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

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We have asked how the human cytomegalovirus major immediate-early 1 (IE1) and 2 (IE2) proteins act to transactivate heterologous cellular and viral promoters. Here we show that transactivation of the human immunodeficiency virus long terminal repeat and the 70,000-molecular-weight heat shock protein (hsp70) promoter by IE1 is TATA box independent and that the IE1 protein does not interact directly with the TATA box-binding factor TFIID. Conversely, transactivation of these promoters by IE2 is TATA box dependent and a direct interaction between IE2 and TFIID occurs, suggesting that IE2 transactivation is mediated through interaction with TFIID.

RNAs expressed from the human cytomegalovirus (HCMV) major immediate-early (IE) promoter-enhancer are differentially spliced at IE times to generate two major protein products: IE1, a 72,000-molecular-weight protein, and IE2, an 80,000-molecular-weight protein (22, 28, 30). These products play a pivotal role in virus infection and have been shown to transregulate both viral and cellular gene expression. It has been shown that IE1 positively autoregulates (3) and IE2 negatively autoregulates (23). In addition, much available data show that IE2 is a transactivator of a number of homologous (HCMV) and heterologous (non-HCMV) promoters (15, 23) and that IE1 can augment IE2 activation (15, 29). We and others have recently shown that IE1 and IE2 are independently able to transactivate the human immunodeficiency virus long terminal repeat (HIV LTR) (2, 33) and the hsp70 promoter (4, 7) as well as the cellular *c-myc* and *c-fos* promoters (7). While it has been suggested that IE1 and IE2 may function as bona fide DNA-binding/transcription factors, it is not known how IE1 or IE2 acts to regulate viral and cellular gene expression. It is known that constitutive promoter sequences, including the TATA box, are sufficient to mediate transactivation of the HIV LTR (2, 3) and the hsp70, *c-fos*, and *c-myc* promoters (7) by IE1 or IE2. Also, like a number of transcription factors (reviewed in reference 19), including adenovirus E1A (12) and the herpes simplex virus tegument protein VP16 (24, 32), IE2 contains independent activation domains (22).

Recently, E1A and VP16 have been shown to positively regulate gene expression by interacting directly with the preinitiation complex (11, 31). E1A contacts sequences in the C terminus of TFIID (11), and VP16, while also binding directly to TFIIB (13), also interacts directly with TFIID (31). We have therefore asked whether the HCMV major IE gene products also mediate transactivation of the HIV LTR and hsp70 promoter via direct interaction with the TATA

box-binding factor TFIID. We show that for transactivation by IE2, a TATA motif is obligatory and that IE2 interacts directly with TFIID. In contrast, transactivation by IE1 does not require a functional TATA element and there is no detectable interaction of IE1 with TFIID.

MATERIALS AND METHODS

Cell culture, transfection, and CAT assays. IBR cells, primary skin fibroblasts immortalized by simian virus 40 (SV40) T antigen (17), were maintained as monolayers in Eagle's minimal essential medium supplemented with 10% fetal calf serum. Cells were split 1:3 every 3 days. Approximately 5×10^6 cells were transfected with 0.5 μ g of chloramphenicol acetyltransferase (CAT) reporter constructs and 3 μ g of effector DNA by calcium phosphate coprecipitation. Cells were harvested 34 h posttransfection, and CAT activity was assayed as described previously (5). After autoradiography, spots were excised and quantified by liquid scintillation counting. Each transfection was repeated at least three times, and representative results are shown.

Plasmids. pSV2IE1 was made by blunt ending a *Bam*HI fragment of pJD083 (1), containing a full-length HCMV IE1 cDNA, into a blunt-ended *Bam*HI-*Eco*RI deletion of pSG5 (6). pHM121, a full-length HCMV IE2 cDNA clone under the control of the SV40 early promoter-enhancer in a Bluescript (Stratagene) vector, was a kind gift of Thomas Stamminger (Erlangen, Germany). The hsp70 CAT constructs and the E1A expression vector pE1A were a generous gift of J. Nevins (25). The HIV LTR CAT constructs were provided by G. Nabel (20). pSV2neo has been previously described (27).

Generation of in vitro translated proteins. Phagemid vectors for E1A (pTM1E1A) and gelsolin (10) were provided by A. Berk (University of California at Los Angeles) and A. Weeds (University of Cambridge), respectively. For IE1, the IE1-coding region of pJD083 was subcloned into pGEM (pGEM IE1). For IE2 expression, the SV40 promoter region of the Bluescript-based IE2 expression vector (pHM121)

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provided by T. Stamminger was deleted with *Hind*III and religated.

Linearized vector DNA (5 μ g) was in vitro transcribed (9), and 20% of the RNA product was used for in vitro translation in the presence of [35 S]methionine, using an in vitro translation kit (Promega) in a 50- μ l reaction mix according to the manufacturer's instructions.

GST fusion proteins. pGEX.TFIID-C was cloned by inserting an *Ssp*I-*Dra*I fragment of pKB104 (8), containing the coding sequence for amino acids 168 to 339 of TFIID, into the *Sma*I site of pGEX-3X (Promega) in frame with the glutathione S-transferase (GST) gene. pGEX.TFIID-N was cloned by polymerase chain reaction amplification of the coding region for amino acids 1 to 163 of TFIID and subsequent cloning into the *Bam*HI site of pGEX-2T (Promega) in frame with the GST gene. GST fusion protein expression and purification were as previously described (26). Briefly, a 40-ml overnight culture of *Escherichia coli* (X-90) containing pGEX fusions was diluted 1:10 in 2 $\times$ YT medium containing ampicillin (100 μ g/ml). After incubation for 1 h at 37°C, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and incubation was continued for a further 5 h. For fusion protein recovery, bacterial cultures were pelleted by centrifugation at 3,000 $\times$ g for 5 min at 4°C and resuspended in 9 ml of MTBPS buffer (140 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, pH 7.3) containing 1 mM phenylmethylsulfonyl fluoride. The bacterial pellet was then lysed on ice by mild sonication, and 1 ml of MTBPS containing 10% Triton X-100 was added before centrifugation at 10,000 $\times$ g for 5 min at 4°C. The supernatant was rocked gently for 15 min at 4°C with 300 μ l of glutathione-Sepharose beads (Promega) which had been washed previously three times in MTBPS and after pelleting at 500 $\times$ g the beads were then resuspended 1:1 (vol/vol, final concentration) in MTBPS. For analysis of bound fusion protein, the beads were boiled in 4 $\times$ sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer and loaded onto SDS-polyacrylamide gels. Proteins were visualized by Coomassie blue stain. Fusion proteins were stored at -70°C in 10% glycerol.

TFIID binding assay. Five hundred nanograms of the GST-TFIID fusion proteins on beads was preincubated with bovine serum albumin (final concentration, 1 mg/ml) at room temperature for 5 min and then rocked for 1 h at room temperature with 2 to 5 μ l of in vitro translated test protein in a final volume of 200 μ l in EBC buffer (140 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 mM sodium orthovanadate, 50 mM Tris HCl, pH 8.0). The beads were then washed three times in 1 ml of NETN buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris HCl, pH 8.0), pelleted at 500 $\times$ g for 30 s, and boiled in 4 $\times$ SDS-PAGE sample buffer. Bound proteins were resolved on SDS-15% polyacrylamide gels. To estimate the proportion of in vitro translated protein "pulled down" by the GST fusion, the exact amount of protein added to the binding reaction was always quantified by SDS-PAGE. Gels were fixed and rocked in a fluorograph for 30 min prior to drying and autoradiography.

Far Western blotting. Crude bacterial extracts were separated by SDS-PAGE on 10% gels and blotted onto nitrocellulose in the absence of methanol. Blots were denatured and renatured as previously described (33) and probed with 100 to 200 μ l of reticulocyte lysate containing in vitro translated [35 S]methionine-labelled IE1 or IE2 in 5 ml of hybridization buffer at 4°C overnight. IE1 and IE2 probes were normalized

FIG. 1. Transactivation of hsp promoter CAT constructs. hsp promoter CAT constructs containing the full hsp70 promoter (a), an hsp70 promoter containing the SV40 TATA motif (b), and an hsp70 promoter containing a mutant TATA motif (c) were cotransfected with pSV2neo (lanes 1) or vectors expressing IE1 (lanes 2), IE2 (lanes 3), or E1A (lanes 4). Thirty-four hours posttransfection, cells were harvested and assayed for CAT activity. Representative assays are shown. Mean fold increases above basal activity ($\pm$ standard deviation) for three experiments were 0 (0), 8.0 ($\pm$ 2.8), 4.0 ($\pm$ 1.0), and 9.0 ($\pm$ 2.5) for panel a, lanes 1 to 4, respectively; 0 (0), 7.0 ($\pm$ 0.7), 4.0 ($\pm$ 1.4), and 3.0 ($\pm$ 0.7) for panel b, lanes 1 to 4, respectively; and 0 (0), 11.0 ($\pm$ 1.5), 2.0 ($\pm$ 0.5), and 2.0 ($\pm$ 0.6) for panel c, lanes 1 to 4, respectively.

for [35 S]methionine incorporation by SDS-PAGE and autoradiography.

RESULTS

Transactivation of the hsp70 promoter is TATA box independent for IE1 but TATA box dependent for IE2. We have previously shown that IE1 and IE2 independently transactivate the hsp70 promoter through basal promoter sequences containing only 50 to 60 bp of DNA sequence upstream of the transcription start site, which includes the TATA box (7). These results implicate the basal promoter sequences as a possible target for IE1 and IE2 transactivation of the hsp70 promoter. We sought, therefore, to examine the role of the TATA box in IE1 and IE2 transactivation. Figure 1c, lane 2, shows that transactivation of the hsp70 promoter by IE1 is not TATA box dependent, since an hsp70 promoter in which the TATA motif has been mutated is still transactivated by an IE1 cDNA expression vector. In contrast, the transactivation of the hsp70 promoter seen with IE2 is abolished if the promoter contains a mutated TATA motif (Fig. 1c, lane 3). As a control, we included in these transfections adenovirus E1A, which has been shown to transactivate only an hsp70 promoter which contains the homologous hsp70 TATA motif (25) (Fig. 1a, lane 4). However, in our hands, a low level of transactivation by E1A of the hsp70 promoter containing the SV40 TATA motif remained (Fig. 1b, lane 4). IE1 and IE2 will transactivate an hsp70 promoter with its genuine TATA element (Fig. 1a, lanes 2 and 3) and also an hsp70 promoter in which the TATA motif has been swapped for an SV40 early promoter TATA element (Fig. 1b, lanes 2 and 3, respectively). Since all expression vectors for IE1, IE2, and E1A were under the control of the SV40 promoter-enhancer, promoter competition effects were ruled out by cotransfection with pSV2neo (Fig. 1, lanes 1).

Transactivation of the HIV LTR is also TATA box independent for IE1 but TATA box dependent for IE2. We also asked whether the dependence of IE1 and IE2 on a TATA element was the same in transactivation of the HIV LTR. IE1 and IE2 are also independently able to transactivate the HIV LTR (2, 33) (Fig. 2a, lanes 2 and 3) through basal promoter sequences (2, 33). Figure 2c shows that the ability of IE2 (lane 3) to transactivate the LTR is also TATA box dependent. However, IE1 will still transactivate a mutant TATA box LTR construct (Fig. 2c, lane 2). As with the hsp70

FIG. 2. Transactivation of HIV LTR constructs. HIV LTR CAT vectors containing the full LTR (a), an LTR containing the SV40 TATA motif (b), and an LTR containing a mutant TATA motif (c) were cotransfected with pSV2neo (lanes 1) or vectors expressing IE1 (lanes 2), IE2 (lanes 3), or E1A (lanes 4). Thirty-four hours posttransfection, cells were harvested and assayed for CAT activity. Representative assays are shown. Mean fold increases above basal activity ($\pm$ standard deviation) for three experiments were 0 (0), 9.0 ($\pm$ 3.0), 8.0 ($\pm$ 1.0), and 6.0 ($\pm$ 1.5) for panel a, lanes 1 to 4, respectively; 0 (0), 10.0 ($\pm$ 2.6), 11.0 ($\pm$ 1.0), and 1.5 ($\pm$ 1.0) for panel b, lanes 1 to 4, respectively; and 0 (0), 9.0 ($\pm$ 3.6), 2.0 ($\pm$ 0.5), and 1.5 ($\pm$ 0.7) for panel c, lanes 1 to 4, respectively.

promoter, an LTR construct containing the SV40 early TATA motif (Fig. 2b) was transactivated by both IE1 (lane 2) and IE2 (lane 3) but not by E1A (lane 4), which requires the homologous HIV LTR TATA motif for transactivation as has been previously shown (15). Again, these results were not the effect of promoter competition (Fig. 2, lanes 1).

IE2, but not IE1, interacts directly with TFIID. Since IE2 transactivation, like E1A transactivation, was TATA box dependent, we asked whether the IE2 product was able to interact directly with the TATA box-binding factor TFIID, as was recently shown for E1A (11). Since the direct binding of E1A was shown to work through the conserved C terminus but not the N terminus of TFIID (7), we constructed fusion proteins consisting of the GST protein linked to either the N- or C-terminal residues of TFIID (Fig. 3) and asked whether *in vitro* transcribed and translated IE1 and IE2 proteins bind to these GST-TFIID fusions. Figure 4A shows the relative amounts of *in vitro* translated, radiolabelled IE2 (lane 2) and IE1 (lane 3). Two additional proteins were used in the assay: E1A as a positive control (lane 5) and gelsolin (a nonnuclear protein) as a negative control (lane 4). We also included a mixture of these proteins in the binding

assay (lane 1). Figure 4B, lane 2, shows that IE2 interacts directly with the C terminus of TFIID but not with the N terminus (Fig. 4C, lane 2), whereas IE1 shows no direct interaction with either fusion protein (Fig. 4B and C, lanes 3). As previously shown (11), E1A interacted directly with the C-terminal (Fig. 4B, lane 5) but not the N-terminal (Fig. 4C, lane 5) half of TFIID, and gelsolin shows no interaction with TFIID (Fig. 4B and C, lanes 4). Similarly, specific binding of IE2 to the C-terminal (Fig. 4B, lane 1) but not the N-terminal (Fig. 4C, lane 1) end of TFIID is also seen if IE2 is presented in the binding assay as a complex mixture of proteins. Since IE2 binds to the C-terminal but not the N-terminal GST-TFIID fusion, it is also clear that IE2 does not bind nonspecifically to GST or the agarose beads used in the assay. Also, confirming specificity, IE2, IE1, and E1A do not bind to a GST-vimentin fusion in this assay (data not shown).

To confirm that IE2 interacts with TFIID, we also carried out far Western blot analysis. Crude bacterial extracts containing GST fusions to the C-terminal or N-terminal ends of TFIID were separated by SDS-PAGE, blotted, and probed with [35 S]methionine-labelled IE1 or IE2. Figure 5B confirms that IE2 binds to the C terminus of TFIID (lane 3) but not the N terminus of TFIID (lane 2). Figure 5A also shows that this interaction is specific for IE2, since IE1 shows no such binding.

DISCUSSION

The HCMV major IE gene products transregulate viral and cellular gene expression. We have shown, by using two promoters which are transactivated by IE1 and IE2 independently, that the mechanisms of promoter transactivation by IE1 and IE2 are very different. IE1 transactivation is mediated via a TATA box-independent mechanism, whereas IE2 transactivation is clearly TATA box dependent.

Throughout these experiments, we have compared the mechanism of transactivation of the HIV LTR and the hsp70 promoter by IE1 and IE2 to that by E1A. Transactivation of the HIV LTR and the hsp70 promoter by E1A depends on a specific TATA motif, and E1A will not transactivate the HIV LTR and the hsp70 promoter containing a heterologous TATA element (20, 25). Similarly, mutation of the TATA element within these promoters abolishes E1A transactivation.

FIG. 3. GST fusion proteins. The schematic shows the chimeric proteins formed by a fusion of GST and the TATA box-binding factor TFIID. Fusions of the N terminus of TFIID to GST contain TFIID amino acids 1 to 163 (pGEX.TFIID-N), and the fusion of the C terminus of TFIID to GST contains amino acids 168 to 339 of TFIID (pGEX.TFIID-C).

FIG. 4. TFIID-binding assays. (A) Input proteins of a mixture of IE1, IE2, and gelsolin (lane 1), IE2 alone (lane 2), IE1 alone (lane 3), gelsolin alone (lane 4) and E1A alone (lane 5). The same amounts of protein were used in the TFIID-binding assays in panels B and C. The multiple bands in panel A, lane 2, are most likely degradation or internal initiation products of IE2 but are clearly distinguishable from IE1, E1A, and gelsolin. (B) Binding of a mixture of IE1, IE2, and gelsolin (lane 1), IE2 (lane 2), IE1 (lane 3), gelsolin (lane 4), and E1A (lane 5) to the C terminus of TFIID (TFIID-C). (C) Binding of a mixture of IE1, IE2, and gelsolin (lane 1), IE2 (lane 2), IE1 (lane 3), gelsolin (lane 4), and E1A (lane 5) to the N terminus of TFIID (TFIID-N). Lanes M contain radiolabelled molecular mass markers (indicated in kilodaltons at left).

tion. As with E1A, transactivation of the HIV LTR and the hsp70 promoter by IE2 also requires a TATA motif, but transactivation is still observed if promoters contain heterologous TATA elements. However, IE1 shows no TATA box dependence, and mutation of the TATA element of the HIV LTR or the hsp70 promoter still results in IE1 transactivation.

This TATA box-independent transactivation by IE1 and the TATA box-dependent transactivation by IE2 correlate well with the observed ability of IE proteins to interact with TFIID. We could show no direct interaction between IE1 and TFIID, but we could show a direct and specific binding of IE2 to the TATA box-binding factor. Like E1A, which has been shown to interact specifically with TFIID (11), IE2 interacts specifically with the C-terminal conserved region of TFIID but not the N-terminal region.

FIG. 5. Far Western analysis. After IPTG induction, crude bacterial extracts containing GST protein (lanes 1), GST-TFIID-N fusion protein (lanes 2), or GST-TFIID-C fusion protein (lanes 3) were separated on 10% polyacrylamide gels, blotted onto nitrocellulose, and probed with [³⁵S]methionine-labelled IE1 (A) or IE2 (B). (C) Coomassie blue staining of the separated proteins prior to blotting.

We have shown that the HCMV major IE proteins mediate transactivation of the HIV LTR and the hsp70 promoter by different mechanisms. IE2 appears to share important functional and structural features with other well-characterized transcriptional activators encoded by double-stranded DNA viruses. Like E1A and VP16, IE2 transactivates transcription without being a typical DNA-binding factor. Again, like E1A (11) and VP16 (31), IE2 binds directly to TFIID (this report), and also like VP16 (24, 32) and pseudorabies virus IE (16), IE2 contains acidic activation domains capable of transactivating a GAL promoter-reported construct in GAL domain swap experiments (22). E1A and VP16, though incapable of binding DNA directly, can be targeted to promoters through direct interaction with other DNA-binding transcription factors (14, 18, 21). Since they both also bind the TATA box-binding factor TFIID, current thinking assumes that they act as a "bridge" between promoter-bound activators and the preinitiation complex. It is possible that IE2 acts in a similar way; besides mapping the specific sites of interaction between the IE2 and TFIID proteins, an analysis of protein-protein interactions between candidate DNA-binding transcription factors and IE2 might give further insight into the mechanism of IE2 transactivation. An alternative possibility—one that doesn't necessarily exclude that mentioned above—is that IE2 functions by increasing the affinity of TFIID for the TATA box. This has been suggested for E1A to explain its ability to activate promoters containing no upstream sequences other than the TATA element (11).

Unlike IE2, E1A, and VP16, IE1 does not appear to work by directly contacting TFIID. However, IE1 does share certain characteristics of this group of transcriptional activators. Like IE2 and E1A, IE1 is able to transactivate promoters containing only a TATA motif plus an additional 10 to 20 bp upstream (7, 34). IE1 also appears not to bind DNA directly and does contain an acidic activation domain in common with IE2, by virtue of a commonly spliced exon domain (22). It therefore remains a possibility that IE1 and IE2 function in a similar fashion but that IE1 contacts a preinitiation complex factor other than TFIID. It would be interesting to determine whether IE1 interacts with other

isolated proteins of the preinitiation complex such as TFIID, as has been shown to be an additional feature of VP16 (13).

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