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Inhibition of the association of RNA polymerase II with the preinitiation complex by a viral transcriptional repressor

(transcription/repression/immediate early protein 2/human cytomegalovirus)

GARY LEE*, JUN WU†‡, PERCY LUU*, PETER GHAZAL†, AND OSVALDO FLORES*§

*Tularik Inc., South San Francisco, CA 94080; and †Departments of Immunology and Neuropharmacology, Division of Virology, The Scripps Research Institute, La Jolla, CA 92037

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ABSTRACT **Transcriptional repression is an important component of regulatory networks that govern gene expression. In this report, we have characterized the mechanisms by which the immediate early protein 2 (IE2 or IE86), a master transcriptional regulator of human cytomegalovirus, down-regulates its own expression. *In vitro* transcription and DNA binding experiments demonstrate that IE2 blocks specifically the association of RNA polymerase II with the preinitiation complex. Although, to our knowledge, this is the first report to describe a eukaryotic transcriptional repressor that selectively impedes RNA polymerase II recruitment, we present data that suggests that this type of repression might be widely used in the control of transcription by RNA polymerase II.**

Substantial progress has been made in the identification and functional characterization of eukaryotic transcriptional activators (for review, see ref. 1). In contrast, the understanding of transcriptional repressors in eukaryotes has received considerably less attention. Studies on transcriptional repression in prokaryotes have led to the hypothesis that eukaryotic repressors might function by a competitive mechanism, in which the DNA-bound repressor blocks access of the basal transcription machinery to promoter DNA. Perhaps for this reason, one of the first eukaryotic repressors to be recognized was the simian virus 40 tumor antigen, which represses transcription by masking the promoter and inhibiting its recognition by the general transcription machinery (2, 3). The precise step at which preinitiation complex formation is blocked by the simian virus 40 tumor antigen is unknown. The *Drosophila* *P*-element transposase (4), the cellular DNA binding protein LBP-1 (5), and the bovine papilloma virus E2 protein (6) represent additional examples of repressors that block binding of the preinitiation complex to DNA. In each case, it was found that the repressor blocked basal transcription factor (TF) IID binding to the TATA box, suggesting that the first step in preinitiation complex formation was the target for inhibition by this class of eukaryotic repressors.

Important contributions to all aspects of eukaryotic gene regulation have come from studies aimed at understanding the coordinated regulation of viral gene expression. Excellent viral model systems include the dual-function activator/repressor proteins encoded by herpesviruses. Human cytomegalovirus (HCMV) is a β-herpesvirus that has become a common and very serious pathogen in immunocompromised patients. In this report, we have studied the mechanism by which the HCMV immediate early protein 2 (IE2) represses transcription of its own promoter, the potent major immediate early enhancer-promoter (MIEP) of HCMV. Transient transfection experiments indicate that repression by IE2 requires a cis-regulatory DNA element referred to as cis-repression signal (crs) located between the "TATA" box and the transcription start site of the HCMV MIEP (7–9). More recently, it was shown that purified IE2 protein binds directly to the crs element (10, 11) and represses transcription of the HCMV MIEP *in vitro* (12, 13). In this work, we have employed a purified *in vitro* transcription system to study the precise mechanism by which IE2 represses transcription. Our observations show that IE2 does not impede promoter recognition by TFIID, yet very effectively blocks recruitment of RNA polymerase II.

MATERIALS AND METHODS

Purification of RNA Polymerase II and General Factors. The RNA polymerase II fractions used in the *in vitro* transcriptions (DEAE 5PW step) and in the DNA binding experiments (IIa form, alkyl-Superose step) were purified as described (14). TFIIA and TFIID were purified as described (15). TFIIF, TFIIH, recombinant TBP, IIB, IIEp34, and IIEp56 proteins were purified as described (16–20).

Expression and Purification of Recombinant IE2 Proteins. Both the full-length IE2 cDNA and a DNA fragment encoding IE2p40 were cloned into a pET-15b vector (Novagen). The DNA fragment encoding IE2p40 was isolated with the use of the polymerase chain reaction (PCR). The conditions used to express and purify the full-length IE2 protein and the IE2p40 protein were identical. Briefly, cultures (BL21) were grown to an OD_{600} of 0.5–1.0 unit and induced with 0.5 mM isopropyl β-D-thiogalactoside (BRL) for 2 h at 37°C. Bacterial extracts were prepared, and the proteins were purified according to the conditions described in the Novagen His·Bind buffer kit protocols. The affinity-purified proteins were then fractionated on a Mono Q column (HR5/5, Pharmacia). The molar concentration was calculated by assuming that these proteins exist as dimers in solution.

***In Vitro* Transcription Reactions.** Transcription reaction mixtures contained TFIID (DE-52, 1.0 μg) or recombinant human TATA binding protein (TBP) (60 ng), recombinant human TFIIB (20 ng), recombinant human TFIIE (100 ng), TFIIF (TSK phenyl, 200 ng), TFIIH (400 ng), RNA polymerase II (DEAE 5PW, 100 ng), and TFIIA (DE-52, 5 μg). TFIIA was not used in reactions reconstituted with TBP. Reaction conditions were as described (17). Reaction mixtures were incubated at 30°C for 45 min or as indicated.

Gel Mobility Shift Assay. The protein components indicated in the figure legend were incubated with a 3′-end-labeled DNA fragment (80 bp) containing the core promoter sequences of the HCMV MIEP (bp −34 to +5) and polylinker sequences of the plasmid Bluescript SK+ (Stratagene). Reaction conditions

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Abbreviations: IE, immediate early protein; HCMV, human cytomegalovirus; MIEP, major immediate early enhancer-promoter; crs, cis-repression signal; TF, transcription factor; TBP, TATA binding protein; TAF, TBP associated factor.

‡Present address: Signal Pharmaceuticals Inc., 555 Oberlin Drive, Suite 100, San Diego, CA 92121.
§To whom reprint requests should be addressed.

were as described (16) and TBP (3 ng), IIB (1 ng), IIF (20 ng), RNA polymerase IIa (alkyl-Superose fraction, 10 ng), and IE2p40 (2 fmol) were added as indicated in Fig. 6. The reaction products were separated by electrophoresis on a 4% polyacrylamide gel [30% (wt/vol) acrylamide/0.8% *N,N'*-methylene bisacrylamide] containing 2.5% (vol/vol) glycerol and 1× TBE.

RESULTS

IE2 Represses the HCMV MIEP in a Transcription System Reconstituted with Purified Factors. To characterize the mechanism of repression by IE2, we developed a purified *in vitro* transcription system that supported accurate transcription initiation from the HCMV MIEP. Simultaneous addition of basal factors with recombinant full-length IE2 resulted in significant transcriptional repression of reactions driven by the wild-type HCMV MIEP (Fig. 1*B*, lanes 1–10). The level of repression increased in proportion to the amount of IE2 protein added. In contrast, IE2 had little effect on transcription reactions containing a mutant HCMV MIEP with four nucleotide substitutions in the crs element (Fig. 1 *A* and *B*, lanes 11–15). These observations are consistent with previous studies indicating that repression by IE2 requires direct binding of IE2 to the crs element (11, 12). Quantitation of this experiment indicated that the concentration of IE2 required to repress 50% of the initial activity was approximately 10 nM, corresponding to a molar ratio of five molecules of IE2 per molecule of template.

IE2 efficiently repressed both TFIID- and TBP-directed basal transcription. Quantitation of this experiment indicated that the extent of repression by IE2 was similar in both conditions (Fig. 1*C*). Since repression does not require the TBP associated factor (TAF)-containing TFIID complex, yet works effectively with TBP alone, we do not believe that IE2 functions by attenuating a TAF-mediated activation step. This interpretation is consistent with the fact that the assay we have utilized represents a minimal activator-independent reaction. The experiments described above show that we can recapitulate transcriptional repression by IE2 with the use of a purified *in vitro* transcription system.

Repression by IE2 Is Dependent on the Position of the crs Element. Because the crs element is located between the TATA box and the transcription start site, it is plausible that the mechanism of repression by IE2 might involve steric hindrance with the basal transcription complex. To investigate this possibility we asked whether the position of the crs element with respect to the TATA box was important for repression. We analyzed the effect of IE2 on HCMV MIEP constructs in which the distance between the crs element and the TATA box was increased by 5, 10, or 15 bp (Fig. 2). To analyze these promoter mutants, we employed run-off assays. As shown in Fig. 2, IE2 retained the ability to repress transcription when the distance between the crs element and the TATA box of the HCMV MIEP was increased from 10 to 15 nt (TATA 5 crs), indicating that IE2 does not require a specific stereo alignment with the TATA box to effect repression. In contrast, the ability of IE2 to repress transcription was dramatically decreased when the crs element was positioned 20 or 25 nt downstream from the TATA box (Fig. 2). IE2 had no effect on transcription of a control template (Fig. 2) and, moreover, IE2 did not repress transcription of templates containing the crs element located 10 bp upstream of the TATA box (data not shown). The fact that repression was dependent on the position of the crs element suggests that the mechanism of repression might involve direct competition between IE2 and a component of the basal complex for binding to the HCMV MIEP.

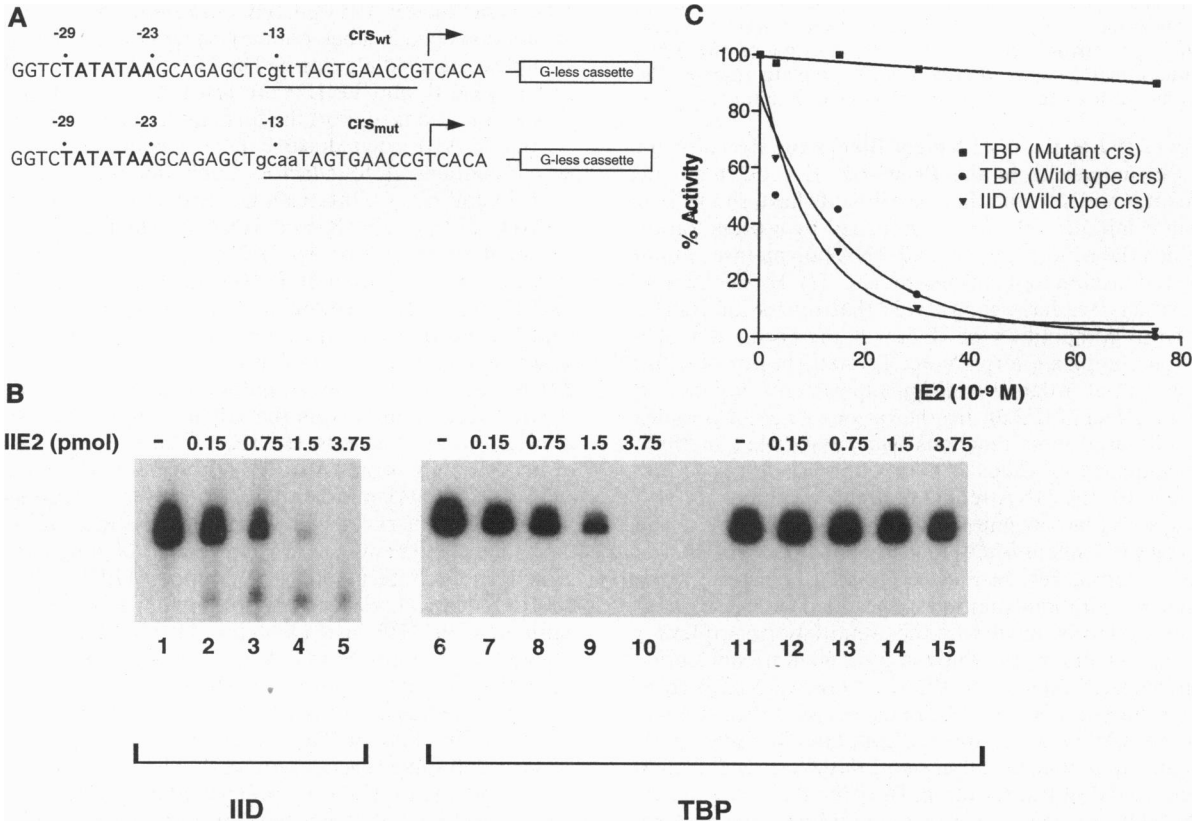

FIG. 1. IE2 represses transcription of the HCMV MIEP in transcription reactions reconstituted with either TFIID or TBP. (*A*) Diagram of the wild-type and mutant HCMV MIEP used in the *in vitro* transcription assays. The nucleotides encompassing the crs element are underlined and the nucleotide substitutions are in lowercase type. (*B*) Transcription reactions were reconstituted. Purified recombinant IE2 protein was added to the reaction mixtures in the amounts indicated above the lanes. (*C*) Quantitative representation of the results shown in *B*.

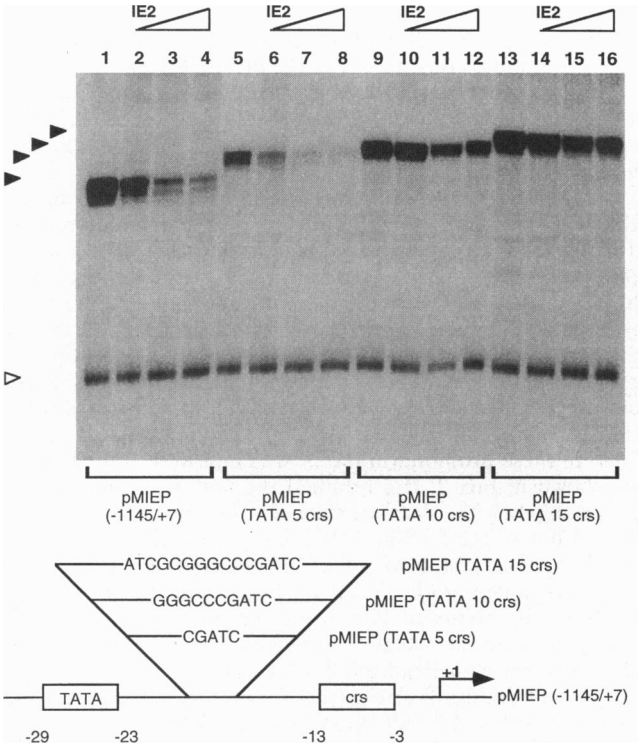

FIG. 2. Repression by IE2 is dependent on the position of the crs element. (*Lower*) A schematic representation of the HCMV MIEP plasmids containing oligonucleotide insertions between the TATA box and the crs element of the HCMV MIEP. (*Upper*) The HCMV MIEP constructs were linearized with the restriction endonuclease *Pvu* II and the products of the transcription reactions were analyzed with a run-off assay. The solid arrowheads indicate the migration positions of the RNA products transcribed from the wild-type and mutant test templates. The open arrowhead indicates the position of the RNA products transcribed from an internal control template. (*Lower*) The DNA template utilized in each set of reactions is indicated.

Binding of IE2 to the crs Element Blocks the Recruitment of RNA Polymerase II to the Promoter. Kinetic, template commitment, and DNA binding experiments have shown that transcription initiation *in vitro* is preceded by a preinitiation step in which the general factors and RNA polymerase II bind in an ordered fashion to the promoter (21, 22). By using crude nuclear extracts, we have shown (13) that repression by IE2 resulted from competition of IE2 with the formation of a preinitiation complex intermediate. To confirm this possibility, we carried out order-of-addition experiments designed to measure the effect of IE2 during the various stages of complex assembly. Transcription reactions were assembled in three consecutive incubation steps as outlined in Fig. 3. In the first step (at time 0), the DNA template and a complete set or a subset of general factors and RNA polymerase II were mixed and allowed to incubate for 30 min to allow the formation of preinitiation complexes. In the second step (at 30 min), the factors that were initially omitted were added with or without IE2 and allowed to associate with the preinitiation complex for an additional 30 min. In the third step (at 60 min), nucleotide triphosphates were added and the reactions were allowed to elongate products for 30 min. The reactions were then stopped and analized. Sarkosyl was added 1 min after the nucleotide triphosphates to a concentration of 0.2% to restrict the analyses to one round of transcription. By using this protocol, we first analyzed transcription reactions containing a complete set of basal factors at time 0 (Fig. 3, lanes 1–3) and found that, as in Fig. 1, IE2 repressed transcription when added at time 0. In contrast, IE2 had no effect on transcription when added after 30 min of preincubation (Fig. 3, compare lanes 1 and 3). These

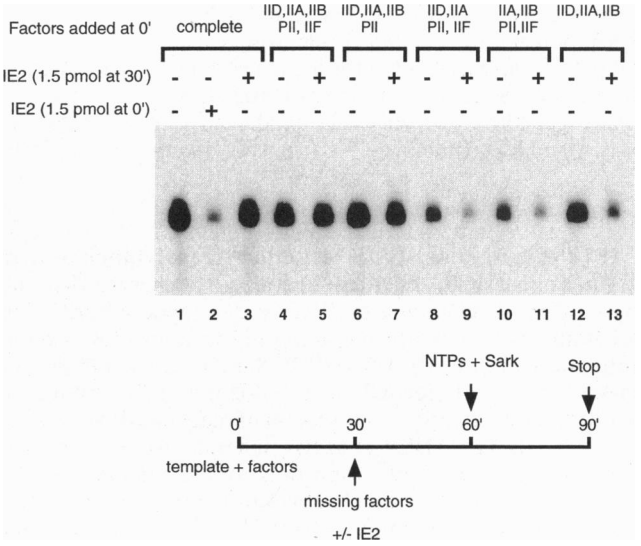

FIG. 3. Preinitiation complex intermediate containing stably bound RNA polymerase II is refractory to repression by IE2. Transcription reactions were assembled as outlined (*Lower*). (*Upper*) Bracketed lanes indicate reactions that received the same combination of factors at time 0. The reactions that received IE2 (1.5 pmol) at time 0 or after 30 min of incubation are indicated by the + symbol. Sark, sarkosyl.

results suggest that initiation-competent complexes are refractory to repression by IE2 and that IE2 might block an intermediate step in complex assembly.

Next, we analyzed the effect of IE2 on transcription after different preinitiation complex intermediates were allowed to form. Addition of IE2 at 30 min had no effect on transcription when only factors IIE and IIH were omitted from the initial incubation (Fig. 3, lanes 4 and 5), suggesting that preinitiation complex intermediates formed by TFIID, TFIIA, TFIIB, RNA polymerase II, and TFIIF were resistant to inhibition by IE2. These conditions support the formation of the TFIID–TFIIA–TFIIB (DAB)–polymerase F (PolF) complex intermediate, which contains all four factors stably bound to the promoter (ref. 23 and Fig. 4). Interestingly, reactions that only received TFIID, TFIIA, TFIIB, and RNA polymerase II were also resistant to repression by IE2 (Fig. 3, lanes 6 and 7). In contrast, preincubation of TFIID, TFIIA, and TFIIB was not sufficient to prevent IE2-mediated repression (Fig. 3, lanes 12 and 13). Furthermore, reactions that were preincubated with a set of factors that would not support the formation of a DAB–Pol preinitiation complex because either TFIIB or TFIID were omitted from the initial preincubation step were sensitive to repression by IE2 (Fig. 3, lanes 8–11). Thus, these results strongly suggest that RNA polymerase II can associate with the DAB complex and thereby prevent subsequent binding of IE2 to the crs element. Furthermore, these results imply that the crs element is accessible for IE2 binding in preinitiation complex intermediates containing TFIID, TFIIA, and TFIIB. Identical results were obtained in experiments reconstituted with TBP instead of the TFIID fraction (data not shown). It is important to note that these experiments indicate that the potential interactions between TFIID TAFs and promoter sequences of the MIEP are not sufficient to counteract IE2 repression. The low levels of specific transcription observed in some reactions are probably due to inactivation of RNA polymerase II during preincubation conditions in which the enzyme cannot associate stably with the promoter (Fig. 3, compare lanes 4–7 with lanes 8–11).

The Physical Association of IE2 and RNA Polymerase II with the HCMV MIEP Is Mutually Exclusive. Our results suggest that repression by IE2 involves competition with RNA

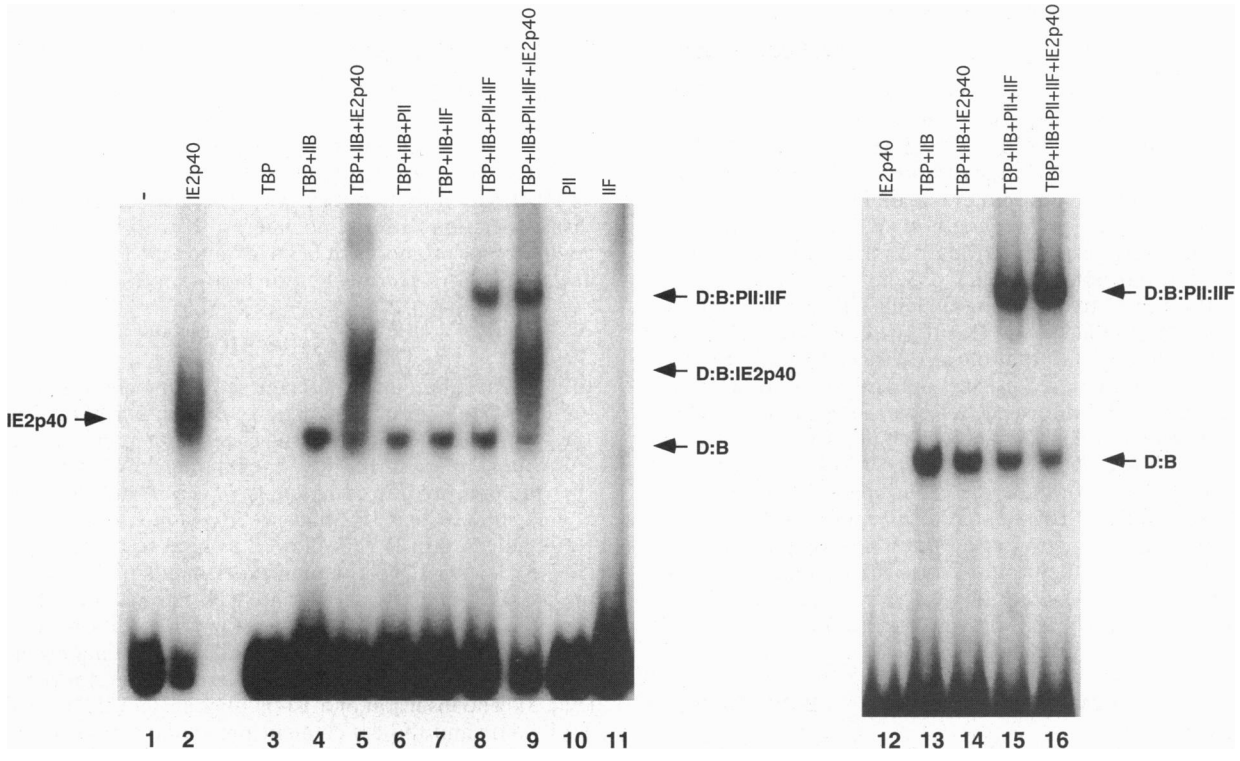

FIG. 4. Binding of IE2 and RNA polymerase II to the HCMV MIEP is mutually exclusive. DNA binding reactions were performed with a DNA fragment containing the HCMV MIE core promoter with the wild-type (lanes 1–11) or a mutated (lanes 12–16) crs element. Factors were added as indicated above the lanes. The mobilities of the different DNA–protein complexes are indicated by solid arrows.

polymerase II for promoter binding. Alternatively, it is possible that both RNA polymerase and IE2 bind the promoter but without any consequence on transcription. To distinguish between these two possibilities, we carried out the gel mobility shift experiments shown in Fig. 4. Binding of the full-length IE2 protein to the crs element produced a slowly migrating complex (data not shown and ref. 11) that was difficult to resolve from preinitiation complexes containing RNA polymerase II. Therefore, we decided to use a naturally occurring truncated version of IE2 (IE2p40) that retains the DNA binding and autoregulation activity of the full-length protein. The role of IE2p40 during the lytic cycle of CMV infection appears to be dedicated to downregulation of the HCMV MIEP. A histidine-tagged IE2p40 fusion protein was expressed in and purified from *Escherichia coli*. The activity of IE2p40 in DNA binding and repression was similar to that of the full-length IE2 protein (data not shown). Addition of IE2p40 resulted in the formation of a specific DNA–protein complex (Fig. 4). In agreement with previous studies using the adenovirus major late promoter, binding reactions with TBP (lane 3), TFIIB (data not shown), TFIIF (lane 11), or RNA polymerase II (lane 10) did not produce specific DNA–protein complexes with a ^{32}P-labeled fragment containing DNA sequences from bp −34 to +5 of the HCMV MIEP. As expected, addition of both TBP and IIB resulted in formation of the TFIID–TFIIB (DB) complex (Fig. 4, lane 4) (24, 25). Addition of IE2p40 to binding reactions containing TBP and TFIIB almost completely shifted the DB complex to a slower migrating complex (DB–IE2p40 complex). This observation indicates, consistent with the functional data shown in Fig. 3, that IE2 can access a promoter associated with TBP and TFIIB. In agreement with the previous experiments using the adenovirus major late promoter, addition of both RNA polymerase II and TFIIF to binding reactions containing TBP and TFIIB resulted in the appearance of both the DB and the DB–PolF complexes (Fig. 4). It has been shown that the DB–PolF complex contains both subunits of TFIIF and at least the largest subunit of RNA polymerase II (23). Interestingly, addition of IE2p40 to binding reactions containing TBP, TFIIB, TFIIF, and RNA polymerase II resulted in the formation of DB–IE2p40 and DB–PolF complexes (lane 9). IE2p40 did not bind to a MIEP fragment containing a mutated crs element (Fig. 4, lane 12) and could not supershift a DB complex formed on the mutated promoter fragment (Fig. 4, lanes 14 and 16). The concentrations of general factors and IE2p40 employed in the DNA binding assay were 20- and 100-fold lower, respectively, than those used in the *in vitro* transcription reactions. Because the concentration of IE2p40 used in the gel-shift experiments was not sufficient to excert repression (data not shown) and the concentration of factors were below saturation (i.e., the DNA probe was in excess), it is not surprising that IE2p40 did not prevent formation of the DB–PolF complex (Fig. 4, lane 9). Unfortunately, the use of higher concentrations of IE2p40 in the DNA binding reactions resulted in DNA–protein aggregates that could not be resolved during electrophoresis (data not shown). The fact that IE2p40 could supershift the DB complex but not the DB–PolF complex indicates that IE2p40 has higher affinity for the DB complex than for the DB–PolF complex and free DNA and strongly suggest that the binding of IE2 and RNA polymerase II to the promoter is mutually exclusive. The most logical interpretation of these observations is that IE2 can cooccupy the MIEP with bound TBP and IIB and thereby prevents the association of RNA polymerase II with the DB complex.

Heterologous DNA Binding Proteins Can Prevent the Recruitment of RNA Polymerase II to the Promoter. To investigate whether this mechanism of repression required specific properties in the repressor protein, we asked whether functionally unrelated nuclear antigen EBNA-1 and BZLF proteins of Epstein–Barr virus could function as transcriptional repressors in the context of the HCMV MIEP. EBNA-1 plays a central role in the maintenance of Epstein–Barr virus latency through its function in episomal DNA replication and transactivation of latency genes (26, 27). BZLF (also known as zta,

zebra, and EB1) is a member of the b-Zip family of transcription factors and regulates the expression of viral genes required for the lytic replication cycle (28).

Derivatives of the MIEP were prepared containing EBNA-1 and BZLF binding sites instead of the crs element. Addition of increasing amounts of BZLF-1 or EBNA-1 proteins had no effect on transcription of the wild-type HCMV MIEP (Fig. 5*A*). When tested on promoters containing their cognate binding sites in place of the crs, both BZLF and $EBNA_{191c}$ proteins behaved as potent transcriptional repressors (Fig. 5*A*). The concentration of BZLF protein required to repress 50% of the initial transcription activity (IC_{50}) was approximately 10 nM (similar to the IE2 IC_{50}). In contrast, the IC_{50} of $EBNA_{191c}$ was 0.5 nM; therefore, 20 times more BZLF and IE2 proteins were required to exert similar levels of repression. It is possible that this difference reflects the fact that the DNA binding affinity for their cognate DNA binding sites is 20 times higher for $EBNA_{191c}$ than for BZLF (10^{-11} M and 2×10^{-9} M, respectively). Order-of-addition experiments as outlined in Fig. 3 indicated that $EBNA_{191c}$ (Fig. 5*B*) and BZLF (data not shown) also repressed transcription by blocking RNA polymerase II binding to the promoter. $EBNA_{191c}$ repressed transcription of the HCMV $MIEP_{EBNA-1}$ template when added simultaneously with RNA polymerase II and the general

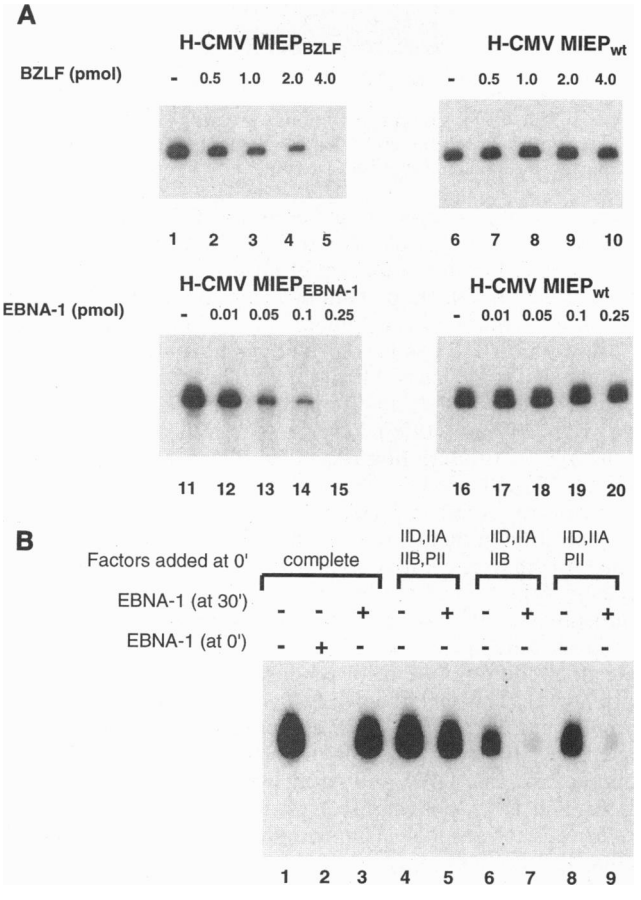

FIG. 5. Heterologous DNA binding proteins can block RNA polymerase II recruitment. (*A*) *In vitro* transcription reactions showing the effect of BZLF and $EBNA_{191c}$ proteins in transcription from the HCMV MIEP hybrid constructs. The DNA template and the amount of BZLF and $EBNA_{191c}$ proteins utilized in each set of reactions are indicated. (*B*) Effect of $EBNA_{191c}$ (0.1 pmol) in transcription reactions containing different preinitiation complex intermediates. Reactions were assembled as outlined in Fig. 3 (*Lower*). The general factors added at time 0 are indicated above each lane. The brackets group reactions that contained the same set of factors at time 0. The + symbol represents reactions that received $EBNA_{191c}$ (0.1 pmol) at either 0 or 30 min as indicated.

factors but not when added after functional preinitiation complexes were allowed to form (Fig. 5*B*). As was the case with IE2, EBNA-1 could not repress reactions that were preincubated with TFIID, TFIIA, TFIIB, and RNA polymerase II but efficiently repressed reactions that were preincubated with a subset of these factors (Fig. 5*B*). Identical results were obtained in experiments reconstituted with TBP instead of the TFIID fraction (data not shown). These observations indicate that association of RNA polymerase II with the preinitiation complex blocks binding of EBNA-1 and that TFIID and TFIIB are sufficient to recruit the polymerase to the promoter.

DISCUSSION

In this report, we have characterized the mechanism by which the HCMV IE2 protein functions as a transcriptional repressor. Two lines of evidence suggested that repression results from interference of IE2 with the binding of RNA polymerase II to the preinitiation complex: (*i*) *in vitro* transcription experiments showed that IE2 had no effect on transcription once RNA polymerase II was allowed to bind to the DB complex (Fig. 3), and (*ii*) DNA binding experiments indicated that the physical association of IE2 and RNA polymerase II to the promoter was mutually exclusive (Fig. 4). Additional evidence supporting this model comes from the experiment showing that the position of the crs element is important for repression (Fig. 2). The insertion of a 10-bp oligonucleotide between the TATA box and the crs element prevented repression by IE2 (Fig. 2). It has been well documented that in TATA containing promoters, the TATA box determines the transcription start site of RNA polymerase II transcription *in vitro* (29). Therefore, the 10-bp insertion shifts the position of the crs element relative to the TATA box and the transcription start site but does not change the position at which RNA polymerase II binds with respect to the TATA box. DNase I footprinting analyses with purified proteins have shown that simultaneous binding of TBP and IE2 results in the complete protection of promoter sequences upstream of the start site of the HCMV MIEP (11). When analyzed independently, the protection pattern of IE2 extended from bp −17 to +10 (10, 11) and that of TBP extended from bp −35 to −15 with respect to start site of the HCMV MIEP (11). We propose that critical DNA contacts made by RNA polymerase II in promoter binding lie within the region from bp −13 to −3 of the HCMV MIEP and that IE2 does not occupy this region when bound to a crs element that is placed 10 bp further downstream. The fact that IE2 cannot repress transcription from a crs element located between bp −3 and +13 relative to the start site suggests that once RNA polymerase II binds the promoter (to form the DB–Pol or DB–PolF intermediates), the preinitiation complex becomes refractory to steric inhibition. If this observation is extrapolated to other promoters, it would suggest that repressors that bind downstream from the region between bp −13 and −3 of a TATA-containing promoter repress transcription of that promoter by a mechanism other than steric interference with the preinitiation complex. It is important to note that binding of IE2 to the promoter region between bp −3 and +13 might disrupt TAF–DNA interactions. Our data does not rule out the possibility that disruption of TAF–DNA contacts might play a critical role in this type of repression, if for example these interactions are important for the stable association of the polymerase with the preinitiation complex *in vivo*. To our knowledge the importance of TAF–DNA contacts on RNA polymerase II recruitment is not known.

Previously, eukaryotic transcriptional repressors that compete with the general transcription machinery for promoter binding have been shown to block TFIID binding (4–6). Several lines of evidence suggest that a repression strategy that depends on blocking TFIID binding would be most efficiently utilized for keeping transcriptionally inactive genes off rather

than for turning active genes off. These include *in vitro* transcription experiments indicating that the TFIID complex remains bound to the promoter after transcription initiation (30) and that binding of TFIID to the TATA box is sufficient to prevent repression by nucleosomes (31), suggesting that transcriptionally active genes contain TFIID complexes stably bound to their promoters. Blockage of a step subsequent to TFIID binding may be the preferred strategy for interference with actively transcribed genes, as the repressor would have access to its binding site each time the polymerase escaped the promoter. This mechanism is suitable for the regulation of the HCMV MIEP as this promoter needs to be on early in the infection cycle and off later in the cycle.

It is surprising that among repressors that block the access of the preinitiation complex to DNA, IE2 represents the first example, to our knowledge, in which the repressor interferes with the RNA polymerase II recruitment step. Even though we did not analyze in detail the effect of core promoter sequences, our data suggest that the only strict requirement for this type of repression is the position of the repressor binding site. This notion is further supported by the fact that two unrelated factors that normally function as activators of transcription and have low and high DNA binding affinities function as potent repressors when their DNA binding sites are placed in a configuration analogous to that of the crs element in the HCMV MIEP. Thus these data suggest that functional analyses of core promoters and identification of relevant DNA binding proteins will reveal new members of this class of eukaryotic repressors.

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