JOURNAL OF VIROLOGY, June 1996, p. 4028–4037 Vol. 70, No. 6
0022-538X/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Human Cytomegalovirus Immediate-Early 2 (IE2) Protein Can Transactivate the Human *hsp70* Promoter by Alleviation of Dr1-Mediated Repression

RICHARD CASWELL, LINDA BRYANT, AND JOHN SINCLAIR*

Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge CB2 2QQ, United Kingdom

Received 10 October 1995/Accepted 14 March 1996

The immediate-early 1 and 2 (IE1 and IE2, respectively) proteins of human cytomegalovirus are known transcription factors, which regulate the expression of viral and cellular genes. Transcriptional activation by IE2 is dependent on the presence of a TATA motif in target promoters, and IE2 can interact directly with the TATA-binding protein (TBP) component of TFIID. TBP is known to be the target for transcriptional repression by the cellular Dr1 protein, and this factor has been shown to repress expression from the *hsp70* promoter in vivo. Since this promoter is up-regulated by IE2, we asked whether the effects of Dr1 can be overcome by IE2. We report here that IE2 can overcome Dr1-mediated repression of the *hsp70* promoter in vivo and that IE2 can interact with Dr1 in vivo and in vitro. We also demonstrate a previously unreported activity of Dr1, inhibition of DNA binding by TBP, and show that IE2 is able to overcome this inhibition in vitro, suggesting a mechanism for the TATA dependency of IE2-mediated *trans* activation.

The most abundantly expressed immediate-early (IE) proteins of human cytomegalovirus (HCMV) are the IE1 (IE72) and IE2 (IE86) proteins (3, 51). These proteins, which arise from differential splicing of transcripts from the major immediate-early promoter-enhancer (40, 49, 50), play a key role in the regulation of HCMV gene expression by autoregulating their own expression from the major IE promoter-enhancer (8, 40, 44, 50) and by *trans* activating the expression of a number of early and late HCMV genes (1, 29, 32, 37, 40, 50). In addition, both IE1 and IE2 are able to up-regulate the expression of a number of cellular genes, such as the c-*myc*, c-*fos*, and *hsp70* genes (5, 11, 15, 20, 22, 24, 39), as well as the human immunodeficiency virus long terminal repeat (2, 16, 52). We have recently shown that IE1, but not IE2, can also *trans* activate the TATA-less human DNA polymerase α promoter (21). The inability of IE2 to *trans* activate this promoter is consistent with our previous observation that IE2 activates the human immunodeficiency virus long terminal repeat and the *hsp70* promoter by a TATA box-dependent mechanism (19); moreover, we and others have shown that IE2 can interact directly with TATA-binding protein (TBP), the TATA box-binding protein of the general transcription factor TFIID, via C-terminal regions of IE2 which are known to be required for transcriptional regulation (6, 14, 19). Conversely, IE1 exhibits no such requirement for a TATA box motif in *trans* activating these promoters (19, 21).

Recent studies investigating the role of cellular factors in the regulation of gene expression have identified a number of transcriptional repressors that act by diverse mechanisms. Some factors, such as the retinoblastoma protein RB, act by binding to and inactivating site-specific transcription factors, such as E2F (12, 23), whereas other factors, such as topoisomerase I (Dr2) and Dr1 (NC2), exert their effects by acting upon components of the basal transcription complex (25, 30, 38). In particular, the ability of Dr1 to repress basal transcription appears to be mediated by the interaction of Dr1 with TBP (25, 53). Dr1 exists in two forms within a cell; one of these is a phosphoprotein, while the other appears to be posttranslationally unmodified, as it comigrates on sodium dodecyl sulfate (SDS)-polyacrylamide gels with bacterially expressed recombinant Dr1 (rDr1) protein (25). Moreover, the two forms behave differently during chromatographic fractionation of nuclear extracts; the phosphorylated form copurifies with the general transcription factor TFIIE, while the unmodified form is associated with TFIID (25). Both the phosphorylated form of human Dr1 (hDr1) and rDr1, which resembles the putatively unmodified form of cellular Dr1, repress transcription in vitro, but they appear to act by different mechanisms. hDr1 is able to interact stably with DNA-bound TBP and to prevent the entry of TFIIA and/or TFIIB into the preinitiation complex, while rDr1 appears to bind to the TFIIA-TBP-DNA complex and to prevent the entry of TFIIB (25). However, although rDr1 does not interact stably with the TBP-DNA complex, rDr1 can bind to TBP in the absence of DNA (25, 53), raising the possibility that rDr1 also influences preinitiation complex formation at the earlier stage of the interaction of TBP with promoter DNA. The ability of different forms of Dr1 to interact with either free or DNA-bound TBP appears to be regulated only by phosphorylation, as dephosphorylation of hDr1 results in the inability of this protein to interact with DNA-bound TBP, i.e., conversion to rDr1-like activity (25, 30). This is consistent with the observation that the unmodified form of cellular Dr1 is associated with TFIID in nuclear extracts (25).

Since Dr1 exerts transcriptional repression by acting upon TBP while IE2 appears conversely to activate gene expression by functional and physical interactions with TBP, we asked whether IE2 is able to overcome Dr1-mediated inhibition of gene expression. Such a derepression mechanism has recently been demonstrated for the adenovirus E1A protein (33); this is of particular interest since E1A and IE2 are known to be alike in their ability to interact with similar regions of TBP (6, 35) and in their requirement of a TATA motif for *trans* activation of target promoters (19, 45). We report here that like E1A, IE2 is able to overcome Dr1-mediated repression of the *hsp70*

* Corresponding author. Mailing address: Department of Medicine, University of Cambridge School of Clinical Medicine, Box 157, Level 5, Addenbrooke's Hospital, Hills Rd., Cambridge CB2 2QQ, United Kingdom. Phone: 44 1223 336850. Fax: 44 1223 336846. Electronic mail address: js@mole.bio.cam.ac.uk.

promoter in vivo. Unlike E1A, however, IE2 does not appear to disrupt the interaction between DNA-bound TBP and hDr1 in vitro but alleviates a previously unreported activity of rDr1, inhibition of DNA binding by TBP, suggesting that one method by which IE2 activates transcription is by a novel derepression mechanism.

MATERIALS AND METHODS

Cell lines and transfection. IBR cells, a simian virus 40 (SV40) T-antigen-immortalized human fibroblast cell line semipermissive for HCMV infection (20), were cultured in minimal essential medium supplemented with 10% serum. Cells were transfected by calcium phosphate coprecipitation with DNAs, as detailed in figure legends, and assayed at 48 h posttransfection for chloramphenicol acetyltransferase (CAT) activity by standard methods. CAT activity, expressed as percent conversion of chloramphenicol, was quantified from thin-layer chromatography plates by using a Hewlett-Packard Instant Imager; the data presented were derived from three independent experiments.

Plasmids. cDNA for hDr1 was synthesized by reverse transcription-PCR of mRNA isolated from HeLa cells. The primers used were derived from the published sequence of Dr1 (25): sense primer, 5′-CAGGAAGGTACTATG GCTTCC-3′, and antisense primer, 5′-ATAGAAACTCAGCTGGTGAA-3′. This PCR product was then used as the template in a second PCR with primers to introduce restriction sites flanking the Dr1 cDNA; different sense primers were used to generate either wild-type Dr1 (5′-TCTGTTGGATCCGCTATAG GTACT**ATGGCT**-3′; the Dr1 coding sequence is in bold) or an epitope-tagged derivative of Dr1 in which the primer introduced sequences coding for an influenza virus hemagglutinin (HA) epitope (13) (5′-TCTGTTGGATCC*ATGGGC TACCCATACGACGTCCCAGATTACGCAGTC***GCTTCCTCGTCTGGCAACG AT**-3′; the HA epitope coding sequence is in italics, and the Dr1 coding sequence is in bold). In both cases, the sequence of the antisense primer was 5′-GCTAT-GAATTCCGAAACTCAGCTGGTGAAT-3′. The resulting cDNAs were blunt ended with T4 DNA polymerase and cloned into the *Sma*I site of pBluescript II KS to generate pBS-Dr1 and pBS-Dr1^{HA}, coding for wild-type and epitope-tagged Dr1, respectively, so that transcription of wild-type Dr1 was under the control of the T7 promoter and that of epitope-tagged Dr1 was controlled by the T3 promoter. The integrity of each clone was confirmed by dideoxynucleotide sequencing, using standard techniques (43). Plasmid pHK3-Dr1^{HA} was made for the expression of epitope-tagged Dr1 in eukaryotic cells by cloning a *Bam*HI fragment from pBS-Dr1^{HA} into the *Bam*HI site of the SV40-based expression vector pHK3 (18). Plasmids pHM121 (for eukaryotic expression of HCMV IE2), pHKIE2mut (for the expression of a deletion mutant of IE2 lacking amino acids 290 to 490), and the *hsp70*-CAT reporter plasmid pHC-77-CAT have been described previously (17, 41, 45).

Expression and purification of recombinant and human proteins. Plasmid pQE10IE2 (for the expression of histidine-tagged IE2) was a kind gift of T. Stamminger (Erlangen, Germany), and purified recombinant IE2 was prepared exactly as described previously (34). Plasmid pGEX2TK-Dr1 was constructed by ligation of a *Bam*HI-*Hin*cII Dr1 cDNA fragment from pBS-Dr1 with *Bam*HI-linearized pGEX2TK (28), followed by filling in of the 3′ *Bam*HI site of the vector with T4 DNA polymerase and recircularization of the recombinant plasmid. Plasmid pGEX2TK-IE2-N, coding for a fusion of glutathione *S*-transferase (GST) to amino acids 1 to 290 of IE2, was made by digesting pGEX2TK-IE2 (6) with *Xho*I and *Eco*RI, filling in of cohesive ends with T4 DNA polymerase, and recircularization of the vector-IE2-N fragment. Plasmid pGEX2TK-IE1 (for the expression of a GST-IE1 fusion) has been described previously (6); pGEX2T-Dr1Δ85-99 (for the expression of an internal deletion mutant of Dr1) was a kind gift of D. Reinberg (University of Medicine and Dentistry of New Jersey, Piscataway) (53). Purified rDr1 and purified recombinant Dr1Δ85-99, IE2-N, and IE1 were obtained by thrombin cleavage of purified GST fusion proteins as previously described (47). All proteins were dialyzed at 4°C against buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 20% (vol/vol) glycerol prior to use in electrophoretic mobility shift assays (EMSAs). Purified TBP, expressed in *Escherichia coli* from a human cDNA clone, was obtained from Promega. Highly purified human TFIIA and hDr1-NC2 preparations were kindly provided by H. T. M. Timmers (University of Utrecht, Utrecht, The Netherlands). In short, TFIIA was isolated from whole-cell extracts of HeLa cells by chromatography over phosphocellulose, DEAE-Sepharose, ammonium sulfate precipitation, Superdex 200 gel filtration, phenyl-Superose, and MonoQ columns. hDr1-NC2 was also purified from HeLa cell extracts by chromatography over phosphocellulose, DEAE-Sephacel, MonoQ, and Superdex 200 columns. TFIIA and hDr1-NC2 activities were assayed by the abilities of proteins to form specific complexes with TBP in EMSAs.

EMSAs. For experiments with the *hsp70* promoter, the probe contained the TATA motif and flanking regions from the human *hsp70* promoter (nucleotides −46 to −2) (5′-GGTCTCCGTGACGACTTATAAAACCCCAGGGGCAAGC GGTCCGG-3′; only the top strand is shown). Various proteins, as indicated in individual figure legends, were preincubated on ice for 5 min in a reaction mixture (volume, 24 μl) containing 10 mM HEPES (pH 7.9), 4 mM $MgCl_2$, 5 mM ammonium sulfate, 8% (vol/vol) glycerol, 2% (wt/vol) polyethylene glycol 8000, 50 mM KCl, 5 mM β-mercaptoethanol, 0.2 mM EDTA, and 0.5 μg of poly(dG) · poly(dC), as described by Maldonado et al. (36), before the addition of 1 μl of labelled probe containing 0.3 pmol (approximately 10 ng) of DNA. Incubation was then continued on ice for 30 min, and complexes were separated by electrophoresis on 4% polyacrylamide gels using Tris-borate-EDTA buffer (40 mM Tris, 40 mM boric acid, 1 mM EDTA [pH 8.2]). Electrophoresis was performed at 70 V at ambient temperature, and complexes were located by autoradiography of dried gels. Experiments with the adenovirus major late promoter (AdMLP) used a probe containing the AdMLP TATA motif and flanking regions (nucleotides −40 to +6) (5′-AAGGGGGGCTATAAAAGGGGGTG GGGGCGCGTTCGTCCTCACTC-3′; only the top strand is shown). The conditions used were identical to those described above. Competition assays were carried out by using either an unlabelled *hsp70* TATA probe or a double-stranded oligonucleotide containing a consensus PEA3 site (5′-TCGAGCAG GAAGTTCGA-3′; only the top strand is shown); as indicated, competitor DNA was preincubated with TBP for 5 min prior to the addition of labelled probe. Control experiments utilized a partially purified NF-1 DNA binding domain expressed in baculovirus-infected insect cells, which was a kind gift of R. Hay (University of St. Andrews). The probe used was a fragment from the human *hsp70* promoter containing sequences from nucleotides −57 to −77, covering the CCAAT motif, and reactions were carried out under the exact conditions described by Jones et al. (26), with the factors detailed in the appropriate figure legend.

Dephosphorylation of hDr1. hDr1 (25 ng) was dephosphorylated by incubation at 37°C for 30 min in a reaction mixture containing 50 mM Tris (pH 9.3), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine, 1 μg of bovine serum albumin (BSA), and (in test samples) 15 U of calf intestinal alkaline phosphatase (CIP; Boehringer Mannheim) in a total volume of 15 μl. Reactions were stopped by quenching on ice, and then the entire sample was used immediately in EMSA reactions as described above. In the case of mock-treated samples, CIP was added subsequent to quenching on ice but prior to use in EMSAs.

Double immunoprecipitation assay. Approximately 5×10^6 HTB96 (U2OS) cells, a human osteosarcoma cell line semipermissive for HCMV infection (46), were transfected with 20 μg of pHK3-Dr1^{HA} by calcium phosphate coprecipitation; 24 h posttransfection, cells were either left uninfected or infected overnight with 5 PFU of HCMV strain AD169 per cell; infected and control uninfected cells were then labelled overnight with 0.25 mCi of [^{35}S]methionine in methionine-free medium containing 1% serum. Cells were harvested and lysed, and extracts were used in double immunoprecipitation assays exactly as previously described (17). Primary immunoprecipitations were carried out by using either 2 μg of monoclonal antibody (MAb) to epidermal growth factor receptor (R-1; Santa Cruz Biotechnology); 2 μg of 12CA5 antibody, which recognizes the HA epitope tag of Dr1^{HA} (Boehringer Mannheim); or 2 μl of anti-IE1/2-specific MAb ID6-6. All secondary immunoprecipitations were performed with 2 μl of ID6-6. Immunoprecipitated proteins were detected by autoradiography after SDS-polyacrylamide gel electrophoresis (PAGE) of washed protein A-Sepharose complexes.

Yeast two-hybrid interaction assay. Experiments were carried out by using the Matchmaker yeast two-hybrid system (Clontech) after the modification of expression vectors as follows. Plasmid pGBT9 was digested with *Eco*RI and *Bam*HI and then ligated with a double-stranded adapter (top strand, 5′-AATTTGGG ATCCCCGGGAATTC-3′; bottom strand, 5′-GATCGAATTCCCGGGGATC CCA-3′). The recombinant plasmid pGBT10 was then digested with *Bam*HI and *Eco*RI and ligated with a *Bam*HI-*Eco*RI IE2 cDNA fragment from pGEX3X-IE2 (6) to generate pGBT10-IE2. Similarly, pGBT10-IE1 was made by cloning the *Bam*HI-*Eco*RI IE1 cDNA fragment from pGEX3X-IE1 (6) into pGBT10. Plasmid pGAD424 was also digested with *Eco*RI and *Bam*HI and then ligated with a double-stranded adapter (top strand, 5′-AATTTGGATCCCCGGG AATTC-3′; bottom strand, 5′-GATCGAATTCCCGGGGATCCA-3′) to yield pGAD426. Plasmid pGAD426-Dr1^{HA} was then made by ligation of a *Bam*HI fragment from pBS-Dr1^{HA} into *Bam*HI-digested pGAD426. All constructs were verified by sequencing. The other plasmids used were supplied with the Matchmaker kit. Yeast transformations and β-galactosidase assays were then carried out in *Saccharomyces cerevisiae* SFY526 exactly as described by the kit manufacturer.

In vitro protein-protein binding assay. Plasmids for in vitro expression of TBP, IE1, full-length IE2, the N terminus of IE2 (amino acids 1 to 290), various truncations of the C terminus of IE2 (amino acids 290 to 390, 290 to 504, 290 to 542, and 290 to 579), and gelsolin have been described elsewhere (6, 19). Plasmids pCJC45, pCJC47, and pCJC48 (for the expression of amino acids 313 to 579, 388 to 579, and 428 to 579 of IE2, respectively) were gifts of G. Hayward (Johns Hopkins University, Baltimore, Md.) (9). These were then used in binding assays with various GST fusion proteins, immobilized on glutathione-Sepharose beads, exactly as previously described (6).

RESULTS

IE2 can overcome Dr1-mediated repression in vivo. Since Dr1 is believed to repress basal transcription in a TBP-dependent mechanism, we asked whether HCMV IE2, which is

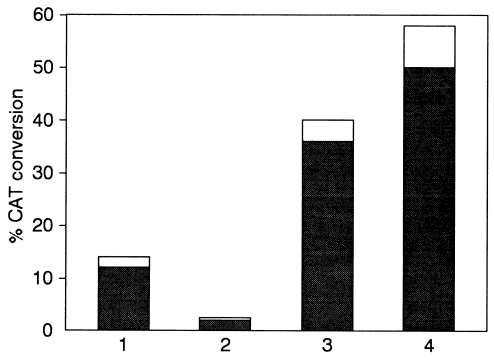

FIG. 1. IE2 can overcome Dr1-mediated repression in vivo. IBR cells were cotransfected with 2.5 μg of the *hsp70* CAT reporter plasmid pHC-77-CAT, along with 4.5 μg of the SV40 promoter control plasmid pHK3 (lane 1), 0.5 μg of pHK3-Dr1^{HA} (expressing epitope-tagged Dr1) and 4 μg of pHK3 (lane 2), 4 μg of pHM121 (expressing IE2) and 0.5 μg of pHK3 (lane 3), or 4 μg of pHM121 and 0.5 μg of pHK3-Dr1^{HA} (lane 4). In each lane, the solid bar represents the mean CAT activity (expressed as percent conversion) from three independent experiments and the open bar shows the standard deviation. The mean activities ± standard deviations were 12% ± 2%, 2% ± 0.5%, 36% ± 4%, and 50% ± 8% for lanes 1 through 4, respectively.

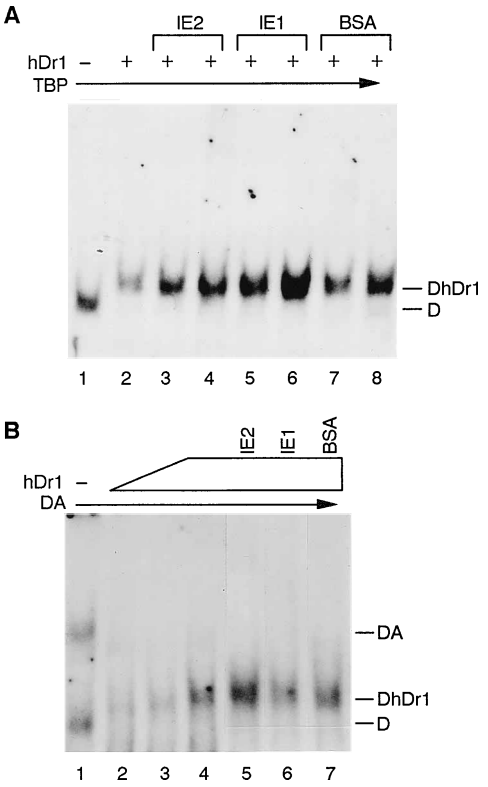

FIG. 2. HCMV IE proteins do not disrupt the DhDr1 complex. (A) EMSA binding reaction mixtures contained 0.5 ng of TBP alone (lane 1) or 0.5 ng of TBP and 20 ng of hDr1 (lanes 2 through 8), along with 75 ng of IE2 (lane 3), 200 ng of IE2 (lane 4), 75 ng of IE1 (lane 5), 200 ng of IE1 (lane 6), 75 ng of BSA (lane 7), or 200 ng of BSA (lane 8). The positions of the D and DhDr1 complexes are indicated on the right. (B) All reaction mixtures contained 0.5 ng of TBP and 20 ng of TFIIA. Lanes 2 through 4 also contained 10, 20, and 30 ng of hDr1, respectively, while lanes 5 through 7 also contained 20 ng of hDr1 along with 75 ng of either IE2 (lane 5), IE1 (lane 6), or BSA (lane 7). The positions of the D, DA, and DhDr1 complexes are indicated on the right.

known to require a functional TATA box motif for activation and which interacts directly with TBP, can overcome the effects of Dr1 in vivo. In transient-transfection experiments using an *hsp70* reporter plasmid, cotransfection of the Dr1 expression vector resulted in a sixfold decrease in expression (Fig. 1; compare lanes 1 and 2), consistent with other reports (33, 53), while IE2 in isolation activated expression by threefold (lane 3), again in agreement with previous observations (19, 20). However, coexpression of both IE2 and Dr1 (lane 4) resulted in a level of CAT activity similar to that seen in the presence of IE2 alone and represented approximately 25-fold activation compared with the activity observed in the presence of Dr1 alone. This suggests that IE2 is not simply *trans* activating the residual activity of the *hsp70* promoter which remains in the presence of Dr1 but actually is able to overcome Dr1-mediated repression of the *hsp70* promoter in vivo, as has recently also been demonstrated for adenovirus E1A (33). This effect cannot be explained simply by IE2 causing a reduction in the level of Dr1 expression. Firstly, IE2 is known to be a modest activator of the SV40 early promoter-enhancer (40), which controls the expression of Dr1 in pHK3-Dr1^{HA}; secondly, immunoprecipitation of epitope-tagged Dr1 from transfected cells showed similar levels of this protein in the presence or absence of cotransfected IE2 (data not shown). It is interesting that the activity observed in the presence of both IE2 and Dr1 was in fact slightly higher, allowing for experimental variation, than that seen in the presence of IE2 alone. Merino et al. (38) have observed that DNA topoisomerase I-Dr2 can act as a repressor of basal transcription in the absence of activators but functions as a coactivator when other activators are present; while the apparent synergy we observed between Dr1 and IE2 in activating the *hsp70* promoter was relatively weak, it is possible that Dr1 also functions as a coactivator with IE2 under the conditions used in these experiments.

IE2 does not displace hDr1 from DNA-bound TBP. Kraus et al. (33) have shown by EMSA that adenovirus E1A is able to disrupt the interaction between DNA-bound TBP and hDr1 in vitro in either the presence or absence of TFIIA. In the light of the ability of both HCMV IE2 and adenovirus E1A to overcome the effects of Dr1 in vivo, we asked whether HCMV IE2 exhibits an activity similar to that of E1A in vitro. In EMSAs using the *hsp70* promoter TATA box as a probe, hDr1 was able to interact stably with DNA-bound TBP, forming the so-called DhDr1 complex (Fig. 2A, lane 2), as previously reported for the AdMLP (25, 33). However, unlike the recently reported activity of E1A, neither IE2 nor IE1 was able to disrupt the DhDr1 complex under these conditions (Fig. 2A, lanes 3 to 6); in fact, both proteins caused a modest nonspecific stabilization of the DhDr1 complex, as discussed below (see Fig. 5).

As previously shown in EMSAs using the AdMLP (25, 33), hDr1 disrupts the interaction of TFIIA with TBP bound to the *hsp70* promoter; Fig. 2B shows that the addition of hDr1 to binding reaction mixtures containing TBP and TFIIA resulted in the loss of the TFIIA-TBP-DNA complex (DA complex) and formation of the DhDr1 complex (lanes 2 to 4). Again, IE2 neither disrupted the DhDr1 complex nor restored the DA complex in these experiments (Fig. 2B, lane 5), in contrast to the reported ability of E1A to restore DA complex formation in the presence of hDr1 (33).

rDr1 and dephosphorylated hDr1 inhibit DNA binding by TBP. Since IE2 did not appear to disrupt the interaction of hDr1 with DNA-bound TBP in vitro, we sought to investigate what other activity of IE2 might account for its ability to overcome Dr1-mediated repression of the *hsp70* promoter in vivo. Bacterially expressed rDr1 has been shown to repress basal transcription in vitro and to interact with free TBP but not

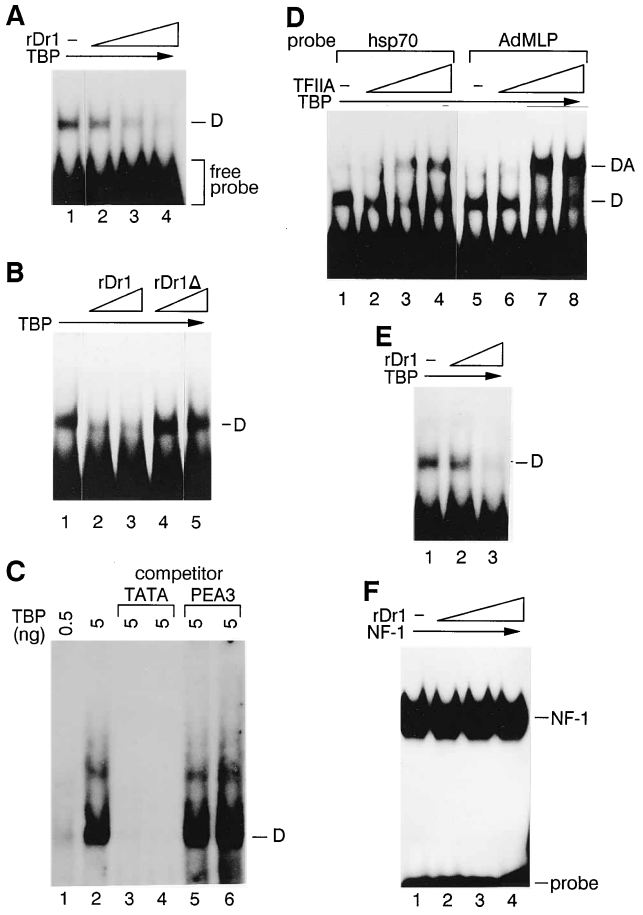

FIG. 3. rDr1 inhibits DNA binding by TBP. (A) EMSA binding reaction mixtures contained either 0.5 ng of TBP alone (lane 1) or 0.5 ng of TBP and either 7.5, 20, or 100 ng of rDr1 (lanes 2 to 4, respectively). (B) All reaction mixtures contained 0.5 ng of TBP and either no rDr1 (lane 1), wild-type rDr1 (40 and 80 ng [lanes 2 and 3, respectively]), or rDr1Δ85-99 (40 and 80 ng [lanes 4 and 5, respectively]). (C) Binding reaction mixtures contained either 0.5 ng of TBP (lane 1) or 5 ng of TBP (lanes 2 through 6); binding to probe was competed with unlabelled *hsp70* TATA probe (10- and 20-fold molar excesses of cold competitor [lanes 3 and 4, respectively]) or by a PEA3 consensus oligonucleotide (10- and 20-fold molar excesses of cold competitor [lanes 5 and 6, respectively]). (D) All reaction mixtures contained 0.5 ng of TBP, along with 3 (lanes 2 and 6), 10 (lanes 3 and 7), or 20 (lanes 4 and 8) ng of TFIIA. Reactions were carried out with either the *hsp70* (lanes 1 through 4) or AdMLP (lanes 5 through 8) TATA probe. (E) Reaction mixtures contained 0.5 ng of TBP and either no rDr1 (lane 1), 10 ng of rDr1 (lane 2), or 30 ng of rDr1 (lane 3); reactions were carried out with the AdMLP TATA probe. (F) Reaction mixtures contained 0.2 μl (100 ng of total protein) of partially purified NF-1 DNA binding domain from baculovirus-infected cells and either no rDr1 (lane 1) or 50, 100, or 200 ng of rDr1 (lanes 2 through 4, respectively). The positions of complexes and probes are indicated on the right of relevant gels.

DNA-bound TBP (25, 53). Interestingly, the unmodified form of cellular Dr1, which comigrates on an SDS-PAGE gel with rDr1, copurifies from HeLa nuclear extracts with the TBP-containing TFIID complex (25), suggesting that this form of cellular Dr1 also interacts directly with TBP prior to its binding to promoter DNA. We asked therefore whether nonphosphorylated forms of Dr1 (rDr1 or dephosphorylated hDr1) have any effect on the ability of TBP to bind to DNA. The results shown in Fig. 3A demonstrate that rDr1 strongly inhibited the ability of TBP to bind to the *hsp70* promoter. The observation of this novel activity of rDr1 relies on the ability to clearly visualize the TBP-DNA complex (the so-called D complex) and therefore would not be observed under conditions in which the D complex is not normally visualized, such as those used by Inostroza et al. (25). Furthermore, this inhibition of D complex formation is dependent on the ability of Dr1 to bind to TBP, as a deletion mutant of Dr1 lacking amino acids 85 to 99, which are required for interaction with TBP (53), did not prevent formation of the D complex (Fig. 3B). Since both the wild-type and mutant rDr1 were expressed in *E. coli* and purified under identical conditions, this experiment also excludes the possibility that an unspecified bacterial contaminant is responsible for the abrogation of DNA binding by TBP. The results of control experiments confirmed that the TBP-DNA complexes we observed were indeed due to specific binding by TBP to the *hsp70* TATA probe (Fig. 3C); at the higher levels of TBP used in this experiment, a more slowly migrating complex can also be seen in lanes 2, 5, and 6, as has been reported previously (27). The addition of TFIIA to binding reaction mixtures resulted in formation of the DA complex with either the *hsp70* or AdMLP TATA probe (Fig. 3D), consistent with the known ability of TFIIA to interact with DNA-bound TBP (4, 36, 42). The results of similar experiments confirmed that the addition of TFIIB to binding reaction mixtures containing TBP resulted in the formation of the DB complex (data not shown). Note that in addition to its inhibition of TBP binding to the *hsp70* probe, rDr1 was able to repress DNA binding by TBP to the AdMLP promoter (Fig. 3E); however, this inhibition was specific for TBP, as rDr1 had no effect on the binding of the NF-1 DNA binding domain to a DNA probe containing a CCAAT motif (Fig. 3F) or on the ability of the purified NF-κB p50 subunit to bind to a κB motif (data not shown).

Dephosphorylation of hDr1 with CIP has been shown to prevent formation of the DhDr1 complex in EMSAs (25, 30). The apparent inability of dephosphorylated hDr1 to interact stably with the TBP-DNA complex resembles the activity of rDr1, which interacts with free, but not DNA-bound, TBP (25, 53). Since we observed that rDr1 can actually inhibit the binding of TBP to DNA, we asked whether dephosphorylated hDr1 also possesses this activity of rDr1. As described previously (25, 30), dephosphorylation was carried out by incubation of hDr1 with CIP, with reaction mixtures then being used in EMSAs. Note that under the conditions used, both the D and DhDr1 complexes were visible in the mock-treated hDr1 sample (Fig. 4, lane 3). The effects of CIP treatment of hDr1 were twofold. Firstly, there was a marked decrease in the intensity of the DhDr1 complex, compared with that seen in the mock-treated hDr1 sample (Fig. 4; compare lanes 3 and 4). This is consistent with previous observations (25, 30) that only phosphorylated hDr1, not dephosphorylated hDr1, can interact stably with DNA-bound TBP to form the DhDr1 complex. The persistence of a low level of DhDr1 complex in the CIP-treated hDr1 sample (Fig. 4, lane 4) indicated that dephosphorylation of hDr1 was not complete; however, more stringent treatment, for instance by prolonged incubation at 37°C, resulted in the loss of activity of hDr1, as measured by its ability to supershift the D complex, even in the absence of CIP (data not shown). The second effect of CIP treatment was to abolish the D complex (Fig. 4; compare lanes 3 and 4). If dephosphorylated hDr1 had no effect on DNA binding by TBP, it would be expected that as dephosphorylation proceeded, there would be a decrease in the intensity of the DhDr1 band and a concomitant increase in the intensity of the D complex, since there would be less hDr1 capable of binding to and supershifting the TBP-DNA complex (the same effect would be observed if there was loss of total hDr1 protein, for instance, by protease contamination of the CIP preparation). However, the observed decreases in the intensities of both the DhDr1 and D complexes indicate that dephosphorylation of hDr1 by CIP not

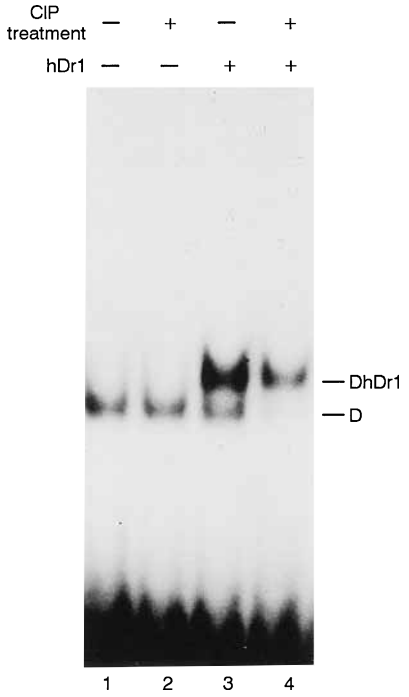

FIG. 4. Dephosphorylated hDr1 inhibits DNA binding by TBP. All EMSA reaction mixtures contained 0.5 ng of TBP and dephosphorylation samples containing either no hDr1 (lanes 1 and 2), 25 ng of mock-treated hDr1 (lane 3), or 25 ng of CIP-treated hDr1 (lane 4). Dephosphorylation reaction mixtures in lanes 2 and 4 were incubated with 15 U of CIP at 37°C for 30 min before use in EMSAs, whereas mock-treated samples (lanes 1 and 3) received 15 U of CIP subsequent to incubation at 37°C and immediately prior to EMSA reactions. The positions of D and DhDr1 complexes are indicated on the right.

only results in the inability of hDr1 to bind to the D complex but actually inhibits the binding of TBP to DNA. As discussed above, this effect would be seen only under conditions in which the D complex was clearly visible. Inspection of the levels of free probe at the bottom of the gel indicates that the effects observed were not due simply to loading anomalies, while control reactions show that preincubation of CIP itself at 37°C in the absence of hDr1 had no effect on the ability of TBP to bind to DNA (Fig. 4, lanes 1 and 2). The effects of CIP treatment of hDr1 were reduced either if lower concentrations of CIP were used or if CIP activity was inhibited by the inclusion of 2 mM sodium orthovanadate–5 mM EDTA in dephosphorylation reaction mixtures (data not shown).

IE2, but not IE1, can alleviate rDr1-mediated inhibition of DNA binding by TBP. As both bacterially expressed rDr1 and dephosphorylated hDr1 inhibit DNA binding by TBP, this suggests a mechanism by which the unmodified cellular form of Dr1 contributes to transcriptional repression and therefore a potential point at which HCMV IE2 is able to overcome Dr1-mediated repression in vivo. In fact, IE2 specifically restored DNA binding to the *hsp70* TATA probe by TBP in the presence of rDr1 (Fig. 5A, lanes 1 through 4), whereas this was not seen with either a truncated IE2 protein (containing amino acids 1 to 290 of IE2; lanes 5 and 6) or IE1 (lanes 7 and 8). This effect cannot be explained by preferential stabilization of the D complex by IE2, as in the absence of rDr1 all the factors tested here gave only modest and comparable stabilization of the D complex (Fig. 5B); indeed, the stabilization caused by IE2 was lower than that seen with the other factors. This stabilization of the D complex is a nonspecific phenomenon (BSA gave rise to

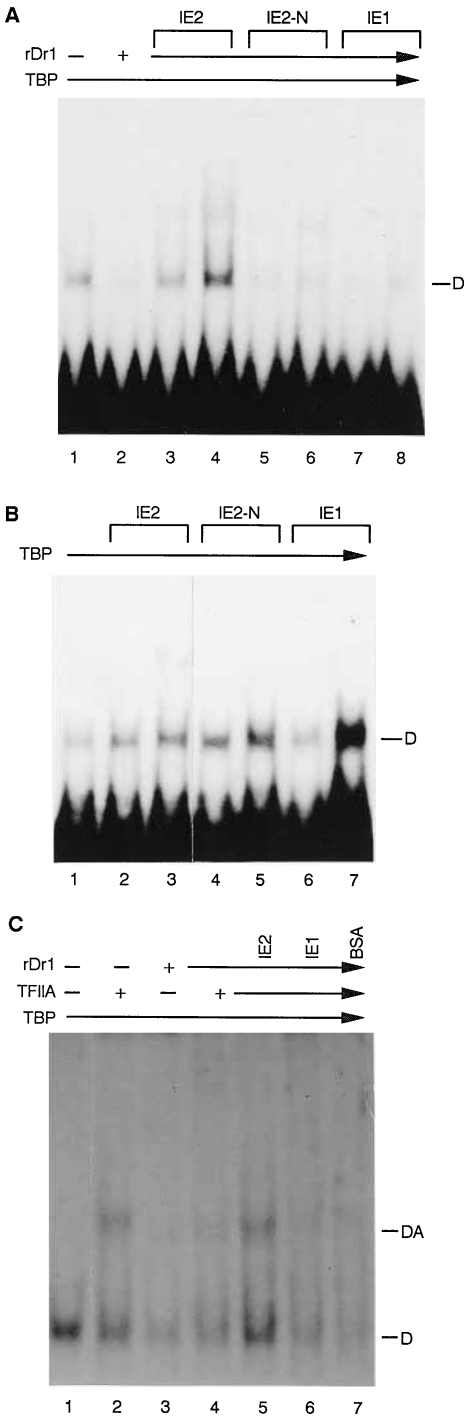

FIG. 5. IE2 overcomes rDr1-mediated inhibition of DNA binding by TBP. (A) EMSA reaction mixtures contained either 0.5 ng of TBP alone (lane 1) or 0.5 ng of TBP and 75 ng of rDr1 (lanes 2 through 8). In addition, reaction mixtures contained either IE2 (50 or 200 ng [lanes 3 and 4, respectively]), IE2-N (50 or 200 ng [lanes 5 and 6, respectively]), or IE1 (50 or 200 ng [lanes 7 and 8, respectively]). (B) Reaction mixtures contained 0.5 ng of TBP alone (lane 1) or 0.5 ng of TBP and either IE2 (50 or 200 ng [lanes 2 and 3, respectively]), IE2-N (50 or 200 ng [lanes 4 and 5, respectively]), or IE1 (50 or 200 ng [lanes 6 and 7, respectively]). (C) All reaction mixtures contained 0.5 ng of TBP, along with either 20 ng of TFIIA (lane 2), 75 ng of rDr1 (lane 3), or both 20 ng of TFIIA and 75 ng of rDr1 (lanes 4 through 7); in addition, reaction mixtures contained 250 ng of either IE2 (lane 5), IE1 (lane 6), or BSA (lane 7). All factors except TFIIA were preincubated on ice for 5 min prior to the addition of probe DNA; TFIIA was added at the same time as was probe DNA, subsequent to preincubation of other factors. The positions of all relevant complexes are indicated on the right of gels.

a similar degree of stabilization of DNA binding by TBP in the absence of rDr1 [data not shown]) and presumably accounts for the observed increase in intensity of the DhDr1 complex seen upon the addition of either IE2, IE1, or BSA to reaction mixtures containing TBP and hDr1 (Fig. 2). Note, however, that the inhibitory effect of rDr1 greatly outweighs this nonspecific effect, as shown by the inability of IE1 or the truncated IE2 protein to restore the D complex in the presence of rDr1 (Fig. 5A, lanes 5 through 8). Thus, the ability of IE2 to restore the D complex (Fig. 5A, lanes 3 and 4) is a specific derepression phenomenon. Results similar to those shown in Fig. 5A were obtained when nonspecific effects were minimized by the inclusion of 1 μg of BSA in all reaction mixtures (data not shown). Note also that under these conditions, IE2 did not appear to form a stable complex with DNA-bound TBP (Fig. 5B, lanes 2 and 3).

As might be expected, rDr1-mediated inhibition of DNA binding by TBP also precluded formation of the DA complex when TFIIA was added to binding reaction mixtures in which TBP and rDr1 had been preincubated (Fig. 5C, lanes 1 through 4). This observation does not conflict with the previously reported ability of rDr1 to interact stably with the DA complex (25), since the addition of rDr1 to preformed DA complexes under the conditions used here resulted in the appearance of a DArDr1 complex (data not shown). The further addition of IE2, but not IE1 or BSA, to reaction mixtures containing TBP, rDr1, and TFIIA resulted in restoration of the D and DA complexes to levels seen in the absence of rDr1 (Fig. 5C, lanes 5 through 7).

IE2, but not IE1, interacts with Dr1 in vivo and in vitro. As IE2 appears to counteract Dr1-mediated phenomena both in vitro and in vivo, we asked whether these effects of IE2 might be mediated by an interaction with Dr1. To test this, we carried out immunoprecipitations from either HCMV-infected or uninfected cells expressing epitope-tagged Dr1 ($Dr1^{HA}$). Cell extracts were immunoprecipitated with either MAb 12CA5 (which recognizes the HA epitope tag), an anti-HCMV IE1/2 antibody, or a negative control antibody (specific for epidermal growth factor receptor) and then reimmunoprecipitated with the anti-IE1/2 antibody. Because of the much higher abundance of IE1 compared with that of IE2 in infected cells (39), double immunoprecipitation with the anti-IE1/2 antibody precipitated mostly the 72-kDa IE1 protein (Fig. 6A, lane 3). However, primary immunoprecipitation with the anti-HA antibody followed by secondary immunoprecipitation with anti-IE1/2 precipitated a protein of the expected size for IE2, i.e., 80 to 86 kDa, rather than for IE1. Therefore, despite the relative abundance of IE1 in HCMV-infected cells, Dr1 is able to interact specifically with IE2 in vivo. Similar results were obtained when identical experiments were performed with primary human fibroblasts (data not shown).

To confirm that the interaction of Dr1 was with IE2 rather than with IE1, we carried out experiments with *S. cerevisiae* using the yeast two-hybrid system. Dr1 was expressed as a fusion to the acidic activation domain (AD) of GAL4, while either IE2 or IE1 was expressed as fusions to the DNA binding domain of GAL4 (GAL4 DBD). Various combinations of plasmids were then tested for their ability to up-regulate expression of a β-galactosidase gene under the control of a promoter bearing GAL4 binding sites. As expected, high levels of β-galactosidase were observed when cells were cotransformed with positive control plasmids encoding DBD-p53 and AD-large T antigen (Fig. 6B), whereas there was no significant enzyme activity when the two fusion vectors expressing either the DBD or AD of GAL4 were transformed. Coexpression of DBD-IE2 and AD-Dr1 resulted in β-galactosidase levels which were approximately 1/10 of those observed with the positive control fusions; however, this activity, which was well within the reliable limits of the assay, was approximately 20-fold higher than that observed upon cotransformation of vectors alone and over 6-fold higher than that seen when the DBD-IE2 plasmid was cotransformed with the AD-expressing vector. Moreover, cotransformation of DBD-IE1 and AD-Dr1 did not activate β-galactosidase expression; these results therefore support the data in Fig. 6A showing that Dr1 is able to interact with IE2, but not IE1, in vivo. The weak β-galactosidase expression observed when DBD-IE2 was cotransformed with the AD-expressing vector may indicate weak, nonspecific interaction between IE2 and the AD of GAL4; alternatively, the increased gene expression could simply be a result of targeting the transcriptional activator IE2 to the promoter by means of the GAL4 DBD. The lack of activity observed when the AD-Dr1 construct was cotransformed with a plasmid expressing a DBD-lamin C fusion implies that interactions which recruit the AD-Dr1 fusion to the promoter are unlikely to be of a nonspecific nature.

To characterize further the nature of the IE2-Dr1 interaction, we carried out in vitro binding assays using various GST fusion proteins. Consistent with previous observations (25, 53), TBP bound to GST-Dr1 in the absence of DNA (Fig. 7A, GST-Dr1 panel, lane 6). As expected from the results of the in vivo assays, IE2 was also able to bind specifically to the GST-Dr1 fusion, although in this assay the binding of full-length IE2 was fairly weak (lane 3′, showing IE2 bound to GST-Dr1, is an overexposure of lane 3). The interaction of IE2 with Dr1 was mediated by residues in the C terminus of IE2 (amino acids 290 to 579), which were both necessary and sufficient for binding (lane 5), whereas the entire N-terminal half of IE2 was dispensable and unable to bind to Dr1 in isolation (lane 4). The weak binding by full-length IE2 to GST-Dr1, compared with that by the C terminus of IE2, parallels similar findings by ourselves and others of IE2 binding to TBP, TFIIB, and RB (6, 17, 48) and presumably indicates that the N-terminal region of IE2 contains sequences which mask or interfere with binding regions in the C terminus of the protein, possibly in relation to the phosphorylation of casein kinase II sites in the N-terminal region, as Sommer et al. have suggested (48). Note that binding was specific for the GST-Dr1 fusion, as inputs showed no binding to either a GST fusion to the N terminus of TBP or GST alone. Furthermore, IE2 appears to contact Dr1 through a region similar to that required for interaction with TBP. Amino acids 85 to 99 of Dr1 are predicted to form part of an amphipathic alpha-helical region which is essential for TBP binding and for repression in vivo (53); deletion of these residues prevented binding not only by TBP, as expected from previous observations (53), but also by the C terminus of IE2 (Fig. 7B), further underlining the specificity of the IE2-Dr1 interaction.

Since it had been shown that the Dr1 binding region of IE2 lies within the C-terminal half of the protein, i.e., between amino acids 290 and 579 (Fig. 7A), further experiments were carried out to delineate the C-terminal and N-terminal boundaries of this region. The removal of C-terminal residues up to and including amino acid 505 did not impair binding to Dr1 (compare the binding of fragments covering residues 290 to 504, 290 to 542, and 290 to 579 [Fig. 8A, GST-Dr1 panel, lanes 2 through 4, respectively]), while further truncation to residue 390 abolished all binding to GST-Dr1 (lane 1). Since we had observed the same pattern for the binding of these IE2 fragments to GST-TBP (though not with fusions to TFIIB or RB) (6, 17), we also tested the abilities of various N-terminal truncations of IE2 to bind to both TBP and Dr1 fusion proteins.

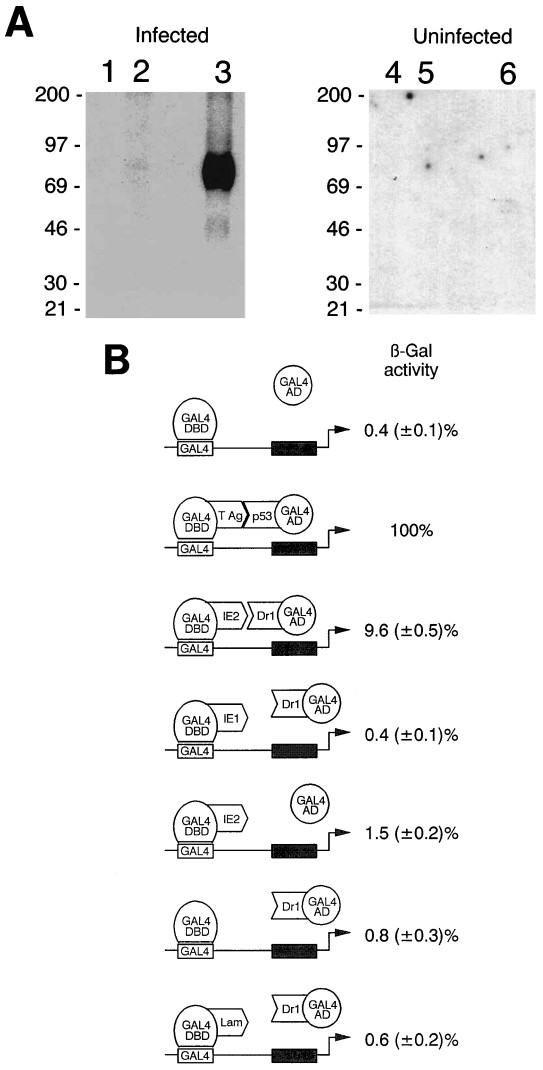

FIG. 6. IE2 interacts with Dr1 in vivo. (A) Human HTB96 cells were transfected with the $Dr1^{HA}$ expression vector pHK3-$Dr1^{HA}$, either infected with HCMV strain AD169 (lanes 1 through 3) or left uninfected (lanes 4 through 6), and labelled with [^{35}S]methionine. Cell extracts were immunoprecipitated with either anti-EGFR (lanes 1 and 4), anti-HA (MAb 12CA5; lanes 2 and 5), or anti-HCMV IE1/2 (lanes 3 and 6) and then subjected to a second round of immunoprecipitation with the anti-IE1/2 antibody. Protein A-Sepharose complexes were resolved by SDS-PAGE, and gels were autoradiographed. The positions of molecular mass (in kilodaltons) markers are indicated on the left. The lanes between lanes 2 and 3 and lanes 5 and 6 were left empty. (B) SFY526 yeast cells were transformed with various combinations of plasmids expressing fusions to either the GAL4 DBD or the GAL4 AD, as shown, and assayed for β-galactosidase activity. The plasmids used were pGBT10 (expressing GAL4 DBD), pGBT10-IE2 (DBD-IE2), pGBT10-IE1 (DBD-IE1), pGAD426 (GAL4 AD), pGAD426-$Dr1^{HA}$ (AD-Dr1), and pLAM5′ (DBD-lamin C; Clontech). The positive control plasmids, expressing DBD-murine p53 and AD-SV40 large T antigen, were pVA3 and pTD1, respectively (Clontech). Assays were carried out in duplicate, and β-galactosidase activities are expressed relative to the p53-T antigen positive control as mean activity ± range.

Figure 8B shows that the residues up to and including residue 312 of IE2 were dispensable for binding to GST-TBP (lane 2), while further deletion up to amino acid 388 abolished binding (lane 3). As with the C-terminal truncations of IE2, the same pattern was observed for binding to GST-Dr1, although the binding was generally somewhat weaker than that seen with GST-TBP, at least as measured by this assay. Therefore, IE2 is able to interact with Dr1 both in vivo and in vitro. While it

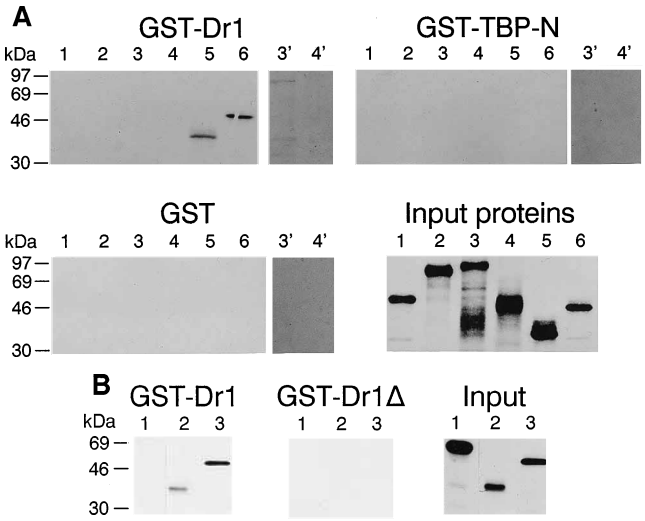

FIG. 7. IE2 binds to rDr1 in vitro. (A) The proteins used in protein-protein binding assays were in vitro-translated gelsolin (lanes 1), IE1 (lanes 2), IE2 (lanes 3), IE2 N-terminal residues 1 to 290 (lanes 4), IE2 C-terminal residues 290 to 579 (lanes 5), and TBP (lanes 6); they were tested for binding to immobilized GST-Dr1, GST-TBP-N, and GST, as shown. In each case, lanes 3′ and 4′ show overexposures of lanes 3 and 4, respectively. (B) Input proteins were tested for binding to GST fusions to either full-length Dr1 (GST-Dr1) or an internal deletion of Dr1 (GST-Dr1Δ85-99 [GST-Dr1Δ]), as shown. The input proteins were gelsolin (lanes 1), IE2 C-terminal residues 290 to 579 (lanes 2), and TBP (lanes 3). In both cases, lanes labelled input were loaded with 20% of the amount of protein used in binding assays. The positions of molecular mass markers are indicated on the left.

seems likely that this interaction is direct, we cannot rule out that other proteins may mediate this interaction in vivo and that such proteins may also be present in the reticulocyte lysate used to generate input proteins for in vitro assays.

The interaction of IE2 with both Dr1 and TBP requires the same or similar regions between amino acids 313 and 504. The deletion of a similar region of IE2 (amino acids 290 to 490) resulted in the inability of the protein to overcome Dr1-mediated repression of the *hsp70* promoter in vivo (Fig. 9); however, since this region also binds TBP (6) and has been shown to be important for transactivation (22, 37, 39, 50), we are at present unable to determine whether this lack of derepression is due solely to an inability to bind Dr1 or to the loss of other important functions of IE2.

DISCUSSION

We and others have shown previously that HCMV IE2 is able to *trans* activate the human *hsp70* promoter by a TATA box-dependent mechanism and that IE2 can interact directly with TBP via the C-terminal regions of IE2 which are important for *trans* regulation (6, 11, 14, 19). We have shown here that overexpression of the transcriptional repressor Dr1 results in reduced expression from the *hsp70* promoter in vivo, concordant with other observations (33, 53), and that this repression can be overcome by coexpression of HCMV IE2. We have also shown that IE2 interacts with Dr1, and similar observations have recently been made for adenovirus E1A (33). However, despite the apparent similarity of IE2 to E1A in this and other respects, for instance, the requirement of a TATA box for *trans* activation and the interaction of both proteins with the basic interrepeat region of TBP (6, 19, 35, 45), the two proteins appear to differ in their interactions with Dr1-containing complexes in EMSAs. While E1A has been reported to

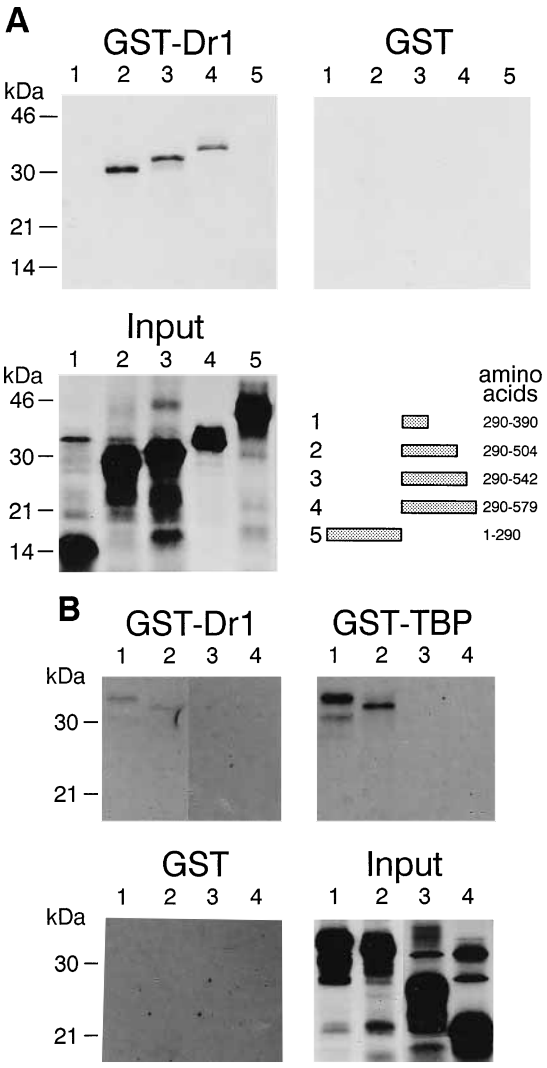

FIG. 8. C-terminal sequences in IE2 confer binding to rDr1. (A) Proteins for protein-protein binding assays were in vitro-translated fragments of IE2 covering residues 290 to 390 (16 kDa; lanes 1), 290 to 504 (30 kDa; lanes 2), 290 to 542 (32 kDa; lanes 3), 290 to 579 (35 kDa; lanes 4), and 1 to 290 (46 kDa; lanes 5); they were tested for binding to immobilized GST-Dr1 and GST, as shown. (B) Input proteins were fragments of IE2 covering residues 290 to 579 (35 kDa; lanes 1), 313 to 579 (31 kDa; lanes 2), 388 to 579 (25 kDa; lanes 3), and 428 to 579 (21 kDa; lanes 4); they were tested for binding to immobilized GST-TBP, GST-Dr1, and GST, as shown. In both cases, lanes labelled input were loaded with 20% of the amount of protein used in binding assays. The positions of molecular mass markers are indicated on the left.

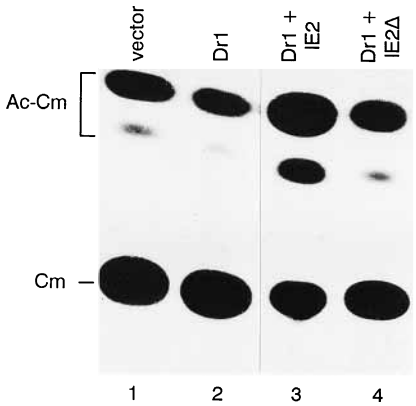

FIG. 9. An IE2 deletion (amino acids 290 to 490) mutant cannot overcome Dr1-mediated repression. Human IBR fibroblasts were cotransfected with 3 μg of the *hsp70* CAT reporter construct pHC-77-CAT, along with 10 μg of pHK3 (lane 1), 5 μg of pHK3-Dr1^{HA} (expressing epitope-tagged Dr1) and 5 μg of pHK3 (lane 2), 5 μg of pHM121 (expressing IE2) and 5 μg of pHK3-Dr1^{HA} (lane 3), or 5 μg of pHKIE2mut (expressing IE2Δ290-490 [IE2Δ]) and 5 μg of pHK3-Dr1^{HA} (lane 4). Cm and Ac-Cm, the positions of chloramphenicol and acetylated chloramphenicol, respectively. Data are representative of three independent experiments.

disrupt the DhDr1 complex in either the absence or presence of TFIIA (33), we have found no such activity for IE2. Nevertheless, since IE2 can overcome Dr1-mediated repression in vivo, we were prompted to look for an alternative mechanism by which this derepression might occur. As a result, we have found that unphosphorylated forms of Dr1, that is, rDr1 and dephosphorylated hDr1, are able to inhibit DNA binding by TBP and that IE2 is able to overcome this inhibition in vitro. The ability of IE2, but not IE1 or the N terminus of IE2 (amino acids 1 to 290), to overcome the inhibition of DNA binding by TBP is consistent with the findings that C-terminal regions of IE2 are required for binding to both rDr1 and TBP in vitro and that IE2, but not IE1, can interact with Dr1 in vivo. Furthermore, these C-terminal regions of IE2 are known to be important for transactivation (22, 37, 39, 50), and an IE2 deletion mutant lacking these residues is unable to overcome Dr1-mediated repression in vivo. These results demonstrate not only a previously unreported activity of Dr1 but suggest a novel mechanism by which IE2 *trans* activates target promoters.

It is known that Dr1 exists in two forms within a cell, one of which is a putatively posttranslationally unmodified form which comigrates with rDr1 on an SDS-PAGE gel (25). This, together with the observation that rDr1 can interact with free TBP, but not DNA-bound TBP (25, 53) (also discussed above), while the unmodified form of cellular Dr1 associates with the TBP-containing TFIID complex in nuclear extracts (25), suggests that rDr1 is in fact identical to the TFIID-associated form of cellular Dr1. Furthermore, since the activity of hDr1 in EMSAs can be converted to that of rDr1 by dephosphorylation (25, 30), this implies in turn that the two cellular forms of Dr1 differ only in their degree of phosphorylation, as Inostroza et al. have suggested (25). Given that rDr1 is known to repress transcription in vitro, it is perhaps surprising that relatively little work has been carried out to examine the role of the unmodified form of cellular Dr1, particularly as it occurs in substantial amounts in the TFIID fraction of nuclear extracts (25). Indeed, the overall contribution of Dr1 and other transcriptional repressors to the regulation of cellular gene expression is still poorly understood. Nevertheless, since such repressors do occur, it seems likely that some activator proteins, whether cellular or viral, may function at least in part by interfering with the activities of these repressors.

We have shown here that nonphosphorylated forms of Dr1, that is, rDr1 and dephosphorylated hDr1, can inhibit DNA binding by TBP and that this inhibition is dependent on the ability of Dr1 to bind TBP. This suggests one mechanism by which not only rDr1 but also the unmodified form of cellular Dr1 acts as a negative regulator of the earliest committed step in preinitiation complex formation, that is, the binding of TFIID to a target promoter, providing in turn a further potential target for up-regulation by viral or cellular activator proteins. Indeed, recent reports have shown that the recruitment of TBP to the TATA box can be a limiting step at some promoters in vivo and that increased recruitment of TBP to the promoter results in increased gene expression (7, 31). Further-

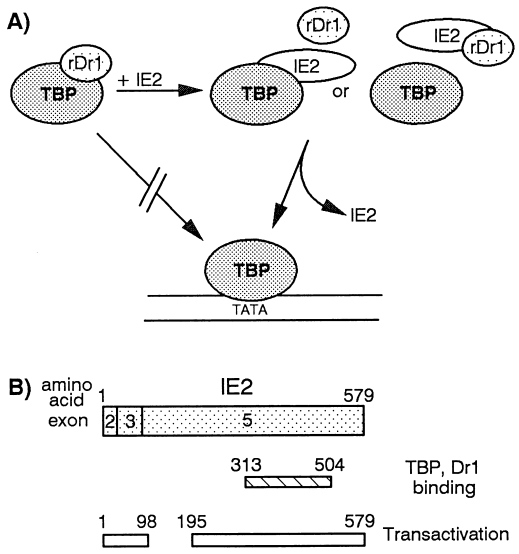

FIG. 10. A possible mechanism for IE2-mediated derepression. (A) IE2 may function by competing with TBP for binding to the region from amino acids 85 to 99 of rDr1; alternatively (or additionally), IE2 could compete with rDr1 for binding to the same site on TBP. (B) IE2 binds to both TBP (6) and rDr1 (this study) via regions which are important for transactivation by IE2 (22, 37, 39, 50).

more, since TBP binds specifically to the TATA box motif, factors which are able to alleviate putative Dr1-mediated repression of DNA binding by TBP might be expected to show a dependency on the presence of a TATA motif to exert their regulatory effects. HCMV IE2 does indeed show such a dependency, suggesting a molecular mechanism by which IE2 could activate TATA-bearing promoters by alleviation of Dr1-mediated repression (Fig. 10A). IE2 appears to interact with both rDr1 and TBP through similar regions (Fig. 10B); therefore, it seems unlikely that a single IE2 molecule could bind both rDr1 and TBP simultaneously in a tripartite complex. Since both TBP and IE2 require amino acids 85 to 99 of Dr1 for binding, it is possible that IE2 could release TBP from rDr1 by competing for the same binding site on rDr1. Alternatively, IE2 could displace rDr1 by competition for the same binding site on TBP; these possibilities are not necessarily mutually exclusive. Recent evidence shows that specific residues within the basic interrepeat region of TBP are crucial for interaction with purified cellular hDr1 (NC2) and that hDr1 and TFIIA compete for binding to this site (30). IE2 also requires the basic region of TBP for binding (6), but it is not known whether precisely the same residues of TBP are involved. Indeed, it is not yet known whether hDr1 and rDr1 bind to the same site on TBP, and the different activities of the two forms of this protein indicate that this may not be the case, as Inostroza et al. have suggested (25). Since in these experiments we were not able to differentiate between the TBP and Dr1 binding domains of IE2 (Fig. 8) or between the IE2 and TBP binding domains of Dr1 (Fig. 7B), it is not yet clear which of these two interactions is the more important, either for IE2-mediated derepression of DNA binding by TBP in vitro or for activation of the *hsp70* promoter in vivo. However, since it has been shown that activator proteins can act at multiple points in the activation of gene expression (10), it is entirely conceivable that both the TBP and Dr1 binding functions of IE2 are necessary for maximal *trans* activation.

In conclusion, we have shown that nonphosphorylated forms of Dr1 can inhibit DNA binding by TBP and that HCMV IE2 can overcome this inhibition in vitro. However, while this observation does provide one possible explanation for the ability of IE2 to counter Dr1-mediated repression of the *hsp70* promoter in vivo and is consistent with the known TATA dependency of IE2, it should be noted that other mechanisms may also be involved. Firstly, it is possible that nonphosphorylated forms of Dr1 act at multiple steps, as it has been suggested that rDr1 inhibits transcription by preventing the entry of TFIIB into the preinitiation complex (25). The experiments presented here do not address this activity of rDr1, nor do they rule out the possibility that IE2 also acts at this point. Secondly, both forms of Dr1, the phosphorylated hDr1 and the rDr1-like TFIID-associated form, are present during in vivo assays; while our results indicate that IE2 is not able to disrupt the interaction of hDr1 with DNA-bound TBP in vitro, we cannot rule out that IE2-mediated derepression of the *hsp70* promoter in vivo may occur by indirect alleviation of this or other as-yet-unknown activities of hDr1. To rule out this latter possibility requires greater understanding of the relative contributions of both forms of cellular Dr1 to transcriptional repression and, in turn, of how the various activities of Dr1 are regulated by phosphorylation within a cell. Finally, it is likely that the ability of IE2 to alleviate Dr1-mediated repression is simply one of a number of mechanisms by which IE2 can activate target promoters. Current understanding of the nature of IE2 indicates that it is a versatile protein, capable of interacting with several cellular transcription factors and up-regulating gene expression by diverse mechanisms. Although it is difficult at present to quantify the contributions of these various effects to transactivation by IE2 as a whole (a problem which is likely to be exacerbated if more than one mechanism operates at the same promoter), these studies provide further insight into the role of IE2 as a multifunctional transactivator protein.

ACKNOWLEDGMENTS

We are most grateful to Marc Timmers for providing purified human TFIIA and hDr1 and for critically reading the manuscript. We also thank D. Reinberg and K. Yeung for the GST-Dr1Δ85-99 fusion construct; A. Berk, C.-J. Chiou, G. Hayward, W. Kaelin, T. Stamminger, and A. Weeds for plasmids; and R. Hay for the preparation of the partially purified NF-1 DNA binding domain.

This work was supported by the Medical Research Council.

REFERENCES

1. **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.
2. **Biegalke, B. J., and A. P. Geballe.** 1991. Sequence requirements for activation of the HIV-1 LTR by human cytomegalovirus. Virology **183:**381–385.
3. **Boshart, M., F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner.** 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41:**521–530.
4. **Buratowski, S., S. Hahn, L. Guarente, and P. A. Sharp.** 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. Cell **56:** 549–561.
5. **Burns, L. J., J. F. Waring, J. J. Reuter, M. F. Stinski, and G. Ginder.** 1993. Only the HLA class I minimal gene promoter elements are required for transactivation by human cytomegalovirus immediate early genes. Blood **81:**1558–1566.
6. **Caswell, R. C., C. Hagemeier, C.-J. Chiou, G. Hayward, T. Kouzarides, and J. Sinclair.** 1993. The human cytomegalovirus 86K 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.
7. **Chatterjee, S., and K. Struhl.** 1995. Connecting a promoter-bound protein to TBP bypasses the need for a transcriptional activation domain. Nature (London) **374:**820–822.
8. **Cherrington, J. M., and E. S. Mocarski.** 1989. Human cytomegalovirus ie1 transactivates the α promoter-enhancer via an 18-base-pair repeat element. J. Virol. **63:**1435–1440.
9. **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.
10. **Choy, B., and M. Green.** 1993. Eukaryotic activators function during multiple streps of preinitiation complex assembly. Nature (London) **366:**531–536.
11. **Colberg-Poley, A. M., L. D. Santomenna, P. P. Harlow, P. A. Benfield, and D. J. Tenney.** 1992. Human cytomegalovirus US3 and UL36-38 immediate-early proteins regulate gene expression. J. Virol. **66:**95–105.
12. **Dalton, S.** 1992. Cell-cycle regulation of the human *cdc2* gene. EMBO J. **11:**1797–1804.
13. **Field, J., J.-I. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I. A. Wilson, R. A. Lerner, and M. Wigler.** 1988. Purification of a *RAS*-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. **8:**2159–2165.
14. **Furnari, B. A., E. Poma, T. F. Kowalik, S.-M. Huong, and E.-S. Huang.** 1993. Human cytomegalovirus immediate-early gene 2 protein interacts with itself and with several novel cellular proteins. J. Virol. **67:**4981–4991.
15. **Geist, L. J., M. M. Monick, M. F. Stinski, and G. W. Hunninghake.** 1994. The immediate early genes of human cytomegalovirus upregulate tumor necrosis factor-α gene expression. J. Clin. Invest. **93:**474–478.
16. **Ghazal, P., J. Young, E. Giulietti, C. DeMattei, J. Garcia, J. Gaynor, R. M. Stenberg, and J. A. Nelson.** 1991. A discrete *cis* element in the human immunodeficiency virus long terminal repeat mediates synergistic *trans* activation by cytomegalovirus immediate-early proteins. J. Virol. **65:**6735–6742.
17. **Hagemeier, C., R. Caswell, G. Hayhurst, J. Sinclair, and T. Kouzarides.** 1994. Functional interaction between the HCMV IE2 transactivator and the retinoblastoma protein. EMBO J. **13:**2897–2903.
18. **Hagemeier, C., and T. Kouzarides.** Unpublished data.
19. **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.
20. **Hagemeier, C. H., S. M. Walker, J. G. P. 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* promoters via basal promoter elements. J. Gen. Virol. **73:**2385–2393.
21. **Hayhurst, G. P., L. A. Bryant, R. C. Caswell, S. M. Walker, and J. H. Sinclair.** 1995. CCAAT box-dependent activation of the TATA-less human DNA polymerase α promoter by the human cytomegalovirus 72-kilodalton major immediate-early protein. J. Virol. **69:**182–188.
22. **Hermiston, T. W., C. L. Malone, P. R. Witte, and M. F. Stinski.** 1987. Identification and characterization of the human cytomegalovirus immediate-early 2 region that stimulates gene expression from an inducible promoter. J. Virol. **61:**3214–3221.
23. **Hiebert, S. W., S. P. Chellapan, J. M. Horowitz, and J. R. Nevins.** 1992. The interaction of Rb with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev. **6:**177–185.
24. **Hunninghake, G. W., B. G. Monks, L. J. Geist, M. M. Monick, M. A. Monroy, M. F. Stinski, A. C. Webb, J.-M. Dayer, P. E. Auron, and M. J. Fenton.** 1992. The functional importance of a cap site-proximal region of the human prointerleukin 1β gene is defined by viral protein *trans*-activation. Mol. Cell. Biol. **12:**3439–3448.
25. **Inostroza, J. A., F. H. Mermelstein, I. Ha, W. S. Lane, and D. Reinberg.** 1992. Dr1, a TATA-binding protein-associated phosphoprotein and inhibitor of class II gene expression. Cell **70:**477–489.
26. **Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. T. Tjian.** 1987. A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. Cell **48:**79–89.
27. **Jupp, R., O. Flores, J. A. Nelson, and P. Ghazal.** 1993. The DNA-binding subunit of human transcription factor IID can interact with the TATA box as a multimer. J. Biol. Chem. **268:**16105–16108.
28. **Kaelin, W. G., Jr., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, D. M. Livingston, and E. K. Flemington.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell **70:**351–364.
29. **Kerry, J. A., M. A. Priddy, and R. M. Stenberg.** 1994. Identification of sequence-specific elements in the human cytomegalovirus DNA polymerase gene promoter required for activation by viral gene products. J. Virol. **68:**4167–4176.
30. **Kim, T. K., Y. Zhao, H. Ge, R. Bernstein, and R. G. Roeder.** 1995. TATA-binding protein residues implicated in a functional interplay between negative cofactor NC2 (Dr1) and general factors TFIIA and TFIIB. J. Biol. Chem. **270:**10976–10981.
31. **Klages, N., and M. Strubin.** 1995. Stimulation of RNA polymerase II transcription initiation by recruitment of TBP in vivo. Nature (London) **374:**822–823.
32. **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.
33. **Kraus, V. B., J. A. Inostroza, K. Yeung, D. Reinberg, and J. R. Nevins.** 1994. Interaction of the Dr1 inhibitory factor with the TATA binding protein is disrupted by adenovirus E1A. Proc. Natl. Acad. Sci. USA **91:**6279–6282.
34. **Lang, D., and T. Stamminger.** 1993. The 86-kilodalton IE-2 protein of human cytomegalovirus is a sequence-specific DNA-binding protein that interacts directly with the negative autoregulatory response element located near the cap site of the IE-1/2 enhancer-promoter. J. Virol. **67:**323–331.
35. **Lee, W. S., C. C. Kao, G. O. Bryant, X. Liu, and A. J. Berk.** 1991. Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. Cell **67:**365–376.
36. **Maldonado, E., I. Ha, P. Cortes, L. Weis, and D. Reinberg.** 1990. Factors involved in specific transcription by mammalian RNA polymerase II: role of transcription factors IIA, IID, and IIB during formation of a transcription-competent complex. Mol. Cell. Biol. **10:**6335–6347.
37. **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.
38. **Merino, A., K. R. Madden, W. S. Lane, J. J. Champoux, and D. Reinberg.** 1993. Topoisomerase I is involved in both repression and activation of transcription. Nature (London) **365:**227–232.
39. **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.
40. **Pizzorno, M. C., P. O'Hare, L. Sha, R. L. LaFemina, and G. S. Hayward.** 1988. *trans*-activation and autoregulation of gene expression by the immediate-early region 2 gene products of human cytomegalovirus. J. Virol. **62:**1167–1179.
41. **Plachter, B., W. Britt, R. Vornhagen, T. Stamminger, and G. Jahn.** 1993. Analysis of proteins encoded by IE regions 1 and 2 of human cytomegalovirus using monoclonal antibodies generated against recombinant antigens. Virology **193:**642–652.
42. **Reinberg, D., M. Horikoshi, and R. G. Roeder.** 1987. Factors involved in specific transcription by mammalian RNA polymerase II: functional analysis of initiation factors IIA and IID and identification of a new factor operating at sequences downstream of the initiation site. J. Biol. Chem. **262:**3322–3330.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Sambucetti, L. C., J. M. Cherrington, G. W. G. Wilkinson, and E. S. Mocarski.** 1989. NFκB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J. **8:**4251–4258.
45. **Simon, M. C., T. Fisch, B. J. Benecke, J. R. Nevins, and N. Heintz.** 1988. Definition of multiple, functionally distinct TATA elements, one of which is a target in the *hsp70* promoter for E1A regulation. Cell **52:**723–729.
46. **Sinclair, J. H.** Unpublished data.
47. **Smith, D. B., and K. S. Johnson.** 1988. Single step purification of polypeptides expressed in *E. coli* as fusions with glutathione-*S*-transferase. Gene **67:**31–40.
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. **Stenberg, R. M., A. S. Depto, J. Fortney, and J. A. Nelson.** 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. J. Virol. **63:**2699–2708.
50. **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. **64:**1556–1565.
51. **Thomsen, D. R., R. M. Stenberg, W. F. Goins, and M. F. Stinski.** 1984. Promoter regulatory region of the major immediate early gene of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **81:**659–663.
52. **Walker, S. M., C. Hagemeier, J. G. P. Sissons, and J. H. Sinclair.** 1992. A 10-base-pair element of the human immunodeficiency virus type 1 long terminal repeat (LTR) is an absolute requirement for transactivation by the human cytomegalovirus 72-kilodalton IE1 protein but can be compensated for by other LTR regions in transactivation by the 80-kilodalton IE2 protein. J. Gen. Virol. **66:**1543–1550.
53. **Yeung, K. C., J. A. Inostroza, F. H. Mermelstein, C. Kannabiran, and D. Reinberg.** 1994. Structure-function analysis of the TBP-binding protein Dr1 reveals a mechanism for repression of class II gene transcription. Genes Dev. **8:**2097–2109.