurify with the general factors.

Although the precise mechanism by which the IE2 86-kDa protein exerts its effects in vivo remains to be elucidated, we propose that this protein might have a dual role. One function would be to remodel the DNA template and to render it more accessible to cellular transcription factors, while a second function might involve specific interactions with these cellular factors. Whether this dual role actually represents a single mode of action involving interaction between common domains of the IE2 86-kDa protein and intermediary proteins or separate functions remains an important question to be addressed.

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