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The Transactivation Domain of Adenovirus E1A Interacts with the C Terminus of Human $TAF_{II}135$

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The CR3 activation domain of the human adenovirus E1A protein stimulates transcription by forming protein-protein interactions with DNA sequence-specific binding factors and components of the TFIID complex. Here, we demonstrate that CR3 can complex with the extreme C-terminal 105 amino acids of the human TATA box binding-factor-associated protein, $hTAF_{II}135$. Furthermore, the C-terminal region of $hTAF_{II}135$ can block transcriptional stimulation from an E1A-inducible promoter in vivo. This ability of the C terminus of $hTAF_{II}135$ to bind CR3 and to inhibit E1A-inducible activation is highly specific. These results demonstrate for the first time that a discrete fragment of a mammalian TBP-associated factor which targets a specific activator can impair the stimulation of transcription.

The human adenovirus 289R E1A transactivating protein can stimulate transcription from a variety of promoters by apparently complexing with both DNA-specific recognition site transcription factors, e.g., ATF-2, SP1, and USF (19, 20), and components of the basal transcription initiation complex (1, 6, 7, 13, 17, 25, 38). Through the formation of such protein-protein interactions, E1A seems to mediate transcription without binding to DNA. The 48-amino-acid conserved region 3 (CR3) of E1A, which is responsible for mediating transactivation (29), appears to target several proteins of the transcription initiation complex (1, 6, 7, 13, 17, 25, 38). CR3 contains a Cys-4 zinc finger which binds to the TATA box-binding protein, TBP (7). Mutations within the CR3 zinc finger region (residues 147 to 177) that eliminate TBP binding correlate with a loss of E1A transactivation function (7). In addition, in vitro studies have revealed an apparent requirement for TBP-associated factors (TAFs) in E1A-mediated stimulation of transcription (38). In these studies, it was found that TBP could not functionally replace purified TFIID, the complex containing TBP and TAFs (for a recent review, see reference 2). Indeed, our recent studies demonstrated that *Drosophila* $TAF_{II}110$ and human $TAF_{II}250$ complex with the CR3 region of E1A (6, 25). In addition, human $TAF_{II}55$ has also been shown to bind CR3 (4). Significantly, interactions between TAFs and other activators have been shown to be essential for modulating transcription both in vitro (3) and in vivo (24, 32).

We previously reported that the CR3 transactivation domain of E1A binds to the C terminus of *Drosophila* $TAF_{II}110$ ($dTAF_{II}110$) (6, 24). Most recently, $hTAF_{II}135$, the human homolog of $dTAF_{II}110$, has been cloned (26, 33). Because modulation of transcription by E1A has been studied in mammalian systems, it was important to determine whether there exists a corresponding E1A-$hTAF_{II}135$ interaction. To examine this question, binding studies using glutathione *S*-transferase fusion proteins and [^{35}S]methionine-labeled proteins generated by in vitro transcription and translation were performed. GST fusion proteins GST-$hTAF_{II}135$ (27), GST-$E1A_{(121\text{-}223)}$ (6), and GST-p50 (gift from I. Verma, Salk Institute) were purified essentially as previously described (6). For in vitro transcription and translation (Promega) of $hTAF_{II}135$ constructs, the T7 RNA polymerase promoter in pET21d or pXJ40 was utilized. Quantitation of the amount of [^{35}S]methionine incorporated was determined by analyzing aliquots of the translation reactions by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and measuring the radioactivity in resolved bands corresponding to the specific $hTAF_{II}135$ polypeptide with a Molecular Dynamics PhosphorImager. The full-length $hTAF_{II}135$ cDNA (pXJ40-$hTAF_{II}135$ (1-1083) has been described previously (26). A *Cla*I digest of pXJ40-$hTAF_{II}135$(1-1083) generated $hTAF_{II}135\Delta C248$. A series of plasmids encoding $hTAF_{II}135$ C-terminal constructs (pET21d578R, pET21dΔN299, pET21dΔN367, pET21dΔN433, and pET21dΔN473) were generated by PCR cloning with pAT6-$hTAF_{II}135$(372-1083), which encodes the C-terminal 711 amino acids of $hTAF_{II}135$, as the DNA template; the 5′ primers contained an appended *Eco*RI site, and the 3′ primer contained the $hTAF_{II}135$ stop codon and an appended *Not*I site. The gel-purified inserts were ligated into *Eco*RI and *Not*I sites of pET21d. $hTAF_{II}135$ polypeptide carboxyl-terminal deletions were produced by utilizing natural restriction sites within the $hTAF_{II}135$ DNA sequences. Specifically, pET21d578R was digested with *Cla*I to produce $hTAF_{II}135$ polypeptide $\Delta C248$, with *Pvu*I to produce $\Delta C75$, and with *Ava*I to produce $\Delta C45$. The E1A constructs (6, 17), BBV-13S, BBV-12S, BBV-$E1A_{(\Delta 140\text{-}146)}$, BBV-$E1A_{(\Delta 147\text{-}153)}$, BBV-$E1A_{(\Delta 154\text{-}159)}$, BBV-$E1A_{(\Delta 160\text{-}168)}$, BBV-$E1A_{(\Delta 169\text{-}174)}$, BBV-$E1A_{(\Delta 175\text{-}179)}$, and BBV-$E1A_{(\Delta 180\text{-}188)}$, were used in in vitro transcription and translation. The assay used to analyze the binding of in vitro-translated proteins to the GST fusion proteins was previously described (25). To normalize the input [^{35}S]methionine incorporated in each binding reaction, an adjusted volume of in vitro-translated $hTAF_{II}135$ polypeptide was used. Bound proteins were eluted from the glutathione-agarose beads by boiling in Laemmli buffer and resolved on an SDS–10 to 20% gradient polyacrylamide gel.

As shown in Fig. 1A, in vitro-translated full-length $hTAF_{II}135$ is able to bind to the GST-$E1A_{(121\text{-}223)}$ fusion protein which includes the CR3 transactivation domain of E1A. Since the C-terminal coding region of $dTAF_{II}110$ is critical for binding E1A (25) and exhibits extensive homology (52% iden-

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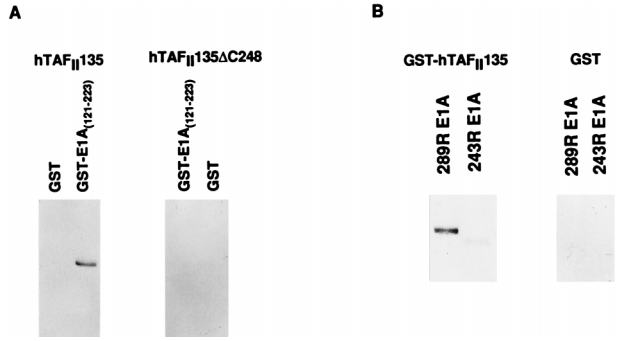

FIG. 1. E1A-hTAF$_{II}$135 interaction requires the CR3 activation domain of E1A and the C-terminal region of hTAF$_{II}$135. (A) The GST-E1A$_{(121-223)}$ fusion protein containing CR3 binds to radiolabeled full-length hTAF$_{II}$135 but not to a mutant of hTAF$_{II}$135 which lacks the C-terminal 248 amino acids. (B) A GST fusion protein containing the carboxyl region of hTAF$_{II}$135 binds to the radiolabeled 289R E1A protein containing CR3 but only negligibly to the 243R E1A protein which lacks CR3; except for CR3, 243R is identical to 289R. Radiolabeled GST-bound proteins were resolved on an SDS–10 to 20% polyacrylamide gel.

tity) with hTAF$_{II}$135 (Fig. 2), we reasoned that the C terminus of hTAF$_{II}$135 might also bind E1A. Therefore, we tested the ability of GST-E1A$_{(121-223)}$ to bind an hTAF$_{II}$135 deletion mutant (hTAF$_{II}$135ΔC248) whose DNA had had 248 codons removed from the region encoding the C terminus of full-length hTAF$_{II}$135. As seen in Fig. 1A, GST-E1A$_{(121-223)}$ completely failed to bind hTAF$_{II}$135ΔC248. This suggested that the C terminus is the only region of hTAF$_{II}$135 required to bind E1A.

In order to confirm that the C terminus of hTAF$_{II}$135 binds directly to the CR3 transactivation domain, a GST fusion protein containing the C-terminal 583 amino acids of hTAF$_{II}$135 was tested for its ability to bind in vitro-translated E1A proteins. As shown in Fig. 1B, the hTAF$_{II}$135 C-terminal fusion protein exhibited strong binding to the 289R E1A protein which contains CR3 but only negligible binding to the 243R E1A protein which is identical to the larger E1A protein except that it lacks CR3. To examine which regions of the E1A CR3 transactivation domain are needed to mediate this E1A-hTAF$_{II}$135 interaction, a series of E1A mutants with sequential amino acid deletions spanning the entire CR3 transactivation domain (6) were translated in vitro and assayed for binding to the GST-hTAF$_{II}$135 C-terminal fusion protein. The results, which are summarized in Fig. 3, indicate that deleting either of the regions of CR3 which flank the C4 zinc finger significantly reduces binding to the hTAF$_{II}$135 C-terminal fusion protein. Relative to the C4 zinc finger, the more distal flanking sequences (residues 140 to 146 or 180 to 188) appeared more sensitive to mutation than did the more proximal flanking sequences (residues 147 to 153 or 175 to 179), producing a bigradient of decreasing hTAF$_{II}$135 binding activity. Deletions within the Cys-4 Zn finger region (residues 147 to 177) of CR3 had no major effect on hTAF$_{II}$135 binding. Interestingly, the Cys-4 Zn finger region has been shown to bind hTBP and is postulated to bind a limiting cellular transcription factor as well (7). Importantly, E1A proteins with mutations in the CR3 regions flanking the Cys-4 Zn finger are not only defective in binding hTAF$_{II}$135 but also are defective in stimulating transcription (7, 23, 35). These results suggest that CR3, and in particular the regions which flank the Cys-4 Zn finger, might contribute to the recruitment of E1A to the basal promoter through interactions with the hTAF$_{II}$135 C terminus.

To fine map the C-terminal region of hTAF$_{II}$135 that interacts with the CR3 region of E1A, in vitro-translated hTAF$_{II}$135 polypeptides generated from deletion constructs were tested for binding to GST-E1A$_{(121-223)}$. For each hTAF$_{II}$135 deletion mutant, a polypeptide of the expected length was synthesized as the major product of the in vitro translation reaction (data not shown). As expected, the 578R C-terminal fragment of hTAF$_{II}$135 was able to bind GST-E1A$_{(121-223)}$ but not GST protein alone or GST-p50, which includes the p50 subunit of NF-κB (Fig. 4A; compare lane 4 with lanes 2 and 3). A series of N-terminal deletions generated from the 578R C-terminal coding region (ΔN299, ΔN367, ΔN433, and ΔN473) were all capable of interacting with GST-E1A (Fig. 4A, lanes 5 to 12). Importantly, the fact that binding still occurs with ΔN473 indicates that an E1A CR3 binding site is contained within the last 105-amino-acid stretch of hTAF$_{II}$135 (summarized in Fig. 4B).

A series of C-terminal deletions (ΔC248, ΔC75, and ΔC45) generated from the 578R hTAF$_{II}$135 construct confirmed that the extreme C terminus of hTAF$_{II}$135 is necessary for binding CR3. Each of these C-terminal deletions abrogated binding to GST-E1A$_{(121-223)}$ (Fig. 4A, lanes 13 to 19). Eliminating the last 248 residues (ΔC248) was expected to abrogate binding to E1A (Fig. 1A). However, failure of the shorter C-terminal deletions of 75 (ΔC75) and 45 (ΔC45) residues to bind E1A clearly substantiated the importance of the last 105 amino acids of hTAF$_{II}$135 for interacting with E1A CR3 (summarized in Fig. 4B).

It was important to demonstrate that binding of CR3 to the C terminus of hTAF$_{II}$135 can occur in vivo and hence may be significant to E1A-mediated transactivation. We reasoned that if the CR3 region targets the C terminus of hTAF$_{II}$135, then E1A transactivation might be blocked in the presence of a polypeptide containing the C terminus of hTAF$_{II}$135. Transfections employing the calcium phosphate precipitation method (9, 10) were performed in duplicate in NIH 3T3 cells, and the total amount of plasmid DNA used per transfection (27 μg) was equalized by the addition of hTAF$_{II}$135 parental plasmid lacking insert. Cell extracts were assayed 48 h posttransfection for reporter chloramphenicol acetyltransferase (CAT)

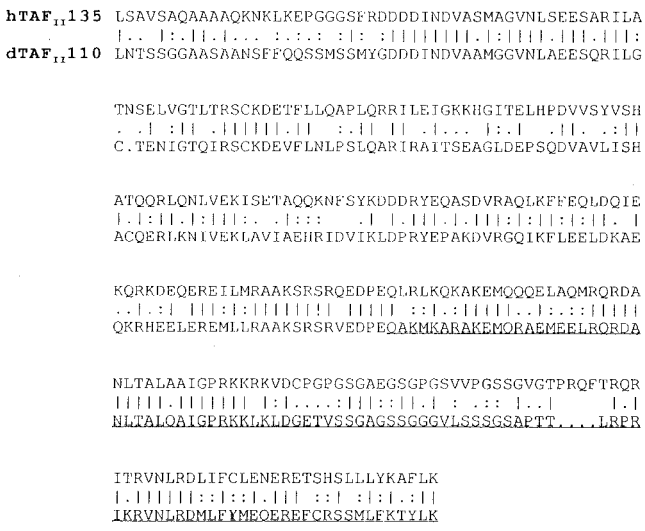

FIG. 2. Sequence comparison of the C-terminal 276 amino acids of hTAF$_{II}$135 and dTAF$_{II}$110. The underlined portion represents the 105-amino-acid region of hTAF$_{II}$135 involved in binding 289R E1A. Vertical lines indicate identical amino acids; double and single dots indicate greater and lesser degrees, respectively, of chemical similarity.

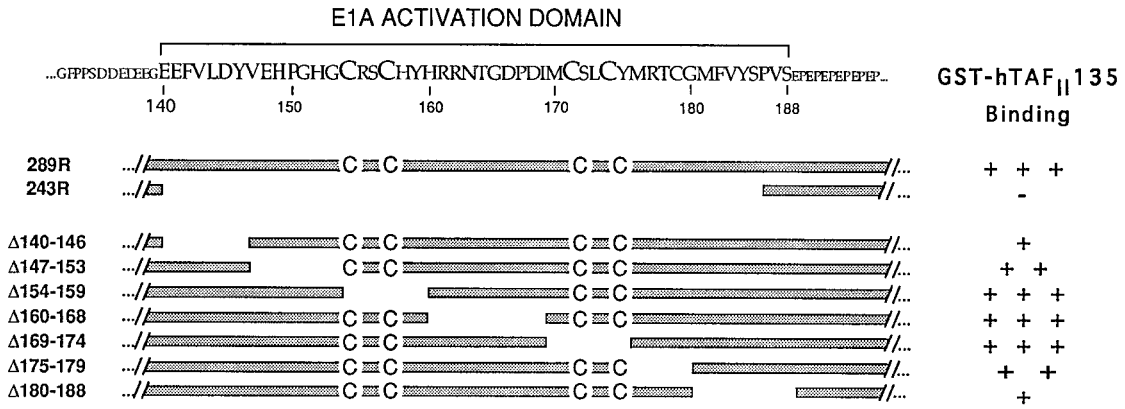

FIG. 3. Regions of E1A CR3 involved in binding the C terminus of hTAF$_{II}$135. The 289R and 243R proteins are identical except that the larger protein contains the Cys-4 zinc finger-containing CR3 transactivation domain, residues 140 to 188. Represented is a series of E1A deletion mutants that span CR3 and a summary of their relative binding abilities to a GST-hTAF$_{II}$135 fusion protein. The 289R E1A mutants, with deletions of residues 140 to 146 and 180 to 188 showed a consistently lower level of binding to GST-hTAF$_{II}$135 than did other deletion mutants.

activity (8). The amount of cell extract used in each CAT assay was normalized by protein concentration (Bio-Rad), and CAT activity was quantitated with a Molecular Dynamics PhosphorImager. To test for inhibition of E1A transactivation, cells were cotransfected with a full-length 289R E1A expression plasmid containing CR3 (pSK-E1A) (35), an E1A-inducible E3 promoter-driven CAT plasmid (pE3CAT) (36), and an hTAF$_{II}$135 expression vector (phTAF135-279C) encoding the 279R C terminus of hTAF$_{II}$135 (26). As shown in Fig. 5A, the ability of pSK-E1A (2 μg) to transactivate the E3 promoter was dramatically inhibited (~75%) with the least amount of transfected phTAF135-279C (1 μg). Inhibition of E1A transactivation to a level approaching basal levels was achieved with slightly higher concentrations of transfected phTAF135-279C. By contrast, a 347-amino-acid N-terminal cDNA-derived fragment (encoded by phTAF135-347N) (26) which does not bind to E1A but which is expressed in transfected cells (data not shown) did not block E1A transactivation (Fig. 5B). Nor was E1A transactivation inhibited by the Gal4 DNA binding domain (37), a non-TAF polypeptide, which does not interact with E1A (20). Conversely, the C-terminal hTAF$_{II}$135 fragment did not affect transcriptional stimulation mediated by the activation domain of VP16 (18, 30), the herpes virus transactivating protein for which there is no apparent interaction with hTAF$_{II}$135 (15) (data not shown). Thus, the in vivo interaction between the C terminus of hTAF$_{II}$135 and E1A appears to be specific.

The C-terminal fragment of hTAF$_{II}$135 most likely inhibited E1A transactivation in vivo by directly targeting CR3. To show that endogenous E1A can interact with the C terminus of hTAF$_{II}$135, nuclear extract (72 μg) prepared from E1A-expressing Ad5-transformed cells (21) was bound to GST or GST-hTAF$_{II}$135 (5 μg). Bound proteins were released and fractionated on an SDS–10 to 20% gradient polyacrylamide gel, transferred onto an Immobilon-P membrane (Millipore) and probed with E1A-specific mouse monoclonal antibody M73 (11). When nuclear extract was incubated with the GST-hTAF$_{II}$135 C-terminal fusion protein, only the 289R E1A protein containing CR3 complexed with the C terminus of hTAF$_{II}$135 (Fig. 6). By contrast, the 243R E1A protein, which is identical to 289R E1A except that it lacks CR3, essentially failed to interact with the C terminus of hTAF$_{II}$135. Taken together, these results strongly suggest that E1A-mediated transactivation involves a specific interaction between CR3 and the C terminus of hTAF$_{II}$135.

In this study, we have demonstrated that residues within the extreme C terminus of hTAF$_{II}$135 are able to bind to the CR3 activation domain of E1A and block its ability to stimulate transcription of an E1A-inducible promoter. Our results indicate that no region other than the 105-amino-acid C terminus of full-length hTAF$_{II}$135 is able to interact with E1A. The predominant feature of the 105-amino-acid C terminus of TAF$_{II}$135 is a serine-, glycine-, and threonine-rich region flanked by stretches of acidic and basic residues (Fig. 4B). The corresponding C terminus of *Drosophila* dTAF$_{II}$110, which was also found to be important for binding 289R E1A (6, 25), shares 57% identity with hTAF$_{II}$135 (26). Significantly, two other viral transactivators, HPV-16 E7 and simian virus 40 T antigen, which can bind to the C terminus of dTAF$_{II}$110 (25) can also bind the C terminus of hTAF$_{II}$135 (unpublished results). Moreover, we anticipate that each of these three oncoproteins will bind to the human B cell-specific hTAF$_{II}$105, since the sequence of this partially cloned TAF revealed that its C terminus is highly homologous to that of hTAF$_{II}$135 (5, 26).

Our finding that the CR3 region is the only segment of E1A which contacts the C terminus of hTAF$_{II}$135 is consistent with growing evidence suggesting that activation domains function, in part, by contacting distinct TAFs (34). Other factors which have been shown to bind CR3 are hTAF$_{II}$250 (6), hTAF$_{II}$55 (4), TBP (7, 13, 17), and certain DNA-specific recognition site transcription factors that include ATF-2, SP1, and USF (19, 20). Significantly, CR3 mutant proteins which fail to bind one or more of these cellular transcription factors are also defective in E1A transactivation (7, 23, 35). While the details of how each of these transcription factors interacts with CR3 need to be elucidated, an analysis of CR3 mutants implies that these factors do not all recognize the same portion of E1A. Interestingly, TBP appears to bind to the Cys-4 zinc finger of CR3 (7), whereas hTAF$_{II}$135 appears to interact with regions of CR3 flanking the Cys-4 zinc finger, as suggested in this study. It is of interest that a limiting cellular factor (CR3BP/LMP1), as defined by genetic analysis, is also critical in E1A activation (7).

The proposal that an interaction between the C terminus of hTAF$_{II}$135 and the CR3 region of E1A is important for E1A

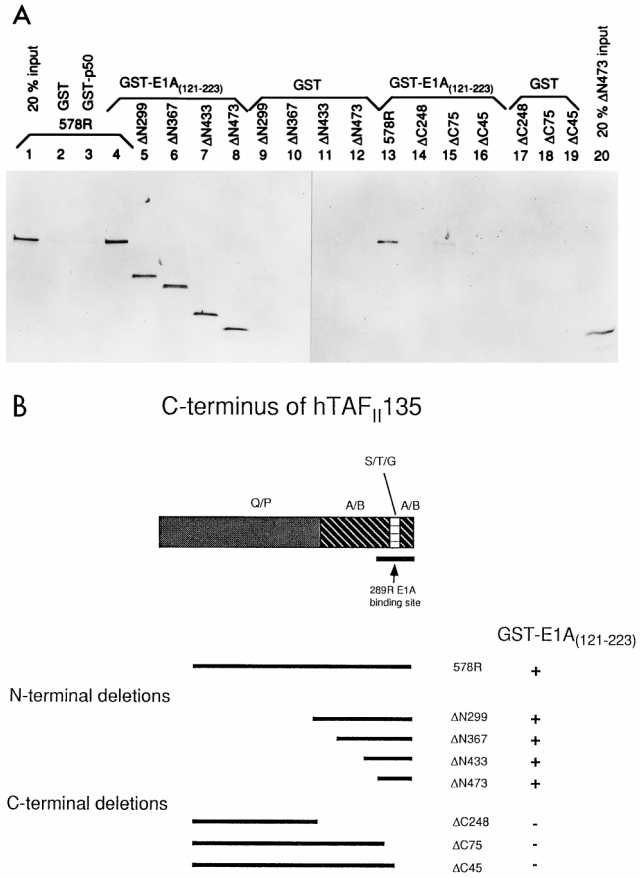

FIG. 4. E1A binds the 105-amino-acid C terminus of $hTAF_{II}135$. (A) N- and C-terminal deletions of the 578R carboxyl region of $hTAF_{II}135$ were generated by in vitro transcription and translation, assayed for binding to GST-$E1A_{(121-223)}$, and resolved on an SDS–10 to 20% polyacrylamide gel. The input counts per minute (20%) for $hTAF_{II}135$ polypeptides 578R and ΔN473 are represented in lanes 1 and 20, respectively. The GST-p50 fusion protein contains the p50 subunit of NF-κB. The slight 578R signal present in lane 15 (ΔC75) is generated from an incomplete restriction digest of pET21d578R. (B) The upper portion represents the C terminus of $hTAF_{II}135$; the E1A binding site is indicated. Shown are regions of $hTAF_{II}135$ rich in serine, threonine, and glycine (S/T/G), glutamine and proline (Q/P), and acidic and basic amino acids (A/B). The lower portion contains a summary of the binding of the $hTAF_{II}135$ polypeptides to GST-$E1A_{(121-223)}$. The binding results are based on multiple experiments, as represented in Fig. 4A.

activation is in concordance with the observation that TAFs within the TFIID complex are needed to support E1A-mediated transactivation (38). In this study, E1A-mediated transactivation in vivo was dramatically inhibited by coexpressing the C-terminal polypeptide of $hTAF_{II}135$. This finding is significant in that it is the first demonstration of how transcriptional stimulation in vivo can be blocked by expressing the portion of a mammalian TAF which contains the binding site for a specific activator. In this study, formation of an $hTAF_{II}135$ polypeptide-E1A complex likely sequesters CR3 and prevents it from targeting components of the transcription initiation complex, including endogenous $TAF_{II}135$. However, we cannot completely rule out the possibility that the C-terminal fragment of $hTAF_{II}135$ may titrate out an additional factor that is necessary for $hTAF_{II}135$-dependent activation. The specificity of the E1A-$hTAF_{II}135$ C-terminal polypeptide interaction was demonstrated by the failure of a N-terminal region of $hTAF_{II}135$ to either bind E1A or to inhibit E1A-mediated transactivation. In addition, we found that coexpressing the C-terminal polypeptide of $hTAF_{II}135$ did not prevent transcriptional stimulation by the acidic activator Gal4-VP16, which has been shown to bind a different human TAF, $hTAF_{II}31$ (15).

Recently, $hTAF_{II}135$ has been shown to interact with two other TAFs, $hTAF_{II}250$ (27) and $hTAF_{II}20/15$ (12, 27), as well as with the transcription factor TFIIA (2). TFIIA stabilizes TFIID binding to the promoter (14, 22) and perhaps induces a conformational change in TFIID (16, 28). Since $hTAF_{II}135$ is also able to bind to both E1A and TFIIA, it is intriguing to consider the possibility that by interacting with $hTAF_{II}135$, E1A stabilizes the TFIID-TFIIA complex to increase the level of activated transcription. It will be important to determine whether the C terminus of $hTAF_{II}135$ interacts with TFIIA or another cofactor which may play a role in E1A-mediated transactivation. In addition to the viral transactivators E1A, T antigen, and E7, the cellular activators SP1 and CREB have also been shown to bind $hTAF_{II}135$ (33). However, unlike these viral transactivators, which all bind to the C terminus of

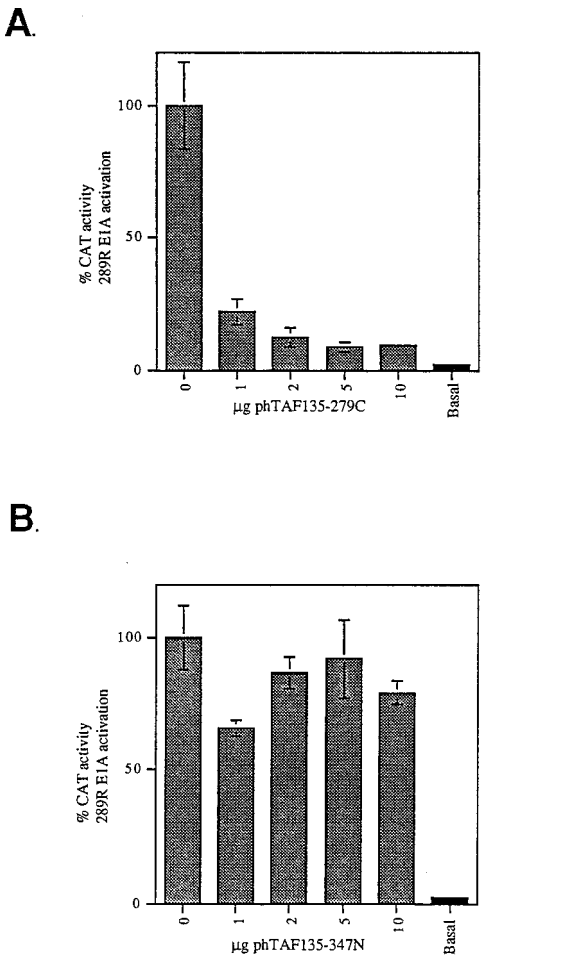

FIG. 5. The C terminus of $hTAF_{II}135$ specifically inhibits E1A transactivation. (A) A C-terminal polypeptide of $hTAF_{II}135$ blocks E1A activation. Constant amounts of the 289R E1A expression plasmid (pSK-E1A; 2μg), an E3 promoter-driven CAT reporter plasmid (pE3CAT; 5 μg), and increasing concentrations of the cytomegalovirus promoter-driven $hTAF_{II}135$ expression vector (phTAF135-279C) encoding the 279R C terminus of $hTAF_{II}135$ were cotransfected into NIH 3T3 cells, and the relative levels of CAT activity were determined. The bar labeled Basal represents the level of CAT activity obtained with pE3CAT alone. (B) A N-terminal polypeptide of $hTAF_{II}135$, encoded by phTAF135-347N, fails to inhibit E1A activation.

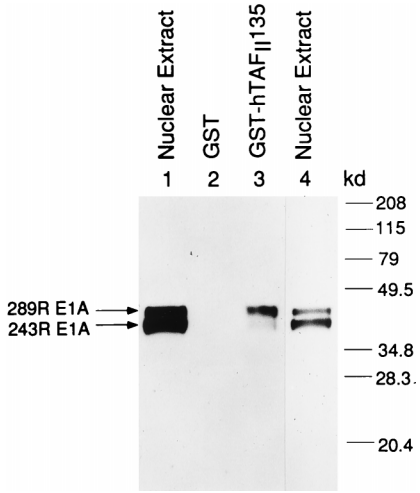

FIG. 6. CR3 of E1A from nuclear extracts is targeted by the C terminus of hTAF$_{II}$135. Nuclear extracts from Ad5-transformed cells (Wt5a) were assayed for binding to the GST-hTAF$_{II}$135 C-terminal fusion protein, and the bound proteins were fractionated on an SDS–10 to 20% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with an E1A-specific mouse monoclonal antibody (M73). Represented are input 289R and 243R E1A proteins from nuclear extracts (lane 1), nuclear extracts incubated with GST alone (lane 2), and nuclear extracts incubated with GST-hTAF$_{II}$135 C-terminal fusion protein (lane 3). Lane 4 is a lower exposure of lane 1 for clearer resolution of the two forms of E1A. The E1A proteins are identical except that 289R contains CR3. kd, kilodaltons.

hTAF$_{II}$135, SP1 and CREB bind to a central region of hTAF$_{II}$135. The ability of different activators to interact with distinct regions of hTAF$_{II}$135 could contribute to synergistically activated transcription, as in the case of the individual activator-TAF interactions described for Bicoid-dTAF$_{II}$110 and Hunchback-dTAF$_{II}$60 (31).

How might interactions between CR3 of E1A and cellular transcription factors facilitate activated transcription? E1A, which does not specifically recognize DNA, apparently localizes to the promoter via interactions between transcription factors bound at sequence-specific DNA recognition sites, e.g., ATF-2 (19, 20), and components of the basal transcription factor TFIID, which include TBP (7, 13, 17), hTAF$_{II}$250 (6), hTAF$_{II}$55 (4), and hTAF$_{II}$135 (this study). This and previous studies suggest that different regions of the CR3 activation domain may contact specific factors of the TFIID complex, i.e., binding of the zinc finger to TBP and the zinc finger flanking regions to hTAF$_{II}$135. Such CR3 interactions could induce conformational changes or chemical modifications of TFIID subunits. Conceivably, E1A mediates transactivation by recruiting or enhancing the stability of TFIID binding to the promoter or by disrupting a negative interaction within the TFIID complex.

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