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Transcription factor TFIIB and the vitamin D receptor cooperatively activate ligand-dependent transcription

(basal transcription factor/nuclear hormone receptor/activator/transfection/vitamin D-responsive element)

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ABSTRACT **The active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], regulates gene transcription through binding to the vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily. Sequence-specific transcription factors, including nuclear hormone receptors, are thought to interact with the basal transcription complex to regulate transcription. In glutathione *S*-transferase fusion-based protein–protein binding assays we found that VDR specifically binds to TFIIB, a component of the basal complex, and that the interaction requires select domains of each protein. To assess the functional significance of this interaction, transfection assays were performed with a 1,25$(OH)_2D_3$-responsive reporter construct. In P19 embryonal carcinoma cells cotransfection of VDR and TFIIB cooperatively activated reporter transcription, while each factor alone gave very low to no activation. This activation was dependent on 1,25$(OH)_2D_3$ and the dose of TFIIB and VDR transfected, demonstrating that a nuclear hormone receptor functionally interacts with TFIIB *in vivo*. In contrast, transfection of NIH 3T3 cells generated strong reporter activation by 1,25$(OH)_2D_3$ in the presence of VDR alone, and cotransfection of TFIIB led to specific dose-dependent repression of reporter activity. Taken together, these results indicate that TFIIB–nuclear hormone receptor interaction plays a critical role in ligand-dependent transcription, which is apparently modulated by a cell-type-specific accessory factor.**

Transcription of RNA polymerase II-specific genes depends on the ordered assembly of basal transcription factors at the promoter region. This event involves binding of TFIID, composed of the TATA-box-binding protein (TBP) and associated factors (TAFs), to the TATA element, after which another set of factors including TFIIA, TFIIB, RNA polymerase II, TFIIE, TFIIF, and TFIIH are recruited into the complex, culminating in the initiation of basal transcription. Spatial and temporal specificity of gene regulation is, however, conferred by sequence-specific transcription factors that bind to distinct DNA elements separate from the basal promoter. These sequence-specific factors are thought to interact with basal factors and modulate their activity (reviews, refs. 1–3). Multiple mechanisms appear to be responsible in this process. Some transcription factors may contact directly one or more basal transcription factors. TBP appears to be capable of binding to a large array of viral and cellular sequence-specific transcription factors (4, 5). Some factors may bind to TFIID through one of the TAFs (5).

Several members of the nuclear hormone receptor superfamily [progesterone receptor, estrogen receptor, COUP-TF, and the thyroid hormone receptor β (TRβ)] have been shown to associate with TFIIB (6–8), a basal factor which is rate limiting in the formation of the preinitiation complex. Indeed, TFIIB was isolated upon copurification with COUP-TF (7). Chimeric activators Gal4-AH and Gal4-VP16 have been shown to associate with TFIIB (9). Association with Gal4-VP16 increases recruitment of TFIIB into the basal complex, resulting in enhanced transcription (10, 11). Interaction with TFIIB is also reported for a factor encoded by *Drosophila* fushi tarazu and a HeLa cell factor LSF (12, 13). TFIIB has been cloned from several species (14–19) and shown to have conserved motifs (see Fig. 1*A*). TFIIB also binds to several basal factors, including TBP, RAP30, and RNA polymerase II, apparently through distinct domains (17, 20–23). We have investigated the role of TFIIB in vitamin D hormone-responsive transcription. Vitamin D is essential for mineral and skeletal homeostasis and regulates many activities, including those of bone cells, skin, and the immune system (24–27). These activities are mediated by a specific nuclear 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] receptor (VDR) that controls the transcription of a number of target genes (28). In this communication we present physical and functional interactions between TFIIB and VDR.

MATERIALS AND METHODS

Plasmids and Construction. Plasmids expressing the glutathione *S*-transferase (GST) fusion proteins of human (h) TFIIB have been described (8). pRS-hTFIIB was prepared by inserting hTFIIB into pRSV2 (29). The hVDR in pSG5 was used for transfection and preparation of *in vitro* translated receptors. *In vitro* transcribed and translated hVDR was prepared by digesting pSG5 hVDR with *Hin*dIII (to generate the wild-type receptor), *Nhe* I (Δ388–427), *Ava* I (Δ356–427), *Ban* II (Δ311–427), *Ava* II (Δ257–427), and *Alu* I (Δ123–427) and then using a commercial translation kit (TNT, Promega). Control reporter pL^{d}40-Luc was prepared by cloning the 40-bp H-2L^d promoter (30) in the pGL2 basic luciferase plasmid (Promega). The vitamin D-responsive reporter VDRE-L^{d}40-Luc was constructed by cloning of oligonucleotides containing four copies of the rat osteocalcin vitamin D-responsive element (VDRE) (31) 5′-tcgaGGGTGAATGAGGACATTA-CTGAagct-3′ (imperfect direct repeat underlined; lowercase, linker) in pL^{d}40-Luc (Fig. 3A).

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Abbreviations: TBP, TATA-box-binding protein; TR, thyroid hormone receptor; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDR, 1,25$(OH)_2D_3$ receptor; GST, glutathione *S*-transferase; h, human; r, rat; VDRE, vitamin D-responsive element; RAR, retinoic acid receptor; RXR, retinoid X receptor; EC, embryonal carcinoma.

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GST-Based Protein–Protein Binding Assay. These experiments are performed as described (8, 10) with modifications. Ten micrograms of GST-hTFIIB and truncated counterparts were coupled to 20 μl of glutathione-Sepharose 4B beads (Pharmacia), and incubated with [^{35}S]methionine-labeled hVDR at 4°C for 1 hr. The beads were then washed, and bound proteins were resolved by SDS/10% or 15% polyacrylamide gel electrophoresis (PAGE). ^{35}S-labeled rat (r) (TRα), human retinoic acid receptor α (hRARα), mouse retinoid X receptor β (RXRβ), β-globin, and luciferase were also tested as controls (29–33).

Transient Transfection. Murine P19 embryonal carcinoma (EC) cells (34) were transfected with 0.5 μg of luciferase reporter, 0.2 μg of pCH110 (Pharmacia), and various amounts of hVDR and hTFIIB expression vectors (35). The amount of total expression vector was kept constant (1 μg) by adding pRSV or pSG5 control vectors. Cells were treated with 10 nM $1,25(OH)_2D_3$ (provided by M. Uskokovic, Hoffmann-La Roche). Cells were harvested 24 hr after transfection, and their activities were normalized by β-galactosidase activity or by protein concentration, which gave comparable results. Luciferase activity was measured as described (35). NIH 3T3 cells were transfected by electroporation using 10 μg of luciferase reporter, various amounts of expression vectors, and pBS (Stratagene) to a total of 60 μg of DNA. After transfection cells were treated with 10 nM or 50 nM $1,25(OH)_2D_3$ for 48 hr and harvested for luciferase assay.

Protein Blot Analysis. Nuclear extracts (30 μg of protein) from P19 EC or NIH 3T3 cells prepared according to Schreiber *et al.* (36) were analyzed by SDS/10% PAGE and immunoblotted with rabbit antibody specific for hTFIIB (directed against a C-terminal peptide; Santa Cruz Biotechnology) and peroxidase-coupled anti-rabbit immunoglobulin as a second antibody (34). Bound antibody was detected by using the Amersham enhanced chemiluminescence system according to the supplier's instructions.

RESULTS

The hVDR Interacts with the C-Terminal Domain of hTFIIB. Since several members of the nuclear hormone receptor superfamily have been shown to interact with TFIIB (6–8), we tested whether hVDR, another member of the superfamily, also interacts with TFIIB by using a GST-based protein–protein binding assay. Fig. 1*A* shows a schematic diagram of the intact hTFIIB and deletion constructs (8, 20) tested. TFIIB has a conserved zinc finger motif (black box in Fig. 1*A*) in the N-terminal region and an imperfect repeat (boxed arrows) in the C-terminal region that resembles that of TBP. In addition, there is a basic α-helical motif at the end of the first repeat (+++). As seen in Fig. 1*B*, *in vitro* transcribed and translated wild-type hVDR bound to the full-length hTFIIB (lane 4), while it did not bind to control beads or control GST (lanes 2, 3). Binding was also detected by employing two constructs with deletions in the N-terminal region of hTFIIB [Δ(4–24) and Δ(45–123)]. In contrast, binding of hVDR was not detected with constructs that contained deletions in the C-terminal region of hTFIIB, lacking the basic helical domain [Δ(178–201); lane 10] or the imperfect repeat [Δ(118–174), Δ(202–269), Δ(273–297), and Δ(238–316); lanes 7, 8, 9, and 11, respectively]. ^{35}S-labeled β-globin and luciferase were tested as negative controls and did not exhibit detectable binding, while ^{35}S-labeled rTRα and hRARα, but not mouse RXRβ (data not shown) exhibited binding (Fig. 1*C*), consistent with previous observations (8). Addition of $1,25(OH)_2D_3$ did not affect hVDR binding to GST-TFIIB under these conditions (data not shown). These results demonstrate that hVDR is capable of directly associating with the C-terminal region of hTFIIB.

The Distal Region of hVDR Is Not Required for Interaction with TFIIB. Fig. 2*A* illustrates hVDR deletion constructs with

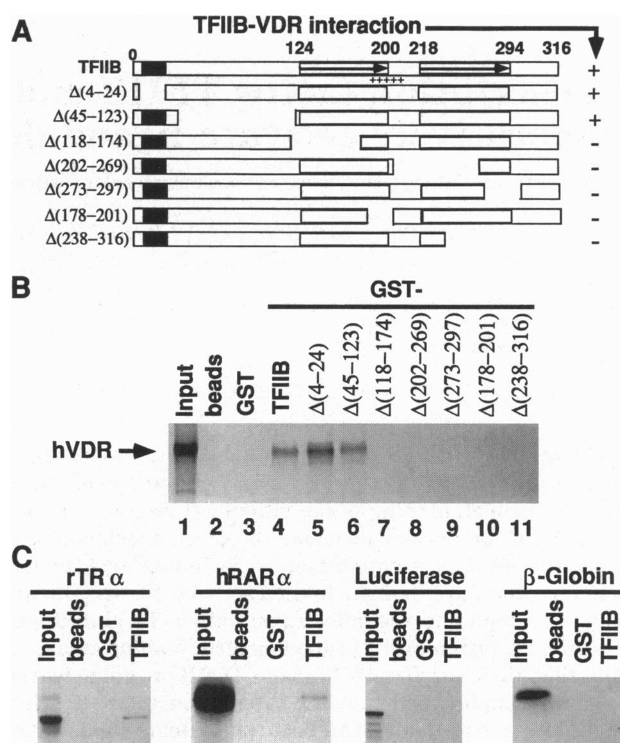

FIG. 1. Binding of hTFIIB to hVDR. (*A*) Schematic diagram of GST-TFIIB fusion constructs. The filled box, open boxes with arrows, and +++ indicate the zinc finger motif, imperfect repeat, and basic α-helical domain, respectively (20). Discontinuous boxes indicate sites of deletion (Δ number indicates the position of deleted amino acids). A summary of TFIIB–VDR interaction is shown on the right. (*B*) Binding of hVDR to GST-TFIIB. ^{35}S-labeled VDR was allowed to react with GST-TFIIB fusion proteins. (*C*) Binding of ^{35}S-labeled rTRα and hRARα tested as positive controls, or luciferase and β-globin as negative controls.

successive C-terminal truncations. These constructs were used to generate hVDR peptides labeled with [^{35}S]methionine (input lanes in Fig. 2*B*) that were then tested for binding to full-length GST-hTFIIB. The control GST beads failed to bind these hVDR peptides. In contrast, C-terminally truncated VDR peptides Δ388–427, Δ356–427, and Δ311–427 showed strong binding to hTFIIB (Fig. 2*B*). The levels of binding by Δ388–427 and Δ356–427 were somewhat greater than those of the intact hVDR and of Δ311–427. Albeit less efficiently, the Δ257–427 deletant also displayed binding. However, the product of a construct containing a further deletion to amino acid 123 failed to bind to GST-TFIIB (Δ123–427). These results indicate that the distal half of the ligand-binding domain of hVDR is not required for binding to hTFIIB. This region is implicated in heterodimerization with RXR (37).

Vitamin D-Dependent Reporter Transcription Is Cooperatively Activated by hVDR and TFIIB. Despite the evidence for the physical interaction between TFIIB and several nuclear hormone receptors observed so far (6–8), the functional significance of these interactions has not been fully determined, since the involvement of TFIIB in ligand-dependent transcription has not been demonstrated. To assess the role of TFIIB in $1,25(OH)_2D_3$-activated transcription, we performed transient transfection assays using a $1,25(OH)_2D_3$-responsive reporter (Fig. 3*A*). This reporter contains four copies of the VDRE from the rat osteocalcin gene (31) connected to the L^d40 basal promoter (30). This reporter does not possess other cis elements, allowing for direct assessment of the functional interaction between the VDRE and the basal promoter. Results of cotransfection assays performed in P19 EC cells are shown in Fig. 3*B*. Transfection of hVDR without hTFIIB

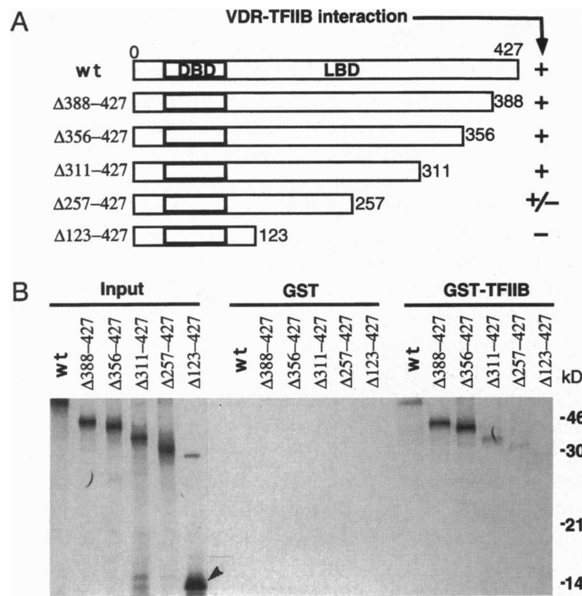

FIG. 2. Binding of truncated hVDRs to full-length TFIIB. (*A*) Schematic diagram of C-terminally truncated hVDRs. The number represents the position of the last deleted amino acid. The DNA-binding domain (DBD) and ligand-binding domain (LBD) are also depicted. A summary of VDR-TFIIB interaction is shown on the right (arrow). wt, Intact (wild type). (*B*) Truncated VDRs with comparable radioactivity (input) were allowed to react with control (GST) or the GST-TFIIB fusion protein. Arrow indicates the translation product of the Δ123–427 truncation.

resulted in a modest level of reporter activation (1.9-fold) upon addition of 10 nM $1,25(OH)_2D_3$. In the absence of $1,25(OH)_2D_3$, luciferase activity was near the background levels in both the presence and the absence of hVDR. Transfection of hTFIIB without hVDR gave a small (<2-fold) enhancement of reporter activity, both with and without ligand, as expected. However, when hVDR and hTFIIB were cotransfected, $1,25(OH)_2D_3$-dependent reporter activity was enhanced dramatically (>30-fold increase relative to the luciferase activity seen by transfection of the control vector). In the absence of $1,25(OH)_2D_3$, cotransfection of TFIIB and hVDR led only to a minor increase in reporter activity (1.9-fold). The control reporter L^{d}40-Luc without VDRE produced a background level of luciferase activity, and cotransfection of hVDR and/or hTFIIB did not measurably affect luciferase activity (data not shown). These results show that TFIIB and VDR functionally synergize to activate $1,25(OH)_2D_3$-dependent transcription in P19 EC cells.

As depicted in Fig. 4*A*, reporter activity was tested by transfecting increasing amounts of hTFIIB and a constant amount of hVDR (250 ng). $1,25(OH)_2D_3$-dependent reporter activation (2.3-fold) was observed even with the lowest amount of hTFIIB transfected (125 ng), and it increased with greater amounts of hTFIIB. The highest activation (34-fold) was observed with 750 ng of hTFIIB. Increasing the dose of hTFIIB to greater than 750 ng (up to 2 μg) neither increased nor decreased the activation (data not shown). Thus, the degree of activation correlated with the dose of transfected hTFIIB, further supporting the functional significance of TFIIB-VDR interaction in transcriptional enhancement. In Fig. 4*B*, reporter activity was measured after transfecting increasing amounts of hVDR along with a constant amount of hTFIIB (750 ng). The highest level of activation (about 35-fold) was observed when the amount of hVDR transfected was 250 ng, the lowest dose tested in these assays. Increasing the amount

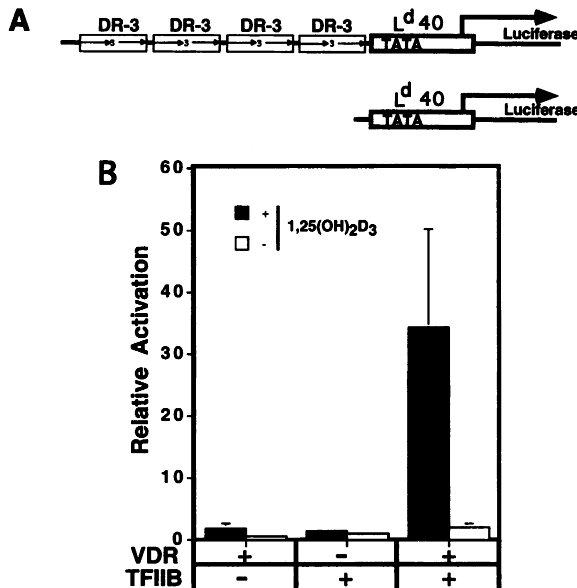

FIG. 3. Cooperative activation of a $1,25(OH)_2D_3$-responsive reporter by hVDR and hTFIIB in P19 EC cells. (*A*) Schematic diagram of reporter constructs. The 40-bp L^{d}40 promoter contained the TATA box and initiation site (30). (*B*) P19 EC cells were transfected with VDR (250 ng) or TFIIB (750 ng) vectors along with 500 ng of VDRE-L^{d}40-Luc and then treated with 10 nM $1,25(OH)_2D_3$. Relative activation was calculated by measuring luciferase activity with addition of VDR and/or TFIIB expression vectors relative to that with the VDRE-L^{d}40-Luc alone. Values represent the average of six experiments ± SD.

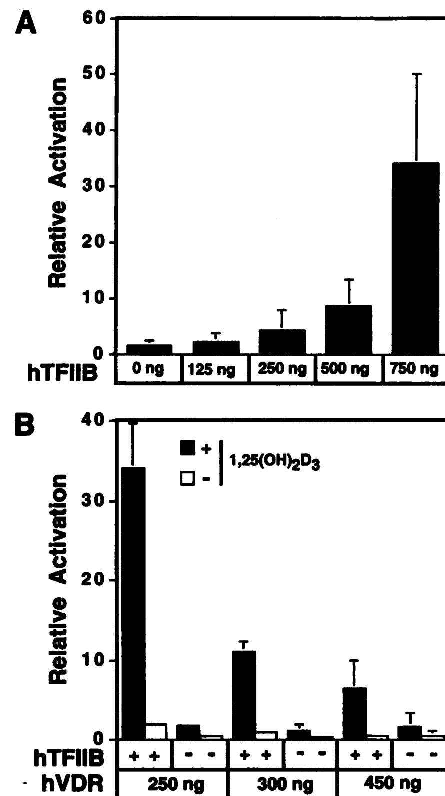

FIG. 4. Concentration-dependent effects of TFIIB (*A*) and VDR (*B*) on ligand-dependent reporter activity in P19 EC cells. (*A*) Cells were transfected with increasing amounts of hTFIIB along with a constant amount of VDR (250 ng) and the VDRE reporter, and then the cells were treated with 10 nM $1,25(OH)_2D_3$. Fold induction was calculated as in Fig. 3. Values are the average of three experiments ± SD. (*B*) Increasing amounts of hVDR were transfected along with a constant amount of hTFIIB (750 ng). Relative reporter activity was calculated as in Fig. 3. Values represent the average of three experiments ± SD.

of hVDR led to a dose-dependent reduction in reporter activity. With the highest dose of hVDR (450 ng), the level of activation was the lowest (<10-fold). Further increasing hVDR to 500 ng did not increase the level of activation (data not shown). This reduction was not caused by ligand sequestration because of excess hVDR, since reporter activation was similarly reduced at 50 nM $1,25(OH)_2D_3$ (data not shown). Increasing amounts of hVDR in the presence of cotransfected TFIIB slightly reduced reporter activity even in the absence of ligand; the basis of this result is not clear at present. In the absence of hTFIIB, increasing amounts of hVDR did not elicit a significant effect on reporter activity. These results indicate that excess hVDR inhibits TFIIB-mediated synergistic reporter activation.

Repression of $1,25(OH)_2D_3$-Dependent Transcription by Excess hTFIIB in NIH 3T3 Cells. To test whether cooperative reporter activation by hTFIIB and hVDR depends on cell type, transfection assays were performed with NIH 3T3 cells. In the absence of $1,25(OH)_2D_3$, transfection of VDRE-L^d40-Luc without the hVDR expression vector yielded essentially background levels of luciferase activity (Fig. 5*A*). Addition of $1,25(OH)_2D_3$ resulted in a modest (<7-fold) but consistent increase in luciferase activity, suggesting that this reporter is weakly stimulated by the endogenous VDR. Transfection of hTFIIB without hVDR appeared to slightly repress reporter activity in the presence of ligand (Fig. 5*A*). However, transfection of hVDR led to a greater than 85-fold enhancement in reporter activity, which was dependent upon addition of $1,25(OH)_2D_3$. The levels of $1,25(OH)_2D_3$-dependent reporter activity in NIH 3T3 cells were consistently greater than those in P19 EC cells (see *Discussion*). Significantly, cotransfection of hTFIIB decreased hVDR activation of reporter transcription; transfection of increasing amounts of hTFIIB (12 and 24 μg) repressed vitamin D-dependent reporter activity in a dose-dependent fashion (Fig. 5*A*). Decreasing the amount of TFIIB further to 6, 3, and 1 μg alleviated the repression but did not generate cooperative activation (Fig. 5*B*). Similar repression was observed when the amounts of VDR were either raised or lowered and tested in combination with various amounts of TFIIB (Fig. 5*B*). Thus, in NIH 3T3 cells excess hTFIIB inhibits $1,25(OH)_2D_3$-dependent reporter activation mediated by hVDR.

The TFIIB-mediated activation found with P19 EC cells and the repression observed with NIH 3T3 cells could be because the amount of TFIIB may be limiting in P19 EC cells, while it may be sufficient in NIH 3T3 cells to allow for activation by VDR. To assess relative levels of endogenous TFIIB in P19 EC cells and NIH 3T3 cells, immunoblot analysis was performed on nuclear extracts from these cells by using antibody specific for TFIIB. As seen in Fig. 5*C*, the levels of TFIIB were comparable in the two types of cells. These results may be consistent with the idea that functional interaction between TFIIB and VDR involves a third factor which is expressed (or active) in a cell-type-specific fashion.

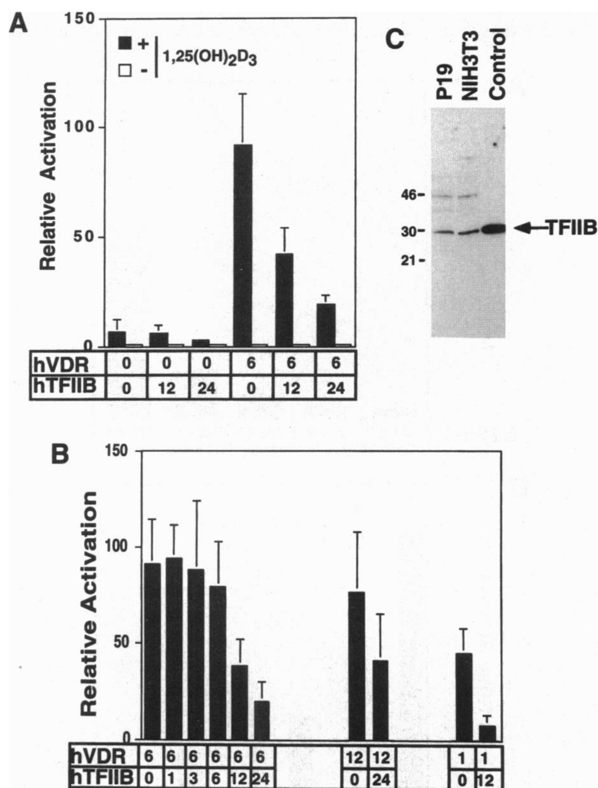

FIG. 5. TFIIB-mediated repression of $1,25(OH)_2D_3$-dependent reporter activation in NIH 3T3 cells. (*A*) NIH 3T3 cells were electroporated with the indicated amounts (in μg) of expression vectors (numbers at the bottom), 10 μg of VDRE-L^d40-Luc, and pBS to a total of 60 μg followed by treatment with 10 nM $1,25(OH)_2D_3$. Relative reporter activity was calculated as above. (*B*) Various amounts of hVDR and hTFIIB (in μg at the bottom) were transfected together with VDRE-L^d40-Luc into NIH 3T3 cells as above. (*C*) Endogenous TFIIB in P19 EC and NIH 3T3 cells. Protein blot analysis was performed with nuclear extracts from each of the cells (30 μg of protein) and analyzed as described in the text. The control lane contained 50 ng of bacterial recombinant hTFIIB. Numbers on the left indicate molecular masses in kDa.

DISCUSSION

The present work demonstrates that hTFIIB and hVDR synergistically enhance $1,25(OH)_2D_3$-dependent reporter activation in P19 EC cells, revealing a functional interaction between a basal factor and a nuclear hormone receptor in ligand-dependent transcription. To our knowledge, this is the first demonstration that TFIIB plays a critical role in ligand-activated transcription *in vivo*. In addition, we show that hVDR and hTFIIB physically interact through specific domains of each protein, thus supporting the observed functional interaction. hVDR required the C-terminal region of hTFIIB for efficient interaction (Fig. 1). This region contains a basic helical domain and a large imperfect repeat (Fig. 1), which are thought to form a distinct protease-resistant core (21, 23). Our results, combined with previous reports (6–8), suggest that nuclear hormone receptors interact with more than one domain of TFIIB. For example, hTRβ is found to interact with the N-terminal as well as C-terminal domain of TFIIB (8). In contrast, the estrogen receptor binds mostly to the C-terminal region of TFIIB (7). The domain of TFIIB required for interaction with hVDR was similar, but not identical, to that required for association with Gal4-VP16, the chimeric activator (10) that has been studied extensively as a model for enhancement of transcription by a sequence-specific factor. The TFIIB domains required for interaction with specific factors appear distinct from those necessary for contacting basal factors, including TBP, RNA polymerase II, and RAP30 (17, 20, 22), indicating that TFIIB interacts with several basal and sequence-specific factors through multiple domains. It should be noted, however, that the domain requirements reported so far rely primarily on observations *in vitro*, which may not apply *in vivo*.

Deletion analysis of hVDR (Fig. 2*B*) suggests that the proximal portion of the ligand-binding domain is required for interaction with TFIIB. TFIIB–VDR interaction, under these conditions, appeared neither to require nor to be affected by the $1,25(OH)_2D_3$ ligand. It should be pointed out here that our deletion data do not exclude the possibility that the N-terminal DNA-binding domain of VDR also takes part in interacting

with TFIIB. A further analysis of VDR mutants specifically lacking this region will be required to answer this question.

By transfection analysis using P19 EC cells we found that hVDR and hTFIIB cooperatively activate VDRE reporter transcription in a $1,25(OH)_2D_3$-dependent fashion. Activation required both hVDR and hTFIIB (Fig. 3B), and the levels correlated with the amount of TFIIB transfected (Fig. 4A). Our results are consistent with the previous finding that TFIIB enhances transcription by the progesterone receptor *in vitro* (7), although ligand dependence was not addressed in this cell-free system. Our results are also in concert with reports describing functional interaction between TFIIB and artificial chimeric activators, Gal4-VP16 and Gal4-AH (9, 11), and support the concept that TFIIB, a component of the basal transcription machinery, plays a major role in communicating with sequence-specific transcription factors. Roberts *et al.* (10) and Choy and Green (11) showed that the Gal4-VP16 activator not only increases TFIIB recruitment into the basal complex but also affects a subsequent step of transcription initiation and that the region of TFIIB necessary for activation by Gal4-VP16 is different from that necessary for basal transcription, suggesting that specific factors act through a mechanism distinct from that employed by basal factors. It is significant that transfection of exogenous TFIIB into NIH 3T3 cells did not elicit cooperative activation, but rather led to dose-dependent repression of reporter transcription activated by hVDR. The difference seen between P19 EC and NIH 3T3 cells was not attributable to a gross difference in endogenous TFIIB levels in the two cell types, as immunoblot analysis (Fig. 5C) indicated that these cells expressed comparable levels of TFIIB. The repression observed with NIH 3T3 cells may be analogous to "squelching," in which an excess of one transcription factor sequesters another factor, reducing the formation of an active transcription complex (38). If this mechanism applies, it is conceivable that a functional interaction between VDR and TFIIB involves an accessory factor, which is expressed (or functional) in a cell-type-dependent fashion. Such an accessory factor may be expressed at different levels in P19 EC and NIH 3T3 cells, and supplementing exogenous TFIIB may differentially affect the kinetics of formation of productive transcriptional complex. There may be more than one cell-type-specific "accessory" protein, which may operate in conjunction with specific transcription factors. Supporting this notion, Wampler and Kadonaga (19) found that excess TFIIB represses some, but not all, basal transcription in a dose-dependent fashion *in vitro*. Intermediary proteins that modulate transcription of target genes have been described for nuclear hormone receptors (39, 40) and for other sequence-specific factors (41, 42).

At present the precise mechanism by which VDR and TFIIB activate transcription in response to ligand is not known; it is possible that the interaction of these two factors leads to increased recruitment of other basal factors. Alternatively, this interaction may cause a shift in conformation of the basal transcription complex, thereby increasing the rate of transcription. The present study serves as a foundation on which to investigate receptor–TFIIB interactions in further detail.

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