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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

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The human cytomegalovirus early promoter for the UL4 gene, which codes for an early viral envelope glycoprotein designated gpUL4, requires immediate-early viral protein two (IE2) synthesis to be activated (C.-P. Chang, C. L. Malone, and M. F. Stinski, J. Virol. 63:281, 1989). We investigated the *cis*-acting and *trans*-acting factors that regulate transcription from this UL4 promoter. In transient transfection assays, the viral IE2 protein negated the effect of an upstream *cis*-acting negative element and enhanced downstream gene expression. A *cis*-acting positive element contributed to the activity of the viral promoter when an upstream *cis*-acting negative element was deleted or when the viral IE2 protein was present. The cellular protein(s) that binds to the *cis*-acting negative element requires further investigation. The cellular protein that binds to the *cis*-acting positive element was characterized. Two DNA sequence-specific protein complexes were detected with DNA probes spanning the region containing the *cis*-acting positive element and human cytomegalovirus-infected human fibroblast cell nuclear extracts. The more slowly migrating complex was labeled complex A, and the faster was labeled complex B. Only complex B was detected with mock-infected cell nuclear extracts. Competition experiments confirmed the specificity of the A and B complexes. The protein bound to the DNA in both the complexes contacts a CCAAT box imperfect dyad symmetry (5′CCAATCACTGG3′). Either CCAAT box within the dyad symmetry could compete for binding the nuclear factor. Mutation of the CCAAT box dyad symmetry resulted in a decrease of the transcriptional activity from the UL4 promoter. A cellular transcription factor, antigenically related to nuclear factor-Y (NF-Y), was found in both complexes A and B. Events associated with viral infection caused phosphorylation of protein complex A. Dephosphorylation of the DNA-binding protein converts complex A to complex B. The effect of phosphorylation of NF-Y is not known.

Human cytomegalovirus (HCMV) is one of the seven human herpesviruses. Although infection by HCMV is usually asymptomatic, the virus can cause serious diseases in infants as well as immunocompromised adults (3, 27). The HCMV double-stranded DNA genome of 230 kb is the largest genome of the seven human herpesviruses and may code for approximately 200 proteins (12). The viral genes are designated alphanumerically within the viral genome (12). Like those of other herpesviruses, the genes of HCMV are transcribed in permissive cells in a sequential and regulated manner. These viral genes have been categorized as immediate-early (IE), early, and late (14, 15, 48, 63, 64). IE gene transcription occurs without de novo viral protein synthesis and is strongly stimulated by specific *cis*-acting elements and *trans*-acting factors. The IE transcription unit downstream of the modulator/enhancer-containing major IE promoter codes for the viral regulatory proteins, designated IE1 and IE2. The IE proteins upregulate transcription from early viral promoters (10, 16, 26, 46, 53, 56–58). Some of the early HCMV promoters contain *cis*-acting elements upstream of the transcription start site and the TATA box that interact with endogenous eucaryotic transcription factors. For example, the TRL4 promoter, which drives the synthesis of a 2.7-kb early mRNA, contains a USF/MLTF binding site, TCACGTGA, between positions −113 and −106 relative to the transcription start site (35, 37). The TRL6 promoter, which drives the synthesis of a 1.2-kb early mRNA, contains an AP-1 binding site, TGACTCA, between positions −75 and −69 (62). The UL112-113 promoter, which drives the synthesis of a 2.2-kb early mRNA, contains upstream ATF sites at positions −193 and −71 (57). Therefore, it is paradoxical that in the HCMV-infected cells these early viral promoters require IE viral protein synthesis for detectable levels of transcription of the downstream gene.

The UL4 promoter, which was originally termed E1.7, drives the synthesis of an early 1.7-kb mRNA. The gene product, gpUL4, is a viral envelope glycoprotein of 48 kDa (10, 11). In this study, we show that the UL4 promoter, which contains a TATA box and an upstream CCAAT box dyad symmetry, is weak when an upstream *cis*-acting negative element is present. The viral IE2 protein can negate the effect of the upstream *cis*-acting negative element and enhance the level of downstream gene expression. In the absence of the upstream negative region, the CCAAT box dyad symmetry is a functional positive regulatory element that confers strong constitutive expression in transiently transfected cells. A cellular transcription factor, NF-Y, binds to the CCAAT box dyad symmetry in the UL4 promoter. While NF-Y is not modified in mock-infected human foreskin fibroblast (HFF) cells, the complex containing NF-Y is modified by phosphorylation in HCMV-infected HFF cells.

MATERIALS AND METHODS

Cell culture and virus. HFF cells were cultured and HCMV (Towne) stocks were prepared as described previously (60).

Plasmid constructions. Plasmid p-220CAT, previously designated pE1.7CAT7, has been described elsewhere (10). Plasmids p-122CAT and p-59CAT were constructed by insertion of

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a 133-bp *Eco*RI-*Nae*I fragment and a 70-bp *Sau*3A-*Nae*I DNA fragment, respectively, with a distal *Xho*I linker and proximal *Hin*dIII linker into the *Xho*I and *Hin*dIII sites of pSVOCAT *Xho*I. The *Nde*I site in pSVOCAT was converted to an *Xho*I site to give pSVOCAT *Xho*I. The 231-bp *Xba*I-*Hin*dIII DNA fragment from p-220CAT was cloned into the *Xba*I and *Hin*dIII sites of pBluescript II KS+ (Stratagene, La Jolla, Calif.) to give plasmid pBXH1. The 131-bp *Eco*RI-*Hin*dIII DNA fragment generated by PCR using primer pair 5′-CGGAATTCTCAGGGGATGATATGGGAagatcagcgctc ATAAGACAAG-3′ and 5′-TTGGGATATATCAACGCTG G-3′ with p-220CAT as template was cloned into the *Eco*RI and *Hin*dIII sites of pBXH1 to give plasmid pBXH1M. Mutant bases are indicated by lowercase letters in the primer sequences. Plasmid p-220M4CAT was constructed by replacing the 231-bp *Xba*I-*Hin*dIII in p-220CAT with the same size *Xba*I-*Hin*dIII fragment from pBXH1M. Plasmid p-81CAT was generated by replacing the 133-bp *Xho*I-*Hin*dIII fragment in p-122CAT with a 92-bp *Xho*I-*Hin*dIII fragment generated by PCR using the primer pair 5′-CTGGACTCGAGAAGGGT TTTT-3′ and 5′-TTGGGATATATCAACGGTGG-3′ with p-122CAT as the template. Plasmid p-122MCAT was constructed by insertion of the M3 DNA (see the description below) into the *Xho*I site of plasmid p-81CAT. Plasmid pBXS was constructed by insertion of a 71-bp *Xho*I-*Sau*3A DNA fragment from p-122CAT into the *Xho*I and *Sma*I sites of pBluescript II KS+ (Stratagene). Plasmids pLink760 and pSVIE2 were described previously (46). All mutations were confirmed by sequencing with the Sequenase kit (United States Biochemical, Cleveland, Ohio) and ^{35}S-ATP (Amersham, Arlington Heights, Ill.).

DNA fragments and oligonucleotides. DNA fragments used in gel mobility shift assays were prepared as follows. The 133-bp DNA fragment from positions −122 to +11 (−122/+11) was isolated from plasmid p-122CAT following digestion of the plasmid with *Xho*I and *Hin*dIII. The pBR DNA fragment (166 bp) was isolated from pBR322 following digestion of the plasmid with *Dde*I. The DNA fragments from positions −122 to −56 (−122/−56) and −119 to −56 (−119/−56) were, respectively, the *Xho*I-*Bam*HI and *Eco*RI-*Bam*HI restriction endonuclease digestion products of plasmid pBXS. Wild-type and mutated DNA fragments (M1, M2, and M3) were generated from pBXS by PCR with M13 reverse primer, 5′-AACAGCTATGACCATG-3′, and specific primer as follows: wild type, 5′-GTCTTATCCAGTGATTGGGT-3′; M1, 5′-GTCTTATCCAGTGAgatctTCCCAT-3′; M2, 5′-GTCT TATagAtctATTGGGTCC-3′; and M3, 5′-TCGAGTCTTAT gagcgctgatctTCCCATATCATCCCCTGAGAAC-3′. The mutated bases are indicated by lowercase letters in the sequences. DNA fragments were isolated from a 3% NuSieve GTG agarose (FMC, Rockland, Maine) gel, following digestion of the PCR products with either *Xho*I (fragments −122/−81, M1, M2, and M3) or *Eco*RI (fragment −119/−81). The double-stranded oligonucleotide NF-Y target sequence was prepared as follows. Complementary oligonucleotides, 5′-ACTTTTAACCAATCAGAAAAAT-3′, and 5′-ATTTTTCT GATTGGTTAAAAGT-3′, were combined, denatured for 2 min at 85°C, and annealed overnight at room temperature. The double-stranded oligonucleotide was purified from single-stranded oligonucleotides by electrophoresis in a 15% polyacrylamide gel.

For generation of end-labeled DNA probes, 50 ng of each DNA fragment was labeled at its 3′ end with either [α-^{32}P]dATP, [α-^{32}P]dGTP, or [α-^{32}P]dTTP (Amersham) and Klenow fragment (Boehringer Mannheim, Indianapolis, Ind.). Unincorporated radionucleotides were removed by a Sephadex G50-150 spin column.

Transfection and CAT assay. HFF cells in 100-mm-diameter plates were transfected with calcium phosphate precipitates containing 10 μg of each reporter plasmid, 5 μg of each effector plasmid, and 20 μg of carrier salmon sperm DNA as described previously (10, 22, 26). A dose-response analysis indicated these concentrations as optimal. Transfections were done at least three times with different HFF cell isolates and different DNA preparations. Cells were harvested 48 h after transfection, and cell extracts were equalized for total protein. The plasmid pCH110 (Pharmacia, Piscataway, N.J.), which contains the simian virus 40 promoter driving expression of the β-galactosidase gene, was used to control for transfection efficiency and variations. The viral IE2 protein activates the simian virus 40 promoter (42) and, consequently, β-galactosidase activity was always enhanced in cells transfected with pSVIE2 and precluded comparison with transfections lacking IE2. Coexpression of β-galactosidase with reporter plasmids revealed that transfection efficiency varied at 1.5 ± 0.6-fold for either pLink760- or pSVIE2-containing transfections. β-Galactosidase activity was assayed as described previously (25). Chloramphenicol acetyltransferase (CAT) assays were carried out as described previously (10, 22). Acetylated derivatives were separated from ^{14}C-chloramphenicol by thin-layer chromatography with a chloroform-methanol (95:5) solvent. The percent conversion was determined by AMBIS (San Diego, Calif.) image acquisition analysis, and standard deviations were calculated. The CAT values obtained in cells transfected with reporter plasmid alone were normalized to β-galactosidase activity to control for variations in transfection efficiency. The data are representative of at least three separate experiments.

Preparation of nuclear extracts. The method and buffers for preparation of nuclear extracts from mock- or HCMV-infected HFF cells were, with some modifications, as described previously (18). Briefly, mock- or HCMV-infected HFF cells were harvested at 48 h after infection, centrifuged to form pellets, suspended in buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.9, containing 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol) with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, leupeptin [5 μg/ml], pepstatin [2 μg/ml], and 1.0 mM TLCK [*N*α-*p*-tosyl-L-lysine chloromethyl ketone]), and allowed to swell at 4°C for 20 min. Protease inhibitors were added to the buffers just before use. Cells were centrifuged, and the pellets were suspended in buffer A with protease inhibitors and then lysed in a Kontes all-glass Dounce homogenizer with a B-type pestle. The crude nuclei were centrifuged to form pellets and suspended in buffer C (20 mM HEPES, pH 7.9, containing 25% [vol/vol] glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, and 0.2 mM EDTA) with the same protease inhibitors. After 30 min at 4°C, a clear supernatant was obtained by centrifugation and dialyzed against buffer D (20 mM HEPES, pH 7.9, containing 20% [vol/vol] glycerol, 0.1 M KCl, and 0.2 mM EDTA) with protease inhibitors. Precipitates were centrifuged to form pellets, and the supernatant was stored as aliquots at −134°C.

Gel mobility shift assay. Either mock- or HCMV-infected nuclear extracts from HFF cells (10 or 20 μg) were incubated at room temperature for 30 min in a reaction mixture of either 12.5 or 25 μl containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1.0 mM EDTA, 5% glycerol, and poly(dI):poly(dC) at 80 ng/μl. For cold competition, 2.5- to 100-fold molar excesses of unlabeled DNA fragments were also in the reaction mixtures. For supershift assays, 0.5 or 1 μl of preimmune or immune serum was in the reaction mixture. Two kinds of

polyclonal antibodies were used in supershift assays. Anti-NF-YB (a gift from R. Mantovani, Institut de Chimie Biologique, Faculte de Medecine, Strasbourg, France) reacts with mouse NF-Y B subunit, and 1218 (a gift from J. Nelson, Oregon Health Science University, Portland) reacts with the IE2 protein of HCMV. The radioactive probe (~50,000 cpm) was added, and the reaction mixture was allowed to incubate at room temperature for another 30 min. Samples were subjected to electrophoresis at 4°C in a 5% polyacrylamide gel containing 0.5× TAE (20 mM Tris-acetate, pH 7.2, containing 1.0 mM EDTA). The gel was then dried and exposed to Kodak X-Omat film (Eastman Kodak Company, Rochester, N.Y.) or Hyperfilm-MP (Amersham).

Footprint assay. Binding reactions were done as described above, except the total reaction volume was 100 μl. In addition, the amounts of extracts, poly(dI):poly(dC), and probe were increased fourfold. In situ footprinting of DNA-protein complexes was performed as described by Kuwabara and Sigman (38).

Potato acid phosphatase (PAP) assay. HCMV-infected HFF cell nuclear extracts were incubated at room temperature for 30 min with either increasing amounts (0.025 to 0.25 U) of PAP (from white potato; Sigma Chemical Company, St. Louis, Mo.) or 0.25 U of PAP plus 2.6 mM $NaMoO_4$ (sodium molybdate, a phosphatase inhibitor) in 10 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, 1.0 mM EDTA, 5% glycerol and poly(dI):poly(dC) (80 ng/μl). One microliter of DNA probe −122/−81 (~30,000 cpm) was added, and the mixtures were incubated at 4°C for 30 min. The samples were subjected to electrophoresis as described above. The counts associated with each band were determined by AMBIS image acquisition analysis.

RESULTS

Negative and positive *cis*-acting elements in the UL4 promoter. The transcription start site from the UL4 promoter has been demonstrated previously (10). Transient transfection experiments demonstrated that the UL4 promoter was transactivated by IE2 alone but not IE1 (10, 46). It has been proposed that IE2 activates transcription from viral early promoters by an anti-repression mechanism (36). To determine whether the UL4 promoter had an upstream *cis*-acting negative element and to test for possible downstream positive elements, we constructed 5′ end deletions upstream of the transcription start site. Various lengths of the UL4 promoter were fused upstream of the CAT reporter gene. Plasmid p-220CAT contains a TATA box, a CCAAT box dyad symmetry, and 121 bp upstream of the CCAAT box dyad symmetry. Plasmid p-122CAT is the same as plasmid p-220CAT, except there is only 23 bp upstream of the CCAAT box dyad symmetry. Plasmids p-220M4CAT and p-122MCAT contain a mutated CCAAT box dyad symmetry but are otherwise similar to p-220CAT and p-122CAT, respectively. Plasmid p-59CAT contains only a TATA box (Fig. 1A). Permissive HFF cells were either transfected with 10 μg of the plasmids described above or cotransfected with 10 μg of the plasmids and 5 μg of either control plasmid pLink760 or IE2-expressing plasmid pSVIE2. Plasmid pLink760 contains the enhancer containing the HCMV major immediate-early promoter only. Plasmid pSVIE2 contains the enhancer containing the HCMV major immediate-early promoter driving expression of the IE2 gene. Expression of the 579-amino-acid IE2 protein from plasmid pSVIE2 has been described previously (46). Cells were harvested 48 h after transfection, and CAT activity was measured as described in Materials and Methods.

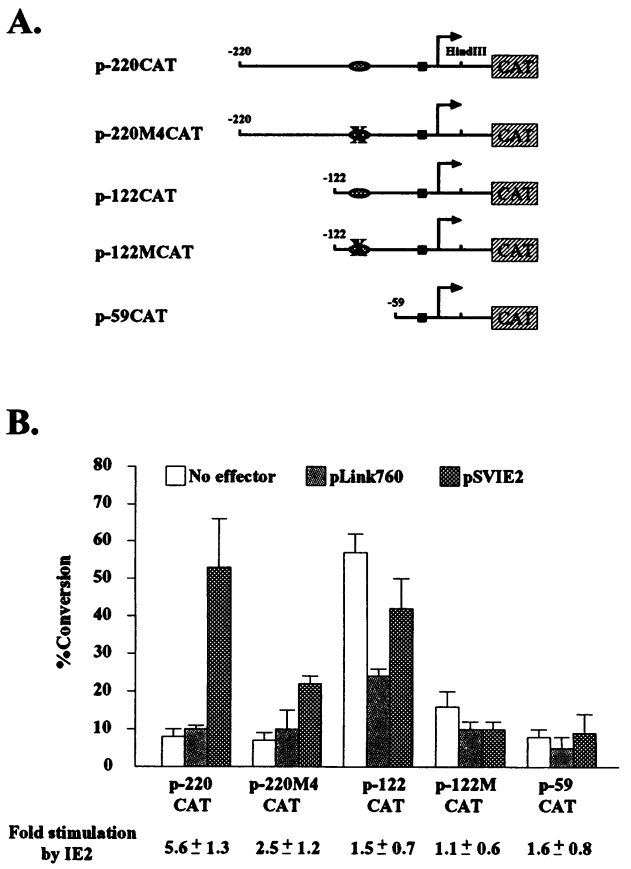

FIG. 1. Expression from the UL4 promoter. (A) Schematic representation of expression plasmids containing various upstream sequences or mutated sequences from the UL4 promoter transcription start site linked to the bacterial CAT gene. The putative TATA box and CCAAT box are designated by the solid black box and crossed oval, respectively. HFF cells were transfected with 10 μg of each plasmid indicated or cotransfected with 5 μg of control plasmid pLink760 or IE2 expression plasmid pSVIE2. Plasmid pCH110, which expresses β-galactosidase, was also cotransfected to control for transfection variations. Cells were harvested 48 h after transfection. The numbers indicate the distance in base pairs from the UL4 promoter. (B) The percent conversion of ^{14}C-chloramphenicol to the 3′-acetylated derivatives and fold stimulation by IE2. 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.

Plasmids p-220CAT and p-59CAT transfected without effector DNA had 8.6 and 6.5% conversion rates, respectively. In contrast, plasmid p-122CAT had 6.9-fold higher CAT activity (Fig. 1). These results suggested the presence of a negative regulatory sequence between positions −220 and −123 and a positive regulatory element between −122 and −60 relative to the UL4 transcription start site. The activity with p-122CAT was reduced approximately twofold when cotransfected with pLink760. Since pLink760 also has CCAAT and TATA boxes, it may have competed for common transcription factors. Mutation of the CCAAT box dyad symmetry in plasmids p-220M4CAT and p-122MCAT reduced the percent conversion. The CCAAT box mutation in plasmid p-220M4CAT did not change the effect of the upstream negative element.

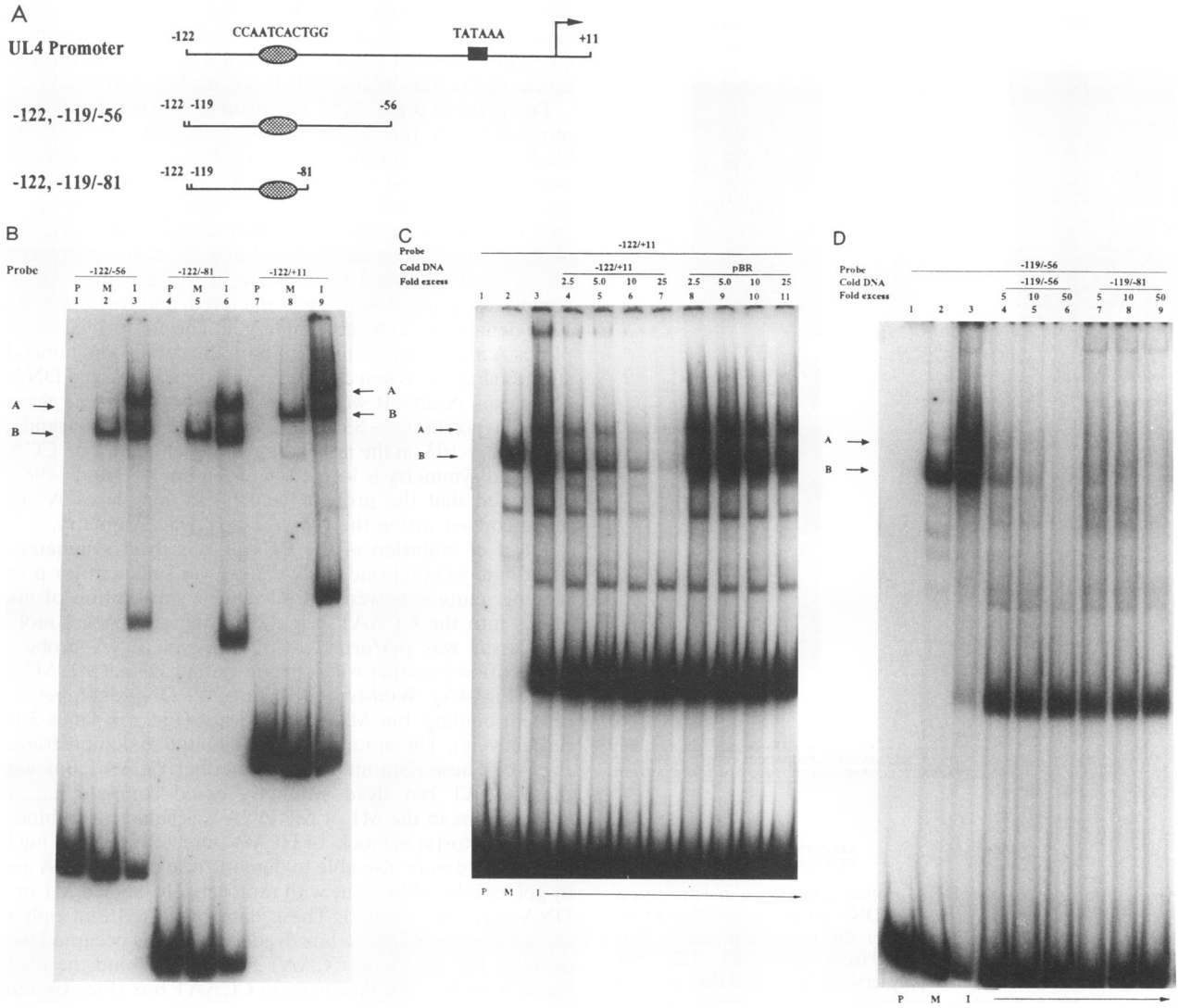

FIG. 2. Specific binding of factors to the UL4 promoter from either mock- or HCMV-infected HFF cell nuclear extracts. (A) Schematic representation of the 133-bp DNA fragment of the UL4 promoter containing the TATA box (solid black box) and the CCAAT box dyad symmetry (hatched oval). DNA fragments containing only the CCAAT box dyad symmetry are also diagrammed. (B) Detection of protein factor(s) in either mock- or HCMV-infected HFF cell nuclear extracts by gel mobility shift assay. Gel mobility shift assays were carried out as described in Materials and Methods. Lanes: 1, 4, and 7, DNA probe (P) alone; 2, 5, and 8, DNA probe plus mock-infected HFF cell nuclear extracts (M); 3, 6, and 9, DNA probe plus HCMV-infected HFF cell nuclear extracts (I). (C) DNA probe −122/+11 plus HCMV-infected HFF cell nuclear extracts in the presence of 2.5- to 25-fold molar excess of the unlabeled homologous DNA fragment (lanes 4 to 7) or nonspecific 166-bp DNA fragment isolated from pBR322 (lanes 8 to 11). (D) DNA probe −119/−56 plus HCMV-infected HFF cell nuclear extracts in the presence of 5- to 50-fold molar excess of unlabeled probe −119/−56 or −119/−81 DNA (lanes 4 to 6 and 7 to 9, respectively). Complexes A and B are indicated by arrows.

The amount of downstream gene expression from p-220CAT cotransfected with pSVIE2 was as high as that from p-122CAT (Fig. 1). Plasmids expressing mutant IE2 proteins had no effect on p-220CAT (pE1.7CAT), as described previously (46). The data suggest that the HCMV UL4 promoter has upstream negative and positive elements. The effect of the positive element on downstream transcription is weak when an upstream *cis*-acting negative element is present. The effect of the negative element can be negated by the viral IE2 protein. A cellular protein(s) binds to the negative element (29a). The identity of the cellular protein(s) requires further characterization. The cellular protein that binds to the positive element was characterized below.

Downstream of position −122 a nuclear factor(s) binds to a sequence in the UL4 promoter. To determine whether the region downstream of position −122 of the UL4 promoter binds a cellular protein factor(s) in mock- or HCMV-infected HFF cell nuclear extracts, several ^{32}P-labeled DNA probes between −122 and +11 were prepared for gel mobility shift assay as described in Materials and Methods. The probe between −122 and +11 contains both the TATA box and the CCAAT box dyad symmetry. The probes between −122 and −56 or −81 contain only the CCAAT box dyad symmetry (Fig. 2A). Gel mobility shift assays detected a DNA-binding factor in nuclear extracts from mock-infected HFF cells designated B complex (Fig. 2B, lanes 2, 5, and 8). A similar

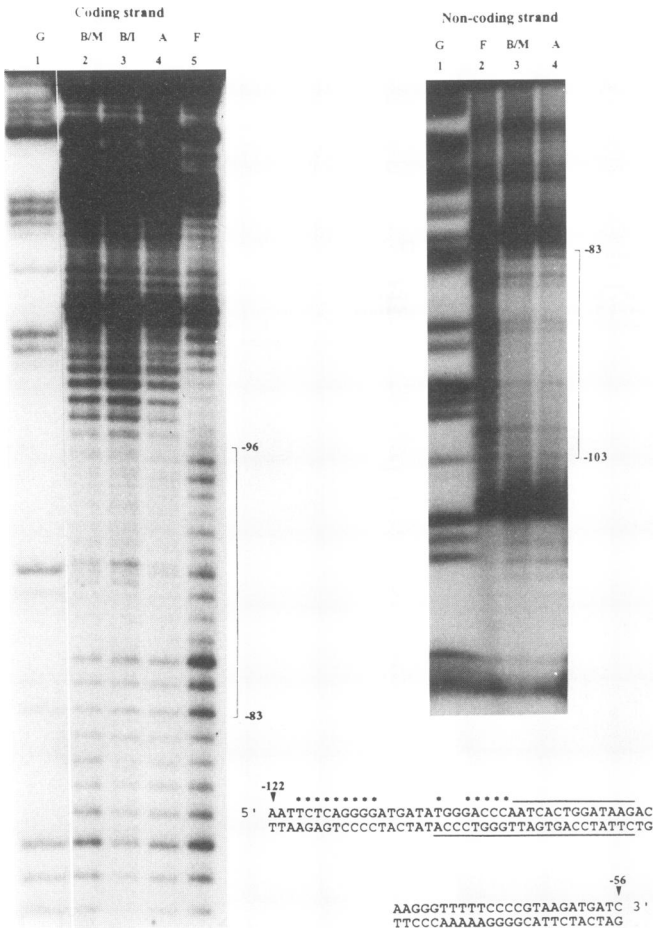

FIG. 3. Footprint of the DNA-protein complexes in situ, using 1,10-phenanthroline-copper ion. The DNA probe was radioactively labeled on either the coding strand or the noncoding strand. The DNA-protein complexes were separated from free probe (−122/−56) by electrophoresis in a 5% native polyacrylamide gel and then cleaved in situ by 1,10-phenanthroline-copper ion. The free probe and the complexes were excised, eluted, and fractionated in a 12% denaturing polyacrylamide gel. Lanes: G, G residue marker prepared by chemical cleavage; F, free probe; B/M, footprint of complex B formed in the presence of mock-infected HFF cell nuclear extracts; B/I, footprint of complex B formed in the presence of HCMV-infected HFF cell nuclear extracts; A, footprint of complex A formed in the presence of HCMV-infected HFF cell nuclear extracts. The regions in complexes A and B protected from chemical cleavage between positions −96 and −83 in the coding strand and between −83 and −103 in the noncoding strand are designated. At the bottom of the figure, the protein binding region is designated by solid lines, and enhanced cleavages of nucleotides due to DNA conformational changes are marked by stars.

complex and an additional more slowly migrating complex designated complex A were detected by the same DNA probes in HCMV-infected HFF cell nuclear extracts (Fig. 2B, lanes 3, 6, and 9). The nonradioactive DNA fragments at −122 to +11, −119 to −56, and −119 to −81 competed efficiently for the formation of complexes A and B (Fig. 2C, lanes 4 to 7; Fig. 2D, lanes 4 to 6 and 7 to 9, respectively). In contrast, the nonspecific 166-bp DNA fragment derived from plasmid pBR322 failed to compete (Fig. 2C, lanes 8 to 11). The DNA-protein complex migrating faster than A and B is due to a nonspecific DNA-binding protein in HCMV-infected cell nuclear extracts. These data suggested that DNA fragments bearing the CCAAT box dyad symmetry bound a protein factor(s) from mock- or HCMV-infected HFF cell nuclear extracts.

Footprint to the CCAAT box dyad symmetry. To determine more precisely the nucleotide contacts made by this DNA-binding protein, footprinting using the 1,10-phenanthroline-copper ion method was performed as described in Materials and Methods. Nucleotides in close contact with the protein factor(s) should be protected from chemical cleavage. Complexes A and B were fractionated in a 5% native polyacrylamide gel and subjected to cleavage in the gel. Free DNA or complexes A and B were excised, eluted, and fractionated in a 12% denaturing polyacrylamide gel. The footprints for complexes A and B were similar for both the coding and noncoding strands (Fig. 3). When compared with free DNA, the DNAs in complexes A and B were protected from chemical cleavage between positions −83 and −96 on the coding strand and −83 and −103 on the noncoding strand (Fig. 3). The CCAAT box dyad symmetry is located between bp −88 and −98. We concluded that the protein factor(s) in complexes A and B made contact within the CCAAT box dyad symmetry.

Effect of mutation of the CCAAT box dyad symmetry. To determine whether one CCAAT box was sufficient for protein binding, mutations were introduced by substitution of nucleotides into the CCAAT box dyad symmetry. A gel mobility shift assay was performed with wild-type DNA probe and competition by either wild-type or mutant DNA (M1, M2, and M3) (Fig. 4A). Wild-type, M1, and M2 DNAs competed for protein binding, but M3 DNA did not (Fig. 4B, lanes 3 to 6, respectively). The amount of probe bound is demonstrated in Fig. 4C. These data indicated that either CCAAT box within the CCAAT box dyad symmetry could compete; i.e., one CCAAT box in the M1 or M2 DNA was sufficient to bind the protein factor(s) in mock- or HCMV-infected HFF cell nuclear extracts. We were also able to demonstrate complexes A and B by gel mobility shift assay with radioactively labeled M1 or M2 DNA (data not shown). These data suggested that only one CCAAT box in CCAAT box dyad symmetry is occupied by the protein. The upstream CCAAT box did not bind the nuclear factor as well as the downstream CCAAT box (Fig. 4B and C, lanes 4 and 5, and data not shown).

A cellular transcription factor, NF-Y, in complexes A and B. A variety of different CCAAT box-binding proteins in the eucaryotic cell have been detected (2, 4, 6, 7, 9, 13, 21, 28, 32, 34, 39, 41, 43–45, 47, 49, 50, 52, 55). The molecular mass of the protein factor(s) bound to the CCAAT box dyad symmetry in the UL4 promoter was determined by UV cross-linking and denaturing gel electrophoresis. A cross-linked protein factor of approximately 69 kDa was detected (data not shown). NF-Y, a CCAAT box-binding factor, consists of a heterodimer of 40- and 32-kDa components (28). NF-Y binds to the CCAAT box of the major histocompatibility complex class II genes and to CCAAT boxes in other genes (4, 19, 28, 47). To determine the relationship between the protein factor(s) in complexes A and B and NF-Y, we synthesized a double-stranded oligonucleotide of the NF-Y target sequence and performed cold competition experiments. The wild-type sequence probe (positions −122 to −81) of the UL4 promoter was used to compete against the homologous sequence (Fig. 5, lanes 4 to 6) as well as the NF-Y target sequence (Fig. 5, lanes 7 to 9) in binding assays.

To test for NF-Y in complexes A and B, a polyclonal anti-NF-YB antibody was obtained (47). Antibodies were added to the DNA-protein binding reaction as described in Materials and Methods. Anti-NF-YB retarded the mobility of

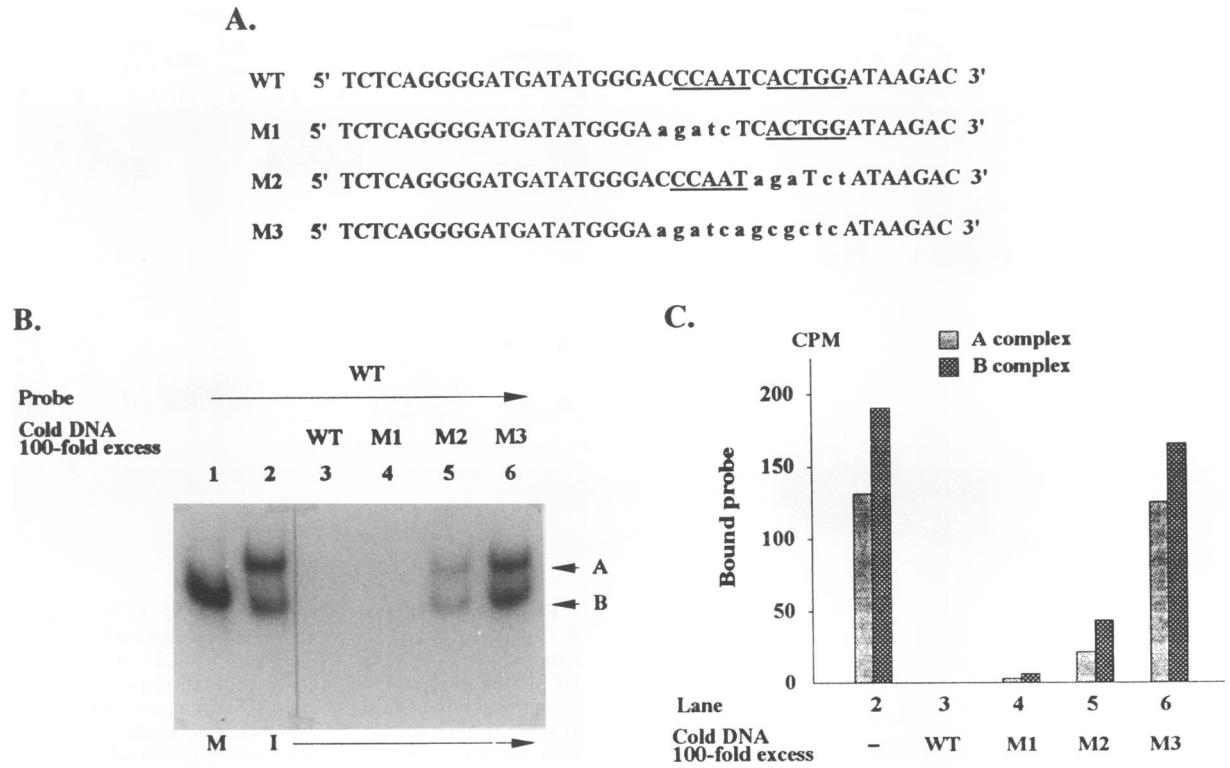

FIG. 4. Effects of mutations in the CCAAT box dyad symmetry on competition for either A or B DNA-protein complex formation. Gel mobility shift assays were performed as described in Materials and Methods. (A) DNA sequences. WT, the wild-type 39-bp DNA fragment containing the CCAAT box dyad symmetry; M1, the mutated 39-bp DNA fragment containing a pentapoint mutation, CCCAA to AGATC, which abolishes the upstream part of the CCAAT box dyad symmetry; M2, the mutated 39-bp DNA fragment containing mutations, CAC to AGA and GG to CT, which abolishes the downstream part of the CCAAT box dyad symmetry; M3, the mutated 39-bp DNA fragment containing a mutation, CCCAATCACTGG to AGATCAGCGCTC, which abolishes the entire CCAAT box dyad symmetry. The CCAAT box dyad symmetry is underlined, and the mutated nucleotides are shown in small letters. (B) Autoradiogram of cold competition assay. Lanes: 1, 39-bp WT DNA probe plus mock-infected HFF cell nuclear extracts; 2 to 6, 39-bp WT DNA probe plus HCMV-infected HFF cell nuclear extracts either with no added competitor (lane 2) or with added 100-fold molar excess of either WT, M1, M2, or M3 DNA (lanes 3 to 6, respectively). Complexes A and B are indicated by arrows. M, mock-infection. I, HCMV-infection. (C) Bar graph of the amount of bound probe without or with cold competitor WT, M1, M2, or M3 DNA. The counts per minute were determined by AMBIS image acquisition analysis. Lanes are the same as the lanes in panel B.

both complexes B and A to give a new complex designated C (Fig. 6, lanes 3 and 4 and lanes 7 and 8, respectively). Preimmune serum and an anti-IE2 polyclonal antibody (1218) had no effect on the complexes (Fig. 6, lanes 5 and 9 and lanes 6 and 10, respectively). A monoclonal anti-NF-YA antibody (anti-NF-Y 1a) (47) also reacted with complexes A and B (data not shown). These results indicated that the complexes contain protein(s) antigenically indistinguishable from NF-Y.

Effect of PAP on complex A. Complex A was detected only with HCMV-infected HFF cell nuclear extracts. The more slowly migrating DNA-protein complex could be due to the interaction of a cellular or virus-specified protein with NF-Y or to posttranslational modifications of NF-Y in the virus-infected cells. An analysis of [^{35}S]Met-Cys-labeled proteins associated with complexes A and B did not detect a unique protein species in complex A (29a). Therefore, we tested for phosphorylation of complex A. HCMV-infected HFF cell nuclear extracts were treated with PAP alone or PAP plus sodium molybdate ($NaMoO_4$, a PAP inhibitor) at room temperature for 30 min before adding the radioactive wild-type DNA sequence probe ($-122/-81$). Incubation of HCMV-infected HFF cell nuclear extracts with increasing amounts of PAP resulted in a decrease in the formation of complex A (Fig. 7, lanes 2 to 7). The decrease in the formation of complex A was inhibited by $NaMoO_4$ (Fig. 7, lane 8). Dephosphorylation of complex A increased the relative amount of complex B (Fig. 7B). The phosphorylation of the protein in complex A increased with time after infection and reached maximum levels by 48 h after infection (29a). These data suggested that complex A in the HCMV-infected HFF cell nuclear extracts resulted from phosphorylation of NF-Y and this modification of NF-Y occurs in HCMV-infected cells but not in mock-infected cells.

DISCUSSION

Even though the UL4 promoter has a consensus TATA box and a functional upstream CCAAT box dyad symmetry and TFII-D and NF-Y are constitutively present in human fibroblast cells, there was a lower level of transcription from the promoter when an upstream *cis*-acting negative element was present. In the absence of the upstream *cis*-acting negative element, our mutation analysis indicated that the CCAAT box dyad symmetry strongly contributed to the constitutive activity of the UL4 promoter, since transcription from the promoter with the CCAAT box region substituted by mutant DNA was

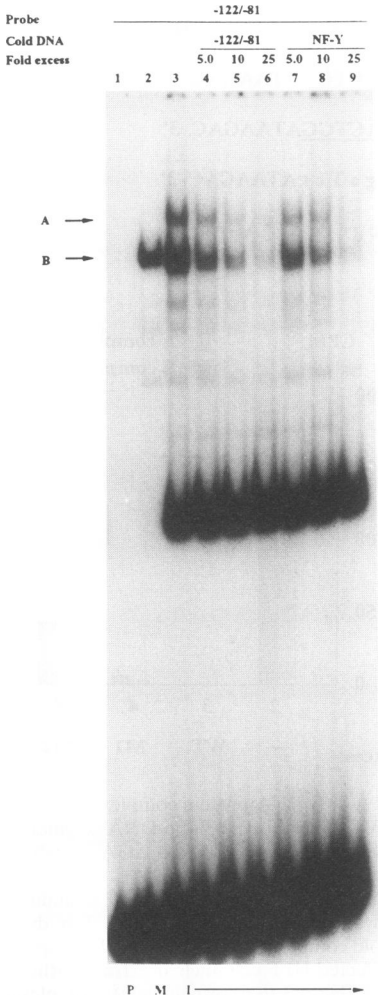

FIG. 5. Cold competition assay with the NF-Y target sequence. Gel mobility shift assays were performed as described in Materials and Methods. Lanes: 1, 42-bp DNA probe alone (−122/−81); 2, 42-bp DNA probe plus mock-infected HFF cell nuclear extracts; 3, 42-bp DNA probe plus HCMV-infected HFF cell nuclear extracts; 4 to 6, same as lane 3, plus increasing molar excesses of unlabeled DNA fragment −122/−81; 7 to 9, same as lane 3, plus increasing molar excesses of unlabeled double-stranded NF-Y target sequence representing the Y box from mouse major histocompatibility complex E_a promoter, 5′-ACTTTTAACCAATCAGAAAAAT-3′. Complexes A and B are indicated by arrows. P, probe; M, mock infection; I, HCMV infection.

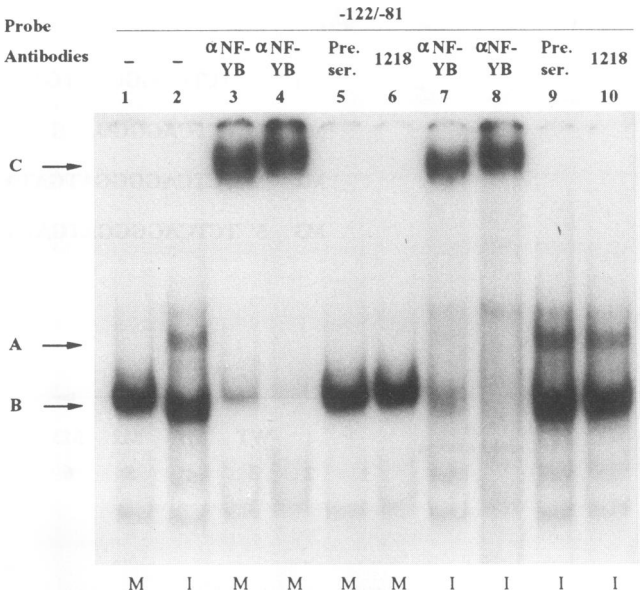

FIG. 6. A cellular transcription factor, NF-Y, in complexes A and B. Complexes A and B were formed by incubation for 30 min at room temperature of a 42-bp DNA probe (−122/−81) with mock- and HCMV-infected HFF cell nuclear extracts, respectively. Anti-NF-YB antibody, preimmune serum, or 1218 serum was added. After 30 min at room temperature, complexes were fractionated by electrophoresis in a 5% native polyacrylamide gel as described in Materials and Methods. Lanes: 1, 42-bp DNA probe plus mock-infected HFF cell nuclear extracts; 2, 42-bp DNA probe plus HCMV-infected HFF cell nuclear extracts; 3 and 4, same as lane 1, plus 0.5 (lane 3) or 1.0 (lane 4) μl of anti-NF-YB antibody; 5 and 6, same as lane 1, plus 1.0 μl of preimmune serum (lane 5) or 1218 (lane 6); 7 and 8, same as lane 2, plus 0.5 (lane 7) or 1.0 (lane 8) μl of anti-NF-YB antibody; 9 and 10, same as lane 2, plus 1.0 μl of preimmune serum (lane 9) or 1218 (lane 10). Complexes A, B, and C are indicated by arrows. Pre. ser., preimmune serum; 1218, anti-IE2 antibody; M, mock infection; I, HCMV infection.

weak. The viral IE2 protein negated the effect of the upstream *cis*-acting negative element. Preliminary data indicate that a cellular protein(s) binds to the negative element(s) (29a). Further experiments are necessary to identify the cellular protein(s).

NF-Y is a heterodimer of 40 (A component) and 32 (B component) kDa (28). Polyclonal antibody to the B component of NF-Y supershifted the DNA-protein complexes formed with either mock-infected or HCMV-infected HFF cell nuclear extracts. Monoclonal antibody to the A component of NF-Y also reacted with the complexes (data not shown). The data imply that only one CCAAT box within the CCAAT box dyad symmetry was required to bind NF-Y. Mutation of one CCAAT box in the dyad symmetry did not prevent protein from binding to the other when the flanking wild-type DNA sequence was present.

A number of CCAAT box binding proteins in eucaryotic cells, such as NF-Y (4, 19, 28, 39, 47), EF1 (20, 44, 52), C/EBP (2, 6, 7, 9, 32, 43, 55), NF-IL6 (1), NF-IL6 beta (34), heteromeric CBF (CBF-A and CBF-B) (28, 44, 45), NF1/CTF (13, 49), CP1 (13, 28), CP2 (13, 41), and HAP2/3/4 (21, 50), have been reported. NF-Y appears to be the same as CP1, EF1, and heteromeric CBF in higher eucaryotes and HAP2/3 in yeast cells (28). The specificity of binding by a CCAAT box-binding protein is determined by both the CCAAT box and the flanking DNA sequence.

In the HCMV-infected cells, the protein associated with complex A was posttranslationally modified by phosphorylation. Since a unique protein associated with complex A was not detected, the presence of the phosphate groups in complex A may have been sufficient to allow electrophoretic separation of the DNA-protein complexes designated A and B. In the infected cells, complex A was detectable as early as 6 h after infection and reached maximum levels by 48 h (data not shown). Detection of complex A did not require viral DNA synthesis (29a). The effect of phosphorylation of NF-Y on transcription from the UL4 promoter is not known. Phosphorylation of a CCAAT box-binding protein CTF, that binds to a CCAAT box upstream of the c-*myc* gene has been reported to

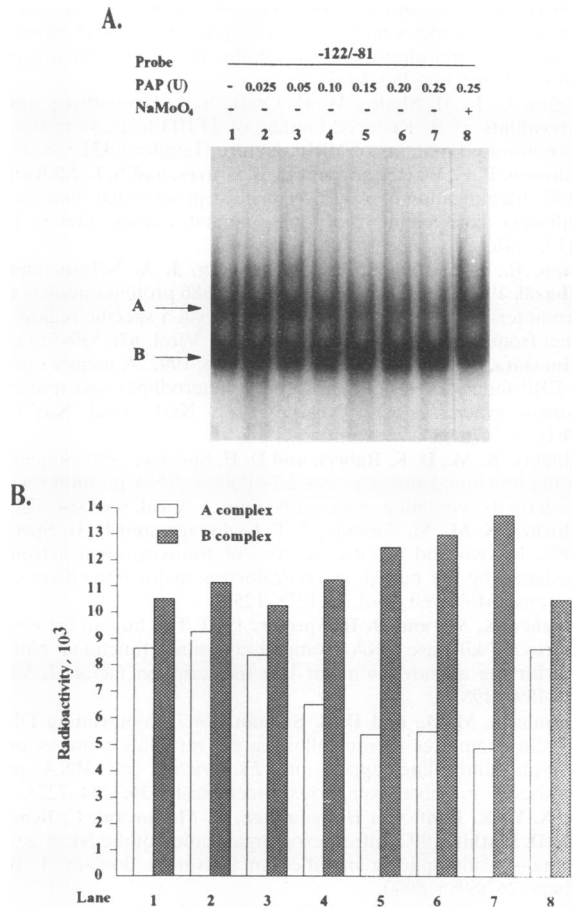

FIG. 7. Effect of PAP on complex A. HCMV-infected HFF cell nuclear extracts were incubated with either PAP or PAP plus $NaMoO_4$ at room temperature for 30 min, prior to DNA-protein binding reactions at 4°C for 30 min. Complexes were fractionated by electrophoresis in a 5% native polyacrylamide gel as described in Materials and Methods. (A) Effect of PAP on complex A. Lanes: 1, 42-bp (−122/−81) DNA probe plus HCMV-infected HFF cell nuclear extracts; 2 to 7, same as lane 1, plus 0.025, 0.05, 0.10, 0.15, 0.20, and 0.25 U of PAP, respectively; 8, same as lane 7, plus 2.6 mM $NaMoO_4$. Complexes A and B are indicated by arrows. (B) Radioactivity (in net counts) associated with complex A or B after PAP treatment. The lanes correspond to the lanes in panel A.

repress the level of downstream transcription (65). Therefore, it is possible that phosphorylation of NF-Y at late times after infection may down regulate the activity of the early viral promoter.

The IE proteins of HCMV strongly activate promoters with upstream CCAAT boxes, such as the heat shock promoter (24, 54), the HLA-A promoter (8), and the viral UL4 promoter (10, 46). When the CCAAT box was deleted from the above promoters, enhancement of transcription by the IE proteins was weaker (8, 23). Although the possibility remains that the IE2 viral protein forms complexes with NF-Y to enhance transcription, we were unable to detect IE2 in complex A by gel mobility shift assay with anti-IE2 antibodies (polyclonal antibody 1218 and monoclonal antibody 810).

The viral IE2 protein independently activates transcription from the viral promoters upstream of the HCMV UL4 and UL112-113 genes (10, 46, 57). The IE2 protein fails to activate independently of IE1 the viral promoters upstream of other HCMV genes such as UL83 (lower tegument), UL54 (DNA polymerase), and UL44 (DNA-binding protein) (16, 58, 59). The IE2 protein of HCMV may have multifunctional activities that can interact with a variety of positive as well as negative transcription factors. Like other viral transactivators, such as E1a (29), ICP4 (17, 30), VP16 (31, 61), Zta (40), and Tat (5, 51), IE2 can bind to the TATA-binding protein TBP in vitro (23, 33). The interaction of IE2 with TBP may explain why this viral protein is a promiscuous transactivator of homologous as well as heterologous viral and cellular promoters. The effect of IE2 on the HCMV UL4 early promoter containing only a TATA box was approximately a twofold increase in downstream expression. The effect of IE2 in the HCMV-infected cells may also be upstream of the TATA box and the transcription start site. By an unknown mechanism, the IE2 protein may be able to negate the effect of the upstream *cis*-acting negative element. The activity of the viral promoter required the positive *cis*-acting element in the presence of IE2, since the amount of downstream expression was significantly reduced when a mutant element was substituted. Transcription from the UL4 promoter was significantly enhanced by the CCAAT box dyad symmetry that binds NF-Y when the upstream *cis*-acting negative element was not present. Further investigation is necessary to determine why HCMV early promoters with *cis*-acting positive elements for binding eucaryotic transcription factors are repressed until HCMV IE protein synthesis.

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