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Dominant Negative Retinoid X Receptor β Inhibits Retinoic Acid-Responsive Gene Regulation in Embryonal Carcinoma Cells

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Retinoid X receptors (RXRs) heterodimerize with multiple nuclear hormone receptors and are thought to exert pleiotropic functions. To address the role of RXRs in retinoic acid- (RA) mediated gene regulation, we designed a dominant negative RXRβ. This mutated receptor, termed DBD⁻, lacked the DNA binding domain but retained the ability to dimerize with partner receptors, resulting in formation of nonfunctional dimers. DBD⁻ was transfected into P19 murine embryonal carcinoma (EC) cells, in which reporters containing the RA-responsive elements (RAREs) were activated by RA through the activity of endogenous RXR-RA receptor (RAR) heterodimers. We found that DBD⁻ had a dominant negative activity on the RARE reporter activity in these cells. P19 clones stably expressing DBD⁻ were established; these clones also failed to activate RARE-driven reporters in response to RA. Further, these cells were defective in RA-induced mRNA expression of *Hox-1.3* and RARβ, as well as in RA-induced down-regulation of Oct3 mRNA. Gel mobility shift assays demonstrated that RA treatment of control P19 cells induces RARE-binding activity, of which RXRβ is a major component. However, the RA-induced binding activity was greatly reduced in cells expressing DBD⁻. By genomic footprinting, we show that RA treatment induces in vivo occupancy of the RARE in the endogenous RARβ gene in control P19 cells but that this occupancy is not observed with the DBD⁻ cells. These data provide evidence that the dominant negative activity of DBD⁻ is caused by the lack of receptor binding to target DNA. Finally, we show that in F9 EC cells expression of DBD⁻ leads to inhibition of the growth arrest that accompanies RA-induced differentiation. Taken together, these results demonstrate that RXRβ and partner receptors play a central role in RA-mediated gene regulation and in the control of growth and differentiation in EC cells.

Retinoic acid (RA), a natural derivative of vitamin A, plays a pivotal role in vertebrate development and in the establishment and maintenance of physiological processes in adult tissues (44, 55). Embryonal carcinoma (EC) cells have been used as a model to study the mechanism of RA action during development (2, 67). RA triggers differentiation of EC cells along different cell lineages and changes the expression of many genes (28, 29). The pattern and kinetics of RA-induced gene expression in these cells parallel, in many cases, those observed during embryonic development. For example, RA-induced expression of a series of *Hox* genes mimics the colinearity rule of restricted spatiotemporal pattern of expression in vivo (66).

The main effectors of RA action are two classes of RA receptors, termed RA receptors (RARs) and retinoid X receptors (RXRs) (7, 22, 25, 40, 41, 52). Both classes of receptors belong to the nuclear hormone receptor superfamily (4, 16). There are different members for each class of receptors (α, β, and γ in mammals), and within each class several isoforms are usually present (38, 47, 72). Whereas RXRs bind with high-affinity 9-*cis* RA (9C-RA), an isomer of RA, RARs bind both all-*trans* RA (t-RA) and 9C-RA isomers (1, 26, 39).

RXRs have been shown to heterodimerize in vitro with several members of the nuclear hormone receptor superfamily, including RARs, thyroid hormone receptors (TR), and the vitamin D receptor (6, 32, 36, 42, 71). We and others have shown that RXR heterodimerization is dependent on a large region of the C-terminal domain (18; see also the references above) and that heterodimerization greatly increases target DNA binding. Cotransfection studies have suggested that the increased binding results in a synergistic increase in target gene transcription (14, 32, 42, 71). Because of their ability to heterodimerize with multiple partners, RXRs are expected to introduce a combinatorial diversity to the nuclear hormone receptors and to exert pleiotropic activities in ligand-mediated gene regulation. RXRs also form homodimers in the presence of 9C-RA, which leads to increased binding to certain RA-responsive elements (73), suggesting an additional role for RXRs upon homodimerization.

Despite extensive studies describing the activities of RXRs in vitro, the existence and function of naturally occurring RXR heterodimers or homodimers in vivo have not been fully elucidated. To this end, we have made use of

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a mutated RXRβ lacking the DNA binding domain (DBD$^-$), which may exert a dominant negative activity, and studied its effect upon RA-induced gene regulation in EC cells. Our results show that this mutated receptor, when introduced into P19 EC cells, inhibits RA-induced transcription of reporters, which is otherwise primarily mediated by endogenous RAR-RXR heterodimers. We also show that stable expression of DBD$^-$ results in reduced factor binding to RA-responsive elements (RAREs) in vitro and inhibits factor occupancy at the endogenous RARβ gene promoter in vivo. DBD$^-$ was found to alter the pattern of endogenous gene expression induced by RA in P19 cells and to inhibit RA-mediated growth regulation in F9 EC cells. These results illustrate a central role for RXRs in RA-mediated regulatory processes in vivo.

MATERIALS AND METHODS

Plasmid construction. (i) Reporters. The basal thymidine kinase (TK) luciferase reporter was constructed by cloning the herpes simplex virus TK gene promoter (−105 to +55) from pBL-CAT8+ (65) into the luciferase construct pGL2 (Promega). Reporter plasmids were constructed by inserting the following oligonucleotides into the *Bgl*II site of the basic TK reporter in the sense orientation: 5′-gatccgctagcAAG GGT TCA CCG AAA GTT CAC TCG CATa-3′ (βRARE tk-Luc, one or two copies), 5′-gatcgATT CAG GTC ATG ACC TGA GGa-3′ (TRE_p tk-Luc, one or two copies; see reference 24). GRE tk-Luc with 5′-agcttTTG TAC AGG ATG TTC TAG TCT AGA TGT ACA GAT GTT CTG-3′ was a gift from L. King (National Cancer Institute, National Institutes of Health). βRARE3 TK luciferase with three copies of 5′-tcgagggtAAG GGT TCA CCG AAA GTT CAC-3′ was a gift from E. Linney (Duke University). The RARβ β-galactosidase reporter fused with a 650-bp upstream region of the murine RARβ gene was constructed from RLZ79 as described elsewhere (56) and kindly provided by A. Zimmer (National Institutes of Health).

(ii) Expression vectors. The mammalian expression plasmid pcx was constructed by excising the *neo* gene fragment from pcxn2. pcxn2, a vector shown to allow high-level expression of an insert cDNA in EC cells, and pcxn-IL2, containing the interleukin-2 cDNA (48), were a generous gift of J. Miyazaki (Tokyo University, Tokyo, Japan). Expression plasmids for mRXRβ and DBD$^-$ used for transient assays were constructed by cloning *Eco*NI-*Acc*I fragments of Rous sarcoma virus (RSV)-RXRβ and RSV-RXRβ D-del (46) into the *Eco*RI site of pcx after fill-in reactions. Expression plasmids for δN2 and δN3 were prepared by inserting the RXRβ deletion constructs N2 and N3 (42) into pcx. For stable transfections, pcxn2 driving the expression of RXRβ or DBD$^-$ were used. The expression vectors for TRα (42) and glucocorticoid receptor (GR) (10) were kindly provided by V. Nikodem (National Institutes of Health) and M. Danielsen (Georgetown University), respectively. hRARβ in pSV-SPORT1 (Bio-Rad Laboratories) was a kind gift from A. DeJean (Pasteur Institute, Paris, France) through A. Zimmer (National Institutes of Health). The β-galactosidase plasmid (pCH110) used for normalizing transfection efficiency was obtained from Pharmacia.

Coimmunoprecipitation. RXRβ, hRARβ, and DBD$^-$ in pExpress (19) were transcribed and translated in vitro by using reticulocyte lysates in the presence or absence of [^{35}S]methionine as suggested by the manufacturer (Ambion). Equal amounts of unlabeled RXRβ or DBD$^-$ (as judged by Western blot [immunoblot] analysis of an aliquot of translated materials) were mixed with labeled RARβ (15 μl from a standard reaction mixture) and incubated with rabbit anti-peptide antisera specific for RXRβ (RM774 [43]) or control preimmune sera overnight at 4°C. Reactions were precipitated with packed protein A-agarose beads (Pharmacia). Precipitated materials were washed, eluted from the beads, resolved by sodium dodecyl sulfate–11% polyacrylamide gel electrophoresis (SDS–11% PAGE), and autoradiographed.

Cell culture and transfections. P19 EC cells were grown in α minimal essential medium supplemented with 10% fetal bovine serum, glutamine, and gentamicin (50 μg/ml). CV1 and F9 tk$^-$ cells were grown in Dulbecco's modified Eagle's medium supplemented as described above. Transfections were performed by the calcium phosphate precipitation method, with *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonate (BES) buffer (8). Unless otherwise indicated, P19 cells were seeded in a 12-well plate (5×10^4 cells per well) 12 h prior to transfection and transfected with a total of 1.1 μg of DNA for 8 to 10 h. Routinely, 200 ng of reporter and 200 ng of expression plasmids were added to a well. The amount of expression vectors was kept constant by adding the control pcx plasmid without insert. pBluescript was used as carrier DNA to adjust the total amount of DNA transfected. After transfection, cells were rinsed and further incubated with fresh medium containing appropriate ligands for 16 to 20 h. Cells were harvested and lysed, and luciferase activity was measured as described elsewhere (63). 9C-RA was dissolved in 2 mM dimethyl sulfoxide and stored in the presence of argon gas at −70°C. t-RA was obtained from Kodak. Other ligands were obtained from Sigma. Luciferase activities were normalized by β-galactosidase activity derived from the cotransfected pCH110 or by protein concentrations. To obtain stable transfectants, P19 cells were transfected with pcxn-2 or pcxn-DBD$^-$ as described above and were selected with G418 (500 μg/ml; Gibco) for 10 to 15 days. Colonies (10 to 12) were isolated from each transfection and were further propagated as separate clones. Expression of DBD$^-$ in these clones was monitored by Western blot analysis (43). Briefly, whole cell lysates derived from 5×10^6 cells in SDS sample buffer were treated with DNase I and RNase A for 15 min at 37°C, electrophoresed in SDS–10% polyacrylamide, blotted onto polyvinylidene difluoride filters, and incubated with culture supernatants containing monoclonal anti-RXRβ antibody MOK 13-17 for 4 h at room temperature. Filters were rinsed and reacted with ^{125}I-labeled sheep anti-mouse immunoglobulin (Amersham). Cells were aliquoted and frozen immediately after the initial confirmation of DBD$^-$ expression. Subsequent analyses were performed with the same frozen aliquots.

RA-resistant F9 cells. The method described by Espeseth et al. (15) was used. Briefly, F9 EC cells (10^5 cells) seeded in a 100-mm-diameter dish were transfected with 10 μg of pcxn2 without insert, pcxn2-RXRβ, or DBD$^-$ for 24 h and were then immediately exposed to G418 (500 μg/ml). After 24 to 36 h, t-RA (1 μM) was added to half of the culture. Cells were fed with fresh G418 and t-RA every 3 to 4 days for up to 3 weeks until macroscopic colonies developed. Plates were fixed with methanol and stained with Giemsa stain (Sigma), and the number of colonies was counted.

EMSA. Untransfected P19 cells and clones established from stable transfection were treated with 1 μM t-RA for 48 h and lysed in 2 volumes of buffer C (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 25% [vol/vol] glycerol) containing a combina-

tion of protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 33 μg of aprotinin per ml, 10 μg of pepstatin per ml). Supernatants obtained from lysates were used for electrophoretic mobility shift assays (EMSA) without dialysis. Extracts (6 μg of protein) were incubated with 10 fmol of ^{32}P-labeled oligonucleotide (10^5 cpm per reaction mixture) in EMSA binding buffer [4 μg of poly (dI-dC) per ml, 20 mM Tris (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 5 mM dithiothreitol, 5% (vol/vol) glycerol] for 30 min at 4°C. The oligomers used were βRARE, 5′-GAT CCG CTA GCA AGG GTT CAC CGA AAG TTC ACT CGC ATA-3′; and UCR (used as a control [17]), 5′-CTG CAG TAA CGC CAT TTT GCA AGG CAT GAA-3′. For competition, extracts were preincubated with a 100× molar excess of unlabeled oligomers for 30 min prior to the addition of labeled probe. For supershift experiments, extracts were preincubated with 4 μl of MOK 13-17 in ascites prior to the addition of probe. Ascites obtained with myeloma cells (immunoglobulin G1) were used as a control. Electrophoresis was performed with 4% nondenaturing polyacrylamide gels.

Ribonuclease protection and RNA blot assays. Cells were treated with 1 μM t-RA for 6, 24, and 72 h, and total RNA was isolated according to standard procedures (9). RNase protection assay was performed according to standard procedures (59) with 25 μg of total RNA and an in vitro-transcribed ^{32}P-labeled probe specific for the RARβ2 isoform (74, 75). For Northern (RNA) blot analysis, 2 μg of poly(A)$^+$ RNA was separated on a 1% agarose gel with 2.2 M formaldehyde and blotted onto nylon filters. Filters were cross-linked by UV and hybridized with random-primed ^{32}P-labeled DNA probes for Oct3 (a *Pst*I-*Xba*I cDNA fragment; 58) or for *Hox-1.3* (an *Eco*RI-*Bgl*II cDNA fragment; 49) in binding buffer (50% formamide, 5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA at pH 7.7], 2× Denhardt solution, 0.1% SDS, 0.1 mg of herring sperm DNA per ml) at 42°C. The final wash was in 1× SSC, (0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C. Filters were exposed for 16 to 24 h at room temperature for Oct3 and for 72 to 96 h at −70°C for *Hox-1.3*.

In vivo footprinting. P19 clones treated with 1 μM t-RA for 24 h were incubated with 0.1% dimethylsulfate for 2 min at room temperature. Genomic DNA was isolated and digested with piperidine. Ligation-mediated PCR was performed with Vent polymerase (13, 21). To generate footprinting of the RARE in the RARβ gene, the following gene-specific primers were used: 5′-TGG CAA AGA ATA GAC-3′ (+57 to +43), 5′-AGA ATA GAC CCC TCC TGC CTG CCT CGG AGC-3′ (+51 to +27), and 5′-ACC CTC CTG CCT CGG AGC TCA CTT CTA C-3′ (+44 to +13) (coding strand); and 5′-ATT GTT TGC AGC TGA G-3′ (−210 to −194), 5′-GAC TCG CTG GCT GAA GGC TCT TGC AAG-3′ (−167 to −142), and 5′-GGC TGA AGG CTC TTG CAG GGC TGC TGG GAG-3′ (−159 to −130) (noncoding strand). Labeled PCR products were run in an 8% sequencing gel and analyzed by autoradiography.

RESULTS

A mutated RXRβ able to dimerize with a partner receptor but unable to bind to a target DNA. We analyzed an mRXRβ construct lacking the DNA binding domain (DBD$^-$; see Fig. 1A) as a candidate for a dominant negative activity that would abrogate the function of both RXR heterodimers and homodimers in vivo. DBD$^-$ carries the intact C-terminal domain shown to be required for dimerization and should form heterodimers with multiple receptor partners (32, 36, 42, 71; see Fig. 1B). This mutant, however, is defective in binding to target DNA and thus unable to *trans*-activate a target gene (46, 63). Dimers formed with DBD$^-$ would not be able to bind to target DNA, and would, in turn, render intact partner receptors nonfunctional. DBD$^-$ may thus exhibit a dominant negative phenotype when expressed at a sufficient level. We also analyzed a second mutated receptor, δN2 (Fig. 1A), in which a large part of the N-terminal domain, the DNA binding domain, and a small proximal part of the C-terminal domain have been deleted, since it has been shown to retain the capacity to heterodimerize with receptor partners (42).

Coimmunoprecipitation assays were performed to confirm the ability of DBD$^-$ to heterodimerize with a partner receptor (Fig. 1B). We chose RARβ as the partner in this assay, since RARβ is induced by RA in EC cells and is likely to be active in RA-mediated gene regulation in P19 cells (72). Radiolabeled, in vitro-translated RARβ was mixed with unlabeled intact RXRβ or DBD$^-$, and the mixtures were precipitated with a rabbit anti-peptide antibody specific for RXRβ (42). Radiolabeled RARβ was efficiently coprecipitated by anti-RXRβ antibody when mixed with either DBD$^-$ or the intact RXRβ (Fig. 1B, lanes 4 and 6). This coimmunoprecipitation was specific, since (i) anti-RXRβ antibody did not precipitate RARβ when mixed with control lysates (lane 2) and (ii) control preimmune sera did not precipitate RARβ when mixed with RXRβ or DBD$^-$ (lanes 5 and 7). These results show that DBD$^-$ is capable of heterodimerizing with RARβ in the absence of target DNA and without requiring a specific ligand, in full agreement with previous reports (42). The same results were obtained by using radiolabeled TRα (data not shown). The amount of radioactive RARβ coprecipitated with DBD$^-$ was consistently less than that coprecipitated with intact RXRβ, suggesting that DBD$^-$ is slightly less efficient than intact RXRβ at forming heterodimers with partner receptors. It is possible that the DNA binding domain is involved in stabilization of RXR dimers. The existence of additional dimerization interfaces in the DNA binding domain has been shown in several steroid receptors and has recently been shown for RXRα (35). DNA binding capacity of DBD$^-$ in the presence of RARβ (or TRα) was tested by EMSA. No binding was observed with DBD$^-$ under the conditions in which binding to several response elements could be readily seen with intact RXRβ mixed with RARβ or TRα (data not shown). The data provide the basis for the dominant negative activity postulated for DBD$^-$.

DBD$^-$ acts as a dominant negative inhibitor of RA-induced reporter transcription in P19 EC cells. RXRβ constructs outlined in Fig. 1A were cloned into the expression plasmid pcx (see Materials and Methods) and tested for their activity following transient transfection into P19 EC cells. As a reporter, we used βRARE tk-Luc, in which the RARE of the RARβ gene was connected to the basal TK promoter and the luciferase gene (12). Results are shown in Fig. 2A and B. Treatment of transfected P19 cells with 9C-RA increased βRARE tk-Luc reporter activity by 200- to 300-fold (Fig. 2B). However, when DBD$^-$ was cotransfected, luciferase activity was reduced in a dose-dependent manner, with the maximum inhibition being >90% of the control (Fig. 2A), while no inhibition was seen with cotransfection of a control plasmid, pcx. t-RA and 9C-RA were equally effective in activating the βRARE tk-Luc reporter, and DBD$^-$ inhibition was observed with either RA at concentrations up to 10 μM (data not shown). The δN2-mutated RXRβ, which also lacks

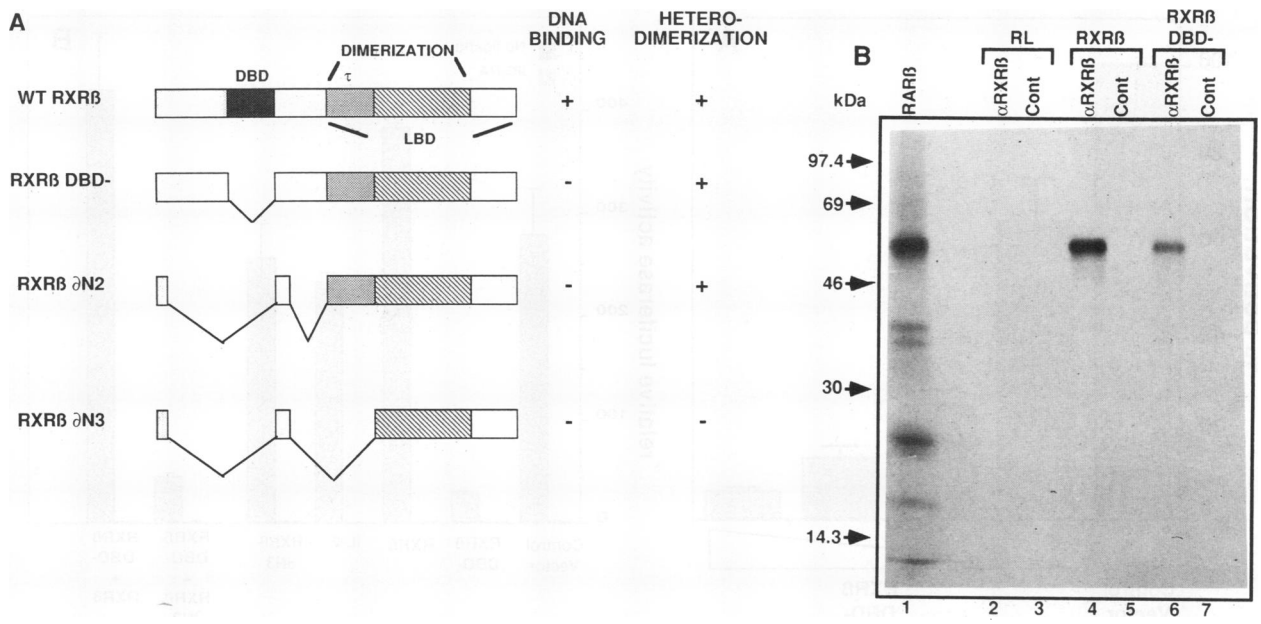

FIG. 1. (A) Schematic representation of mutated RXRβ constructs. Summarized to the right are activities of each deletion for target DNA binding and for heterodimerization. Construction of the mutated receptors is described elsewhere (43, 46). (B) Coimmunoprecipitation of RARβ and DBD⁻. In vitro-translated, ^{35}S-labeled RARβ (lane 1) was mixed with control reticulocyte lysates (RL; lanes 2 and 3) or with unlabeled in vitro-translated wild-type RXRβ (lanes 4 and 5) or DBD⁻ (lanes 6 and 7) (in vitro translated) and reacted with anti-RXRβ antibody (αRXRβ) or normal rabbit sera (Cont).

DNA binding activity and is capable of heterodimerizing with a partner but has a more extensive N-terminal deletion, inhibited βRARE reporter activity in a similar fashion (data not shown). At the same levels of expression vectors, cotransfection of DBD⁻ caused >80% inhibition, whereas intact RXRβ resulted in a slight increase in luciferase activity and luciferase activity was not affected when cells were cotransfected with plasmids expressing the lymphokine IL-2 or the δN3 construct, which has a more extensive deletion in the C-terminal domain that abrogates dimerization, confirming the specificity of DBD⁻ inhibition (Fig. 2B). In addition, DBD⁻ inhibited RA-induced activation of TRE_p tk-Luc reporter (data not shown). These results indicate that DBD⁻ and δN2, both capable of dimerizing with a partner receptor but unable to bind to target DNA, inhibit RA-induced activation of reporter transcription, consistent with the proposed dominant negative property. This inhibition is likely to be attributed to impaired function of endogenous RXRs, since the addition of exogenous intact RXRβ restored reporter activity to a level comparable with that seen with the control plasmids (Fig. 2B). As expected, δN3, unable to bind to target DNA or to heterodimerize, failed to restore reporter activation.

The DBD⁻-mediated inhibition observed is likely caused by inhibiting the function of RAR-RXR heterodimers in P19 cells. In order to ascertain whether DBD⁻ exerts an inhibitory activity upon RAR(s), reporter assays were performed in CV-1 cells transfected with exogenous RARβ. CV-1 cells were chosen because they have been reported to contain low levels of endogenous RARs (12). As seen in Fig. 2C, RA-induced βRARE tk-Luc activity was enhanced when exogenous RARβ was cotransfected. However, when DBD⁻ was cotransfected with RARβ, the activity was reduced to a level slightly lower than that prior to RARβ transfection. Cotransfection of intact RXRβ, up to the maximal amounts of expression vector used for DBD⁻, resulted in a strong increase in reporter activity, whereas δN3 had no effect (data not shown). These results show that DBD⁻ specifically inhibits the activity of RAR(s).

To study whether DBD⁻ exerts an inhibitory effect on transcription of other hormone-responsive reporters, we tested thyroid hormone-dependent *trans*-activation of the TRE_p tk-Luc reporter and glucocorticoid-dependent transactivation of the GRE tk-Luc reporter following cotransfection of P19 cells with the TRα and GR, respectively. RXRβ heterodimerizes with TRα (42) but not with the GR under comparable conditions. Cotransfection of DBD⁻ led to strong inhibition on TRE_p tk-Luc reporter activity, but not on GRE tk-Luc reporter activity, indicating that DBD⁻-mediated inhibition occurs independently of RA and selectively on receptors that dimerize with RXR (data not shown).

To assess whether DBD⁻ inhibits RA activation of RAREs in the context of a natural gene, we tested the activity of the murine RARβ2 promoter driving expression of the β-galactosidase reporter in P19 cells. This reporter construct contains a 650-bp fragment of the mRARβ upstream sequence, including the RARE used above, that has been shown to be active in transgenic mouse embryos (56, 74). As seen in Fig. 3, both 9C-RA and t-RA were effective in activating this reporter at 50 nM, the lowest concentration tested. 9C-RA at 1 μM gave a maximum enhancement (>20-fold) in reporter activity. t-RA at 1 μM was also effective, but slightly less so than 9C-RA. A combination of 9C-RA and t-RA both at 50 nM gave a level of enhancement similar to that seen by either RA alone and did not show an additive effect, consistent with the report by Durand et al. (14). However, in all cases cotransfection of DBD⁻ resulted in a strong reduction (>70%) in reporter activity, indicating

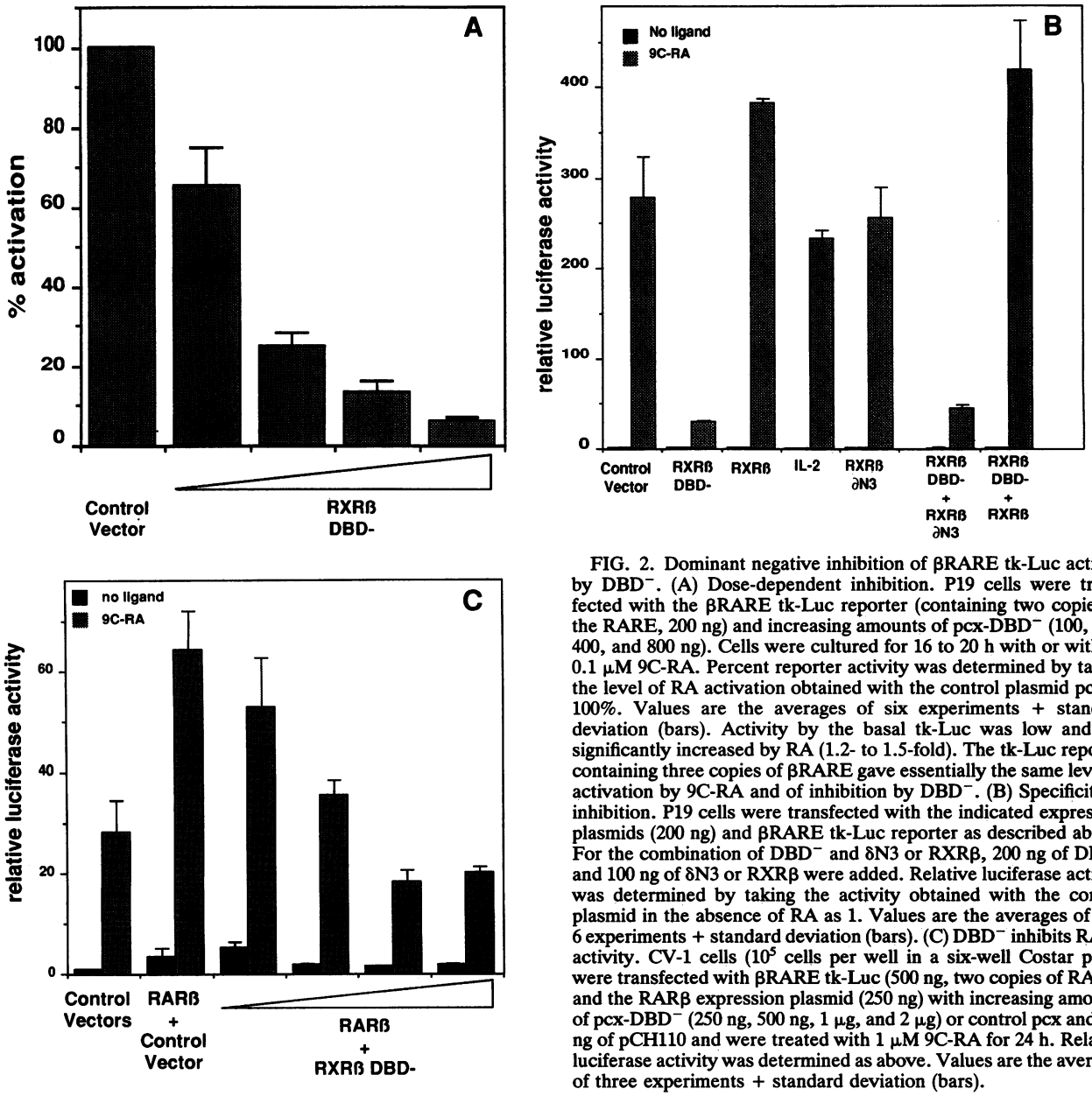

FIG. 2. Dominant negative inhibition of βRARE tk-Luc activity by DBD$^-$. (A) Dose-dependent inhibition. P19 cells were transfected with the βRARE tk-Luc reporter (containing two copies of the RARE, 200 ng) and increasing amounts of pcx-DBD$^-$ (100, 200, 400, and 800 ng). Cells were cultured for 16 to 20 h with or without 0.1 μM 9C-RA. Percent reporter activity was determined by taking the level of RA activation obtained with the control plasmid pcx as 100%. Values are the averages of six experiments + standard deviation (bars). Activity by the basal tk-Luc was low and not significantly increased by RA (1.2- to 1.5-fold). The tk-Luc reporter containing three copies of βRARE gave essentially the same level of activation by 9C-RA and of inhibition by DBD$^-$. (B) Specificity of inhibition. P19 cells were transfected with the indicated expression plasmids (200 ng) and βRARE tk-Luc reporter as described above. For the combination of DBD$^-$ and δN3 or RXRβ, 200 ng of DBD$^-$ and 100 ng of δN3 or RXRβ were added. Relative luciferase activity was determined by taking the activity obtained with the control plasmid in the absence of RA as 1. Values are the averages of 3 to 6 experiments + standard deviation (bars). (C) DBD$^-$ inhibits RARβ activity. CV-1 cells (10^5 cells per well in a six-well Costar plate) were transfected with βRARE tk-Luc (500 ng, two copies of RARE) and the RARβ expression plasmid (250 ng) with increasing amounts of pcx-DBD$^-$ (250 ng, 500 ng, 1 μg, and 2 μg) or control pcx and 100 ng of pCH110 and were treated with 1 μM 9C-RA for 24 h. Relative luciferase activity was determined as above. Values are the averages of three experiments + standard deviation (bars).

that DBD$^-$ inhibits transcription from an RA-inducible, natural promoter.

Stable transfectants expressing DBD$^-$. To study the effect of DBD$^-$ on endogenous gene expression, P19 cells were stably transfected with the pcxn2 plasmid (48; see Materials and Methods) containing DBD$^-$. As a control, cells were also transfected with the pcxn2 plasmid without insert. Upon selection with G418, similar numbers of colonies arose from transfections with the two plasmids (colony-forming efficiency, approximately 10^{-4} to 10^{-5}). Colonies were propagated individually and established as clones. Expression of the DBD$^-$ protein in these clones was monitored by immunoblot analyses using a monoclonal antibody, MOK 13-17, specific for RXRβ (43) (Fig. 4A). DBD$^-$ migrated as a truncated protein of M_r 36,000 to 38,000, which could be distinguished from the intact RXRβ, encoded by the endogenous gene, of M_r 44,000. About 30% of G418-resistant clones derived from the DBD$^-$ construct expressed detectable levels of DBD$^-$ proteins. As seen in Fig. 4A, levels of DBD$^-$ proteins in most of the clones were in great excess relative to the level of the endogenous RXRβ. Three randomly selected clones expressing DBD$^-$ (δ clones) and three clones derived from transfection with the control plasmid were further analyzed for their responsiveness to RA. Figure 4B shows the results of reporter assays following transient transfection of βRARE tk-Luc. Expression of this reporter was enhanced after RA treatment in all control clones to a degree similar to that seen in parental cells. In contrast, all three δ clones showed a reduction in RA-induced βRARE tk-Luc activity (15 to 30% of control). Both reports containing two or three copies of RAREs showed similarly reduced activity in the δ clones. In addition, RA induction of TREp tk-Luc activity was strongly reduced in these clones, con-

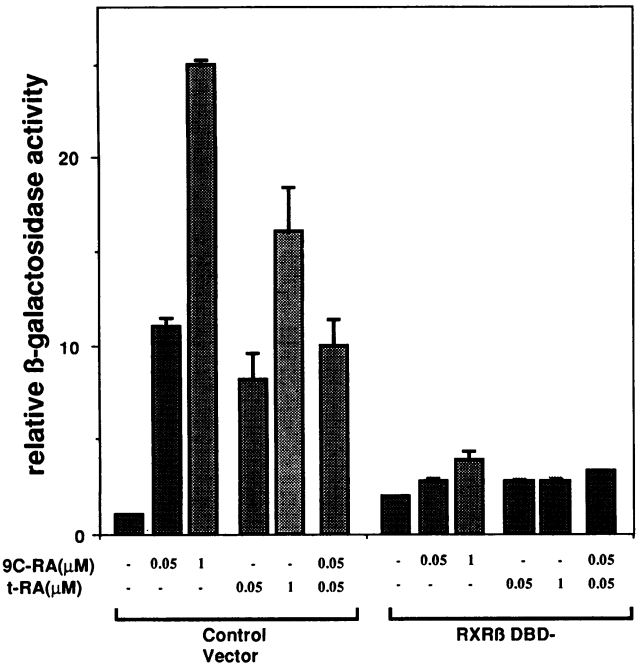

FIG. 3. DBD⁻ represses RA activation of the natural RARβ gene promoter. P19 cells (10^5 cells per well in a six-well Costar plate) were transfected with the RARβ-*lacZ* reporter (0.75 μg) and DBD⁻ (2 μg) or control pcx (2 μg). Cells were then incubated for 20 h with 9C-RA (50 nM, 1 μM), t-RA (50 nM, 1 μM), or both (50 nM plus 50 nM), and the β-galactosidase activity was measured and normalized by lysate protein concentrations. Values are the averages of four experiments + standard deviation (bars).

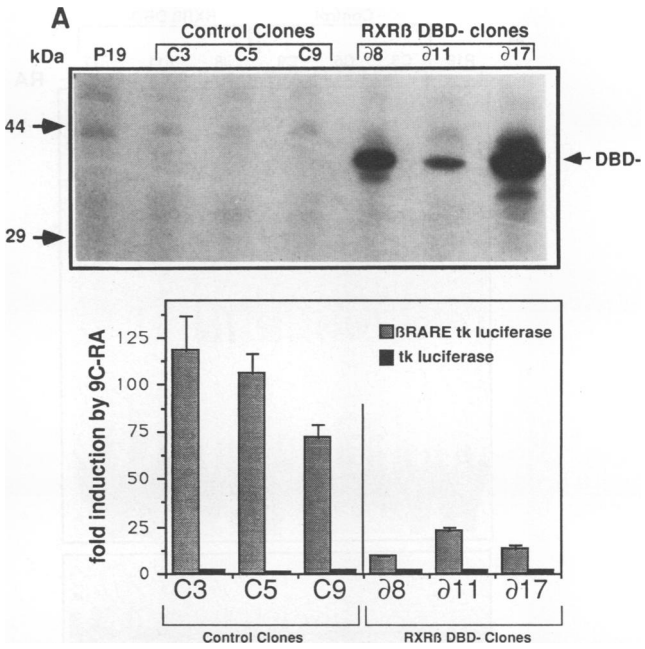

FIG. 4. (A) Immunoblot analysis of DBD⁻ protein expression in P19 clones stably transfected with pcxn2-DBD⁻. Whole-cell lysates prepared from clones transfected with pcxn2-DBD⁻ (δ) or with the control pcxn2 without insert (C) were fractionated by SDS-PAGE and immunoblotted with the anti-RXRβ antibody MOK13-17. The arrow marks the position of DBD⁻. The lower bands in the δ clones are most likely proteolytic degradation products of the overexpressed DBD⁻. (B) βRARE tk-Luc reporter activity in P19 clones stably expressing DBD⁻. Stable P19 transfectants expressing DBD⁻ proteins were transiently transfected with βRARE tk-Luc (three copies of the RARE) and stimulated by 9C-RA. Fold induction was determined as luciferase activity obtained with the ligand relative to the activity without the ligand. Values are the averages of three experiments + standard deviation (bars).

sistent with transient transfection data in parental P19 cells (data not shown). The activity of the basal tk-Luc reporter was similar for all control and δ clones and was not affected by RA treatment (data not shown). These results indicate that stable expression of DBD⁻ inhibits RA-responsive reporter activity in a dominant negative manner.

RA-induced RARE-binding activity contains RXRβ and is repressed in DBD⁻ clones. To examine RARE-binding factors expressed in DBD⁻ clones, EMSA were performed. Whole-cell extracts from parental P19 cells, three control and three δ clones, were prepared before and after RA treatment and were tested by using the βRARE probe. The RARE sequence in this probe was identical to that in the βRARE tk-Luc used above. The results are presented in Fig. 5. Extracts prepared without RA treatment produced weak bands specific for RARE (asterisk and arrow in Fig. 5A), regardless of whether they were from control or δ clones or from parental cells. In control and parental P19 cells, the intensity of the lower band (arrow) was markedly increased following RA treatment (Fig. 5A, lanes 2, 4, and 6) while the intensity of the upper band (asterisk) was unchanged. Both the complexes were inhibited by competition with unlabeled RARE but not an unrelated competitor oligonucleotide, UCR (Fig. 5C). In contrast, RA treatment of all three δ clones resulted in only a modest to undetectable increase in RARE binding (Fig. 5A, lanes 10, 12, and 14). All the extracts tested exhibited comparable levels of binding activity for a control unrelated probe, UCR, irrespective of RA treatment (Fig. 5B); the UCR probe binds a ubiquitous C_2H_2 zinc finger protein, UCRBP, whose expression is unaffected by RA in EC cells (17). To determine whether RXRβ is involved in RARE-binding activity in these cells, the effect of anti-RXRβ antibody was tested. MOK 13-17 (used for the Western assay described above) or a control antibody was added to parental P19 cell extracts in the EMSA binding reaction. As seen in Fig. 5D, MOK 13-17 completely abolished RARE-binding activity while control antibody had no effect, indicating that both the upper (asterisk) and lower (arrow) complexes contain RXRβ, most likely heterodimerized with a partner receptor. RA treatment also increased binding activity for other RAREs in parental cells and control clones. These activities were also abolished by anti-RXRβ antibody and were not increased by RA treatment in δ clones (data not shown). These results show that RXRβ is a component of both constitutive and RA-induced factor-binding activity in P19 cells and that this binding is repressed by DBD⁻ expression. Similar, RA-induced factor-binding activity involving RXRβ has been observed in N-Tera2 EC cells (63).

DBD⁻ expression inhibits RA-induced factor occupancy in the endogenous RARβ gene. Expression of the RARβ gene is induced following RA treatment in various EC cells (72). We have recently observed, by genomic footprinting, that the RARE of the mRARβ gene becomes strongly occupied following RA treatment in P19 EC cells, although it is unoccupied prior to RA addition. Detailed kinetics and other characteristics of RA-induced in vivo footprinting of the

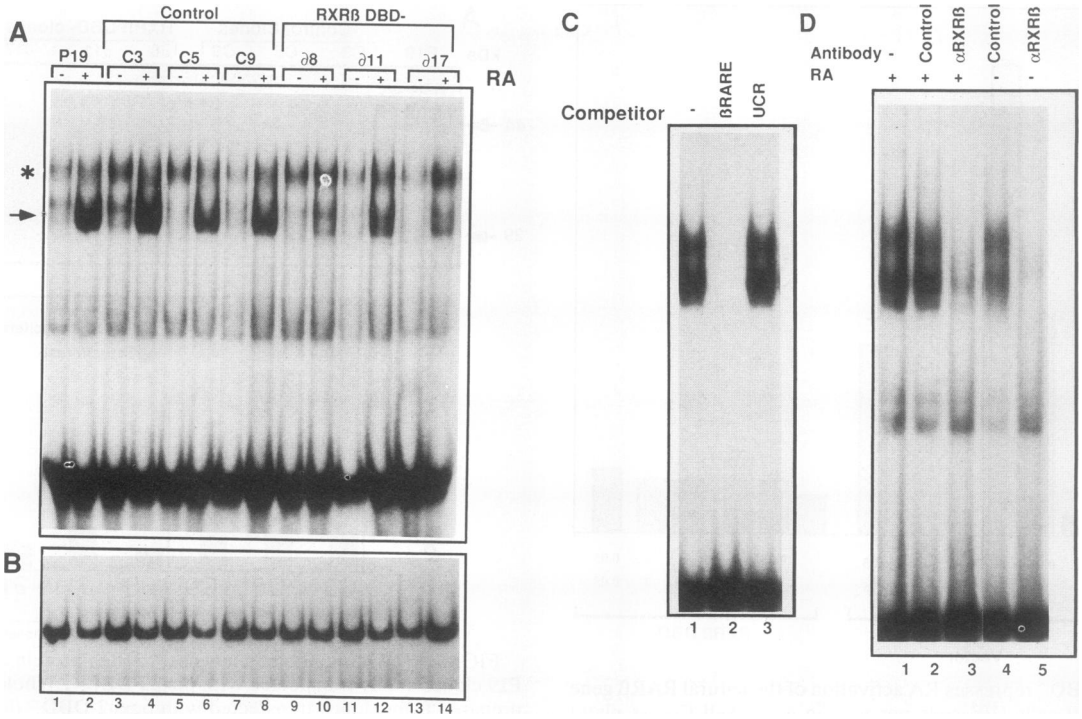

FIG. 5. Reduced RARE factor binding activity in DBD⁻ clones. Whole-cell extracts from parental P19 cells, control transfectants, and δ clones with (+) or without (−) RA treatment were analyzed in EMSA using ^{32}P-labeled βRARE oligomer (A) or control UCR (B) as a probe. The arrow and the asterisk indicate RARE specific complexes. (C) Competition experiments were performed with extracts from RA-treated parental P19 cells by using a 100-fold molar excess of βRARE or UCR. The same competition pattern was observed with all of the control and δ clones (data not shown). (D) Effect of anti-RXRβ antibody on the formation of βRARE complexes. Extracts from RA-treated (+) or untreated (−) parental P19 cells were incubated with 4 μl of MOK13-17 ascites or isotype matched, control mouse ascites (Control) prior to the addition of labeled βRARE probe.

RARβ gene will be presented elsewhere. Here, we performed genomic footprinting of the gene for δ clones (see Materials and Methods). The RARE is present from −56 to −36 bp relative to the transcription start site in the murine RARβ-2 promoter and is identical to the RARE used above (Fig. 2, 5, and 6). Representative results obtained with a δ clone (δ17) and control clone (C3) are shown in Fig. 6. Prior to RA treatment, the RARE was not protected either in the control or the δ clones, indicating that before RA treatment factors are not bound to this element at a significant level in vivo. However, after RA treatment, strong protection was observed in control clones in G residues at −53, −52, and −41 in the coding strand and at −49 and −38 in the noncoding strand. Parental P19 cells exhibited the identical pattern of protection (data not shown). In stark contrast, RA treatment of the δ17 clone resulted in no protection for any of the G residues in the RARE. No significant protection was observed in other DBD⁻ clones treated with RA (not shown), consistent with the results of EMSA shown in Fig. 5. These results show that P19 cells expressing DBD⁻ are unable to induce factor binding to the endogenous RARE in response to RA.

Expression of DBD⁻ interferes with the RA-mediated regulation of endogenous genes. To further assess the functional consequence of DBD⁻ expression, we have examined mRNA expression of three representative genes known to be regulated by RA in EC cells, namely Oct3, *Hox-1.3*, and RARβ. The expression of RARβ was examined by RNase protection assay using a probe that detects the β2 isoform of the RARβ gene. In control clones, RARβ mRNA was

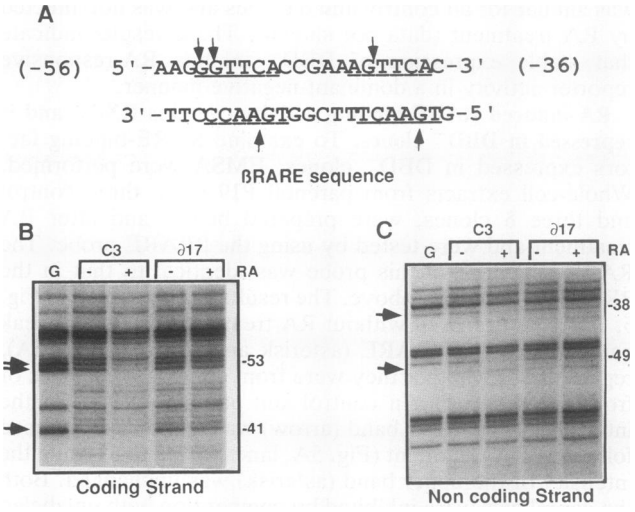

FIG. 6. In vivo footprinting of the endogenous RARβ gene in a δ clone. (A) Schematic representation of the RARE sequence in the mRARβ gene. The RARE direct repeat is underlined. Arrows indicate protected G residues in the control clone after RA treatment. (B and C) Control (C3) and δ clone (δ17) untreated (−) or treated (+) with 1 μM t-RA for 24 h were subjected to in vivo footprinting for the coding (B) or noncoding (C) strand. G, control G ladders revealed by purified DNA prepared from parental P19 cells. The arrows indicate protected G residues in the control clone after RA treatment.

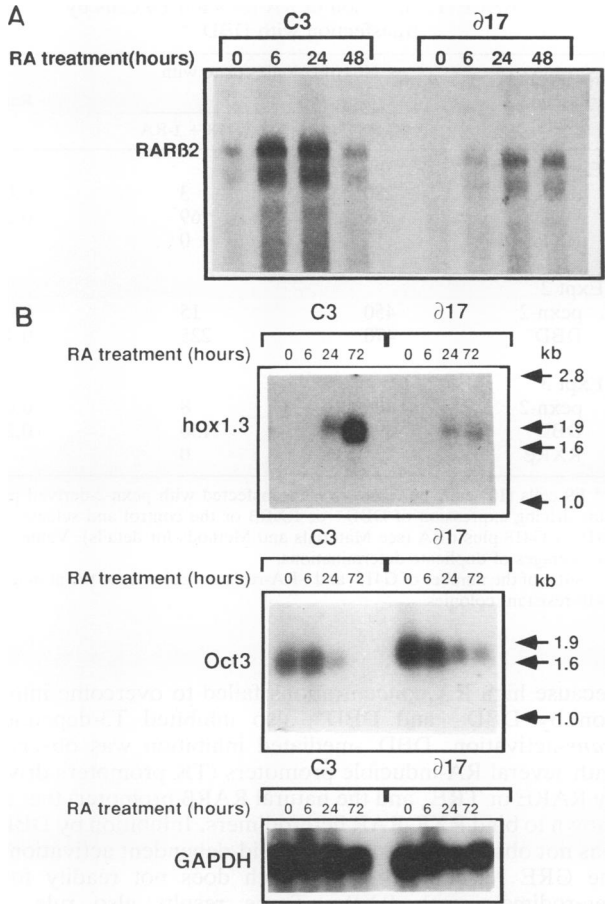

FIG. 7. Expression of endogenous *Hox-1.3*, Oct3, and RARβ genes in a δ clone. (A) RNase protection analysis. Total RNA (25 μg) from the above-mentioned clones were analyzed by using an in vitro-transcribed probe specific for the murine RARβ2 isoform. Shown is the 186-bp protected band generated after hybridization with the probe and digestion with RNase A+T_1. (B) RNA blot hybridization. Poly(A)$^+$ RNA (2 μg) prepared from control (C3) or δ (δ17) clones treated with t-RA (1 μM) for the indicated periods of time were hybridized with ^{32}P-labeled Oct3 or *Hox-1.3* probes or with a GAPDH probe used as a control.

undetectable before RA treatment but was strongly induced by 6 h of treatment, and the mRNA level was similar up to 24 h of RA treatment (Fig. 7A). However, in the δ clones only a very low level of RARβ mRNA was induced at 6 h. The levels increased by 24 h, albeit at a reduced level, and did not decline by 48 h. The amount of RNA used for each lane was comparable, as monitored by expression of GAPDH mRNA tested as an internal control for the RNase protection assays (data not shown). These data were reproducibly observed with multiple preparations of RNA from different δ clones. These results indicate that DBD$^-$ inhibits the induction of RARβ mRNA and this inhibition is more prominent in an early stage of RA treatment (less than 24 h). Figure 7 examines levels of Oct3 and *Hox-1.3* mRNAs in a control (C3) and a δ clone (δ17) by Northern analysis. *Hox-1.3* is a homeo box gene induced following RA treatment in F9 cells (45). Oct3 is an octamer-binding transcription factor containing the POU homeo domain and is expressed exclusively in developing germ cells, embryonic stem cells, and undifferentiated EC cells (50, 58, 61). *Hox-1.3* mRNA was undetectable prior to RA treatment in the control clone but was induced by 24 h, and its levels increased further by 72 h. However, in the δ clone *Hox-1.3* mRNA, although induced by 24 h at a low level, did not significantly increase thereafter; at 72 h, the mRNA levels were approximately 10% of those in the control clone (Fig. 7B). Oct3 mRNA expression is rapidly down-regulated following RA treatment in several EC cells (51). As expected, Oct3 mRNA levels were high prior to RA treatment both in the control and the δ clone. After 24 h of RA treatment, in the control clone the level of Oct3 was sharply decreased and became <1% of that seen before RA treatment 72 h after the addition of the hormone. A similar decline was noted for the parental P19 cells (data not shown). Oct3 mRNA levels in the δ17 clone fall somewhat more slowly, since its level remained at >10% of that before RA treatment even 72 h after the addition of the hormone. Thus, in the δ clone RA induction of *Hox-1.3* expression was strongly inhibited, while Oct3 mRNA expression was down-regulated with a slower kinetics than in the control clone.

RXRβ DBD$^-$ expression inhibits RA-induced growth arrest in F9 EC cells. In order to further delineate the extent of the DBD$^-$ effects on RA-mediated regulatory processes in EC cells, we exploited the phenomenon of RA-induced growth arrest. It has been documented that cellular proliferation is inhibited in some EC cells when they undergo differentiation in response to RA (2). The degree of growth inhibition differs among EC cells, however. For example, at certain RA concentrations P19 cells undergo differentiation while rapidly proliferating. On the other hand, RA treatment leads to almost complete cessation of proliferation in F9 EC cells, resulting in cell death within 2 to 3 weeks following RA treatment. In this context, Espeseth et al. (15) reported that F9 cells expressing a mutated RARα lacking part of the C-terminal domain are deficient in their responsiveness to RA and become resistant to growth inhibition by RA. Here, we have tested whether expression of DBD$^-$ leads to a similar deficiency in RA responsiveness and to generation of RA-resistant cells. F9 cells were transfected with either the pcxn2 plasmid with DBD$^-$ or with the control pcxn2 as described above. An additional transfection was performed with the pcxn2 plasmid driving expression of intact RXRβ. Cells were selected by G418 alone or by G418 plus t-RA (1 μM) for 2 to 3 weeks. At the end of the culture, colonies were stained with Giemsa stain and counted (Fig. 8 and Table 1). Comparable numbers of colonies (280 to 408) were produced when selected by G418 alone by transfection with DBD$^-$ and with the control plasmid. However, when selected by the G418 plus t-RA, only a few colonies emerged by transfection with the control plasmid. In contrast, transfection with DBD$^-$ resulted in a large number of colonies surviving the selection (Fig. 8). These results were observed reproducibly in three separate experiments (see the summary in Table 1), in which t-RA-resistant cells ranged from 22% to 47% of the G418-resistant cells after transfection of DBD$^-$. This number may be consistent with the observation that about 30% of G418-resistant cells expressed the DBD$^-$ protein after stable transfection in P19 cells (see above). These results demonstrate that F9 cells expressing DBD$^-$ fail to arrest cell growth in response to RA and indicate that RXRβ and its dimerization partners are involved in RA-mediated growth regulation in F9 EC cells. It is noteworthy that only a few G418-resistant colonies were produced when cells were transfected with the plasmid driving expression of intact RXRβ, either in the presence or absence of RA (Fig.

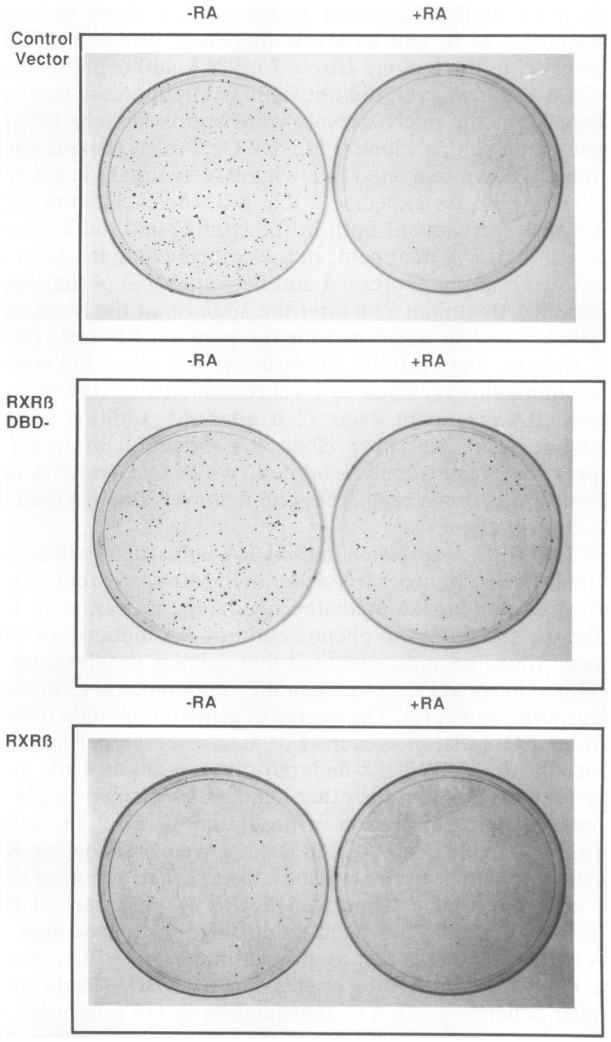

FIG. 8. Generation of RA-resistant F9 colonies following transfection of DBD$^-$. F9 cells were transfected with the control pcxn2, DBD$^-$, or wild-type RXRβ and were selected by G418 in the presence (+) or absence (−) of 1 μM t-RA in the presence of G418 for 3 weeks. Plates (100 mm in diameter) were fixed and stained with Giemsa stain.

8, bottom panel; Table 1). These data indicate that overexpression of RXRβ is harmful to the growth of F9 cells.

TABLE 1. Selection of RA-resistant F9 cells by transfection with DBD$^-$[a]

Expt no. and plasmid nature	No. of colonies/plate with resistance to:		Ratio[b]
	G418	G418 + t-RA	
Expt 1			
pcxn-2	280	3	0.01
DBD$^-$	308	69	0.22
RXRβ	15	0	
Expt 2			
pcxn-2	450	15	0.03
DBD$^-$	470	225	0.47
Expt 3			
pcxn-2	408	8	0.019
DBD$^-$	395	150	0.37
RXRβ	34	0	

[a] F9 cells (10^5 cells per plate) were transfected with pcxn-2-derived plasmids driving expression of DBD$^-$ or RXRβ or the control and selected for G418 or G418 plus t-RA (see Materials and Methods for details). Values are the averages of duplicate determinations.

[b] Ratio of the number of G418- and t-RA-resistant colonies to the number of G418-resistant colonies.

DISCUSSION

We show that DBD$^-$ elicits a dominant negative effect in P19 EC cells and inhibits RA-induced reporter transcription mediated by RAR-RXR heterodimers. Further, DBD$^-$ profoundly alters the course of gene regulation, leading to an EC cell phenotype defective in RA-regulated growth and differentiation. The dominant negative effect was observed with DBD$^-$ and δN2, both of which lack the DNA binding domain but carry the intact dimerization domain. Since δN2 lacks a large region of the N-terminal domain (Fig. 1A), the observed inhibitory effect is not likely to be achieved through the N-terminal domain. On the other hand, inhibition was not observed with δN3, which had an additional C-terminal deletion and was deficient in dimerization. The inhibition was not due to ligand sequestration by excess DBD$^-$, because high RA concentrations failed to overcome inhibition by DBD$^-$ and DBD$^-$ also inhibited T3-dependent *trans*-activation. DBD$^-$-mediated inhibition was observed with several RA-inducible promoters (TK promoters driven by RARE or TRE_p and the natural RARβ promoter) that are shown to bind RXR-RAR heterodimers. Inhibition by DBD$^-$ was not observed for glucocorticoid-dependent activation of the GRE reporter by GR, which does not readily form heterodimers with RXRs. These results also rule out squelching of general transcription factors as the basis of the observed inhibition (23). Since the dominant negative effect was reversed by addition of intact RXRβ, it was most likely caused by interference against the endogenous RXR dimers.

The most plausible mechanism for the observed effect is the formation of DBD$^-$-containing dimers defective in DNA binding. That DBD$^-$ inhibits the induction of βRARE reporter activity by transfected RARβ in CV-1 cells (Fig. 2C) as well as the induction of TRE_p reporter activity by transfected TRα strongly favors the formation of functionally inactive DBD$^-$-RAR (or TR) heterodimers as the basis of the negative effect.

The dominant negative activity of DBD$^-$ described here is reminiscent of previously reported inhibitory effects seen by receptors deficient in DNA binding. Forman et al. (20) and Barettino et al. (3) described different chicken c-*erbA* (TR) constructs lacking the DNA binding domain which exert a dominant negative effect on ligand-dependent transcription mediated by c-*erbA*-α and by RARs. In the latter system, this inhibition has been shown to be due to the recruitment of RXRs into inactive dimers, a situation reciprocal to that of DBD$^-$ dimers presented in this work. More recently, Schen and al. (60) reported that an RARβ construct that does not have the DNA binding domain acts as a dominant negative repressor in P19 cells and that the RARβ dimerization domain is necessary for this negative effect. These reports further support the idea that DBD$^-$ is acting through the formation of nonfunctional heterodimers.

EMSA data in Fig. 5 show that RA treatment induces factor binding to the βRARE in P19 cells. Similar RA induction of factor binding was observed for the TRE_p and

region II of major histocompatibility complex class I genes not only in P19 EC cells but also in N-Tera2 EC cells (63), suggesting that induced factor binding to RAREs is a common prerequisite for RA-induced transcription. We show that RA induction of RARE binding activity is greatly reduced in clones expressing DBD$^-$. In agreement with EMSA results, in vivo footprinting analysis (Fig. 6) shows that the RARE in the endogenous RARβ gene is unoccupied in P19 cells before RA addition and that strong occupancy ensues following RA treatment. However, this RA-induced in vivo occupancy was not observed in DBD$^-$ clones, providing conclusive evidence that expression of DBD$^-$ inhibits RA-induced factor binding to the endogenous RARE.

Since both constitutive and RA-induced RARE-binding activities were completely abrogated by antibody specific for RXRβ (Fig. 5), most, if not all, RARE-binding activities are likely to contain RXRβ. These results are in agreement with the observation that RXRβ is abundantly expressed in P19 EC cells (71) and that this antibody eliminates the RA-induced major histocompatibility complex region II binding activity in N-Tera2 cells (63). The receptor partners that heterodimerize with RXRβ and activate transcription in P19 cells would be, for the most part, RARs (63). RARα and RARγ have been shown to be expressed constitutively in P19 cells, while RARβ is induced after RA treatment (33, 54, 72). RA treatment also augments RARα levels (37). A similar pattern of RAR expression has been reported for F9 cells (72). As proposed (33), constitutively expressed RARs are likely to be involved in the initial activation of the RARβ gene, presumably after heterodimerization with RXRβ. Once expressed, RARβ would also be able to form functional heterodimers with RXRβ, as is seen in Fig. 1B. Thus, the observed dominant negative effect may involve multiple members of RARs during RA-induced differentiation. The molecular identity of the receptor partners dimerizing with RXRβ is currently being investigated in our laboratory.

Stable transfectants expressing DBD$^-$ allowed us to study RNA expression of three endogenous genes known to be regulated by RA. Following RA treatment of parental P19 cells, *Hox-1.3* and RARβ mRNA are rapidly induced, while Oct3 expression is down-regulated. mRNA expression of all three genes was clearly altered in DBD$^-$ clones, as levels of *Hox-1.3* and RARβ mRNA were reduced, while Oct3 down-regulation was delayed in these cells.

Hox-1.3 mRNA levels were much lower in DBD$^-$ clones than those in control cells throughout 72 h of RA treatment, although the kinetics of induction was unaffected. Since RA induction of *Hox-1.3* mRNA is most likely to be transcriptional (45, 48a, 49), it is reasonable to assume that this inhibition is largely due to reduced transcription of *Hox-1.3*. Several *Hox* genes analyzed in detail have been shown to have a canonical RARE, responsible for RA-induced transcription (34, 53). It is likely that many *Hox* genes are, in fact, regulated by RXR-RAR heterodimers and hence affected by DBD$^-$. *Hox-1.3* expression is reported to be unaffected in F9 cells expressing a dominant negative RARα (15), indicating that DBD$^-$ acts in a manner distinct from that of this mutated RARα. RARβ mRNA levels were also reduced in δ clones. The repression was more pronounced in early stages of RA treatment. Jonk et al. (30) reported that RARβ mRNA levels increase with a biphasic mode after RA treatment in P19 cells, suggesting that multiple regulatory events are involved in inducing the expression of this gene. That DBD$^-$ was less efficient in inhibiting RARβ expression in the later stage may be indicative of such heterogeneity in RARβ gene regulation. Figure 7 also suggests that RA-induced down-regulation of Oct3 mRNA levels is delayed in DBD$^-$ clones. Down-regulation of Oct3 expression is an early event following RA treatment and has been attributed to two distinct upstream elements, neither of which seems to bind RARs, acting in concert (51). In this case, it is possible that other mechanisms not involving RAR-RXR have a prominent role (see below).

It should be mentioned that DBD$^-$ did not completely inhibit RA regulation of these genes. Incomplete inhibition could result if the level of DBD$^-$ expressed in these cells was not sufficient to titrate all of the relevant partner receptors and thus if a small fraction of receptors were free to form functional heterodimers. Alternatively, RA treatment of P19 cells may activate other pathways of transcription that do not directly depend on RARs or RXRs and that may be active in controlling the expression of these genes. It is of note that RA treatment of EC cells leads to induction of AP-1 activity and of c-*jun* mRNA (70) that may be mediated by a response element distinct from a classical RARE (31). Further, ectopic expression of c-*jun* has been shown to induce several differentiation genes also known to be induced by RA and to up-regulate RARβ promoter activity (11). It is conceivable that the AP-1 or other pathways of transcription might have contributed to the residual RA effect seen in these cells.

Whatever contributions by other pathways may exist, it is clear that DBD$^-$ expression results in global alteration of RA-regulated growth and differentiation in EC cells. About 30% of G418-resistant F9 colonies stably transfected with DBD$^-$ failed to undergo growth arrest and continued to proliferate in the presence of RA, while only very few colonies survived after transfection with the control plasmid. Consistent with the involvement of RXRβ in growth regulation, few stable P19 transfectants could be established with the plasmid containing intact RXRβ. Furthermore, we have not been able to produce stable RXRβ transfectants in other EC cells including N-Tera2 cells (data not shown), suggesting that overexpression of RXRβ leads to growth inhibition in EC cells.

An inhibition of growth arrest similar to that resulting from DBD$^-$ expression has been reported by a dominant negative RARα (15); since this mutated RAR is likely to be defective in dimerization, its mechanism of action is also likely to be distinct from that of DBD$^-$ (see above). However, RA-induced growth arrest of F9 cells has not been observed with RARα′, a dominant negative RAR found in RAC65 cells that were originated from P19 EC cells (54). This receptor has a C-terminal deletion that impairs RA binding but not dimerization (60). If so, the growth inhibition observed with DBD$^-$ in these experiments may involve a heterodimer partner other than RARα.

Two additional derivatives from RAR or RXR that exhibit a dominant negative effect have been described (14, 68). These mutated receptors also have C-terminal truncations and are in theory able to bind to target DNA regardless of their competence in dimerization. Since they appear to elicit regulatory effects distinct from that of DBD$^-$, a complementary use of the two types of deletion constructs may provide further understanding of RA-mediated pathways of gene regulation.

The members of many transcription factor families act as dimers (27). Thus, factors that are deficient in DNA binding activity but form dimers may occur naturally. Such factors may exert negative autoregulation in vivo (for an example, see reference 57). Several lines of evidence indicate that

some members of the nuclear hormone receptor superfamily lack their DNA binding activities and that they are involved in modulating hormone-ligand-mediated gene expression. Leroy et al. (37) reported RAR isoforms that potentially produce receptors without the first zinc finger. Similarly, the ecdysone-inducible E75 gene in *Drosophila melanogaster* has two overlapping transcription units that generate two receptor proteins, E75A and E75B (64). E75A contains both zinc fingers, whereas E75B contains only the second. Moreover, truncated forms of TRs lacking the DNA binding domain in avian erythroid cells have been described (5). An estrogen receptor (ER) isoform (ERδE3) which has a deletion of the second zinc finger and which may have a negative effect if it forms homodimers with an intact ER has been isolated from the human breast cancer cell line T47D (69). It is possible that a naturally occurring, functional counterpart of DBD^-, which plays an important role in regulating various functions associated with RXRs, exists.

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