The retinoid X receptor (RXR) influences gene activation through heterodimeric and homodimeric association with DNA and associates with TATA binding protein, TAF110, and cAMP response element-binding protein-binding protein; yet the molecular mechanisms responsible for gene activation by RXRs remain incompletely defined. Since the general transcription factor IIB (TFIIB) is a common target of sequence-specific transcriptional activators, we suspected that RXR might regulate target genes via an interaction with TFIIB. Co-immunoprecipitation, far Western analysis, and glutathione S-transferase binding studies indicated that murine RXRβ (mRXRβ) was capable of binding to human TFIIB in vitro. Functional analysis with a dual-hybrid yeast system and cotransfection assays revealed the interaction of mRXRβ with TFIIB to be ligand-dependent in vivo. Truncation experiments mapped the essential binding regions to the carboxyl region of mRXRβ (amino acids aa 254–389) and two regions in the carboxyl region of TFIIB (aa 178–201 and aa 238–271). Furthermore, the A390–410 mRXRβ mutant bound to TFIIB in vitro but was not active in the dual-hybrid yeast system, suggesting that the extreme carboxyl region of RXR was required for in vivo interaction with TFIIB. These data indicate that interaction of mRXRβ with TFIIB is specific, direct, and ligand-dependent in vivo and suggest that gene activation by RXR involves TFIIB.

Nuclear hormone receptors are ligand-dependent transcription factors that regulate numerous cellular functions, including growth, development, differentiation, reproduction, and metabolism (Refs. 1–3 and references therein). Ligand binding to nuclear hormone receptors leads to assembly of a functional preinitiation complex (PIC) 1 and subsequent RNA transcription in vitro (4, 5). Activated transcription of RNA polymerase II (RNAPII) genes requires binding of transcription factor IID (TFIIF) to the TATA element, which is facilitated by TFIIB, and stepwise assembly of the following transcription factors: TFIIB, RNAPII/TFIIF, TFIIE, and TFIH (reviewed in Refs. 6 and 7). TFIIB includes the TATA binding protein (TBP) associated factors (TAFs) (Ref. 7, and references therein). Interaction of nuclear receptors with the basal transcription complex may occur directly (8–14) or in association with TAFs (15) or other cofactors (Refs. 16–25; reviewed in Refs. 26 and 27). Ligand binding to the receptor results in recruitment of factors that facilitate assembly of an activated complex (28–30) or, conversely, the release of repressor molecules, such as SMRT (16) or N-CoR (17, 18) that inhibit transcription (16–18, 31–32). A significant advance was the demonstration that TFIIB interacts with the estrogen receptor (10), progesterone receptor (10), chicken ovalbumin upstream promoter-transcription factor (10), thyroid hormone receptor (TR) (11, 31), and vitamin D receptor (VDR) (12–14). The interaction of nuclear hormone receptors with TFIIB is significant since recruitment of TFIIB to the PIC by transcriptional activators is believed to be a critical, rate-limiting step for PIC assembly (7, 33, 34) and TFIIB has been suggested to play a critical role in gene activation by nuclear hormone receptors (12, 31, 32, 35, 36).

The retinoid X receptor (RXR) is a unique member of the steroid-thyroid-retinoid nuclear receptor family due to its ability to act as a homodimer or function as a heterodimer partner with other nuclear receptors (reviewed in Ref. 2). Specifically, RXRs have been shown to form heterodimers with the TR (37–42), retinoic acid receptor (37–42), VDR (38, 41), peroxisome proliferator-activated receptor (43, 44), chicken ovalbumin upstream promoter-transcription factor (45, 46), Arp-1 (47), farnesoid-x-activated receptor (48), ubiquitous receptor (49), liver-X receptor α (50), and NGFI-B/NURR1 (51). RXRs are activated by the 9-cis-isomer of retinoic acid (9-cis-RA) (52, 53) and contain specific transcriptional activation domains in the N terminus and C terminus, termed AF-1 and AF-2 (which includes the τ or τ4 region), respectively (54–58). Some studies have suggested that RXRs principally function as “silent partners” to augment DNA binding (2, 59, 60), whereas other reports indicate an active role in gene regulation (48, 50, 51, 61–63). An in vivo role for RXR in gene regulation was confirmed by disordered development observed in mice that lacked the receptor (Refs. 1 and 2 and references therein) and by the ability of a dominant negative mutant of RXR to prevent differentiation of P-19 embryonal cells (64). Given the potential involvement of RXR in the function of many receptors, ligand-dependent transcription by the RXR must be tightly regulated.

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1 The abbreviations used are: PIC, preinitiation complex; TFIID, TATA binding protein/TFIID; TBP, TATA binding protein; TAF, TATA associated factors; RNAPII, RNA polymerase II; VDR, vitamin D receptor; RSV, Rous sarcoma virus; RXR, retinoid X receptor; aa, amino acid(s); PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; DTT, dithiothreitol; RA, retinoic acid; h, human; m, murine; TR, thyroid hormone receptor; LBD, ligand-binding domain; CBP, cAMP response element-binding protein-binding protein.
to ensure that specific cellular responses are properly initiated (reviewed in Refs. 26, 62, and 65).

The specific molecular steps governing the temporal and spatial specificity of ligand-dependent gene activation by RXRs remain to be precisely defined. RXRs have been shown to interact with transcriptional co-factors including TAFII30 (15), SUG1 (24), and TIF1 (25). Studies in yeast revealed that RXRs could associate with either TBP or TAFII110 (58, 67), but these associations could be altered in the presence of a specific RXR agonist (67), suggesting that conformational states of the RXR ligand-binding domain (LBD) could determine the ability of the receptor to bind specific transcription factors in vitro. In a cell-free system, addition of RXR to the PIC reproducibly enhanced TFIIB-TBP complex formation (27); however, a RXR-TFIIB-TBP complex was not observed in native gel electrophoresis, suggesting that in vitro the complex might be unstable. Since RXRs have been shown to bind proteins in the transcription complex, it is possible that RXRs might actively augment binding of receptor proteins to the transcription complex (i.e. not as a silent partner), perhaps in a holocomplex with CBP (29, 30). In support of this mechanism, several proteins interact in a ligand-dependent manner with the carboxyl (AF-2 or τ) region of nuclear hormone receptors that heterodimerize with RXRs (21–25). Also against an indirect or silent partner role for RXR is the observation that a dominant negative RXR mutant (e.g. ΔAF-2 or τ) could abrogate the ability of a heterodimer to augment gene activation (64, 66, 69, 70, 71); since RXRs associate weakly, or not at all with SMRT (16) or N-CoR (17, 18), trans-repression is not easily explained. Similarly, an indirect mechanism of action does not explain the ability of RXR to function as a homodimer (68). Furthermore, studies have specifically revealed the ability of liganded RXR to alter the response of a heterodimer complex associated with DNA (63, 72). These observations suggest that RXRs may actively participate in assembly of the transcription complex.

In an effort to clarify some of the molecular mechanisms of gene activation by RXRs, we have tested the interaction of the general transcription factor TFIIB with mRXRβ. Using several experimental approaches, we observed the association of mRXRβ with TFIIB to be ligand-dependent, specific, and direct. Furthermore, interaction required the carboxyl region of mRXRβ and two distinct regions within TFIIB. These data support the conclusion that RXR may contribute directly to the recruitment of essential transcription factors involved in ligand-dependent gene activation.

MATERIALS AND METHODS
Plasmids—Murine RXRα cDNA was released by NcoI digestion of pEXpress-RXRβ (73) and subcloned into pAS1-CYH2 (74) to produce pRXRβ-pAS1-CYH2. Unless otherwise stated, experiments were performed with the mRXRβ receptor isoform. Similarly, NcoI fragments of either wild-type or deletion mutants of mRXRβ (73) were subcloned to pGEX (Pharmacia Biotech Inc.) to generate GST-RXR plasmids. Expression of appropriately sized GST-RXR wild-type, or mutant, proteins was confirmed by PAGE. For the construction of TFIIB-GAL4-AD, pGEX2T-htFIIB (13) was digested with BamHI, Klenow filled, digested with EcoRI, and the fragment subcloned into pACTII, which had been XbaI cleaved, Klenow filled, and EcoRI digested. For construction of TFIIB-GAL4-AD (-), the primers 5′-CAATCAACAT CGAGTTGTCG ATGGCTGCT GCAGCTGGTT G-3′ and 5′-CCGATGC AGCTGGTGC ATGGCTGCT GCAGCTGGTT G-3′ corresponding to the 5′ end of hFIIB, and 5′-TCCGAGCTGC GAGAT TCGTGTCG ATAGGTTGTC AC-3′ corresponding to the 3′ end of TFIIB, except for the dc3 mutant for which the following 3′ primer was used, 5′-CGTACGCGGA ATCCGGATCC TAAAG GGC TATATGTGTA GTGGC-3′. Polymerase chain reaction products were subcloned into the BamHI/EcoRI site of pACTII. For the construction of the following pACTII-pACTII-GAL4 showing 5′ GAL4 5′-GCTG TGTTAT CCGCAATAC GA CGTACTAG TGGAGGC CGCCAGGCG 3′, 3′ GAL4 3′-CCCCAGGCG 3′, and 3′ GAL4 3′-CCCCAGGCG 3′, the primer was used for the construction of the Δ1, Δ2, Δ3, Δ1 Δ5 pACTII-pACTII plasmids. All plasmid constructions were verified by dyeo sequencing.

Yeast Dual-hybrid System and Immunoblot Analysis—The yeast strain Y190 MATa, leu2-3,112, ura3-52, trp1-100, his3-120, ade2-101, gal4-52, ura3-GAL-lacZ, LYS2::GAL1-HIS3, cyh2′ was transformed using a standard lithium acetate method. Transformants underwent two rounds of selection on standard dextrose (SD) plates supplemented with histidine and adenine, were grown to saturation in appropriately supplemented SD media at 30 °C, re-incubated to fresh medium, and grown to mid-log phase overnight. Either 1 μM 9-cis-RA or vehicle (Me2SO) was added to cells cultured in light-protected tubes for 6 h. Cells were pelleted at 2,000 rpm for 10 min at 4 °C, resuspended in breaking buffer (100 mM Tris-Cl, pH 8, 1 mM dithiothreitol (DTT), 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride), and lysed by vortexing at 4 °C with cold, glass beads six times for 15 s at 15-s intervals. Protein extracts were quantified with the Bio-Rad protein assay (Bio-Rad). β-Galactosidase activity was detected using 30 μg of protein yeast extract, the Galactolite chemiluminescence assay (Tropix, Bedford, MA) with a Monolight 2010 luminoimeter (Analytical Luminescence Laboratory, San Diego, CA). TFIIB in yeast extracts was detected by Western analysis using antibodies directed against hTFIIB (sc-4001, Santa Cruz Biotechnology, Santa Cruz, CA) and an enhanced chemiluminescence detection method (ECL, Amersham Corp.). Immunoblot analysis of yeast proteins containing mRXRβ was performed with monoclonal antibody (13.17) essentially as described (40) or with anti-GAL4-DBD (CLONTECH, Palo Alto, CA) as noted.

Co-immunoprecipitation Assays—Antibody-protein complexes were precipitated using 20 μl of pre-equilibrated protein A-Sepharose beads as described, with slight modifications (75), and then washed extensively. Bound products were eluted in sample buffer and analyzed by 12% SDS-PAGE and autoradiography.

Far Western Analysis—Following electrophoretic transfer, nitrocellulose filters were denatured in 6 M guanidine hydrochloride and renatured by stepwise dilutions to 0.187 M guanidine HCl in HBB (25 mM Hepes-KOH, pH 7.4, 2.5 mM NaCl, 50 mM MgCl2, 1 mM DTT). Filters were blocked 12–14 h in HBB with 5% nonfat milk and 0.05% IPEGAL, and 1 mM MgCl2, 1% nonfat milk, 0.05% IPEGAL, and 1 mM EDTA, 2.5 mM MgCl2, 1% nonfat milk, 0.05% IPEGAL, and 1 mM DTT) (21). 500,000 cpm/ml of 32P-labeled GST-RXR or GST fusion proteins2 were added, and incubation was continued at 4 °C for 12 h. Filters were washed in HBB three times and subjected to autoradiography.

Gluatathione-Sepharose Binding Assays—20 μl of glutathione-Sepharose beads coated with bacterially expressed GST fusion proteins were mixed with equal amounts of 5′-labeled in vitro translated RXR or TFIIB and luciferase, added in the presence of 1 μM 9-cis-RA, and rocked at 4 °C for 1 h. Beads were collected, washed extensively in HBB (40 mM Hepes, 75 mM KCl, 0.5 mM EDTA, 5 mM MgCl2, 1 mM DTT, 1 mM 4-aminoethylbenzenesulfonyl fluoride hydrochloride, 0.5 mg/ml bovine serum albumin, and 0.05% Nonidet P-40) containing 1 μM 9-cis-RA or vehicle (Me2SO). Bound proteins were eluted in sample buffer and analyzed by 10% SDS-PAGE and autoradiography. Input lanes show one-tenth of loaded lysate.

For precipitation of TFIIB from nuclear extracts, 25 μg of Namalwa human B lymphocyte nuclear extract was prepared as described (40) and incubated with 10 μl of coated beads at 4 °C for 1.5 h in 20 mM Hepes, 20% glycerol, 0.2 mM EDTA, 50 mM KCl, 0.5% bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride. Beads were collected and washed extensively in 20 mM Hepes, 20% glycerol, 0.2 mM EDTA, 100 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, and 0.05% Triton X. Bound proteins were eluted by boiling and loaded onto 12% SDS-PAGE, blotted to nitrocellulose, and examined using immunoblot analysis.

Cell Culture and Transient Transfection—Murine P19 embryonal carcinoma cells were grown in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (20%), and gentamicin (50 mg/ml) and plated at a cell density of 3 × 105 cells/ml 24 h prior to transfection by calcium-phosphate precipitation. 0.5 μg of CRBPII-tk-luciferase (which contains two copies of the rat CRBPII-RXRε element) or tk-luciferase reporter (64) was added to
Expression vectors pExpress-RXRβ (73) and pRSV2-hTFIIB (12), as indicated. RSV-β-galactosidase (0.5 mg) was used to control for transfection efficiency (70). Cells were treated with 1 μM 9-cis-RA or vehicle (MeSO), harvested 24 h after transfection, and extracts normalized for β-galactosidase activity.

Partial Proteolysis Assay—35S-Labeled TFIIB was incubated with 7 μg of S9 nuclear proteins prepared from cells infected with wild-type baculovirus (control) or recombinant mRXRβ baculovirus. In other experiments, labeled TFIIB was added to a 20-μl bed volume of Sepharose beads coated with bacterially expressed GST, or GST-RXR, fusion proteins. After binding for 2 h at 4 °C, trypsin (50 or 500 ng) was added for 5 min at 30 °C and the reaction stopped with 1 × SDS, and products were resolved by 12–15% SDS-PAGE and subjected to autoradiography.

RESULTS

Interaction of RXR with TFIIB Is Direct—Since other nuclear hormone receptors have been shown to interact with TFIIB (10–14), and TFIIB is a common target of sequence-specific transcriptional activators (6, 7), we tested whether RXR could precipitate TFIIB from proteins extracted from mammalian cell nuclei. Sepharose beads were coated with either bacterially expressed GST-RXR (RXR) fusion proteins or control proteins (GST), incubated with nuclear proteins, and washed, and immunoblots of bound proteins were performed using antibodies directed against TFIIB. As shown (Fig. 1A), TFIIB could be specifically precipitated from extracts prepared from mammalian cell nuclei using GST-RXR-coated beads. In control experiments, beads that lacked the RXR fusion protein failed to bind TFIIB, thus supporting the specificity of the interaction. TFIIB precipitated from extracts of nuclear proteins co-migrated with endogenous TFIIB detected in immunoblot analysis of nuclear proteins and recombinant TFIIB (Fig. 1A), thus suggesting that RXR could precipitate TFIIB from mammalian cell extracts (see below).

To examine the specificity of the in vitro interaction between RXR and TFIIB, co-immunoprecipitation experiments (75) were performed using in vitro transcribed and translated proteins with antibodies directed against either TFIIB or mRXRβ (40). As shown in Fig. 1B (lane 3), 35S-labeled in vitro transcribed and translated RXR and TFIIB were both precipitated by anti-TFIIB antibody, consistent with an interaction between the two proteins. The anti-TFIIB antibody precipitated labeled mRXRβ in the presence of TFIIB (lanes 3 and 5) but not in the absence of TFIIB (lane 6). Conversely, anti-RXR antibody precipitated labeled TFIIB, but only in the presence of RXR. The assay was specific since neither the anti-TFIIB nor anti-RXR antibodies precipitated a control protein, in vitro translated luciferase (lanes 2, 4, 5, and 8); and conversely, a control antibody (lane 1) did not precipitate labeled RXR, TFIIB, or luciferase.

A possibility not addressed by co-immunoprecipitation experiments, or GST “pull down” experiments with nuclear proteins, was that an endogenous factor(s) present in reticulocyte lysates (or mammalian nuclear extracts) functioned as a molecular bridge to mediate the interaction between RXR and TFIIB, such as CBP (29, 30). To address this concern we tested whether RXR binding proteins in the bacterial extracts, suggesting that the probe extracts themselves did not contain bridging factors (not shown). Far Western experiments (Fig. 1C) were performed using purified recombinant 32P-labeled GST-RXR fusion protein and recombinant hTFIIB protein. As a positive control, we observed that 32P-labeled GST-RXR protein detected RXR (i.e. RXR-RXR homodimers) in S9 extracts containing recombinant RXR (Fig. 1C, lane 1) but not in control S9 extracts (lane 2).

Consistent with a direct interaction between the two proteins, labeled RXR detected a strong band in lanes containing TFIIB (lanes 3–5), but no binding was observed to a 32P-labeled control protein (lanes 6–8). Specificity of RXR binding was further substantiated in a related series of experiments demonstrating
that $^{32}$P-labeled GST-RXR detected TR, but only if the C terminus of the labeled IRX molecule was intact. As expected, migration of TFIIIB detected by $^{32}$P-labeled GST-RXR in far Western experiments precisely matched migration of $^{35}$S-labeled TFIIIB in co-immunoprecipitation experiments (Fig. 1A) and Western analysis of TFIIIB using anti-TFIIIB antibodies (see above). Although co-migration is not conclusive proof of identity between two proteins, Fig. 1B and C together with A provide substantial evidence that RXR and TFIIIB interact in vitro, and far Western analysis revealed that additional factors were not essential for the interaction.

**TFIIIB Augments RXR-dependent Reporter Activity in Mammalian Cells**—Since mRXRα could associate with TFIIIB in vitro, we examined whether overexpression of both TFIIIB and RXR might augment RXR-dependent reporter activity in mammalian cells. To test this possibility, transient transfections were performed in undifferentiated P-19 embryonal carcinoma cells with mRXRα and hTFIIIB expression constructs and a CRBPII-tk-luciferase reporter (left panel) or a control tk-luciferase reporter that lacked an RXRE (right panel). Reporter activity of extracts is expressed in fold induction (mean ± S.E.) over tk-luciferase values and represents data from at least four independent experiments performed in triplicate in the presence of 1 μM 9-cis-RA. Reporter activity was corrected for transfection efficiency using RSV-$\beta$-galactosidase. Amounts of added expression plasmids (μg) for TFIIIB or RSV-Δ control are indicated. A constant amount of mRXRα expression plasmid (0.5 μg) was added as indicated by the shaded bar.

![Graph](image)

**Fig. 2.** TFIIIB augments RXR-dependent reporter activity in mammalian cells. Transient transfections were performed in P19 embryonal carcinoma cells with mRXRα and hTFIIIB expression constructs and a CRBPII-tk-luciferase reporter (left panel) or a control tk-luciferase reporter that lacked an RXRE (right panel). Reporter activity of extracts is expressed in fold induction (mean ± S.E.) over tk-luciferase values and represents data from at least four independent experiments performed in triplicate in the presence of 1 μM 9-cis-RA. Reporter activity was corrected for transfection efficiency using RSV-$\beta$-galactosidase. Amounts of added expression plasmids (μg) for TFIIIB or RSV-Δ control are indicated. A constant amount of mRXRα expression plasmid (0.5 μg) was added as indicated by the shaded bar.

**Interaction between RXR and TFIIIB**—We examined the interaction between RXR and TFIIIB in vitro—using the yeast dual-hybrid system (reviewed in Ref. 77), since yeast do not contain RXRs (78), and RXRs have been shown to function in yeast (78, 79). A Saccharomyces cerevisiae strain (Y190) containing an integrated lacZ reporter was transformed with expression plasmids (Fig. 3A) encoding full-length mRXRα fused to the GAL4 DNA binding domain and full-length hTFIIIB fused to the GAL4 activation domain (AD). Reconstitution of this two-hybrid system would permit quantitative analysis of reporter activation and not colony survival alone (77). Furthermore, the incorporation of an integrated lacZ reporter permitted paired examination of ligand effects from individual colonies, thus controlling for variability in levels of receptor expression as determined by immunoblot analysis.

Following transformation several colonies were chosen, subjected to two rounds of dual selection, and analyzed by Western analysis to confirm similar levels of fusion protein expression. Yeast colonies that expressed both TFIIIB and RXR showed a 6-fold increase in reporter activity in the presence of 9-cis-RA (Fig. 3B, lane 4), but in the absence of ligand (open bars) reporter activity did not differ from RXR alone (lane 3). Reporter activity in yeast colonies that expressed only TFIIIB (lane 2) showed no significant increase in reporter activity in the presence or absence of ligand. Not surprisingly, yeast that expressed mRXRα coupled to the GAL4-DBD did show a slight increase in reporter activity (Fig. 3B, lane 3), likely due to the interaction of RXR (see below) with transcription factors endogenously present in yeast. The magnitude of activation observed with RXR and TFIIIB in our particular system resembled levels of activation reported by others for the ligand-dependent association of LBD-RXR and GAL4-TAF110 in yeast (67) and other transcription factors (80). To confirm that differences in reporter activity did not reflect variation in expression of fusion proteins, immunoblot analyses were performed on extracts prepared from yeast colonies subjected to reporter studies (Fig. 3B, lower panel). The ligand-dependent increase in reporter activity observed in the presence of both RXR and TFIIIB, but not for either construct alone, is consistent with a ligand-dependent interaction between RXR and TFIIIB in vivo.

Alternatively, ligand-dependent activation of the lacZ reporter could occur if TFIIIB were able to squelch negative regulator (repressor) of RXR present in yeast or if TFIIIB were acting through intermediary yeast protein complexes (29, 81). Although far Western analysis suggested that additional proteins were not required for the interaction, it was possible that formation of a ternary complex in vivo could confound inter-
Interaction between RXR and TFIIB

The Carboxyl Region of TFIIB Interacts with RXR—We next sought to define the specific regions of TFIIB required for interaction with mRXRβ via GST binding assays (11). Glutathione-Sepharose beads were coated with intact, or truncated, GST-TFIIB fusion proteins and incubated with 35S-labeled mRXRβ. As a control for binding specificity, 35S-labeled luciferase was added to binding reactions. Labeled RXR did not bind to beads coated with GST alone nor did labeled luciferase bind to any of the GST-TFIIB-coated beads (Fig. 4A). Beads coated with the Δ1, Δ2, Δ3, Δ5, and dN1 GST TFIIB mutant proteins bound mRXRβ as avidly as wild-type TFIIB. In contrast, beads coated with protein corresponding to the a1 (Δ178–201) and dc3 (Δ238–316) TFIIB deletion mutants bound less avidly to RXR, suggesting that regions from amino acids 178–201 and 238–316 of TFIIB were required for interaction with RXR, in complete agreement with the GST binding data. Also in agreement with GST binding data, deletion of the C terminus of TFIIB (aa 238–316, dc3) was associated with reduced activation in vitro (see also Fig. 5A).

To confirm these observations, we analyzed a series of TFIIB mutants with the dual-hybrid system. As shown in Fig. 4B, the Δ116 mutant (which encompasses regions deleted with the TFIIB dN1 and Δ1 mutants), Δ2, and Δ5 TFIIB mutants showed a slight reduction (30–40%) in ligand-dependent reporter activity observed in the presence of the GAL4-AD construct (that lacked TFIIB) resembled levels of basal levels in the absence of TFIIB (GAL4 AD only) or with the dc3 and Δ5 TFIIB mutants. The slight increase in ligand-dependent reporter activity observed in the presence of the GAL4-AD construct (that lacked TFIIB) resembled levels of activation observed with addition of mRXR-GAL4-DBD alone (Fig. 3B), was not dependent upon TFIIB, and was attributed to association of RXR with endogenous yeast transcription factors. The dual-hybrid approach supported the conclusion that the N terminus of TFIIB (aa 1–175) was not required for interaction with RXR, in complete agreement with the GST binding data. Also in agreement with GST binding data, deletion of the C terminus of TFIIB (aa 238–316, dc3) was associated with reduced activation in vitro. Results with the Δ3 mu-
Interaction between RXR and TFIIB

Fig. 4. Analysis of regions within TFIIB required for binding to mRXRβ. A, in vitro binding analysis. In vitro translated, 35S-labeled full-length mRXRβ was incubated with glutathione-Sepharose beads coated with bacterially expressed GST-TFIIB fusion proteins (11), washed, and bound products resolved in SDS-PAGE and exposed to autoradiography. Proteins retained on GST-0 (bracket 1) or GST-TFIIB fusion protein (wild-type TFIIB; bracket 2) coated beads are as indicated. Alternatively, beads were coated with fusion proteins containing mutants of TFIIB: Δ1 (390–410) (bracket 3), Δ2 (178–201) (bracket 4), Δ3 (238–316) (bracket 5), Δ5 (390–297) (bracket 6), dN1 (34–24) (bracket 7), α1 (Δ178–201) (bracket 8), and dc3 (Δ259–316) (bracket 9). Addition of 1 μM 9-cis-RA is indicated by α. Input proteins are one-tenth of added lysates. **S-Labeled luciferase was included in all reactions as a negative control. B, deletion analysis of TFIIB in the dual-hybrid system. Left panel, schematic representation of TFIIB mutants analyzed. + indicates that yeast colonies had similar expression of RXR-GAL4-DBD by immunoblot analysis (data not shown). Right panel, reporter activity (β-galactosidase) for each TFIIB mutant construct is indicated in relative light units (mean ± S.E.) over background values, normalized for protein, and represents triplicate determinations from at least six independent colonies. Immunoblot analysis revealed differences in expression of the wild-type TFIIB, and TFIIB mutant fusion proteins did not account for the observed changes in reporter activity (data not shown). Far right column, fold induction is reported for each TFIIB mutant construct in the presence of ligand (1 μM 9-cis-RA; black bars) versus control (vehicle; open bars) in the absence of ligand. Even in the absence of TFIIB, slight reporter activity was observed with GAL4-AD alone (GAL4 AD only).

The C Terminus of RXR Interacts with TFIIB—To map the regions of TFIIB required for binding to RXR, we used intact and mutant GST-RXR fusion proteins immobilized on Sepharose beads (Fig. 5A). Beads coated with full-length GST-RXR fusion protein specifically bound labeled TFIIB (lane 2). No binding was observed to beads coated with GST alone. Also, minimal binding to TFIIB was observed with a ligand-binding domain RXR mutant (ΔLBD). Furthermore, an N-terminal deletion mutant (ΔAF-1) and an AF-2 deletion mutant of RXR (Δ390–410) also bound labeled TFIIB, although the AF-1 mutant showed reduced binding in the absence of ligand. These data suggest that amino acids 254–389 of RXR are required for in vitro binding to TFIIB.

Next, we tested the RXR deletion mutants using the dual-hybrid system. To minimize variability in RXB1 expression between yeast strains, the respective RXR mutant plasmids were transformed into one parent yeast strain expressing satisfactory levels of TFIIB GAL4-AD, thus generating five distinct combinations, of which six individual yeast colonies of each combination were analyzed. Both full-length RXR (wild type) and the deletion construct lacking the DNA binding domain of RXR (ΔDBD) showed preservation of a ligand-dependent interaction between TFIIB and RXR (fold inductions of 14.5 and 29.4, respectively, Fig. 5B). In agreement with in vitro binding studies, the ΔLBD mutant showed no ligand-dependent reporter activity and only a slight increase in basal reporter activity. The increase in basal reporter activity in the absence of ligand suggested relief of transcriptional repression, perhaps signifying loss of binding of a repressive factor to the C terminus of RXR (as observed for other nuclear receptors; see Refs. 16–18). Deletion of the RXR N terminus showed a reduction in maximal levels of reporter activity but preservation of a ligand-dependent response (fold induction = 11), consistent with the in vitro binding studies. Although the RXR mutant with deletion of the extreme C terminus (aa 390–410) bound avidly to TFIIB in vitro (as was reported for VDR, see Ref. 12), the deletion mutant showed no increase in reporter activity, with or without ligand, in keeping with the dominant negative phenotype displayed by this mutant receptor in mammalian cells (55, 56, 70, 71). With the sole exception of the Δ390–410 mutant, the dual-hybrid data are in agreement with the in vitro binding results and revealed that amino acids 254–389 of RXR are essential for in vivo interaction with TFIIB.

In addition, we performed partial proteolysis assays (82, 83) to determine whether RXR could alter proteolysis of TFIIB, since such an effect would support an in vitro association between the two molecules. Also, our mapping analysis (Fig. 5, A and B) localized the region of TFIIB involved in binding with RXR to include a region common to interaction with VP-16 (84), and partial proteolysis assays suggested that VP-16 could induce a conformational change in TFIIB (82, 83). As shown in Fig. 5C, digestion of TFIIB in the presence of baculovirus expressed recombinant RXR resulted in a pattern of cleavage

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different from that observed with wild-type baculovirus control protein. An alteration in digestion pattern was also observed for TFIIB in the presence of bacterially expressed RXR but not GST control protein (not shown). These data are further support for the interaction of RXR with TFIIB and are consistent with either an induced conformational change in TFIIB or protection from proteolytic cleavage due to binding of RXR to TFIIB (82, 83).

**DISCUSSION**

Several nuclear hormone receptors have been shown to interact in vitro with the general transcription factor, TFIIB (10–14, 31), recruitment of which is thought to be a rate-limiting step in pre-initiation complex formation (7, 33, 34). The current view is that nuclear receptors interact with a complex of proteins involving co-activators, co-repressors, integrators, and other transcription factors (reviewed in Refs. 26, 27, and 65). In this report, we provide substantial evidence using both in vivo and in vitro methods to show that mRXRβ may interact with hTFIIB in a direct and ligand-dependent manner to promote gene activation, an observation of significance due to the central role of RXRs in gene regulation in vivo (1, 2, 48, 50, 51, 61–63).

**FIG. 5.** Analysis of regions of mRXRβ required for interaction with TFIIB. A, in vitro binding analysis of RXR deletion mutants to full-length 35S-labeled TFIIB. Sepharose beads were coated with bacterially expressed fusion protein consisting of GST-mRXRβ (bracket 2); or deletion mutants of mRXRβ, including an N terminus mutant Δ22–75 (ΔAF-1) (bracket 3); ligand-binding domain mutant, Δ255–410 (ΔLBD) (bracket 4); and extreme C-terminal mutant Δ390–410 (ΔAF-2) (bracket 5). As a control, beads were coated with GST alone (bracket 1). Beads were incubated in the absence or presence of 1 μM 9-cis-RA (indicated by − or +, respectively), with equal amounts of 35S-labeled TFIIB and luciferase. Bound proteins were resolved in SDS-PAGE. Input proteins show one-tenth of loaded lysate. B, deletion analysis of mRXRβ interaction with wild-type TFIIB in the dual-hybrid system. Left panel, schematic representation of RXR mutants analyzed. + indicates that immunoblot analysis of colonies functionally tested showed similar expression of TFIIB-GAL4-AD in yeast colonies (data not shown). Immunoblot analysis using antibodies directed against mRXRβ (13.17) or GAL4-DBD (CLONTECH, Palo Alto, CA) for the ΔLBD RXR mutant confirmed expression of RXR wild-type and mutant fusion proteins (not shown). Right panel, reporter activity (β-galactosidase) of the corresponding RXR mutants shown in relative light units (mean ± S.E.) over background values was normalized for protein and represents triplicate determinations from at least six yeast colonies. Far right, fold induction for each construct in the presence of ligand (1 μM 9-cis-RA; black bars) divided by activity in the absence of ligand (open bars) is indicated. C, partial proteolysis of RXR-TFIIB complex. An equal amount of labeled 35S-labeled TFIIB was incubated with nuclear extracts containing baculovirus expressed wild-type (WT) (lanes 1 and 2) or RXR (lanes 3 and 4) proteins, and after incubation, 50 ng of trypsin was added to lanes as indicated by −. Addition of 2 μM 9-cis-RA (ligand) was added as indicated (+). Reactions were resolved in SDS-PAGE and subjected to autoradiography. Brackets indicate different digestion patterns observed (lane 4) of a representative experiment.
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isoform-specific manner with TFIIB. Our inability to demon-

strate involvement of the N terminus of mRXR mutant. In the case of the TR (at least in vitro) the N terminus of the hTRβ has been shown to interact with the C terminus of TFIIB, whereas the C terminus of the LBD of hTRβ interacts with the N terminus of TFIIB (11), as supported by experiments in a cell-free system (28). Baniahmad et al. (11) observed that two regions in the C terminus of TR (aa 168–259 and 260–465) interacted with TFIIB, and interaction of the latter region was not hormone-dependent. In comparison, Fondell et al. (31) showed that the C terminus (aa 213–410) of hTRα interacted with both TFIIB and TBP, whereas the N terminus (albeit weakly) and the C terminus of hTRα interacted with the C terminus of TFIIB (aa 244–316). The findings with different TR isoforms suggest that different receptors may interact in an isoform-specific manner with TFIIB. Our inability to demonstrate involvement of the N terminus of mRXRβ in binding to TFIIB may be specific for murine RXRα, since mRXRβ has not been found to possess an N-terminal amino acid region homologous to that in TR (36) that interacted with TFIIB.

Evidence also suggests that TFIIB may present different interfaces for binding to different nuclear receptors, activators, and co-activators. For instance, interaction of RXR, and some other nuclear receptors, does not involve regions of overlap with binding of TFIIB to TFIIF (RAP30) which appears to involve the first 111 aa of TFIIB (33). However, studies have mapped the binding motifs of TBP and RNAPII to regions of TFIIB (33) common to regions utilized in binding of TFIIB to RXR (Fig. 5) and other receptors (11, 13, 14, 31, 32). In addition, it has been shown that other sites in TFIIB are important for co-activator binding (6, 7, 82, 84–87), such as for TAFII40 (aa 195–217) and VP16 (aa 165–217 and aa 269–296) (85). That interfaces of TFIIB involved in binding to RXR differ (in part) from those observed for TR suggests that both RXR and TR might jointly contact TFIIB through distinctly different, but not mutually exclusive, binding interfaces within TFIIB. More definitive proof of this notion will require additional experiments. From the perspective of RXR, different ligands (e.g., LG100268 and LG1007540) have been shown to quantitatively and qualitatively affect interaction of RXR with TBP and TAF110 (67), suggesting that differential interaction of the receptor with basal transcription machinery could be dependent upon the conformation of the LBD (67). These findings support the idea that RXR may augment binding of the heterodimeric complex to the PIC. Thus, our results suggest that RXR may enhance the ability of TR or RAR (for instance) to recruit TFIIB to the PIC, in support of findings in cell-free transcription models (28).

It should be mentioned that MacDonald et al. (13) demonstrated that VDR interacts with TFIIB using a dual-hybrid yeast system but did not observe an interaction between hRXRα or mRXRβ with TFIIB, although RXRs were not the principal focus of the study. The critical difference between the experiments of MacDonald et al. (13) and our experiments is that the interaction between RXR and TFIIB in yeast was entirely dependent on 9-cis-RA, a variable not reported in that study (13), and ligand has been noted to be important for binding to other transcription factors (29, 58, 67; see also below). Additionally, it is likely that other significant differences between the two model systems may account for the differences observed between results of MacDonald et al. (13) and our findings.

Our observations with TFIIB, and those of others with TBP and TAF110 (58, 67), suggest that RXR interacts specifically with distinct components of the basal transcription machinery to augment transcription. In most instances, the in vitro mapping data agreed with in vitro findings, but the exceptions serve to elucidate distinct features of receptor function. First, the association of RXR and TFIIB was not ligand-dependent in vitro but was ligand-dependent in vivo. This observation was supported by results of Baniahmad et al. (11) and Tong et al. (28) with TR; likewise, we observed only a slight increase in binding of mRXRβ to TFIIB in vitro in the presence of ligand. It is possible that failure of the bacterially expressed, recombinant RXR protein to assume a proper conformation or damage to (or proteolysis of) the receptor during purification affected our ability to detect ligand-dependent differences in vitro. In support of the necessity of ligand for association of RXR and TFIIB in vivo, a ligand-dependent interaction in yeast was also observed between TBP and TBP (58, 67), CBP (29), and TAF110 (58, 67). Overall, the necessity of ligand for in vivo interactions suggests that specific conformation is necessary in order for mRXRβ to associate with TFIIB.

Second, we observed an interaction between the Δ390–410 RXR deletion mutant and TFIIB in vitro but not in vivo, suggesting that in vivo the extreme carboxyl region of RXR is required to permit interaction with TFIIB (Fig. 5B), as was observed for TBP (58). Since the Δ390–410 RXR deletion mutant has previously been shown to bind ligand (71), this suggests that ligand binding alone is not sufficient to promote interaction of RXR with TFIIB. Our results are consistent with dominant negative activity reported for the Δ390–410 mutant (55, 56, 66, 69, 70, 71) and support other studies (66, 69) suggesting that the mechanism involves binding of the receptor to DNA. In addition, our results suggest the mutant is unable to interact properly with TFIIB in vivo. Specifically, use of the dual-hybrid system to characterize this mutant strongly argues against other mechanisms, since DNA binding and nuclear localization of the Δ390–410 mutant to the promoter were ensured by the inclusion of the GAL-4DBD in the construct. Others (9, 15) have drawn similar conclusions based on observations with mutant estrogen receptor constructs and TFIIB. An alternate explanation for dominant negative activity of the Δ390–410 RXR deletion mutant in vivo could be that an AF-2 binding factor (e.g., CBP, TIF1, ERAP-160, or RIP 140) is required in vivo for stabilization of RXR interaction with TFIIB. In support of this alternate explanation, it has been suggested that yeast proteins, such as SUG1, interact with basal transcription factors TBP (84) and hTAF110 (15) as well as the AF-2 region of nuclear receptors (23, 24), consistent with the notion that binding between TFIIB and RXR might be influenced by factors present in yeast. Further experiments are required to definitively distinguish between these two possibilities.

Since it had been shown that the acidic activator, VP-16, induced a conformational change in TFIIB (82, 83) and bound to a similar region of TFIIB, we performed a partial proteolysis assay to test whether RXR may induce a conformational change within TFIIB. Using in vitro translated TFIIB and baculovirus/or bacterially expressed RXR proteins, we observed that RXR induced an altered proteolytic digestion pattern of TFIIB. These data suggest that one possible action of nuclear receptors may be to induce a conformational change in TFIIB which, as suggested for VP16, then exposes other binding interfaces of TFIIB to other transcription factors, possibly TAF110 (82, 85), TBP (9, 31, 86, 87), TFIID (8, 34, 85, 88),
TFIIF, and RNAPII (89). These results raise the question of whether gene activation by nuclear hormone receptors might result not simply from a passive assembly of transcriptional proteins due to acidic, proline-rich, or glutamine-rich regions, but rather as the active alteration of protein interfaces of TFIIF which in turn present tissue protein interfaces with affinity to other transcription factors, thus influencing assembly of the PIC.

In summary, the demonstration that RRX can be interacting with TFIIF in a ligand-dependent manner is consistent with the notion that RRX may contribute to recruitment of TFIIF to heterodimeric DNA-bound receptor complexes as well as homodimeric receptor complexes.

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