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The retinoblastoma-susceptibility gene product binds directly to the human TATA-binding protein-associated factor $TAF_{II}250$

(transcription factor TFIID/protein–protein interaction)

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ABSTRACT **RB, the protein product of the retinoblastoma tumor-suppressor gene, regulates the activity of specific transcription factors. This regulation appears to be mediated either directly through interactions with specific transcription factors or through an alternative mechanism. Here we report that stimulation of Sp1-mediated transcription by RB is partially abrogated at the nonpermissive temperature in ts13 cells. These cells contain a temperature-sensitive mutation in the TATA-binding protein-associated factor $TAF_{II}250$, first identified as the cell cycle regulatory protein CCG1. The stimulation of Sp1-mediated transcription by RB in ts13 cells at the nonpermissive temperature could be restored by the introduction of wild-type human $TAF_{II}250$. Furthermore, we demonstrate that RB binds directly to $hTAF_{II}250$ *in vitro* and *in vivo*. These results suggest that RB can confer transcriptional regulation and possibly cell cycle control and tumor suppression through an interaction with TFIID, in particular with $TAF_{II}250$.**

RB, the protein product of the retinoblastoma-susceptibility gene, is a nuclear protein whose inactivation is associated with the etiology of a subset of human cancers (1). Although the mechanism through which RB suppresses tumorigenesis is unknown, RB has been demonstrated to regulate the activity of specific transcription factors in either a positive or a negative manner (2–8). In particular, RB negatively regulates the activity of the E2F-1 and Elf-1 through direct protein interaction (8–11), whereas RB positively regulates Sp1-mediated transcription through an unknown mechanism (6, 12).

Sp1-mediated transcription *in vitro* has been shown to be conferred through specific TATA-binding protein (TBP)-associated factors (TAFs) which compose the TFIID component of the transcription complex. In particular, Sp1 interacts with *Drosophila* $TAF_{II}110$ ($dTAF_{II}110$) through a region within transactivation domain B of Sp1 (13, 14). $TAF_{II}110$ interacts with $TAF_{II}250$, which in turn interacts directly with TBP (15–18), thus bridging Sp1 with TBP. $TAF_{II}250$ was shown to be identical to CCG1, first identified as a regulator of the cell cycle (17–20). A temperature-sensitive mutation in CCG1 ($TAF_{II}250$) in the Syrian hamster cell line ts13 results in a block to cell cycle progression in G_1 at the nonpermissive temperature, presumably due to altered expression of important cell cycle regulatory genes (21–23). Recent experiments have shown that $TAF_{II}250$ interacts with a variety of other TAFs, including $TAF_{II}150$, $TAF_{II}110$, $TAF_{II}60$, $TAF_{II}30\alpha$, and $TAF_{II}30\beta$ (15), thus making it an important component of the TFIID complex.

In this study, we sought to identify the mechanism through which RB stimulates Sp1-mediated transcription. We mapped the domain(s) in Sp1 important for conferring the effect of RB *in vivo*, using GAL4-Sp1 fusions. A region in Sp1 similar to the region important for its interaction with $TAF_{II}110$ was shown to be important for stimulation of Sp1-mediated transcription by RB. In addition, the effect of RB on Sp1-mediated transcription was partially abolished at the nonpermissive temperature in ts13 cells. Moreover, RB was shown to bind directly to $TAF_{II}250$ both *in vitro* and *in vivo*. These results suggests that RB can regulate transcription and possibly the cell cycle and suppression of tumorigenesis through an interaction with $TAF_{II}250$.

MATERIALS AND METHODS

Plasmids. The GAL4-Sp1 fusion plasmids contain the indicated Sp1 sequences in the pSG424 vector, fused in frame to the GAL4 DNA-binding domain (aa 1–147) as described (14). The G5BCAT reporter construct contains five GAL4 binding sites upstream of the E1B TATA box fused to the chloramphenicol acetyltransferase (CAT) reporter gene (6). pSG147 and GAL4-VP16 constructs have been described (6). Human RB was expressed from a simian virus 40 expression vector (SVE) (24). Human $TAF_{II}250$ ($hTAF_{II}250$) either with or without an influenza hemagglutinin (HA) epitope tag was expressed from a cytomegalovirus (CMV) expression vector (22). The glutathione *S*-transferase (GST)-RB-(379–928) fusion was provided by W. Kaelin (25). The GAL4-RB expression plasmid was constructed by inserting the 4.5-kb *Eag* I (filled in)–*Sac* I fragment from the human RB expression plasmid into *Sma* I and *Sac* I sites of pSG424. The GAL4-RB plasmid encodes almost the entire RB (aa 10–928) fused in frame to the GAL4 DNA-binding domain. The GAL4-RB(706) and GAL4-RB (dl 817–839) plasmids express a GAL4-RB fusion protein with a single amino acid change from cysteine to phenylalanine at aa 706 and a deletion of 817–839, respectively.

Cell Culture, Transfection, and CAT Assays. NIH 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% calf serum. tk-ts13 and C-33A cells were maintained in DMEM plus 10% fetal bovine serum. The pts250 cells were generated by transfection of a CMV-HA-$TAF_{II}250$ expression vector into tk-ts13 cells, followed by selection for growth at 39.6°C. Expression of the epitope-tagged $TAF_{II}250$ protein in the rescued pts250 population was confirmed by Western analysis using an anti-HA antibody.

A calcium phosphate precipitation procedure (6) was used for all transfections. Cell extracts were prepared 48 hr after the addition of the DNA. For normalization of transfection efficiencies, 0.5 μg of a luciferase expression plasmid (pSV_2-luc) was included in each cotransfection as an internal control. The

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Abbreviations: TBP, TATA-binding protein; TAF, TBP-associated factor; CAT, chloramphenicol acetyltransferase; GST, glutathione *S*-transferase; HA, hemagglutinin; CMV, cytomegalovirus.

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acetylated and nonacetylated forms of [^{14}C]chloramphenicol were separated by thin-layer chromatography and β emissions were counted directly on a Betagen counter (Betagen, Waltham, MA).

GST-RB Binding Assays. GST and GST-RB-(379–928) were expressed in *Escherichia coli* DH5α, bound to glutathione-Sepharose 4B beads (Pharmacia), and incubated with the human and *Drosophila* TAFs, which were generated by *in vitro* transcription/translation. One microgram of plasmid DNAs encoding the respective TAFs was transcribed/translated in a 25-μl reaction mixture for 90 min at 30°C with either the T7 or the T3 coupled transcription/translation system (Promega). After the addition of 100 μl of binding buffer (100 mM KCl/25 mM Hepes, pH 7.6/0.1 mM EDTA/12.5 mM $MgCl_2$/10% glycerol/0.1% Nonidet P-40/1 mM dithiothreitol/0.1 mM phenylmethanesulfonyl fluoride/0.2 mM sodium metabisulfite), precipitated proteins were pelleted by centrifugation. Fifty microliters of the resulting supernatant was incubated with 20 μl of packed beads carrying ≈1 μg of GST-RB-(379–928) (see Fig. 3*A*) or 2 μg of GST. The binding was performed by constant nutating for 4 hr at 4°C. After five washes with binding buffer, bound proteins were boiled in Laemmli sample buffer, separated by SDS/PAGE (varying from 8% to 15%), and, after fixing/drying of the gel, visualized by autoradiography. Exposures were taken at different times. At least two independent *in vitro* binding experiments were performed for each TAF. The percentage binding was determined by analyzing each gel twice on a PhosphorImager (Molecular Dynamics).

Far Western Blot Analysis. This was performed as described (18).

Immunoprecipitation and Western Blot Analysis. Sixteen micrograms of RB or GAL4 fusion plasmid was cotransfected with 8 μg of a HA-tagged hTAF$_{II}$250 CMV expression vector

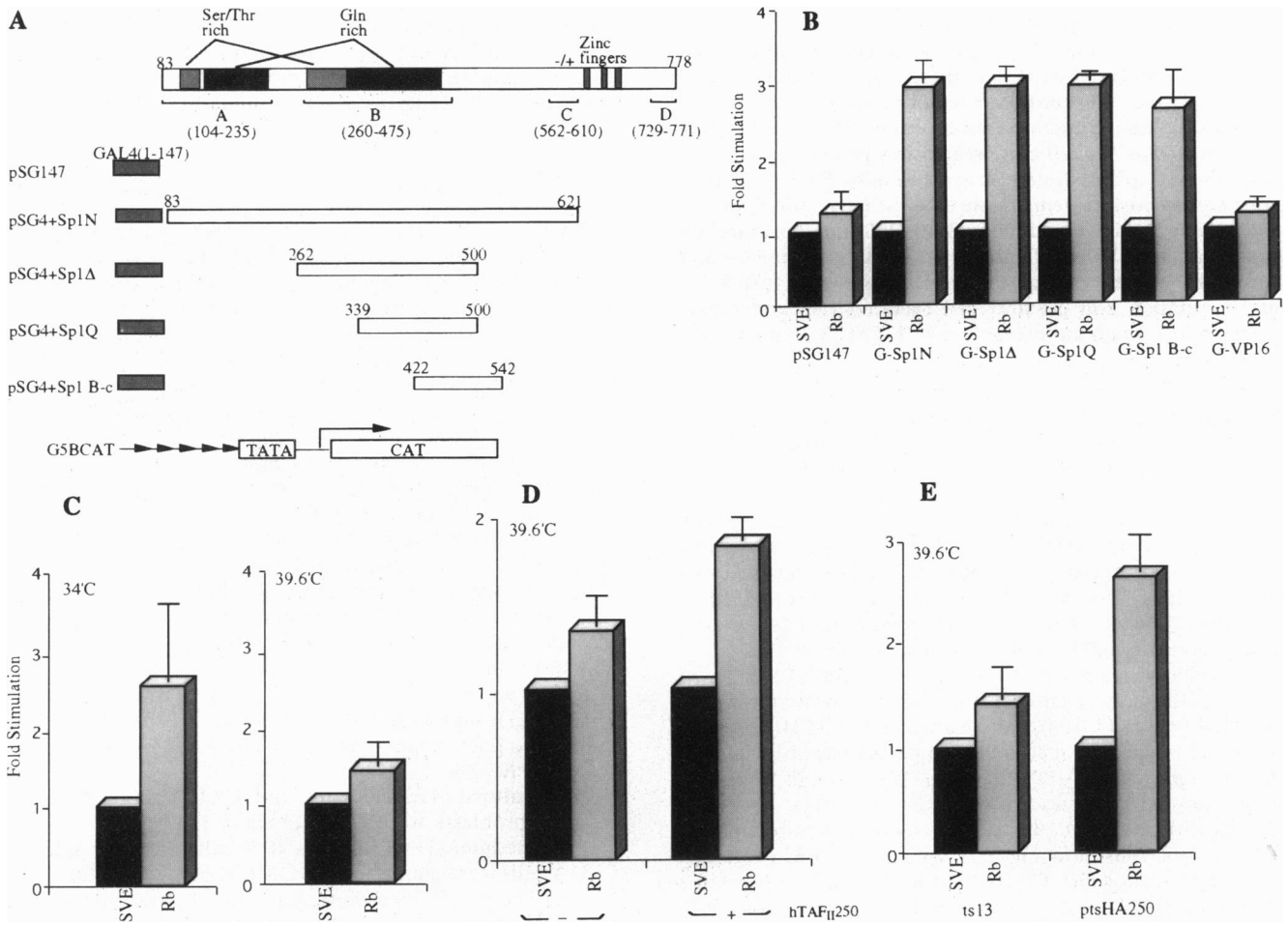

FIG. 1. (*A*) Carboxyl-terminal region of transactivation domain B of Sp1 is sufficient for mediating the stimulation by RB. The structures of the GAL4-Sp1 constructs and the reporter plasmid G5BCAT are shown. (*B*) Stimulation of GAL4-Sp1-mediated transcription by RB. One-tenth microgram of the GAL4-Sp1 constructs was cotransfected with 2 μg of the GAL4-dependent reporter G5BCAT and 5 μg of either an RB expression vector or the SVE control plasmid into NIH 3T3 cells. Four-tenths microgram of pSG147 and 0.05 μg of GAL4-VP16 construct were used as control plasmids. Results are presented as the average fold stimulation from three separate experiments, with the CAT activity from the cotransfections with SVE for each GAL4 construct given a value of 1. The absolute level of CAT activity conferred by pSG147 was 20-fold lower than that for the GAL4-Sp1 constructs. (*C*) Ability of RB to stimulate GAL4-Sp1-mediated transcription in tk-ts13 cells is reduced at the nonpermissive temperature. GAL4-Sp1N, G5BCAT, and either the RB expression vector or SVE were cotransfected into tk-ts13 cells. Following transfection, the cells were switched to either the permissive temperature (34°C) or the nonpermissive temperature (39.6°C). The data are presented as the average fold stimulation from three separate experiments with the CAT activity of SVE lanes given a value of 1. The levels of CAT at the different temperatures were normalized separately. (*D*) Cotransfection of hTAF$_{II}$250 expression vector stimulates RB-mediated stimulation of GAL4-Sp1-mediated transcription. Two and five-tenth micrograms of CMV-hTAF$_{II}$250 expression plasmid was added to the GAL4-Sp1, G5BCAT, and RB cotransfection into tk-ts13 cells and the level of CAT expression at 39.6°C was determined 48 hr later. Results shown are an average of three experiments. (*E*) RB stimulates Sp1-mediated transcription in a tk-ts13 population expressing wild-type TAF$_{II}$250 (pts250). GAL4-Sp1N, G5BCAT, and either the RB expression vector or SVE were cotransfected into tk-ts13 or pts250 cells. Following transfection, the cells were switched to the nonpermissive temperature (39.6°C). The data are presented as the average fold stimulation from three separate experiments with the CAT activity of SVE lanes given a value of 1.

into 6×10^5 C-33A cells by calcium phosphate precipitation. Two days after transfection, cultures were lysed in 1 ml of ELB [50 mM Hepes, pH 7.0/250 mM NaCl/5 mM EDTA/0.1% Nonidet P-40/1 mM dithiothreitol/1 mM Na_3VO_4 containing leupeptin (1 μg/ml), aprotinin (1 μg/ml), phenylmethanesulfonyl fluoride (50 μg/ml), and 7-amino-1-chloro-3-tosylamido-2-heptanone ("tosyllysine chloromethyl ketone," TLCK, 50 μg/ml)]. After clarification, cell lysates were incubated with either an anti-GAL4 antibody (provided by Ivan Sadowski, University of British Columbia) or an anti-RB antibody (C-15; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Protein A-Sepharose was then included for another 2 hr at 4°C. The immunocomplexes were pelleted by centrifugation for 10 min at 4°C and then washed twice with ELB. Samples were boiled in 1× Laemmli sample buffer, subjected to SDS/6% PAGE, and then analyzed by immunoblotting with 12CA5 (24), IF8 (Santa Cruz Biotechnology), or anti-GAL4 antibodies as indicated.

RESULTS

Sp1-mediated transcription is stimulated by coexpression of RB in transient assays in a variety of cell types (6, 12). However, unlike E2F (11), MyoD (3), and Elf-1 (8), no direct association between RB and Sp1 has been observed. To determine the mechanism through which RB regulates Sp1-mediated transcription, the domain(s) in Sp1 sufficient to confer stimulation by RB was mapped by using GAL4-Sp1 fusions (Fig. 1*A*). The ability of RB to stimulate transcription mediated by pSG4+Sp1Q and pSG4+Sp1B-c (Fig. 1*B*) suggests that a region within transactivation domain B of Sp1 between aa 422 and 500 is sufficient to confer stimulation of GAL4-Sp1-mediated transcription by RB. This region of Sp1 has been demonstrated to interact with $dTAF_{II}110$ (13, 14), which in turn binds to $TAF_{II}250$ (15, 16), thereby bridging Sp1 with TBP.

The ability of RB to regulate Sp1-mediated transcription was examined in the Syrian hamster cell line ts13 (21, 22), which has a temperature-sensitive mutation in $TAF_{II}250$ (23). The GAL4-Sp1 expression vector and G5BCAT reporter plasmid were cotransfected into ts13 cells either with or without a simian virus 40-based RB expression plasmid and the cells were grown at either the permissive or the nonpermissive temperature. The stimulation of GAL4-Sp1-mediated transcription by RB was partially abolished at the nonpermissive temperature (39.6°C; Fig. 1*C*). The addition of a $TAF_{II}250$ expression vector to the cotransfection resulted in an increase of RB stimulation at 39.6°C (Fig. 1*D*). Similarly, RB was able to stimulate Sp1-mediated transcription at 39.6°C in a population of ts13 cells stable expressing wild-type $TAF_{II}250$ (pts250; Fig. 1*E*). Taken together, these results suggest that RB can regulate Sp1-mediated transcription, in part, through a $TAF_{II}250$-dependent pathway.

One possible mechanism through which RB could regulate $TAF_{II}250$-dependent transcription is through direct protein interaction. To determine whether RB interacts directly with $TAF_{II}250$, $TAF_{II}110$, and/or other TAFs, all cloned TAFs—i.e., $hTAF_{II}250$ (18), $dTAF_{II}150$ (26), $dTAF_{II}110$ (13), $dTAF_{II}80$ (27), $hTAF_{II}70$ (28), $hTAF_{II}32$ (R. Klemm, J. Goodrich, S. Zhow, and R. Tjian, personal communication), $dTAF_{II}30\alpha$ (29), and $dTAF_{II}30\beta$ (29)—were *in vitro* transcribed, translated, and incubated with beads carrying GST-RB-(379–928). As shown in Fig. 2, $hTAF_{II}250$ specifically bound with high affinity to GST-RB-(379–928) whereas $dTAF_{II}150$ and $dTAF_{II}80$ bound weakly to GST-RB-(379–928). Other TAFs—in particular, $dTAF_{II}110$, which has been shown to strongly interact with Sp1 (13, 14)—did not interact with RB. To investigate whether the observed interaction

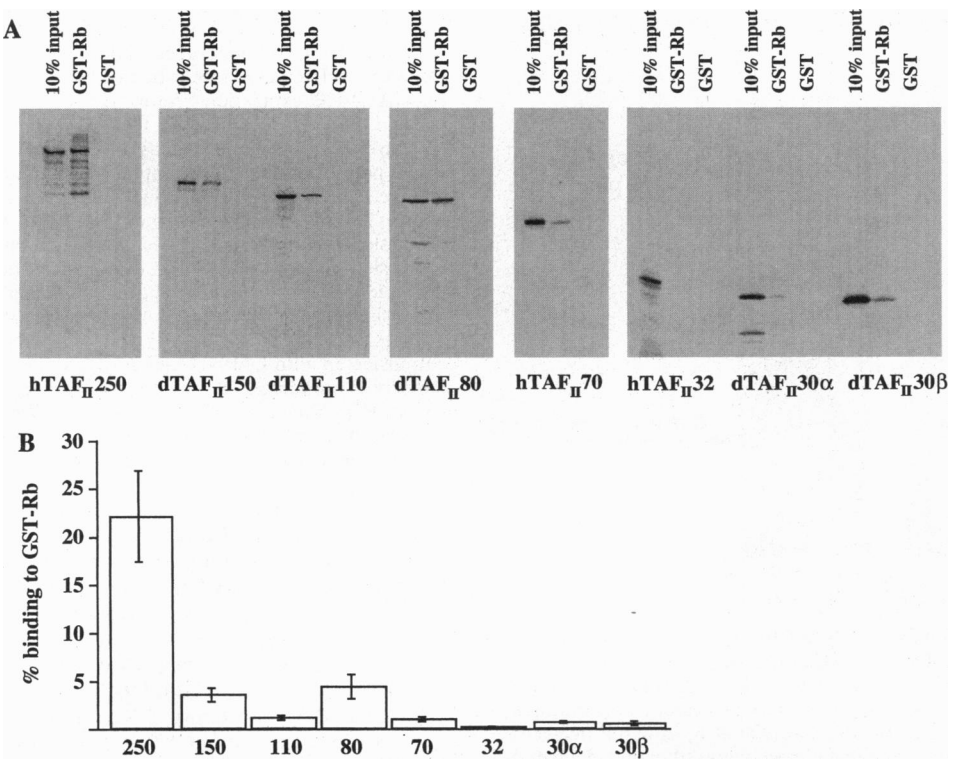

FIG. 2. *In vitro* binding of TAFs to GST-RB. (*A*) GST-RB-(379–928) (25) and GST proteins bound to glutathione beads were incubated with ^{35}S-labeled human (h) and *Drosophila* (d) TAFs: $hTAF_{II}250$; $dTAF_{II}150$; $dTAF_{II}110$; $dTAF_{II}80$; $hTAF_{II}70$; $hTAF_{II}32$, the human homologue of $dTAF_{II}40$; $dTAF_{II}30\alpha$; and $dTAF_{II}30\beta$. The most intense band of each lane corresponds to the full-length protein of the appropriate expected size of the respective TAF. Each set of autoradiograms shows 10% of the input proteins that were incubated with the beads (10% input) and the binding of the individual TAFs (as indicated) to either GST-RB-(379–928) or GST beads. (*B*) Column diagram shows the percentage binding of each individual TAF to GST-RB-(379–928).

between hTAF$_{II}$250 and RB is direct, a Far Western analysis (18) using labeled hTAF$_{II}$250 as a probe was performed. hTAF$_{II}$250 bound specifically to purified GST-RB-(379–928) (Fig. 3*B*, lane 2) as well as to GST-RB-(379–928) in the crude lysate (Fig. 3*B*, lane 1). No binding of hTAF$_{II}$250 to proteins in the crude lysate or the truncated forms of GST-RB protein was observed, demonstrating the specificity of this interaction and suggesting that hTAF$_{II}$250 may interact with the carboxyl-terminal region of RB.

To determine whether RB associates with hTAF$_{II}$250 in mammalian cells, vectors expressing full-length RB fused to the DNA-binding domain of GAL4 and a HA-tagged hTAF$_{II}$250 protein (HA-hTAF$_{II}$250) were cotransfected into C-33A cells, a cervical carcinoma cell line containing a RB protein with a 4-aa deletion in the B domain (30). In these experiments, the GAL4 DNA-binding domain was used as an epitope tag for RB. Extracts from the transfected cells were immunoprecipitated with a GAL4 antibody (Fig. 4 *A* and *B*) or a RB antibody (Fig. 4*C*) and subjected to Western analysis using a HA antibody to detect HA-hTAF$_{II}$250. HA-hTAF$_{II}$250 was coimmunoprecipitated with GAL4-RB only in cells cotransfected with GAL4-RB and HA-hTAF$_{II}$250 vectors (Fig. 4*A*, lane 4). No association with hTAF$_{II}$250 was observed in cells cotransfected with plasmid expressing either the GAL4 DNA-binding domain alone (pSG147) or GAL-Sp1 (Fig. 4*B*, lanes 1 and 2). Moreover, a GAL4-RB mutant, GAL4-RB(dl817–839), was significantly reduced in its ability to bind hTAF$_{II}$250 (Fig. 4*B*, lane 5). Interestingly, the GAL4-RB(706) point mutant, which is unable to bind to either viral oncoproteins (31) or E2F (11), was still able to bind to hTAF$_{II}$250 (Fig. 4*B*, lane 4; data not shown). These results and preliminary mapping studies suggest that the TAF$_{II}$250-binding domain(s) in RB is different from the viral oncoprotein- and E2F-binding domains. We also have demonstrated that wild-type RB binds to hTAF$_{II}$250, by coimmunoprecipitation using a RB polyclonal antibody (Fig. 4*C*, lane 3). The reason why GAL4-RB binds more efficiently than RB to TAF$_{II}$250 is unknown.

DISCUSSION

We have used both *in vitro* and *in vivo* analysis to demonstrate that RB can bind directly to TAF$_{II}$250 (CCG1) to potentially

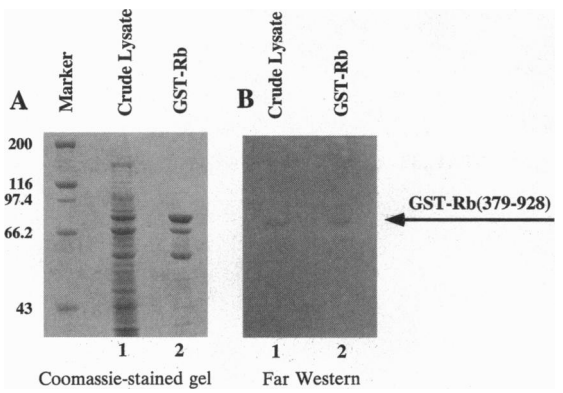

FIG. 3. hTAF$_{II}$250 binds directly to RB. (*A*) Coomassie-stained gel showing a crude lysate of *E. coli* DH5a expressing GST-RB-(379–928) (lane 1) and purified GST-RB-(379–928) (lane 2). Marker sizes are given at left in kilodaltons. (*B*) Far Western analysis demonstrating a direct binding of hTAF$_{II}$250 to GST-RB-(379–928). A duplicate gel of that shown in *A* was transferred to a nitrocellulose filter and incubated with ^{35}S-labeled hTAF$_{II}$250. The arrow points to the full-length GST-RB-(379–928) proteins in the crude lysate (lane 1) and to the purified GST-RB-(379–928) protein (lane 2). Note that neither any of the GST-RB-(379–928) breakdown products (or incomplete translation products) nor any significant protein in the crude lysate interacts with hTAF$_{II}$250.

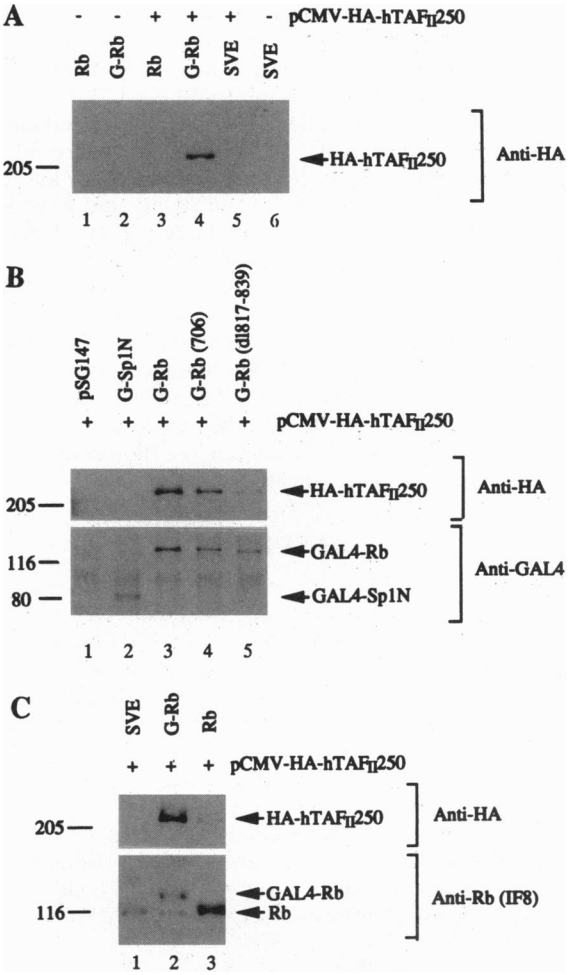

FIG. 4. (*A*) Coimmunoprecipitation of hTAF$_{II}$250 with GAL4-RB. Cell extracts from C-33A cells transfected with either RB (lanes 1 and 3), GAL4-RB expression vector (lanes 2 and 4), or SVE control plasmid (lanes 5 and 6) with (+) or without (−) an HA-tagged hTAF$_{II}$250 expression plasmid as indicated were immunoprecipitated with a polyclonal anti-GAL4 antibody. The immunoprecipitates were analyzed by immunoblotting using an anti-HA antibody (12CA5). (*B*) hTAF$_{II}$250 binds specifically to GAL4-RB. A CMV-HA-hTAF$_{II}$250 expression vector was cotransfected with the indicated GAL4 fusion expression plasmids into C-33A cells. The extracts were immunoprecipitated with an anti-GAL4 antibody and analyzed by immunoblotting using an anti-HA (*Top*) or anti-GAL4 antibody (*Lower*). (*C*) hTAF$_{II}$250 binds to GAL4-RB and RB *in vivo*. C-33A cells were cotransfected with CMV-HA-hTAF$_{II}$250 plus either a GAL4-RB or RB expression vector. The cell extracts were immunoprecipitated with an anti-RB polyclonal antibody (C-15; Santa Cruz Biotechnology) and analyzed by immunoblot with an anti-HA (*Upper*) or anti-RB monoclonal antibody (IF8, Santa Cruz Biotechnology; *Bottom*).

stimulate Sp1-mediated transcription. In addition, we have further demonstrated that TAF$_{II}$250 associates with RB in a yeast two-hybrid system (data not shown). TAF$_{II}$250 binds directly to TBP (18), where it has been suggested to regulate the activity of specific transcription factors, including those important for regulating the cell cycle (22). Thus TAF$_{II}$250 represents an attractive target for cell growth regulators such as RB as well as p107 and p130, which also can bind to TAF$_{II}$250 (Z.S., J. Adnane, S.R., and P.D.R., unpublished). It is important to note that GST-RB also was able to interact with TAF$_{II}$150 and TAF$_{II}$80 *in vitro*, albeit at a lower level than with TAF$_{II}$250. Since dTAF$_{II}$150 and dTAF$_{II}$80 were used it is possible that RB could bind with a higher affinity to the human homologues of these two *Drosophila* TAFs.

Although we have demonstrated an interaction between RB and TAF$_{II}$250 in several independent assays, the mechanism

through which RB binding to $TAF_{II}250$ stimulates Sp1-mediated transcription is not obvious. However, the failure to demonstrate a direct interaction between RB and Sp1 (data not shown) and the observation that a minimal transactivation domain of Sp1 able to bind $TAF_{II}110$ is sufficient to confer activation by RB suggest that RB stimulates transcription by interacting with other components of the transcription complex. The results in ts13 cells, which harbor a temperature-sensitive mutation in $TAF_{II}250$, suggest that at least one of the targets for regulation of Sp1-mediated transcription by RB is $TAF_{II}250$. It is possible that RB either modulates the interaction between $TAF_{II}250$ and other TAFs [e.g., the homologue of $dTAF_{II}110$ with which Sp1 interacts (13, 14)], or modulates another $TAF_{II}250$ function. RB repression of E2F-1-mediated transcription also is, in part, $TAF_{II}250$-dependent, since repression by RB is alleviated at the nonpermissive temperature in ts13 cells (Z.S. and P.D.R., unpublished work). In addition, initial mapping studies suggest that the $TAF_{II}250$-binding domain in RB is distinct from the E2F-binding domain (9). Thus it is possible that for certain transcription factors such as E2F, RB can bind to $TAF_{II}250$ and to the factor simultaneously, acting as a bridge between TFIID and the transcription factors. This possible mechanism is different than that required for the regulation of Sp1, since RB has not been shown to bind to Sp1 directly.

Our results have demonstrated that a target for the retinoblastoma tumor suppressor is $TAF_{II}250$, an essential component of the transcriptional complex. Since $TAF_{II}250$ appears able to modulate transcription of cell cycle regulatory genes (22), the interaction of $TAF_{II}250$ with RB may be important in mediating cell cycle control and possibly tumor suppression. Recently, it was found that the E1A-associated protein p300, which functions as a coactivator, shares homology to $TAF_{II}250$ in a short region called the bromodomain (32). Taken together, these results suggest that coactivators like $TAF_{II}250$ and p300 are important targets for cell growth regulators, tumor suppressors, and oncogenes.

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