An in vitro system for human cytomegalovirus immediate early 2 protein (IE2)-mediated site-dependent repression of transcription and direct binding of IE2 to the major immediate early promoter

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ABSTRACT  In vivo, negative autoregulation of the strong major immediate early promoter (MIEP) of human cytomegalovirus requires the viral immediate early 2 protein (IE2) and a cis element located from position −13 through position −1 relative to the transcription start site. We have established an in vitro transcription system that reproduces the specificity of IE2-mediated negative autoregulation. The carboxy-terminal 290-amino acid fragment of IE2 was purified as a bacterial fusion protein. Addition of this chimeric protein to the cell-free system specifically repressed transcription from the MIEP containing the wild-type cis-acting repressor element but not from a mutated template in which the cis element had been replaced by heterologous DNA. Control protein and a mutant IE2 fusion protein containing two specific amino acid substitutions in a putative zinc finger motif did not repress the MIEP in vitro. Using conditions defined by this functional assay, we demonstrated by mobility-shift experiments that IE2 binds directly and specifically to DNA bearing the cis-acting repressor element. In addition, IE2 bound to the MIEP in the in vitro transcription reaction mixture.

Transcription from the human cytomegalovirus (HCMV) major immediate early promoter (MIEP) is constitutive and extremely efficient in many cell types and in cell-free extracts (1–3). The MIEP influences the transcription of two immediate early (IE) genes designated IE1 and IE2. IE2 codes for several protein isoforms. The largest IE2 (82 kDa) has 579 amino acid residues. A 40-kDa IE2 isoform consists of amino acids 242–579 (B. Plachter, personal communication; refs. 4 and 5). IE2 transactivates several cellular promoters as well as homologous or heterologous viral promoters (6–11). In addition, IE2 negatively autoregulates the MIEP (9, 10). Repression by IE2 depends on the presence of a cis-acting repressor element from position −13 through position −1 relative to the transcription start site of the MIEP (12–14). It is orientation independent and, when moved to a similar position in heterologous enhancer-containing promoters, confers IE2 responsiveness (12–14). By transient transfection of either permissive human fibroblast cells or nonpermissive HeLa or Vero cells, the presence of this element in the MIEP allows IE2 to mediate approximately 6-fold repression of reporter gene expression (12–14).

The precise mechanism of IE2-mediated repression is not yet understood. We have expressed the carboxy-terminal 290 amino acids of IE2 as a bacterial fusion protein and have established an in vitro system that reproduces the specificities of repression as defined by in vivo studies. In addition, using conditions functionally tested by the in vitro transcription system, we have performed gel mobility-shift assays that provide evidence that the carboxy-terminal 290 amino acids of IE2 are sufficient to mediate direct and site-dependent binding to the MIEP. Our data suggest that the mechanism for IE2-mediated repression of the MIEP involves direct interaction between the wild-type IE2 and the cis-acting repressor element.

MATERIALS AND METHODS

Recombinant Plasmids. To produce the pKS760E (wild-type promoter) and pKSd1REE (mutant promoter) templates used in run-off transcription assays, the 1-kilobase (kb) EcoRI fragment from pCAT760 (2) or pCAT760d1RE (12), respectively, was subcloned into the EcoRI site of the p Bluescript II phagemid vector KS+ (Stratagene).

Constructs used to produce recombinant proteins were based on the plasmid vector pMAL-c (New England Biolabs), which expresses the cloned sequence of interest fused to the Escherichia coli maltose binding protein by induction with isopropyl β-d-thiogalactoside (IPTG). Plasmid pMAL-cXS has a 1.4-kb Xho I fragment (filled-in with Klenow fragment)/Sal I fragment from exon 5 of IE2 inserted into the Stu I/Sal I cloning sites of pMAL-c. Plasmid pMALcHL was constructed in the same manner as pMALcXS except that the 1.4-kb insert was isolated from the mutant plasmid HL446,452 (K. C. Yeung, M. Leatham, and M. F. S., unpublished work).

Purification of Recombinant Proteins. The protein fusion and purification system was obtained from New England Biolabs and used according to manufacturer instructions with the following modifications: (i) batch purification was performed and (ii) the elution buffer was 10 mM Hepes, pH 8.0/10% (vol/vol) glycerol/0.1 mM EDTA/30 mM maltose.

Preparation of HeLa Cell Nuclear Extracts. Nuclear extracts were prepared by a modification of the method of Lee and Green (15). Extracts were diluted, rather than dialyzed, with an equal volume of buffer D at the final step (16). In some experiments, the HeLa cell nuclear extracts were obtained from Promega.

In Vitro Run-off Transcription and Analysis of RNA Products. Standard 20-μl reaction mixtures were 0.25 μg of truncated template DNA in 25 mM Hepes, pH 8.0, containing 0.2 mM EDTA, rRNasin at 1 unit/μl (Promega), 1.25 mM creatine phosphate, 100 mM NaCl, 10 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 10% (vol/vol) glycerol, 0.5 mM each ATP, CTP, and GTP, 0.05 mM UTP, 10 μCi of [α-32P]UTP (800 Ci/mmol; 1 Ci = 37 GBq), and 30–60 μg of HeLa cell nuclear extract. Reactions were incubated for 60 min at 30°C then terminated by addition of 100 μl of 4 mM Tris-HCl, pH 7.5, containing 7.6 M urea, 0.5% SDS, 10 mM EDTA, 0.14 M NaCl, and 5 μg of yeast tRNA. Run-off products were phenol extracted, ethanol precipitated, fractionated in 6% polyacrylamide/7 M urea gels, and exposed to Kodak X-Ormat AR

Abbreviations: HCMV, human cytomegalovirus; MIEP, major immediate early promoter; IE, immediate early; IPTG, isopropyl β-d-thiogalactoside; r+, recombinant.

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Signals were quantitated by densitometry using both a Beckman DU-8 Gel Scan Copusset module and an LKB Ultroscan XL laser densitometer.

**Mobility-Shift Assay.** Standard 10-μl binding reaction mixtures were 25 mM Hepes, pH 8.0, containing 0.2 mM EDTA, 100 mM NaCl, 10 mM KCl, 8 mM MgCl₂, 1 mM dithiothreitol, 10% (vol/vol) glycerol, 2 μg of sheared salmon sperm DNA, and 2–5 × 10⁶ cpm of probe. Competition experiments included unlabeled DNA at 30-50-fold molar excess over labeled probe DNA. Antibody supershift assays included rabbit polyclonal antiserum (1218) to IE2 (7) or rabbit preimmune serum labeled probe included rabbit antiserum (1218). Upon addition of probe, reactions were incubated for 15 min at 30°C. Bound probe was separated from free probe by electrophoresis in a 5% acrylamide gel with 225 mM Tris borate, pH 8.3/0.5 mM EDTA running buffer at room temperature. Gels were dried and the shifted complexes were detected by autoradiography.

**RESULTS**

**In Vitro Transcription from Wild-type and Mutant DNA Templates.** To demonstrate that the *in vivo* requirement for the cis-element, located upstream of position –13 through position +1, is faithfully maintained *in vitro*, we constructed two promoter templates. Template pKS760E contained the wild-type HCMV MIEP and retains the cis-acting repressor ele-

![Fig. 1](image-url) **Fig. 1.** *In vitro* transcription from wild-type and mutant templates. (A) Schematic representation of the promoter constructs used in the *in vitro* transcription assays. The open box labeled RE represents the cis-acting repressor element, and the arrow indicates the initiation site and direction of transcription. The hatched box represents the chloramphenicol acetyltransferase gene sequence. An expanded view of sequences from the wild-type and mutant promoters is shown for the region between the underlined *Ssr I* and *HindIII* sites. The wild-type sequence of the cis-acting repressor element is boxed from –13 through –1. The transcription initiation site is labeled +1. The heterogeneous DNA sequence of the mutant promoter is shown in lower-case letters. The templates are otherwise identical. Predicted run-off transcript size in nucleotides is listed for each template. (B) Autoradiogram of run-off transcripts synthesized *in vitro* on truncated DNA templates. RNAs were analyzed by electrophoresis in a 6% acrylamide/7 M urea gel. Lanes: 1, molecular weight standards (2-μP-end-labeled *Hind III* restriction fragments of pBR322); 2, no added DNA; 3, EcoRI-cut pKS760E (12.5 μg/ml); 4, EcoRI-cut pKS1REE (12.5 μg/ml). Numbers on the right indicate sizes of the run-off transcripts in nucleotides.

![Fig. 2](image-url) **Fig. 2.** Expression of wild-type and mutant IE2 proteins as maltose-binding fusion proteins. (A) The *Xho I/Sal I* DNA fragment encoding amino acids 290–579 was subcloned into the pMAL-c polycoding site to give the recombinant plasmid designated pMal-cXS. DNA mutated in the *Cys 5*/*His 5* motif was cloned in the same way to give the recombinant plasmid designated pMal-cH. The mutations in the *Cys 5*/*His 5* motif converted the histidine residues at amino acid positions 446 and 452 to leucine residues. The pMal-c vector produces a BGal fusion product. The recombinant proteins and their molecular masses are indicated to the right. (B) Total cell lysate fractionated by SDS/polyacrylamide gel electrophoresis followed by Coomassie blue staining. Lanes: 1, molecular weight standards (× 10⁻³); 2, *E. coli* TBI cell lysate; 3–5, total cell lysates from TBI cells transformed with various expression vector DNAs and induced with IPTG for 2.5–3.0 hr. (C) Analysis of affinity-purified fusion proteins by SDS/polyacrylamide gel electrophoresis followed by Coomassie blue staining. Lanes: 1, molecular weight standards (× 10⁻³); 2–4, 2 μg each of purified fusion proteins r-IE2, r-BGal, and r-IE2H.
this study, both promoter constructs were cleaved at a common downstream EcoRI restriction endonuclease site. Based on the position of the transcription initiation site mapped previously (17), the truncated wild-type template would be predicted to generate a 268-nucleotide run-off transcript and the mutant template a 282-nucleotide run-off transcript. When these truncated templates were transcribed in vitro with HeLa cell nuclear extracts, both promoters demonstrated efficient levels of transcription (Fig. 1B). The sizes of the RNAs were as predicted.

Expression and Purification of the Recombinant Viral Proteins. pMALcXS consists of wild-type sequences from IE2 subcloned into pMAL-c such that the carboxyl-terminal 290 amino acids of the 579-amino acid IE2 protein are expressed in the fusion product (Fig. 2A). Cells transformed with the pMALcXS construct produce a 70-kDa recombinant (r-) IE2 (r-IE2) (Fig. 2B, lane 3). The pMALcHL construct expresses a mutant form of IE2. The two histidine residues at amino acid positions 446 and 452 in the cysteine-histidine (Cys2His2) motif have been specifically replaced by leucine residues (Fig. 2A). The pMALcHL construct produces a 70-kDa mutant IE2 (r-IE2HL) (Fig. 2B, lane 5). The pMAL-c expression vector (Fig. 2A) expresses a 50-kDa β-galactosidase (βGal) fusion protein, r-BGal (Fig. 2B, lane 4).

The recombinant proteins were purified from bacterial lysates and appeared to be up to 90% pure by Coomassie blue staining (Fig. 2C).

Effect of r-IE2 on Transcription from the Wild-Type or Mutant MIEP. We investigated the effect of IE2 on the MIEP of HCMV by quantitating the amount of RNA synthesized in vitro from the pKS760E template at different doses of r-IE2. Keeping the molar amount of DNA template constant in all transcription reactions and increasing the amount of purified r-IE2 added, we detected a concentration-dependent repression of transcription (Fig. 3A, lanes 1–4). Quantitation of the bands by scanning densitometry estimated the degree of repression to be 2.5-fold with 4 pmol of r-IE2 (compare lanes 1 and 2), 5-fold with 8 pmol of r-IE2 (compare lanes 1 and 3), and >10-fold with 12 pmol of r-IE2 (compare lanes 1 and 4).

In contrast to the results seen with r-IE2, the addition of r-BGal at any dose did not significantly affect transcription of the wild-type MIEP (Fig. 3B). These results support the

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**Fig. 3.** Effect of wild-type IE2 protein on *in vitro* transcription from wild-type and mutant templates. (A) Concentration-dependent repression of transcription by r-IE2. Increasing amounts [0, 4, 8, and 12 pmol (lanes 1–4, respectively)] of r-IE2 were added to the standard run-off transcription assay. Each assay contained 0.1 pmol of pKS760E promoter construct. Analysis of transcription products was as described in Materials and Methods. (B) Dose–response curve of r-BGal on pKS760E. Increasing amounts [0, 4, 8, and 12 pmol (lanes 1–4, respectively)] of r-BGal were added to the standard run-off transcription assay. Each assay contained 0.1 pmol of pKS760E promoter construct. (C) Effect of deletion of the cis-acting repressor element on repression of transcription of the MIEP. *In vitro* transcription run-off products were analyzed in a 6% acrylamide/7 M urea gel. Lanes: 1–3, 0.1 pmol of pKS760E promoter construct; 4–6, 0.1 pmol of pKSd1REE mutant construct; 1 and 4, buffer control; 2 and 5, 6 pmol of r-IE2; 3 and 6, 6 pmol of r-BGal. Arrows, sizes of run-off transcripts in nucleotides.

![Fig. 4](image-url)  
**Fig. 4.** Effect of IE2 wild-type and mutant protein on *in vitro* transcription from wild-type and mutant templates. *In vitro* transcription products were analyzed in a 6% acrylamide/7 M urea gel. Lanes: 1–3, 0.1 pmol of pKS760E promoter construct; 4–6, 0.1 pmol of pKSd1REE mutant construct; 1 and 4, 0.42 μg of r-BGal; 2 and 5, 0.42 μg of r-IE2; 3 and 6, 0.42 μg of r-IE2HL. Arrows, sizes of run-off transcripts in nucleotides.
interpretation that the transcriptional repression observed for r-IE2 is due to the activity of the viral protein.

It was then necessary to determine whether r-IE2-mediated repression in vitro was still dependent on the cis-acting repressor element. For these assays, the fusion proteins were used at 6 pmol since this dose most consistently produced levels of repression similar to that observed in vivo. In the presence of r-IE2, transcription from the wild-type pKS760E template was reduced ≈6.5-fold while that from pKSd1REE was not reduced (Fig. 3C, compare lanes 1 and 2 and also lanes 4 and 5). r-BGal did not have a significant effect on transcription from either promoter (Fig. 3C, compare lanes 1 and 3 and also lanes 4 and 6).

Repression of the MIEP Requires Functional r-IE2. r-IE2HL contains specific mutations in the Cys$_2$His$_2$ motif of IE2 that eliminate repressive function of IE2 in vivo (K. C. Yeung and M.F.S., unpublished data). The effect of r-IE2HL on in vitro transcription from both wild-type and mutant promoter templates was compared to that of r-IE2. On pKS760E, r-IE2 caused a 4.1-fold repression of transcription whereas the mutations of r-IE2HL abolished repressive activity in vitro (Fig. 4, compare lanes 2 and 3). pKSd1REE showed no repression of transcription but a slight (1.7-fold) stimulation in the presence of r-IE2 (Fig. 4, compare lanes 4 and 5) and no effect by the presence of r-IE2HL (Fig. 4, lane 6). The negative control (r-BGal, lanes 1 and 4) contained the same mass of protein as r-IE2 or r-IE2HL.

r-IE2 Binds Directly and Specifically to DNA Bearing the cis-Acting Repressor Element. Having established an in vitro system that supports specific repression of the MIEP by r-IE2, we then used the assay to investigate direct interactions between r-IE2 and the cis-acting repressor element. We
performed gel mobility-shift assays with r-IE2 and probe bearing the cis-acting repressor element (RET7H) or probe from which the cis element had been deleted and replaced by heterologous DNA (ΔT7H). r-IE2 bound only to probe RET7H (Fig. 5A, compare lanes 1 and 4). The control protein, r-BGal, did not retard the mobility of either probe (Fig. 5A, lanes 2 and 5), and the mutant viral protein, r-IE2HL, also was unable to bind to either probe tested (Fig. 5A, lanes 3 and 6). The specificity of r-IE2 binding was further demonstrated by competition assays. Binding of r-IE2 to the RET7H probe was reduced only in the presence of unlabeled RET7H competitor DNA. Quantitated by scanning densitometry, unlabeled RET7H DNA produced a 6.3-fold reduction in binding at 25-fold excess whereas ΔT7H as unlabeled competitor did not affect binding (Fig. 5A, lanes 7–10).

To demonstrate the presence of r-IE2 in the shifted complex, a polyclonal serum (1218) raised against the IE2 protein or a preimmune control serum was added to the binding reactions. Supershifted complexes were observed only with the 1218 antisera (Fig. 5B, compare lane 2 to lanes 3 and 5). This supershift was dependent on the presence of r-IE2 protein as 1218 antisera alone did not affect the mobility of RET7H probe (Fig. 5B, lane 4).

Finally, we examined the DNA binding activity of r-IE2 in the context of our functional in vitro transcription system. We included HeLa nuclear extracts in the binding reactions to determine whether r-IE2 binding to the cis-acting repressor element still occurred in the presence of transcriptionally active extract. We could clearly demonstrate the appearance of a uniquely shifted band in reactions containing r-IE2 that was not evident in reactions with HeLa extract alone or extract containing r-BGal (Fig. 5C).

**DISCUSSION**

Regulation of gene expression occurs by positive or negative mechanisms or by a combination of both. In vivo studies have described IE2-mediated repression of the MIEP and its requirement for a cis-acting repressor element. Our approach to defining the viral or cellular proteins involved in IE2-mediated repression was to develop an in vitro transcription system that supports the specificity of IE2-mediated repression observed in vivo. In vitro systems developed for transcriptional regulators such as Drosophila engrailed (18) and yeast α-2 homeodomain proteins (19) as well as simian virus 40 large tumor antigen (20) enabled investigators to study effects of repressors on the assembly and stability of transcription complexes and identify critical protein domains or nucleic acid residues that interact during selective regulation of promoter activity.

Based on results of in vivo studies, several models for the mechanism of IE2-mediated repression have been proposed. The simplest model evoked direct binding of the cis-element by IE2. Previously, we and others have been unable to demonstrate by gel shift analysis specific binding of in vitro-translated or bacterially expressed and purified IE2 to the cis-acting repressor element. Now, using reagents tested in our functional in vitro transcription assay, we provide evidence that the carboxy-terminal 290 amino acids of IE2 expressed as r-IE2 are sufficient to mediate sequence-specific direct binding to DNA. Therefore, both the IE2 isoform of 82 kDa (amino acids 1-579) and the late IE2 isoform (amino acids 242-579) can potentially bind to the cis-acting repressor element in the MIEP. Mutant protein, r-IE2HL, which fails to repress the wild-type MIEP in in vitro transcription assays, is also unable to bind DNA as measured by mobility-shift analysis, suggesting a role for the putative zinc finger motif in IE2 association with the cis-acting repressor element.

Binding of r-IE2 to the cis-acting repressor element, which is positioned near the transcription initiation site, suggests repression involves a blockade of, or interaction with, the RNA polymerase II transcription complex. Protein–protein interactions between transcription factor IID and the HCMV IE2 protein have recently been reported (21). Reconstitution of IE2-mediated repression of the HCMV MIEP in vitro and evidence that r-IE2 can bind directly to the cis-acting repressor element should allow us to identify relevant interactions between viral and cellular factors involved in IE2-mediated repression. Investigation into the mechanism by which IE2 mediates significant repression of transcription from a very strong enhancer-containing promoter should contribute to our understanding of herpesvirus latent and productive infections and transcriptional control of eukaryotic promoters.

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