JOURNAL OF VIROLOGY, Dec. 1995, p. 7612–7621 Vol. 69, No. 12
0022-538X/95/$04.00+0

Binding of Cellular Repressor Protein or the IE2 Protein to a *cis*-Acting Negative Regulatory Element Upstream of a Human Cytomegalovirus Early Promoter

LIPING HUANG AND MARK F. STINSKI*

Department of Microbiology, School of Medicine, University of Iowa, Iowa City, Iowa 52242

Received 15 June 1995/Accepted 29 August 1995

We have previously shown that the human cytomegalovirus early UL4 promoter has upstream negative and positive *cis*-acting regulatory elements. In the absence of the upstream negative regulatory region, the positive element confers strong transcriptional activity. The positive element contains a CCAAT box dyad symmetry and binds the cellular transcription factor NF-Y. The effect of the negative regulatory element is negated by the viral IE2 protein (L. Huang, C. L. Malone, and M. F. Stinski, J. Virol. 68:2108, 1994). We investigated the binding of cellular or viral IE2 protein to the negative regulatory region. The major *cis*-acting negative regulatory element was located between −168 and −134 bp relative to the transcription start site. This element could be transferred to a heterologous promoter, and it functioned in either orientation. Mutational analysis demonstrated that a core DNA sequence in the *cis*-acting negative regulatory element, 5′-GTTTGGAATCGTT-3′, was required for the binding of either a cellular repressor protein(s) or the viral IE2 protein. The cellular DNA binding activity was present in both nonpermissive HeLa and permissive human fibroblast cells but more abundant in HeLa cells. Binding of the cellular repressor protein to the upstream *cis*-acting negative regulatory element correlates with repression of transcription from the early UL4 promoter. Binding of the viral IE2 protein correlates with negation of the repressive effect.

Human cytomegalovirus (HCMV) is a ubiquitous pathogen causing asymptomatic infections or severe complicating infections in utero as well as in immunosuppressed patients (1, 20). HCMV contains a double-stranded DNA genome of 230 kbp. The viral genes, which may code for approximately 200 proteins, have been divided into three temporally regulated classes, immediate early (IE), early, and late (6, 9, 10, 39, 55, 56). IE genes are expressed after entry of the virus into the host cell and require specific *cis*-acting elements in the viral promoters as well as cellular and viral *trans*-acting factors. Two IE transcripts, which code for viral regulatory proteins, designated IE1 and IE2, are transcribed from the modulator/enhancer-containing major IE promoter (MIEP). Even though some of the viral early promoters have *cis*-acting positive regulatory elements upstream of the TATA box and the transcription start site such as AP-1 (54), ATF (50), NF-Y (23), and USF (30), transcription of the early genes is weak in the absence of the IE proteins. After IE viral protein synthesis, these viral early promoters are strongly activated (5, 28, 39, 49, 56). Although several different HCMV IE proteins could have a role in the transcriptional activation of these viral early promoters, the viral IE2 protein is sufficient to strongly activate the UL4 and UL112-113 promoters in transiently transfected cells (5, 23, 29, 45). The IE1 protein alone has little effect on these promoters, but it can act synergistically with the IE2 protein for the strongest activation of transcription (5, 23, 29, 38, 49, 50).

It has been demonstrated that the viral IE2 protein has at least two functions: (i) it negatively autoregulates its own promoter (7, 34, 37, 41, 51), and (ii) it positively regulates viral or cellular promoters (5, 16, 17, 19, 29, 38, 42). Negative autoregulation by the IE2 protein is DNA sequence and position dependent (34). The AT-rich *cis*-repression signal, 5′-CGTT TAGTGAACC-3′, located between −13 and −1 bp relative to the transcription start site of the MIEP, is required for IE2 protein binding and repression of transcription in vitro (32, 37) or in vivo (7, 34, 41). Like other viral transactivators, such as adenovirus E1a, herpes simplex virus VP16 and ICP4, Epstein-Barr virus Zta, and human immunodeficiency virus Tat (3, 21, 25, 26, 33, 40, 53), the HCMV IE2 protein interacts with the TATA-box-binding protein (4, 16, 17, 27, 48). In vitro, the IE2 protein can interact with eucaryotic transcription factors such as Rb, SP-1, TEF-1, and TFIIB (4, 15, 36, 48).

In constructs containing the UL4 promoter of HCMV with only an upstream TATA box, the enhancement effect of the IE2 protein was only about twofold (23). In contrast, when the UL4 promoter had either 220 or 2,200 bp upstream, the basal activity was still low, but the viral IE2 protein enhanced transcription from the promoter 6- to 50-fold, respectively (23, 38). We suggested that one effect of the IE2 protein may be mediated by the negation of a negative regulatory region upstream of the CCAAT box dyad symmetry as well as by direct interaction with TFIID (23) or other eucaryotic transcription factors (36).

In this report, we defined a *cis*-acting negative regulatory element upstream of the CCAAT and TATA boxes of the UL4 promoter. A cellular protein(s) bound to this *cis*-acting negative regulatory element. Purified viral IE2 protein bound to the same element. Transient transfection assays suggest that binding of the cellular protein(s) correlates with repression of transcription from the UL4 promoter. Purified viral IE2 protein affected the binding of the cellular repressor protein(s) to the *cis*-acting negative regulatory element.

MATERIALS AND METHODS

Cell culture. Human foreskin fibroblast (HFF) cells were cultured as described previously (52). HeLa cells were grown in Eagle's minimal essential medium supplemented with 10% newborn calf serum.

* Corresponding author. Phone: (319) 335-7792. Fax: (319) 335-9006. Electronic mail address: CMDMFS@VAXA.WEEG.UIOWA.EDU.

Plasmids. Plasmids p-220CAT and p-122CAT were described previously (5, 23). Plasmids p-195CAT and p-138CAT were constructed by deleting the 231-bp *Xba*I-*Hin*dIII fragment in plasmid p-220CAT and inserting 206- and 149-bp DNA subfragments, respectively. The subfragments were generated by PCR using the antisense primers 5′-GC TCTAGAGCTGCCCACTG-3′ for the 206-bp DNA and 5′-TTCGGCTCTAGACCGGGG GATAG-3′ for the 149-bp DNA. Sense primer 5′-TTGGGATATATCAACGGTGG-3′ was used, and plasmid p-220CAT was the template. Plasmids p-220M6CAT, p-220M8CAT, p-220M9CAT, p-220M10CAT, and p-220M11CAT were constructed by replacing the wild-type (WT) 231-bp *Xba*I-*Hin*dIII fragment in plasmid p-220CAT with the same-size *Xba*I-*Hin*dIII fragments bearing the site-specific mutations (see Fig. 3A). All mutations in the expression plasmids were confirmed by dideoxy-chain termination sequencing (U.S. Biochemical Corporation, Cleveland, Ohio). [^{35}S]dATP (>600 Ci/mmol) was purchased from Amersham (Arlington Heights, Ill.).

For construction of plasmid p19CAT, the promoter was derived from the 86-bp *Hin*cII-*Xba*I DNA fragment of pCAT760d1RE, which lacks the IE2-mediated *cis*-repression signal, and this fragment was inserted into the blunt end of the *Xba*I site of plasmid pCAT-Basic (Promega, Madison, Wis.). A 19-bp sequence (35) containing the ATF binding site (underlined), 5′-CCCATT GACGTCAATGGGG-3′, was inserted into the blunt end of the *Pst*I site of plasmid pCAT-Basic (Promega). Plasmids p2UL4R-19CAT, p2UL4RR-19CAT, and p4UL4R-19CAT were constructed by insertion of sequences containing two copies of the UL4 promoter repressor site (site 2 DNA sequence, underlined), 5′-CTAGAGTATCCGGTTTGGAATCGTTCGGCTCTGGTCCGGGCTAGCT AGAGTATCCGGTTTGGAATCGTTCGGCTCTGGTCCGGGCTAGATC-3′, into the blunt end of the *Hin*dIII site of plasmid p19CAT. Plasmids p2UL4R-19CAT and p2UL4RR-19CAT have two copies of the site 2 DNA sequence in the WT and reversed orientations, respectively. Plasmid p4UL4R-19CAT has four copies in the WT orientation. Plasmid p2mUL4R-19CAT was generated by insertion of two copies of mutated site 2 DNA sequence in the WT orientation (5′-AGCTTAGTAcCCatgagtc AAgacgTtGaCTCaGGTCCGGG-3′) into the *Hin*dIII site of plasmid p19CAT. Mutated nucleotides are shown in lowercase letters. Plasmids pSVIE2 and pSVIE2HL were described previously (38, 60).

Preparation of nuclear extracts. HFF and HeLa cell nuclear extracts were prepared as described previously (12, 23).

Footprint assay. The 118-bp *Xba*I-*Eco*O109I DNA fragment (−220 to −102) generated from plasmid p-220CAT was labeled at its 3′ end with either [^{32}P]dGTP or [^{32}P]dCTP, using the Klenow fragment of *Escherichia coli* DNA polymerase I (New England Biolabs, Beverly, Mass.). Unincorporated deoxynucleoside triphosphates were removed by gel filtration through a Sephadex G50-150 spin column. HeLa cell nuclear extract (5.0 or 7.5 μg) and 25,000 cpm of probe were incubated on ice for 30 min in 10 μl of DNA binding buffer G (10 mM Tris-HCl [pH 7.5] containing 50 mM NaCl, 1.0 mM EDTA, 1.0 mM dithiothreitol [DTT], and 5% glycerol) with 1.0 μg of poly(dI-dC) · poly(dI-dC). In-solution footprinting of DNA-protein complexes was as described by Kuwabara and Sigman (31). Briefly, the DNA-protein complexes were treated with 1,10-phenanthroline–copper ion on ice for 5 min. The reactions were stopped by adding 5.8 μl of 0.1% 2,9-dimethyl-1,10-phenanthroline and incubated on ice for 5 min. DNA was denatured by adding 12.0 ml of sequencing loading buffer containing 92% formamide, 10 mM EDTA, and 0.3 M β-mercaptoethanol and boiled for 3 min. The DNA fragments were fractionated by electrophoresis in a 15% denaturing polyacrylamide gel. Autoradiography was with Kodak X-Omat film (Eastman Kodak Company, Rochester, N.Y.).

EMSA. The 118-bp WT and mutated (M8, M9, M10, and M11) *Xba*I-*Eco*O109I DNA fragments were isolated from plasmids p-220CAT, p-220M8CAT, p-220M9CAT, p-220M10CAT, and p-220M11CAT, respectively. The 36-bp WT and mutant site 2 DNA fragments were generated by PCR using the primers 5′-GCAGGTATCTAGAGTATCCG-3′ and 5′-CCTCACGCTAGCCCGGACC AGAGCCG-3′ and plasmids p-220CAT and p-220M6CAT, respectively, as templates. After *Xba*I and *Eco*O109I or *Nhe*I restriction endonuclease digestion, probes were made by filling in the 3′ end of WT *Xba*I-*Eco*O109I or *Xba*I-*Nhe*I DNA fragments with [^{32}P]dCTP and the Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs). The electrophoretic mobility shift assay (EMSA) was done with 12.8 fmol of DNA probe (−220 to −102) or 16.8 fmol of site 2 DNA probe (−168 to −134) at room temperature for 15 min. For cellular protein binding assays, 2.0 μg of HeLa cell nuclear extract was preincubated with 2 μg of poly(dI-dC) · poly(dI-dC) for 15 min in 15 μl of DNA binding buffer G. Alternatively, 1.75 μg of HeLa or 1.0 mg of HFF cell nuclear extract was preincubated with 1.0 mg of sheared salmon sperm DNA at room temperature for 10 min in 15 μl of 25 mM Tris-HCl (pH 8.0) containing 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 0.01% Nonidet P-40, and 9% glycerol (buffer I). For the recombinant IE2 (r-IE2) protein binding assay, 5.8 pmol of r-IE2 was preincubated with 2 μg of sheared salmon sperm DNA at room temperature for 10 min in 15 μl of 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 8.0) containing 0.2 mM EDTA, 100 mM NaCl, 10 mM KCl, 8.0 mM $MgCl_2$, 1.0 mM DTT, and 5% glycerol. For cold competition assays, 10- or 50-fold molar excesses of nonradioactively labeled DNA fragments were added to the preincubation reaction mixtures. For supershift assay, 2.5 and 5.0 μl or 0.5 to 5.0 μl of 1:5 diluted preimmune serum and polyclonal anti-NF-Y antibody (a gift from R. Mantovani, Institut de Chimie Biologique, Faculté de Médecine, Strasbourg, France) or polyclonal anti-IE2 antibody (1218; a gift from J. Nelson, Oregon Health Science University, Portland), respectively, were added to the preincubation reaction mixtures. For cellular protein and r-IE2 protein competition assays, 1.75 μg of HeLa cell nuclear extract was preincubated with 1.0 μg of sheared salmon sperm DNA at room temperature for 10 min in 15 μl of DNA binding buffer I, and then 16.8 fmol of site 2 DNA probe (−168 to −134) was added to the reaction mixture at room temperature for another 15 min. Finally, r-IE2 or r-IE2HL protein (0.4, 0.8, or 1.2 μg) was added at room temperature for 15 min. All samples were subjected to electrophoresis at room temperature in a 5% polyacrylamide gel containing 0.5× Tris-borate-EDTA (pH 8.3) buffer. Hyperfilm-MP (Amersham) was used for autoradiography.

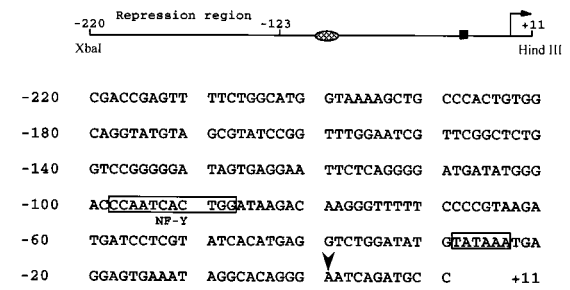

FIG. 1. Schematic representation and DNA sequence of the UL4 promoter. The TATA box and the NF-Y binding site are designated by the square and oval, respectively. The region that represses downstream transcription was reported to be between −220 and −123 bp (24). The arrow indicates the transcription start site.

UV cross-linking assay. The UV cross-linking assay was performed by the method of Williams et al. (58). The WT site 2 DNA fragment was substituted with bromodeoxyuridine by PCR, using the primers used for EMSA (see above) and plasmid p-220CAT as the template. The PCR product was digested with restriction endonucleases *Xba*I and *Nhe*I and labeled with [^{32}P]dCTP by the Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs). HeLa cell nuclear extract (3.5 μg) was preincubated with 2 μg of poly(dI-dC) · poly(dI-dC) at room temperature for 10 min in 25 μl of 10 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, 1.0 mM EDTA, 1.0 mM DTT, and 5% glycerol, and then 0.16 pmol bromodeoxyuridine-substituted 36-bp site 2 DNA probe was added. For cold competition assays, a 50-fold molar excess each of nonradioactively labeled WT and mutated site 2 DNA (−168 to −134; see above) was added to the preincubation reaction mixtures. Open 1.5-ml vials (Costar, Cambridge, Mass.) covered by Saran Wrap were placed under a 312-nm UV lamp (Fisher Biotech, Pittsburgh, Pa.) at a distance of 5 cm and irradiated at room temperature for 30 min. Samples were mixed with 25 μl of 125 mM Tris-HCl (pH 6.8) containing 20% glycerol, 4% sodium dodecyl sulfate (SDS), 2% β-mercaptoethanol, and 0.001% bromophenol blue. After boiling for 2 min, the proteins were subjected to electrophoresis in a denaturing SDS–7% polyacrylamide gel.

Transfection and CAT assay. HeLa and HFF cells in 100-mm-diameter plates were transfected with calcium phosphate precipitates containing 5 or 10 μg of each reporter plasmid and 10 or 15 μg of carrier salmon sperm DNA. Plasmid pCH110 (5 μg; Pharmacia), which expresses β-galactosidase driven by the simian virus 40 promoter, was included to control for transfection efficiency and variations. Transfections were done at least three times. Cells were harvested 48 h after transfection, and cell lysates were equalized for total protein. β-Galactosidase activity was measured as described previously (18). Chloramphenicol acetyltransferase (CAT) assays were carried out as described previously (5, 14). Acetylated derivatives were separated from [^{14}C]chloramphenicol by thin-layer chromatography. The percent conversion was determined by AMBIS image acquisition analysis (San Diego, Calif.), and standard deviations were calculated. The CAT values obtained in cells transfected with reporter plasmids alone were normalized to β-galactosidase activity to control for variations in transfection efficiency.

RESULTS

Cellular protein binding to the repression region of the UL4 promoter. A negative regulatory region was identified between −220 and −123 bp upstream of the transcription start site of the HCMV UL4 promoter. The viral IE2 protein negated the repressive effect of this region, which correlated with a significant increase in downstream transcription (23). Downstream of the repression region is a CCAAT box dyad symmetry that binds NF-Y and positively regulates the UL4 promoter (23). The locations and sequences of these negative and positive *cis*-acting elements are shown in Fig. 1.

To determine whether the negative regulatory sequence binds a cellular protein(s), nuclear extracts from HeLa cells were prepared for footprinting assay, using the 1,10-phenanthroline–copper ion method as described in Materials and Methods. A 118-bp ^{32}P-labeled DNA probe from −220 to −102 bp was used in the presence of 1 μg of poly(dI-dC) · poly(dI-dC) (Fig. 1). Three protein protection regions, designated sites 1, 2 and 3, were detected (Fig. 2A). On the noncoding strand, the protected nucleotides in site 1 mapped between −215 and −195 bp, with enhanced cleavage at −204 and −205 bp. The protected nucleotides in site 2 mapped between −166 and −151 bp on the noncoding strand. Protection on the coding strand was also detected between −169 and −139 bp. The protected nucleotides in site 3 mapped between −123 and −116 bp on the coding strand (Fig. 2). These data suggested that cellular proteins in a nuclear extract bind to the DNA sequence in the negative regulatory region of the UL4 promoter.

Cellular protein binding to the site 2 DNA sequence. The 118-bp DNA probe (−220 to −102) was used in an EMSA with HeLa cell nuclear extract. To increase the specificity of cellular protein binding to the viral DNA probe, the poly(dI-dC) · poly(dI-dC) concentration was twofold higher than in footprint assays. The higher concentration of poly(dI-dC) · poly(dI-dC) inhibited cellular protein binding to sites 1 and 3 (data not shown). Two DNA-protein complexes were detected and designated complexes X and Y (Fig. 3B, lane 1). To define the site in the 118-bp DNA probe that reacted with cellular protein to generate DNA-protein complex X or Y, we used cold competition assays with DNA sequences mutated in site 1, 2, or 3 (Fig. 3A). The M8 DNA fragment is mutated in sites 1 and 2, M9 is mutated in sites 1 and 3, M10 is mutated in sites 2 and 3, and M11 is mutated in sites 1, 2, and 3 (Fig. 3A). HeLa cell nuclear extract was incubated with a 10- or 50-fold molar excess of each nonradioactively labeled competitor DNA (WT, M8, M9, M10, or M11) prior to the addition of the WT 118-bp DNA probe. The WT and M9 DNA fragments competed efficiently for the formation of complexes X and Y (Fig. 3B, lanes 2 and 3 and lanes 6 and 7, respectively). In contrast, M8, M10, and M11 DNA fragments competed poorly even at a 50-fold molar excess (Fig. 3B, lanes 4 and 5, lanes 8 and 9, and lanes 10 and 11, respectively). These data indicated that the binding of cellular protein(s) to site 2 generated DNA-protein complexes X and Y. The cold competition results were confirmed by using radioactively labeled M8, M9, M10, and M11 DNA fragments as probes in an EMSA. Only the M9 DNA probe, which contains an intact site 2 DNA sequence, detected DNA-protein complexes X and Y.

To confirm that the site 2 DNA sequence generates DNA-protein complexes X and Y, nuclear extracts were prepared from nonpermissive HeLa cells and permissive HFF cells for EMSA with a 40-bp DNA probe (5′-CTAGAGTATCCGGTTTGGAATCGTTCGGCTCTGGTCCGGG-3′) containing the site 2 DNA sequence (underlined) but not the site 1 or site 3 DNA sequence. The 40-bp site 2-containing DNA probe was able to detect DNA-protein complexes X and Y in either HeLa or HFF cell nuclear extracts (Fig. 4, lanes 2 and 3). The minor band under complex X with the HeLa cell nuclear extract was not always detectable. Approximately sixfold more nuclear extract from HFF cells was required to detect DNA-protein complex X or Y. These data suggest that the site 2 cellular DNA-binding protein is present in both cell types, but the protein binding activity is more abundant in the nonpermissive HeLa cells than in the permissive HFF cells.

Cellular protein in complexes X and Y. The approximate molecular weight of the cellular protein(s) bound to the site 2

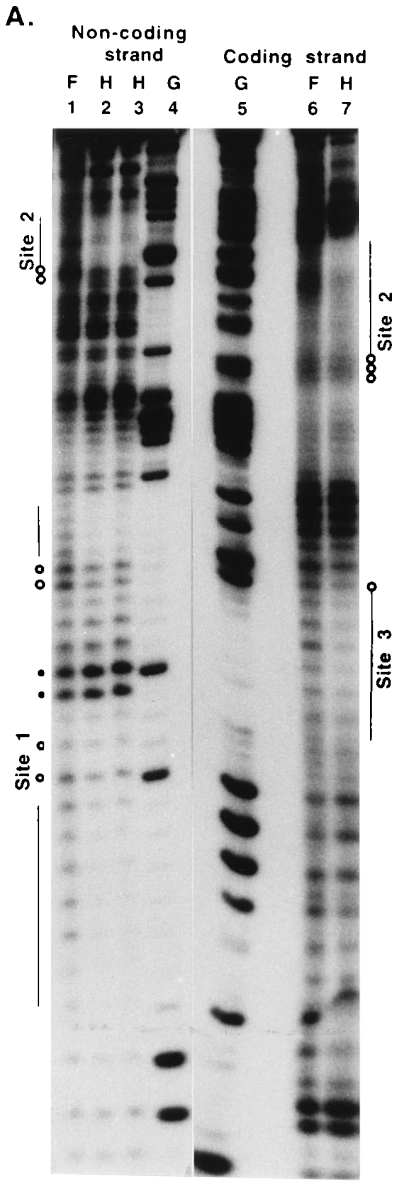

FIG. 2. Cellular protein binding sites in the repression region of the UL4 promoter. (A) Footprint of the DNA-protein complexes. The *Xba*I-*Eco*O109I DNA fragment (−220 to −102) was radioactively labeled on either the coding or noncoding strand. Protein binding sites were detected by the 1,10-phenanthroline–copper ion cleavage assay as described in Materials and Methods. Lanes: 1 and 6, free probe (F) of the noncoding and coding strands, respectively; 2 and 7, 5 μg of HeLa nuclear extract (H) plus DNA probe of the noncoding and coding strands, respectively; 3, 7.5 μg of HeLa nuclear extract plus probe of the noncoding strand; 4 and 5, G residue marker prepared by chemical cleavage. (B) Summary of regions protected from cleavage by cellular protein. The fully protected nucleotides are underlined. The partially protected nucleotides are indicated by open circles. Enhanced cleavages of nucleotides are indicated by solid circles. The protein binding regions are designated sites 1, 2 and 3.

A.

```
                -215                      -169                                      -139       -116
           -220  |                   -195  |                                         | -127     | -102
WT   CTAGAG|--GAGTTTTCTGGCATGGTAAAA--CGTATCCGGTTTGGAATCGTTCGGCTCTGGT--TGAGGAATTCTC--|
      XbaI          Site 1                          Site 2                          Site 3

M8   CTAGAG|--GAcagcTCgaGCggGGcAgct--CGTATCCGtagactcgagcgaCGGCTtTGGT--TGAGGAATTCTC--|

M9   CTAGAG|--GAcagcTCgaGCggGGcAgct--CGTATCCGGTTTGGAATCGTTCGGCTCTGGT--cctaGAtcTagg--|

M10  CTAGAG|--GAGTTTTCTGGCATGGTAAAA--CGTATCCGtagactcgagcgaCGGCTtTGGT--cctaGAtcTagg--|

M11  CTAGAG|--GAcagcTCgaGCggGGcAgct--CGTATCCGtagactcgagcgaCGGCTtTGGT--cctaGAtcTagg--|
```

B.

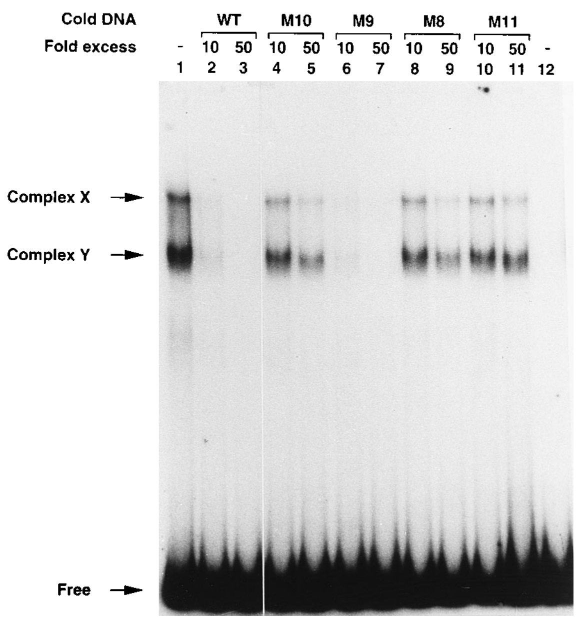

FIG. 3. EMSA and cold competition with WT and mutant repression region DNA. (A) WT and mutated *Xba*I-*Eco*O109I DNA fragments (−220 to −102) from the UL4 promoter. Sites 1, 2, and 3 are shown in nucleotides. The remaining nucleotides are shown as dashes (see details in Fig. 2B). The numbers are relative to the transcription start site. The *Xba*I restriction endonuclease site is indicated. The mutated nucleotides are shown in lowercase letters. (B) Autoradiogram of EMSA and cold competition assay. Lanes: 1, WT DNA probe (−220 to −102) plus HeLa cell nuclear extract; 2, 4, 6, 8, and 10, probe plus HeLa cell nuclear extract in the presence of 10-fold molar excesses of nonradioactively labeled WT, M10, M9, M8, and M11 DNAs, respectively; 3, 5, 7, 9, and 11, probe plus HeLa cell nuclear extract in the presence of 50-fold molar excesses of nonradioactively labeled WT, M10, M9, M8, and M11 DNAs, respectively; 12, probe alone. Complexes X and Y and the free probe are indicated by arrows.

DNA sequence in the negative regulatory region of the UL4 promoter was determined by the method of Williams et al. (58) and as described in Materials and Methods. A protein(s) of approximately 102 kDa was detected (Fig. 5, lane 2). When a 50-fold molar excess of nonradioactively labeled WT site 2 DNA fragment was added, it competed efficiently for protein binding (Fig. 5, lane 3). In contrast, a mutated (lowercase letters) site 2 DNA (5′-CTAGAGTATCCGtagactcgagCgaCG GCTCTGGTCCGGG-3′) fragment did not compete (Fig. 5, lane 4). The cellular DNA-binding protein of approximately 102 kDa was also detected in HFF cell nuclear extracts (24). These data suggest that a cellular protein binds to site 2 and is present in both DNA-protein complexes.

Downregulation of transcription by the site 2 DNA sequence. To determine whether the site 2 DNA sequence has a functional significance in permissive HFF cells, CAT reporter constructs with various lengths of the negative regulatory DNA sequence upstream of the CCAAT and TATA boxes and transcription start site of the UL4 promoter were analyzed for repression of transcription. Plasmid p-220CAT contains the entire negative regulatory sequence (−220 to −123). Plasmids p-195CAT and p-138CAT contain 73 bp and 20 bp of the negative regulatory sequence from −195 to −123 bp and from −138 to −123 bp, respectively (Fig. 6A). HFF cells were trans-

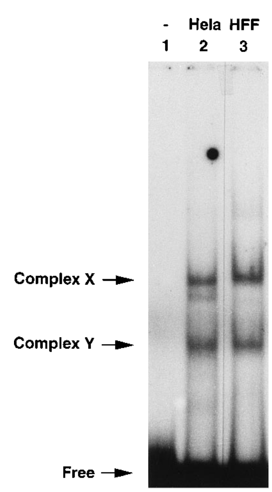

FIG. 4. Binding of cellular protein(s) to the site 2 DNA sequence of the UL4 promoter. Nuclear extract was prepared from HeLa or HFF cells. EMSA was performed as described in Materials and Methods. Lanes: 1, WT site 2 DNA (−168 to −134) probe alone; 2, probe plus HeLa cell nuclear extract (1.75 μg); 3, probe plus HFF cell nuclear extract (10 μg). Complexes X and Y and the free probe are indicated by arrows.

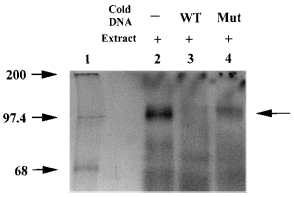

FIG. 5. Size of cellular protein bound to the site 2 DNA. The site 2 DNA (−168 to −134) was radioactively labeled and bromodeoxyuridine substituted. UV cross-linked cellular protein was fractionated in a denaturing SDS–7% polyacrylamide gel as described in Materials and Methods. Lanes: 1, ^{14}C-protein size standards (positions are indicated in kilodaltons at the left); 2, protein in complexes X and Y; 3, competition with a 50-fold molar excess of the nonradioactively labeled site 2 DNA (−168 to −134); 4, competition with a 50-fold molar excess of the mutant (Mut) site 2 DNA (−168 to −134); see mutant DNA sequence in Fig. 3 and in Materials and Methods). The major protein band is designated by an arrow.

fected with 10 μg of reporter plasmids per plate, and CAT activity was measured as described in Materials and Methods. Plasmids p-220CAT and p-195CAT had mean percent conversion rates of 6.2 and 8.9, respectively. Plasmid p-138CAT had CAT activity fivefold higher than that of p-220CAT (Fig. 6B). The data suggested that site 2 DNA contains a regulatory element between −195 and −139 relative to the transcription start site. Site 3 had a twofold repressive effect on the UL4 promoter (24). The effect of site 3 requires further investigation.

To demonstrate that the site 2 DNA sequence is a functional negative regulatory element, we transferred WT and mutated site 2 DNA sequences upstream of a heterologous promoter containing an upstream ATF binding site as described in Materials and Methods. Plasmids p2UL4R-19CAT and p2UL4R R-19CAT have two copies of the WT site 2 DNA sequence in opposite orientations. Plasmid p4UL4R-19CAT has four copies of the WT site 2 DNA sequence. Plasmid p2mUL4R-19CAT has two copies of a mutated (lowercase letters) site 2 DNA sequence (5′-CTAGAGTATCCGtagactcgagCgaCGGC TCTGGTCCGGG-3′). Schematic diagrams of the plasmids are shown in Fig. 7A. Transfections were carried out with 5 or 10 μg of the plasmids described above in nonpermissive HeLa or permissive HFF cells. Expression of CAT activity from p2UL4R-19CAT was repressed by the WT site 2 DNA sequence but not by the mutated site 2 DNA sequence in either HeLa or HFF cells (Fig. 7B). In HeLa cells, CAT expression from plasmids containing two copies of the WT site 2 DNA sequence in the WT and reversed orientations was repressed 6.8- and 7.0-fold, respectively. CAT expression from a plasmid containing four copies of the WT site 2 DNA sequence was also repressed (Fig. 7B). In HFF cells, in which EMSA demonstrated that there was less of the cellular repressor protein(s) than in HeLa cells (Fig. 4), repression was 2.6-, 2.4-, or 2.9-fold for plasmid p2UL4R-19CAT, p2UL4RR-19CAT, or p4UL4R-19CAT, respectively (Fig. 7B). The plasmid containing a mutated site 2 DNA, p2mUL4R-19CAT, demonstrated no repression of CAT expression in either HeLa or HFF cells (Fig. 7B). When plasmid p2UL4R-19CAT was cotransfected with either a plasmid expressing mutant IE2 protein (pSVIE 2HL) or WT IE2 protein (pSVIE2), there was a 5.3-fold stimulation in CAT expression in cells containing WT IE2 protein. Without the upstream *cis*-acting negative element, there was approximately a twofold stimulation (24). The data suggest that the site 2 DNA sequence is a functional *cis*-acting negative regulatory element that acts in either orientation, and as previously described (23), the IE2 protein can negate the repressive effect on the UL4 promoter.

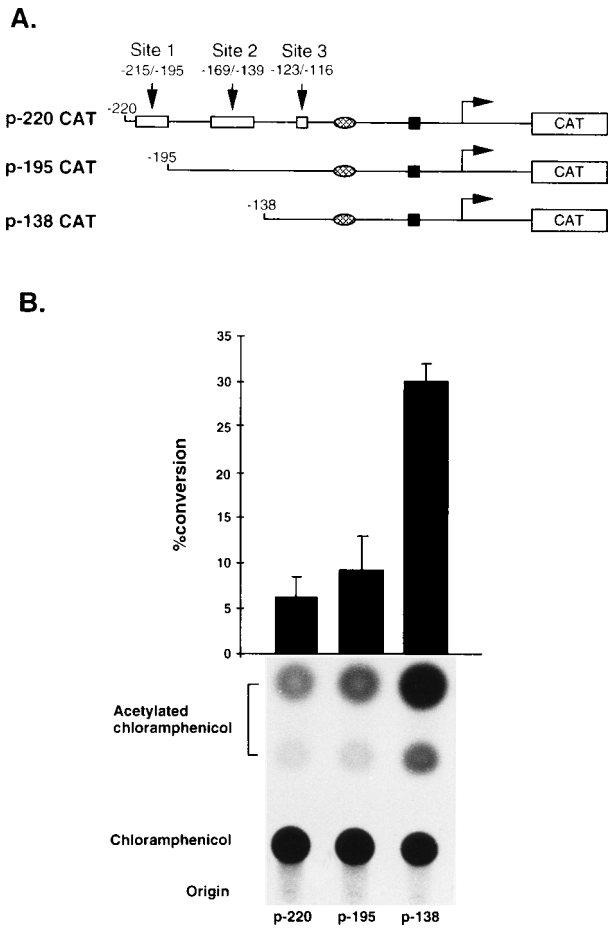

FIG. 6. Deletion of the repressor region in the UL4 promoter. (A) Schematic representation of expression plasmids containing cellular protein binding site 1, 2, or 3 upstream of the UL4 promoter transcription start site linked to the bacterial CAT gene. The TATA and CCAAT boxes are designated by squares and ovals, respectively. The rectangles indicate the locations of protein binding sites 1, 2, and 3 (see Fig. 2). The numbers indicate the distance in base pairs from the transcription start site of the UL4 promoter. (B) Relative percent conversion of [^{14}C]chloramphenicol to the 3′-acetylated derivatives. HFF cells were cotransfected with 10 μg of test plasmid and 5 μg of internal control plasmid pCH110 (Pharmacia). Transfections were done at least three times. The results of the CAT assays were averaged, and the standard deviations were determined. The CAT values were normalized to β-galactosidase activity to control for variations in transfection efficiency as described in Materials and Methods.

IE2 protein binding to the *cis*-acting negative regulatory element. Transient transfection experiments have demonstrated that the UL4 promoter is transactivated by the viral IE2 protein of HCMV (5, 23, 38). We proposed that in addition to positively affecting the TATA-box-containing UL4 promoter, the IE2 protein negates the effect of the *cis*-acting negative regulatory element (23). The IE2 DNA-binding protein may effect binding of the cellular repressor protein(s) to the *cis*-acting negative regulatory element. To test this hypothesis, we compared the DNA sequence of the *cis*-acting negative regulatory element of the UL4 promoter with the *cis*-repression signal of the MIEP. A region of 65% homology between the *cis*-repression signal and the *cis*-acting negative regulatory element of the UL4 promoter was found. As demonstrated previously, the IE2 protein could bind to the *cis*-repression signal and functionally repress transcription from the MIEP (37). Repression of the MIEP requires only the C-terminal amino acids of the IE2 protein (residues 290 to 579) (8, 37).

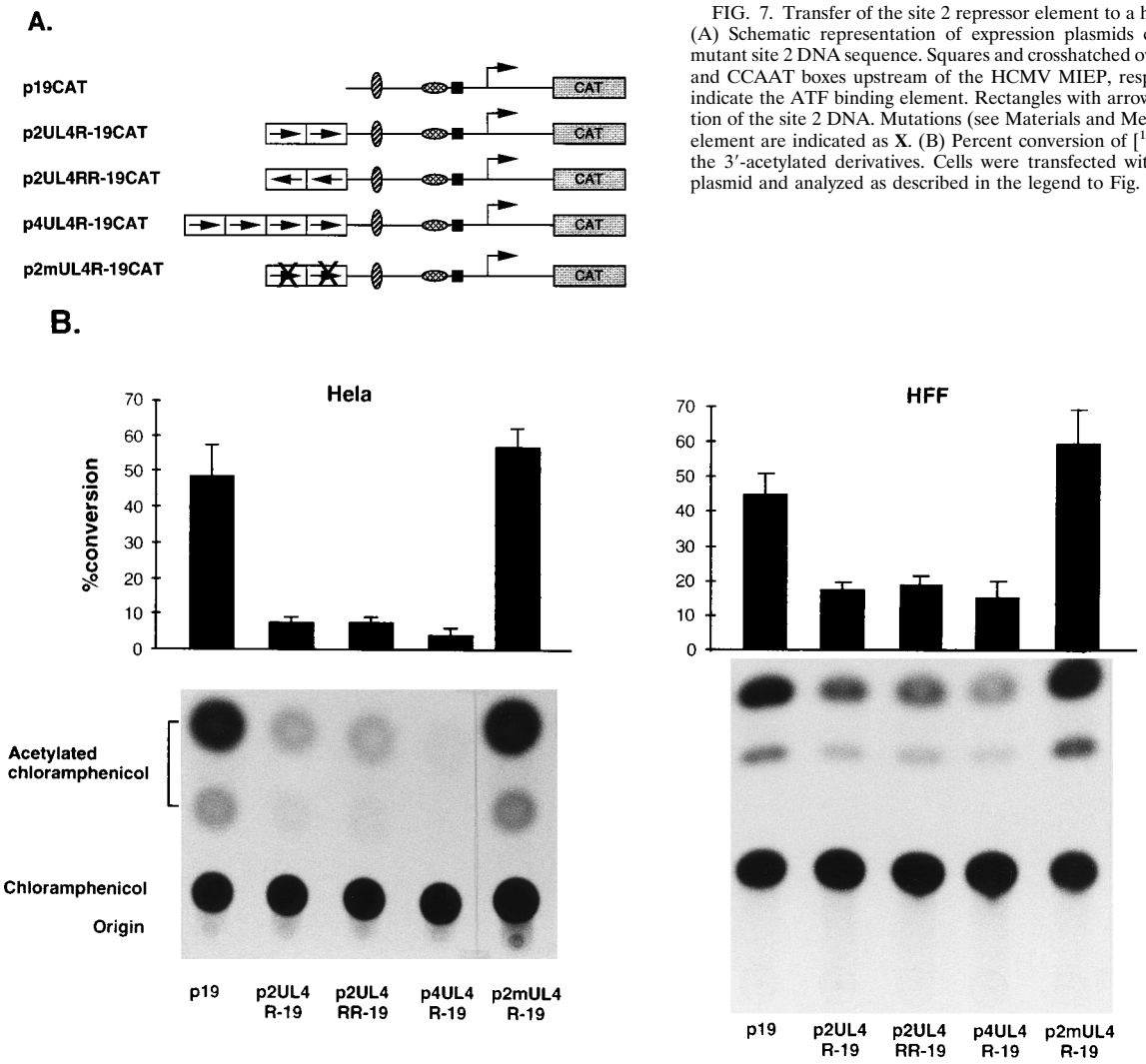

FIG. 7. Transfer of the site 2 repressor element to a heterologous promoter. (A) Schematic representation of expression plasmids containing the WT or mutant site 2 DNA sequence. Squares and crosshatched ovals indicate the TATA and CCAAT boxes upstream of the HCMV MIEP, respectively. Striped ovals indicate the ATF binding element. Rectangles with arrows indicate the orientation of the site 2 DNA. Mutations (see Materials and Methods) in the repressor element are indicated as **X**. (B) Percent conversion of [^{14}C]chloramphenicol to the 3′-acetylated derivatives. Cells were transfected with 5 or 10 μg of each plasmid and analyzed as described in the legend to Fig. 5.

To test whether the IE2 protein binds to the *cis*-acting negative regulatory element of the UL4 promoter, EMSA was performed with a maltose-IE2 fusion protein (r-IE2) containing amino acids 290 to 579 of the IE2 protein as described in Materials and Methods. In addition, a maltose-IE2 protein mutated in the putative C_2H_2 zinc finger motif (r-IE2HL) that fails to bind the *cis*-repression signal was used as a control. Figure 8A demonstrates that r-IE2 and r-IE2HL were purified to greater than 90% purity. ^{32}P-labeled DNA probe (−220 to −120) from the UL4 promoter was used in an EMSA. A DNA–r-IE2 complex was detected (Fig. 8B, lane 2). A cold competition assay with a 10- or 50-fold molar excess of nonradioactively labeled WT, M8, M9, M10, or M11 DNA fragment (Fig. 3A) demonstrated that WT DNA and site 2-containing M9 DNA inhibited formation of the DNA-protein complex at 50-fold molar excesses (Fig. 8B; compare lanes 2 and 8). M8, M10, or M11 DNA competed poorly (Fig. 8B; compare lanes 6, 10, and 12).

To confirm that the DNA-protein complex detected in EMSA contained IE2 antigen, a supershift assay with anti-IE2 antibody 1218 was performed. The antibody was incubated with r-IE2 before ^{32}P-labeled DNA probe (−220 to −102) was added. With increasing amounts of rabbit polyclonal serum 1218, the amount of DNA–r-IE2 complex was decreased and a new, slowly migrating complex (supershift) was detected (Fig. 8C; compare lane 3 with lanes 9 to 12). A rabbit polyclonal preimmune serum or an anti-NF-Y serum had no effect on the DNA–r-IE2 complex, but the serum did enhance r-IE2 binding activity (Fig. 8C; compare lanes 3 with lanes 4 to 7). Serum 1218 alone did not bind to the DNA probe (Fig. 8C, lane 8). These results indicate that r-IE2 can specifically bind to the *cis*-acting negative regulatory element of the UL4 promoter.

To determine whether the binding of cellular repressor protein(s) to site 2 DNA and r-IE2 protein binding are mutually exclusive, crude nuclear extract was added to the DNA-protein binding reaction mixture containing a ^{32}P-labeled 40-bp site 2 DNA probe (−168 to −134). After incubation at room temperature for 15 min as described in Materials and Methods, purified r-IE2 or mutant r-IE2HL protein was added. In the presence of 0.4 μg of r-IE2 protein, the relative abundance of complexes X and Y was significantly reduced even in the presence of excess probe (Fig. 9; compare lane 2 with lane 3). In contrast, 0.4 μg of r-IE2HL protein had little to no effect on complexes X and Y (Fig. 9; compare lane 2 with lane 4). These data suggest that cellular protein does not exclude the binding of r-IE2 protein to site 2 DNA. Whether these proteins bind to

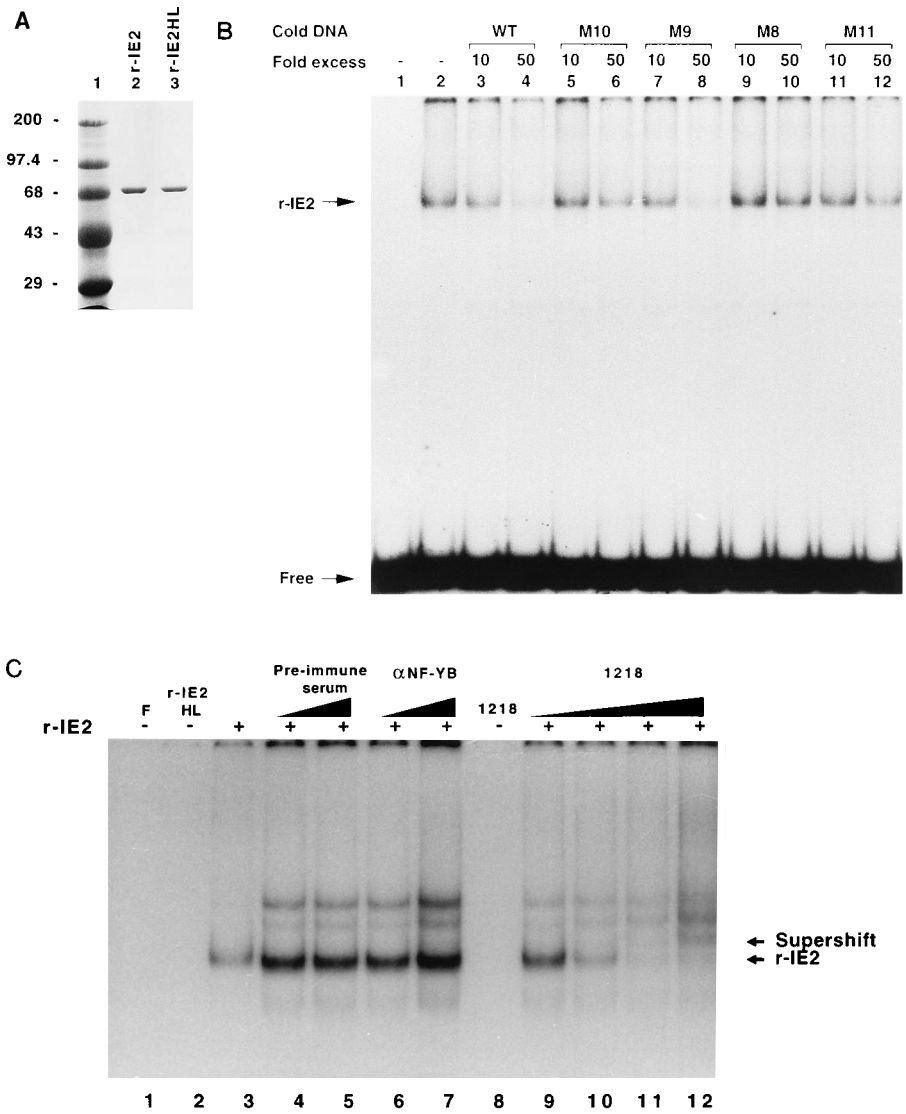

FIG. 8. Binding of the IE2 protein to site 2 DNA of the UL4 promoter. (A) WT and mutant IE2 affinity-purified fusion proteins. r-IE2 fusion protein is 70 kDa in size and contains IE2 amino acids 290 to 579. r-IE2HL contains the same amino acids as r-IE2, but the histidines at positions 446 and 452 were converted to leucines. Construction of r-IE2 and r-IE2HL fusion proteins was previously described (38). Lanes: 1, ^{14}C-protein size standards (positions are shown in kilodaltons at the left); 2, 11.6 pmol of r-IE2; 3, 11.6 pmol of r-IE2HL. (B) EMSA and cold competition assay with WT and mutant repression region DNAs. The assays were carried out as described for Fig. 3. WT and mutated DNA fragments are described in Fig. 3A. Lanes: 1, free WT DNA probe (−220 to −102); 2, probe plus r-IE2; 3, 5, 7, 9, and 11, probe and r-IE2 plus 10-fold molar excesses of nonradioactively labeled WT, M10, M9, M8, and M11 DNA fragments, respectively; 4, 6, 8, 10, and 12, probe and r-IE2 plus 50-fold molar excesses of nonradioactively labeled WT, M10, M9, M8, and M11 DNA fragments, respectively. r-IE2–DNA complex and free probe are indicated by arrows. (C) Supershift of the r-IE2–DNA complex by anti-IE2 antibody 1218. EMSA was carried out as described in Materials and Methods. Lanes: 1, WT DNA free probe (F; −220 to −102); 2, probe plus 5.8 pmol of r-IE2HL; 3, probe plus 5.8 pmol of r-IE2; 4 and 5, probe plus 5.8 pmol of r-IE2 in the presence of 2.5 and 5.0 μl of 1:5 diluted preimmune serum, respectively; 6 and 7, probe plus 5.8 pmol of r-IE2 in the presence of 2.5 and 5.0 μl of 1:5 diluted anti-NF-YB polyclonal antibody, respectively; 8, probe plus 5 μl of 1:5 diluted antibody 1218; 9, 10, 11, and 12, probe plus 5.8 pmol of r-IE2 in the presence of 0.5, 1.25, 2.5, and 5.0 μl of 1:5 diluted antibody 1218. The r-IE2–DNA complex and supershifted complex are indicated by arrows.

the same site or overlapping binding sites is presently not known. The viral IE2 protein may negate the effect of the cellular repressor protein on transcription from the UL4 promoter by competing for the same binding site or an overlapping binding site.

DISCUSSION

Eucaryotic cellular repressor proteins have been reported to downregulate transcription by binding to *cis*-acting negative regulatory elements. In HeLa cells, repressor proteins of 38, 97, 98, and 128 kDa have been reported to bind to the *cis*-acting negative regulatory elements in the long terminal repeat of human immunodeficiency virus, upstream of the alpha-1 acid glycoprotein gene, the enhancer repeats of BK virus, and upstream of the epidermal growth factor receptor promoter, respectively (22, 43, 57, 59). The role of cellular repressor proteins in HCMV gene regulation as it relates to latent and lytic infection is poorly understood. Transcription from HCMV early promoters in HFF cells is weak prior to viral IE protein synthesis (5, 23, 55). We have described a *cis*-acting negative regulatory element between −168 and −134 bp relative to the transcription start site of the early UL4 promoter. Downregulation of transcription by an upstream negative reg-

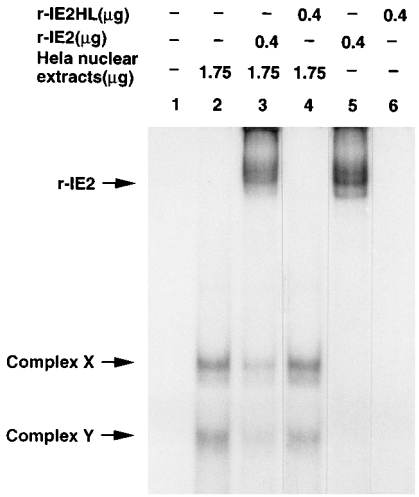

FIG. 9. Cellular repressor protein and IE2 protein binding to the *cis*-acting negative regulatory element. Probe was incubated with 1.75 μg of HeLa cell nuclear extract or DNA binding buffer at room temperature for 10 min, and then either WT r-IE2 or mutant r-IE2HL protein was added. EMSA was done after 15 min of incubation at room temperature as described in Materials and Methods. Lanes: 1, WT site 2 DNA probe (−168 to −134) alone; 2, probe plus HeLa cell nuclear extract; 3, probe plus HeLa cell nuclear extract and then 0.4 μg of r-IE2 protein; 4, probe plus HeLa cell nuclear extract and then 0.4 μg of r-IE2HL protein; 5, probe plus DNA binding buffer and then 0.4 μg of r-IE2 protein; 6, probe plus DNA binding buffer and then 0.4 μg of r-IE2HL protein. Complexes, X, Y, and r-IE2–DNA are indicated by arrows.

ulatory region was negated by the viral IE2 protein (23). Footprinting assays, EMSAs, and cold competition assays detected a *cis*-acting negative regulatory element that binds either a cellular repressor protein(s) or the viral IE2 protein. Deletion mutagenesis followed by a transient transfection assay demonstrated that transcription from the UL4 promoter increased fivefold in the absence of the upstream *cis*-acting negative regulatory element.

A protein(s) of approximately 102 kDa in nuclear extract from either HeLa or HFF cells was found to bind to the *cis*-acting negative regulatory element of the UL4 promoter. Whether the cellular protein(s) corresponds to the cellular repressor factors described above remains to be determined. Site-specific mutagenesis demonstrated that the core sequence of the negative regulatory element, 5′-GTTTGGAATCGTT-3′, is required for protein binding in vitro and repression of downstream gene expression in vivo in transiently transfected cells.

The binding of the cellular repressor protein(s) to the upstream *cis*-acting negative regulatory element may interfere with the activity of the transcription factor NF-Y, which binds to the downstream CCAAT box, or TFIID, which binds to the TATA box. When the *cis*-acting negative regulatory element was transferred upstream of a heterologous promoter containing a downstream ATF binding site, it functioned as well as when in the UL4 promoter. Therefore, it is more likely that binding of the cellular repressor protein(s) to the upstream *cis*-acting negative regulatory element interferes with binding of TFIID or the transcription initiation complex.

Some DNA sequence-dependent repressor proteins negatively regulate transcription by competing for the same site as a positive transcription factor. For example, c-Rel competes for the NF-κB site and CCAAT box displacement protein competes for the CCAAT box binding site (44, 46). Other repressor proteins bind to the DNA sequence near a positive regulatory element and occlude binding of the positive factor. For example, the E2 protein of human papillomavirus occludes the binding of the transcription factor SP-1 (13). Some repressor proteins can bind a DNA sequence upstream of a TATA box and downstream of a positive regulatory element and consequently block interaction between the transcription initiation complex and the positive transcription factor (46). Lastly, some repressor proteins bind elements known as silencers that are distance and orientation independent. Their presence may affect coactivator protein interaction with the transcription initiation complex (44).

Although the *cis*-acting negative regulatory element of the UL4 promoter functions in either HeLa or HFF cells, it is conceivable that HFF cells contain less of the cellular repressor protein(s) than HeLa cells. In both HeLa and HFF cells, the cellular repressor protein may be associated with another protein by protein-protein interaction. Both DNA-protein complexes X and Y contain a cellular protein(s) of approximately 102 kDa (24). Complex X presumably has another protein because of its slower electrophoretic mobility. However, we were not able to UV cross-link this protein to the DNA. The function of this putative protein-protein interaction is not known.

The footprinting assay used in this study was designed to detect any binding of cellular proteins to the DNA probe containing the negative regulatory region. Three protection regions were detected under these conditions. Binding of cellular proteins to sites 1 and 3 was eliminated by decreasing the amount of HeLa cell nuclear extract and increasing the amount of poly(dI-dC) · poly(dI-dC). These data suggest that with the binding conditions used, sites 1 and 3 bind lower-affinity proteins than site 2. In transient transfection experiments, deletion of the site 1 DNA sequence did not significantly affect repression of the UL4 promoter. The putative binding of a cellular protein(s) to site 3 had only a twofold repressive effect on the UL4 promoter (24). A comparison of the site 2 DNA sequence with that of site 3 detected a common sequence, 5′-GGAATPyNTPy-3′. Further experiments are necessary to determine the significance of cellular protein bound to site 3 and the relationship between sites 2 and 3.

The HCMV IE2 protein is a DNA-binding protein that recognizes AT-rich sequences in the minor groove of the DNA molecule (32). It binds to the *cis*-repression signal between the TATA box and the transcription initiation site in the MIEP and represses transcription (34, 37). A comparison of the *cis*-repression signal DNA sequence with the *cis*-acting negative regulatory element upstream of the UL4 promoter detected homology of 65%. We demonstrated that the IE2 protein or the cellular repressor protein(s) binds to the DNA containing a *cis*-acting negative regulatory element upstream of the UL4 promoter. Since the level of the cellular repressor protein(s) does not decrease with infection (24), a likely mechanism for transactivation of the UL4 promoter by the IE2 protein is negation of the effect of the *cis*-acting negative regulatory element. In vivo, the IE2 protein may have a higher DNA-binding affinity than the cellular repressor protein. The relative affinities of the cellular repressor protein(s) or IE2 protein binding to the *cis*-acting negative regulatory element can be determined once the cellular repressor protein(s) is purified. The viral IE2 protein also binds upstream of the HCMV UL112-113 promoter (2, 45). Whether a cellular repressor protein binds to the same site upstream of the UL112-113 promoter is not known.

In the absence of the upstream *cis*-acting negative regulatory element (plasmid p-122CAT), the CCAAT box dyad symmetry of the UL4 promoter strongly contributed to the constitutive

activity (23). In transient transfection experiments, the IE2 protein stimulated CAT expression from the UL4 promoter containing the *cis*-acting negative regulatory element (plasmid p-220CAT) to the same level as that from the UL4 promoter without the *cis*-acting negative regulatory element (23). Therefore, the binding of the IE2 protein to the *cis*-acting negative regulatory element negates the repressive function of the cellular repressor protein(s) and allows NF-Y to enhance downstream transcription from the UL4 promoter.

The upstream regions of the early viral promoters in the HCMV genome may have a variety of binding sites for both cellular repressor proteins and eucaryotic transcriptional activation proteins. The IE2 protein of HCMV may be a multifunctional protein. By binding to the viral DNA template, it may negate the effect of cellular repressor proteins. Likewise, the viral IE2 protein bound to the DNA template could interact with eucaryotic transcription factors to further enhance the level of downstream transcription. The relative abundance of cellular repressor proteins and HCMV IE2 proteins presumably affects the relative amount of HCMV replication in various cell types of the human host.

ACKNOWLEDGMENTS

We thank R. Roller, C. M. Stoltzfus, and L. Turek for critical review of the manuscript. We thank members of our laboratory for help and encouragement.

This work was supported by Public Health Service grants AI-13562 and HL37121.

REFERENCES

1. **Alford, C. A., and W. J. Britt.** 1990. Cytomegalovirus, p. 1981–2010. *In* B. N. Fields, D. M. Knipe, et al. (ed.), Virology. Raven Press, Ltd., New York.
2. **Arlt, H., D. Lang, S. Gebert, and T. Stamminger.** 1994. Identification of binding sites for the 86-kilodalton IE2 protein of human cytomegalovirus within an IE2-responsive viral early promoter. J. Virol. **68:**4117–4125.
3. **Berkhout, B., and K. T. Jeang.** 1992. Functional roles for the TATA promoter and enhances in basal and Tat-induced expression of the human immunodeficiency virus type 1 long terminal repeat. J. Virol. **66:**139–149.
4. **Caswell, R., C. Hagemeier, C. J. Chiou, G. Hayward, T. Kouzarides, and J. Sinclair.** 1993. The human cytomegalovirus 86-K immediate early (IE) 2 protein requires the basic region of the TATA-box binding protein (TBP) for binding and interacts with TBP and transcription factor TFIIB via regions of IE2 required for transcriptional regulation. J. Gen. Virol. **74:**2691–2698.
5. **Chang, C.-P., C. L. Malone, and M. F. Stinski.** 1989. A human cytomegalovirus early gene has three inducible promoters that are regulated differentially at various times after infection. J. Virol. **63:**281–290.
6. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison III, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154:**125–169.
7. **Cherrington, J. M., E. L. Khoury, and E. S. Mocarski.** 1991. Human cytomegalovirus *ie*2 negatively regulates a gene expression via a short target sequence near the transcription start site. J. Virol. **65:**887–896.
8. **Chiou, C. J., J. Zong, I. Waheed, and G. S. Hayward.** 1993. Identification and mapping of dimerization and DNA-binding domains in the C terminus of the IE2 regulatory protein of human cytomegalovirus. J. Virol. **67:**6201–6214.
9. **DeMarchi, J. M.** 1981. Human cytomegalovirus DNA: restriction enzyme cleavage maps and map location for immediate early, early and late RNAs. Virology **124:**390–402.
10. **DeMarchi, J. M., C. A. Schmidt, and A. S. Kaplan.** 1980. Patterns of transcription of human cytomegalovirus in permissively infected cells. J. Virol. **35:**277–286.
11. **Depto, A. S., and R. M. Stenberg.** 1989. Regulated expression of the human cytomegalovirus pp65 gene: octamer sequence in the promoter is required for activation by viral gene products. J. Virol. **63:**1232–1238.
12. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
13. **Dong, G., T. R. Broker, and L. T. Chow.** 1994. Human papillomavirus type 11 E2 proteins repress the homologous E6 promoter by interfering with the binding of host transcription factors to adjacent elements. J. Virol. **68:**1115–1127.
14. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
15. **Hagemeier, C., R. Caswell, G. Hayhurst, and J. Sinclair.** 1994. Functional interaction between the HCMV IE2 transactivator and the retinoblastoma protein. EMBO J. **13:**2897–2903.
16. **Hagemeier, C., S. Walker, R. Caswell, T. Kouzarides, and J. Sinclair.** 1992. The human cytomegalovirus 80-kilodalton but not the 72-kilodalton immediate-early protein transactivates heterologous promoters in a TATA box-dependent mechanism and interacts directly with TFIID. J. Virol. **66:**4452–4456.
17. **Hagemeier, C., S. M. Walker, P. J. Sissons, and J. H. Sinclair.** 1992. The 72K IE1 and 80K IE2 proteins of human cytomegalovirus independently transactivate the c-fos, c-myc and hsp70 promoter via basal promoter elements. J. Gen. Virol. **73:**2385–2393.
18. **Herbomel, P., B. Bourachot, and M. Yaniv.** 1994. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell **39:**653–662.
19. **Hermiston, T. W., C. L. Malone, P. R. Witte, and M. F. Stinski.** 1987. Identification and characterization of the human cytomegalovirus immediate-early region 2 gene that stimulates gene expression from an inducible promoter. J. Virol. **61:**3214–3221.
20. **Ho, M.** 1982. Cytomegalovirus biology and infection. Plenum Publishing Corp., New York.
21. **Horikoski, N., K. Maguire, A. Kralli, E. Maldonado, D. Reinberg, and R. Weinmann.** 1991. Direct interaction between adenovirus E1A protein and the TATA box binding transcription factor IID. Proc. Natl. Acad. Sci. USA **88:**5124–5128.
22. **Hou, X., A. C. Johnson, and M. R. Rosner.** 1994. Identification of an epidermal growth factor receptor transcriptional repressor. J. Biol. Chem. **269:**4307–4312.
23. **Huang, L., C. L. Malone, and M. F. Stinski.** 1994. A human cytomegalovirus early promoter with upstream negative and positive *cis*-acting elements: IE2 negates the effect of the negative element, and NF-Y binds to the positive element. J. Virol. **68:**2108–2117.
24. **Huang, L., and M. F. Stinski.** Unpublished data.
25. **Imbalzano, A. N., and N. A. DeLuca.** 1992. Substitution of a TATA box from a herpes simplex virus late gene in the viral thymidine kinase promoter alters ICP4 inducibility but not temporal expression. J. Virol. **66:**5453–5463.
26. **Ingles, C. J., M. Shales, W. D. Cress, S. J. Triezenberg, and J. Greenblatt.** 1991. Reduced binding of TFIID to transcriptionally compromised mutants of VP16. Nature (London) **351:**588–590.
27. **Jupp, R., S. Hoffmann, R. M. Stenberg, J. A. Nelson, and P. Ghazal.** 1993. Human cytomegalovirus IE86 protein interacts with promoter-bound TATA-binding protein via a specific region distinct from the autorepression domain. J. Virol. **67:**7539–7546.
28. **Klucher, K. M., D. K. Rabert, and D. H. Spector.** 1989. Sequences in the human cytomegalovirus 2.7-kilobase RNA promoter which mediate its regulation as an early gene. J. Virol. **63:**5334–5343.
29. **Klucher, K. M., M. Sommer, J. T. Kadonaga, and D. H. Spector.** 1993. In vivo and in vitro analysis of transcriptional activation mediated by the human cytomegalovirus major immediate-early proteins. Mol. Cell. Biol. **13:**1238–1250.
30. **Klucher, K. M., and D. H. Spector.** 1990. The human cytomegalovirus 2.7-kilobase RNA promoter contains a functional binding site for the adenovirus major late transcription factor. J. Virol. **64:**4189–4198.
31. **Kuwabara, M. D., and D. S. Sigman.** 1987. Footprinting DNA-protein complexes in situ following gel retardation assays using 1,10-phenanthroline-copper ion: *Escherichia coli* RNA polymerase-*lac* promoter complexes. Biochemistry **26:**7234–7238.
32. **Lang, D., and T. Stamminger.** 1994. Minor groove contacts are essential for an interaction of the human cytomegalovirus IE2 protein with its DNA target. Nucleic Acids Res. **22:**3331–3338.
33. **Lieberman, P. M., and A. J. Berk.** 1991. The Zta trans-activator protein stabilizes TFIID association with promoter DNA by direct protein-protein interaction. Genes Dev. **5:**2441–2454.
34. **Liu, B., T. W. Hermiston, and M. F. Stinski.** 1991. A *cis*-acting element in the major immediate-early (IE) promoter of human cytomegalovirus is required for negative regulation by IE2. J. Virol. **65:**897–903.
35. **Liu, B., and M. F. Stinski.** 1992. Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 *cis*-acting element. J. Virol. **66:**4334–4444.
36. **Lukac, D. M., J. R. Manuppello, and J. C. Alwine.** 1994. Transcriptional activation by the human cytomegalovirus immediate-early proteins: requirements for simple promoter structures and interactions with multiple components of the transcription complex. J. Virol. **68:**5184–5193.
37. **Macias, M. P., and M. F. Stinski.** 1993. 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. Proc. Natl. Acad. Sci. USA **90:**707–711.
38. **Malone, C. L., D. H. Vesole, and M. F. Stinski.** 1990. Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins. J. Virol. **64:**1498–1506.

39. **McDonough, S. H., and D. H. Spector.** 1983. Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD169. Virology **125:**31–46.
40. **Olsen, H. S., and C. A. Rosen.** 1992. Contribution of the TATA motif to Tat-mediated transcriptional activation of human immunodeficiency virus gene expression. J. Virol. **66:**5594–5597.
41. **Pizzorno, M. C., and G. S. Hayward.** 1990. The IE2 gene products of human cytomegalovirus specifically down-regulate expression from the major immediate-early promoter through a target located near the cap site. J. Virol. **64:**6154–6165.
42. **Pizzorno, M. C., M. A. Mullen, Y. N. Chang, and G. S. Hayward.** 1991. The functionally active IE2 immediate-early regulatory protein of human cytomegalovirus is an 80-kilodalton polypeptide that contains two distinct activator domains and a duplicated nuclear localization signal. J. Virol. **65:**3839–3852.
43. **Reifel-Miller, A. E., D. T. Berg, and B. W. Grinnell.** 1991. Repression of enhancer activity by the adenovirus E1A tumor protein(s) is mediated through a novel HeLa cell nuclear factor. J. Biol. Chem. **266:**13873–13882.
44. **Renkawitz, R.** 1990. Transcriptional repression in eukaryotes. Trends Genet. **6:**192–197.
45. **Schwartz, R., M. H. Sommer, A. Scully, and D. H. Spector.** 1994. Site-specific binding of the human cytomegalovirus IE2 86-kilodalton protein to an early gene promoter. J. Virol. **68:**5613–5622.
46. **Skalnik, D. G., E. C. Strauss, and S. H. Orkin.** 1991. CCAAT displacement protein as a repressor of the myelomonocytic-specific gp-91-phox gene promoter. J. Biol. Chem. **266:**16736–16744.
47. **Smith, C. A., P. Bates, R. Rivera-Gonzalez, B. Gu, and N. A. DeLuca.** 1993. ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB. J. Virol. **67:**4676–4687.
48. **Sommer, M. H., A. L. Scully, and D. H. Spector.** 1994. Transactivation by the human cytomegalovirus IE2 86-kilodalton protein requires a domain that binds to both the TATA-box binding protein and the retinoblastoma protein. J. Virol. **68:**6223–6231.
49. **Spector, D. H., K. M. Klucher, D. K. Rabert, and D. A. Wright.** 1990. Human cytomegalovirus early gene expression. Curr. Top. Microbiol. Immunol. **154:** 21–45.
50. **Staprans, S. I., D. K. Rabert, and D. H. Spector.** 1988. Identification of sequence requirements and *trans*-acting functions necessary for regulated expression of a human cytomegalovirus early gene. J. Virol. **62:**3463–3473.
51. **Stenberg, R. M., J. Fortney, S. W. Barlow, B. P. Magrane, J. A. Nelson, and P. Ghazal.** 1990. Promoter-specific *trans* activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. J. Virol. **26:**1556–1565.
52. **Stinski, M. F.** 1978. Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. J. Virol. **26:**686–701.
53. **Stringer, K. F., C. J. Ingles, and J. Greenblatt.** 1990. Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature (London) **345:**782–786.
54. **Wade, E. J., K. M. Klucher, and D. H. Spector.** 1992. An AP-1 binding site is the predominant *cis*-acting regulatory element in the 1.2-kilobase early RNA promoter of human cytomegalovirus. J. Virol. **66:**2407–2417.
55. **Wathen, M. W., and M. F. Stinski.** 1982. Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection. J. Virol. **41:**462–477.
56. **Wathen, M. W., D. R. Thomsen, and M. F. Stinski.** 1981. Temporal regulation of human cytomegalovirus transcription at immediate early and early times after infection. J. Virol. **38:**446–459.
57. **West, M., J. Mikovits, G. Princler, Y.-L. Liu, F. W. Ruscetti, H.-F. Kung, and Raziuddin.** 1992. Characterization and purification of a novel transcriptional repressor from HeLa cell nuclear extracts recognizing the negative regulatory element region of human immunodeficiency virus-1 long terminal repeat. J. Biol. Chem. **267:**24948–24952.
58. **Williams, M., A. Brys, A. M. Weiner, and N. Maizels.** 1992. A rapid method for determining the molecular weight of a protein bound to nucleic acid in a mobility shift assay. Nucleic Acids Res. **20:**4935–4936.
59. **Yang, T.-H., W.-H. Tsai, Y.-M. Lee, H.-Y. Lei, M.-Y. Lai, D.-S. Chen, N.-H. Yeh, and S.-C. Lee.** 1994. Purification and characterization of necleolin and its identification as a transcription repressor. Mol. Cell. Biol. **14:**6068–6074.
60. **Yeung, K. C., C. M. Stoltzfus, and M. F. Stinski.** 1993. Mutations of the human cytomegalovirus immediate-early 2 protein defines regions and amino acid motifs important in transaction of transcription from the HIV-1 LTR promoter. Virology **195:**786–792.