Transactivation and repression of the α-fetoprotein gene promoter by retinoid X receptor and chicken ovalbumin upstream promoter transcription factor

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ABSTRACT

Retinoic acid (RA) is widely involved in the control of cell proliferation and differentiation, as well as embryo pattern formation. Transcription of the oncodevelopmental protein, α-fetoprotein (AFP), is stimulated by retinoic acid (RA) in neoplastic cells. To study RA regulation of AFP gene expression, the 5′-flanking region of AFP gene was cloned and analyzed. In the present study, transfection of deletion mutants and sequence analysis revealed a retinoid X receptor response element (AFP-RXRE) located at position −139 to −127 of the AFP promoter. Synthetic AFP-RXRE was ligated into a reporter construct with the heterologous promoter and chloramphenicol acetyltransferase (CAT). AFP-RXRE conferred a marked RA responsiveness in the cotransfection with retinoid X receptor (RXR), but not with retinoic acid receptors (RARs). Consistent with these data, only RXR bound to AFP-RXRE with high affinity in the mobility shift assays. Chicken ovalbumin upstream promoter transcription factor (COUP-TF), an orphan member of the steroid/thyroid hormone superfamily, also demonstrated specific binding activity to AFP-RXRE *in vitro*. In cotransfection assays, COUP-TF dramatically repressed the transactivation of RXR on AFP-RXRE. The mechanism of repression by COUP-TF may involve the mutual occupancy of the AFP-RXRE binding site between RXR and COUP-TF.

INTRODUCTION

Retinoic acid, a vitamin A derivative, exerts potent effects on many physiological processes including cell proliferation and differentiation, regulation of pattern formation in developing and regenerating limbs, and suppression of carcinogenesis (1−3). The cellular responses to RA are thought to be mediated by two families of nuclear retinoid transcription factors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), which are members of the steroid/thyroid hormone receptor superfamily (4−6). The nuclear retinoid receptors are ligand activated, sequence specific, and function as dimeric transcription regulators (7). Until now three related RARs (RARα, β, and γ) and RXRs (RXRα, β, and γ) have been characterized (8−13). Two nuclear retinoid receptors, RARs and RXRs, differ from each other in their primary structure and ligand specificity (7). RARs bind all-*trans*-RA and 9-*cis*-RA. However, RXRs only show binding affinity for 9-*cis*-RA (14−16). In addition, the diversity of RARs and RXRs is reflected in their binding site, the RA response elements (7). RARs prefer a five nucleotide spacing between the two direct repeat half sites of A/GGGTCA, and RXRs prefer a one nucleotide spacing (17, 18). Another receptor, chicken ovalbumin upstream promoter transcription factors (COUP-TFs), is an orphan member of the steroid/thyroid hormone receptor superfamily (19−21). Recent studies have revealed that the binding site for COUP-TF is an imperfect A/GGGTCA direct repeat with one nucleotide spacing which is similar to the response element of retinoid X receptor (18, 22, 23). It has been demonstrated that COUP-TF represses the transactivation of other steroid/thyroid hormone receptors on their cognate hormone response elements (18, 24−26).

α-Fetoprotein (AFP) is a serum oncodevelopmental protein which is expressed primarily in the fetal liver and yolk sac. The serum level of AFP decreases rapidly after birth and is barely detectable in the adult (27−29). In adult life, however, the expression of AFP may reactivate under physiological or pathological conditions, such as liver regeneration, hepatocarcinomas, and germinal tumors in adults (30). Various agents, such as hormones and retinoic acid, also regulate the synthesis of AFP in specific fetal tissues or neoplastic cells (31, 32). In cell culture, AFP gene expression was shown to be affected by retinoic acid. RA regulated the expression of the AFP gene in embryonal carcinoma and hepatoma cells (33−35). RA induced the differentiation of mouse F9 teratocarcinoma stem cells into the visceral endoderm. Induction of AFP gene expression was one of the major biochemical changes during this differentiation process (33, 34). In addition, RA was capable of activating the expression of the AFP gene in both rat McA-RH 8994 and McA-RH 7777 hepatoma cells (35 and our unpublished data). It has been reported that RA regulation of AFP gene expression was at the level of transcription in McA-RH 8994 cells (35). However, until now little is known about the mechanism of AFP gene regulation by RA.

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In this study, we identified a retinoid X response element (RXRE) in the promoter of AFP gene which is specifically activated by RXR and not RAR. We also showed that the COUP-TF specifically interacted with this element *in vitro*. Furthermore, we demonstrated that COUP-TF can repress the transactivation of RXR on the RXRE in the AFP promoter region.

MATERIALS AND METHODS

DNA plasmid construction

Expression plasmids pAFPCAT and pAPCAT were constructed by cloning a portion of the rat α-fetoprotein 5' flanking region, from -7040 to $+7$ and -320 to $+7$, into the pGEMCAT vector respectively (36). A series of deletion mutant plasmids, p(-6.3)AFPCAT (containing 6.3 kb of AFP 5' flanking region from -6266 to $+7$), p(-3.1)AFPCAT (-3127 to $+7$), p(-1.9)AFPCAT (-1855 to $+7$), and p(-107)APCAT (-107 to $+7$), were generated from pAFPCAT and pAPCAT using the Erase-a-Base system (Promega, Madison, WI). In this system, the double cut pAPCAT was generated by *Sph*I and *Eco*RI for 3' overhang and 5' overhang, respectively. Two complementary oligonucleotides of AFP-RXRE sequence (-139 to -127), 5'-GATCCTGACCCCTGTGCTA-3' and 5'-GATCTAGCAC-AGGGGTCAG-3', flanked with *Bam*HI and *Bgl*II restriction sites were synthesized and annealed. pRXRE-tkCAT was generated by inserting a single copy of the AFP-RXRE oligonucleotide into the unique *Bam*HI site upstream of the construct pBLCAT$_2$ (37). The position of deletion mutants and the identity of the inserted oligonucleotides were confirmed by sequencing.

Transfection assay

A monkey kidney cell line, CV1, was cultured in DMEM supplemented with 10% charcoal-treated fetal bovine serum. The transient transfections were performed by using the modified calcium phosphate coprecipitation method (38), which has been previously described (39), with 0.6 μg of reporter, 0.2 μg of a pECE receptor expression plasmid, and/or 0.2 μg of pRShCOUP-TFI (kindly donated by Dr Ming-Jer Tsai, Department of Cell Biology, Baylor College of Medicine, Houston, TX), 0.6 μg of β-galactosidase expression vector (pCH110) as an internal control, and 2.6 μg of pBluescript as carrier plasmid to a final DNA amount of 4 μg. Cells were transfected for 10 hr, and after the DNA precipitates were washed, cells were incubated for an additional 36 hr with or without all-*trans* or 9-*cis* RA as indicated in figures. Cell extracts were prepared for β-galactosidase and CAT activity assay as described (40, 41). Mouse F9 teratocarcinoma cells and rat hepatoma 7777 cells were kept in α-MEM and Ham's F12 medium supplemented with 10% charcoal-treated fetal bovine serum respectively as previous described (34,39). Transfection of F9 and hepatoma 7777 cells were carried out in a similar fashion as CV1 except that 1.5 μg of reporter vector, 1.5 μg of pCH110, and 0.5 μg of pECE receptor expression vector were used.

In vitro translation of COUP-TFI and tCOUP-TFI

The plasmids encoding the full length of COUP-TFI (pCOUP-TFI) and truncated COUP-TFI (pT7β-tCOUP-TFI) were linearized at the *Eco*RI site (24). RNA was synthesized *in vitro* using SP6 RNA polymerase following the instructions of Stratagene. RNA was translated *in vitro* using the reticulocyte lysate translation kit from Promega.

Gel retardation assays

AFP-RXRE oligonucleotides were end-labeled with [^{32}P]dCTP using the Klenow fragment of DNA polymerase I, for use as probes. Bacteria expressed RARγ and RXRα (obtained from Dr X.K.Zhang, Cancer Center, La Jolla Cancer Research Foundation, La Jolla, CA), and *in vitro* translated COUP-TFI and tCOUP-TFI were used in the binding assay. DNA-binding experiments were performed in 20 μl reaction mixtures containing 10 mM HEPES, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 2.5 mM MgCl$_2$, 10% glycerol, 1 μg poly(dI-dC), RXRα, RARγ and/or 1 μl of *in vitro* translated COUP-TFI and tCOUP-TFI, and 0.5 ng of labeled DNA probe. After 20 min of incubation in room temperature, samples were subjected to 5% non-denaturing polyacrylamide gels in 0.5$\times$TBE buffer at 4°C. Competition experiments were performed by the addition of unlabeled specific oligonucleotides before the addition of the labeled DNA probe. For antibody up-shifting experiments, either anti-RXR-flag antibody (RXRα was synthesized in bacteria with a flag peptide obtained from Dr X.K.Zhang) or anti-COUP-TFI antibody (obtained from Dr M.J.Tsai) were incubated with the reactions prior to the addition of probe.

RESULTS

Delineation of a RXRE in the AFP promoter

It has been previously reported that the expression of the AFP gene is regulated by RA in mouse F9 teratocarcinoma and rat hepatoma McA-RH 8994 cells (33–35). Recently we have also observed that RA can enhance AFP transcription in rat hepatoma 7777 cells by Northern blot analysis. Furthermore, CAT gene transcription mediated by 7 kb of the AFP 5'-flanking region can be activated by RA after stable transfection into mouse F9 cells and transient transfection into rat McA-RH 7777 hepatoma cells. As shown in Figure 1A, three putative retinoic acid response elements were identified. They are located at -6327 to -6313, -1972 to -1842 and in the promoter region (-320 to $+7$). Two upstream RARE-like sequences were characterized and will be reported elsewhere.

Data from transfection studies also implied that there may be a *cis*-acting RA response element in the promoter region since RA enhanced CAT activity by approximately 5 fold (Fig. 1A). Inspection of the AFP promoter region (-320 to $+7$) revealed two tandem repeats of A/GGGTCA, and its degenerated hexad half-site sequences, from -139 to -127 with one nucleotide spacing which is highly similar with the retinoid X response element (RXRE) (Fig. 1B). To further examine the regulation of the AFP gene by RA, a reporter construct (pAPCAT) containing a portion of AFP gene promoter (-320 to $+7$) ligated to the CAT gene was transiently transfected into F9 teratocarcinoma cells. Cotransfection with RXRα expression vector pECE-RXRα, pAPCAT showed a 4.3-fold induction of CAT activity (Fig. 1C). To examine further the function of this sequence, one deletion mutant, pAP(-107)CAT, was constructed. The sequence between -320 to -108 was deleted from the parent reporter plasmid pAPCAT. The RA responsiveness of this mutant plasmid was determined by transfecting it into F9 cells. As shown in Fig. 1C, the 4.3-fold RA responsiveness of pAPCAT in F9 cells cotransfected with RXRα was abolished in the mutant pAP(-107)CAT in which the sequence from -320 to -107 with the RXRE sequence had been deleted.

To further demonstrate that this RXRE sequence in the AFP promoter can confer RA responsiveness on a heterologous promoter, a single copy of an oligonucleotide containing the imperfect A/GGGTCA direct repeat (nucleotides −139 to −127) was inserted into pBLCAT₂ upstream of the thymidine kinase promoter, and designated as pRXRE-tkCAT. The construct pRXRE-tkCAT was transiently transfected into CV1 cells to test the RA responsiveness with or without cotransfection of RARα or RXRα expression vectors. In the absence of cotransfected nuclear retinoid receptor, the RA response was very low (Fig. 2A). When the cells were cotransfected with the RXRα expression vector, CAT activity increased significantly. RA induced CAT expression increased nearly 7-fold when compared with untreated cells. This induction was also found to occur in an orientation independent fashion (Fig. 2A and data not shown). However, control plasmid pBLCAT₂ did not show obvious RA

responsiveness with the cotransfection of RXR in comparison with the results from pRXRE-tkCAT (Fig. 2A). In the case of cotransfection of RARα, RARβ, or RARγ with pRXRE-tkCAT showed a weak RA induction as compared with control pBLCAT₂ plasmid (Fig. 2B). Both basal and RA induced CAT activities were high in control plasmid pBLCAT₂ indicating RARE-like element maybe present in vector pBLCAT₂. However, this RA *cis*-acting element in pBLCAT₂ responds weakly to RXR (Fig. 2A).

It has recently been demonstrated that the cognate ligand for RXRs is 9-*cis* RA, a stereoisomer of all-*trans* RA (14−16). To further characterize the AFP-RXRE sequence located in the AFP promoter region, the dose-dependent effects of all-*trans* RA and 9-*cis* RA on the CAT activity of pRXRE-tkCAT was studied. Results shown in Figure 2C demonstrate that a 1000 fold increase in the concentration of all-*trans* RA is required to induce the same
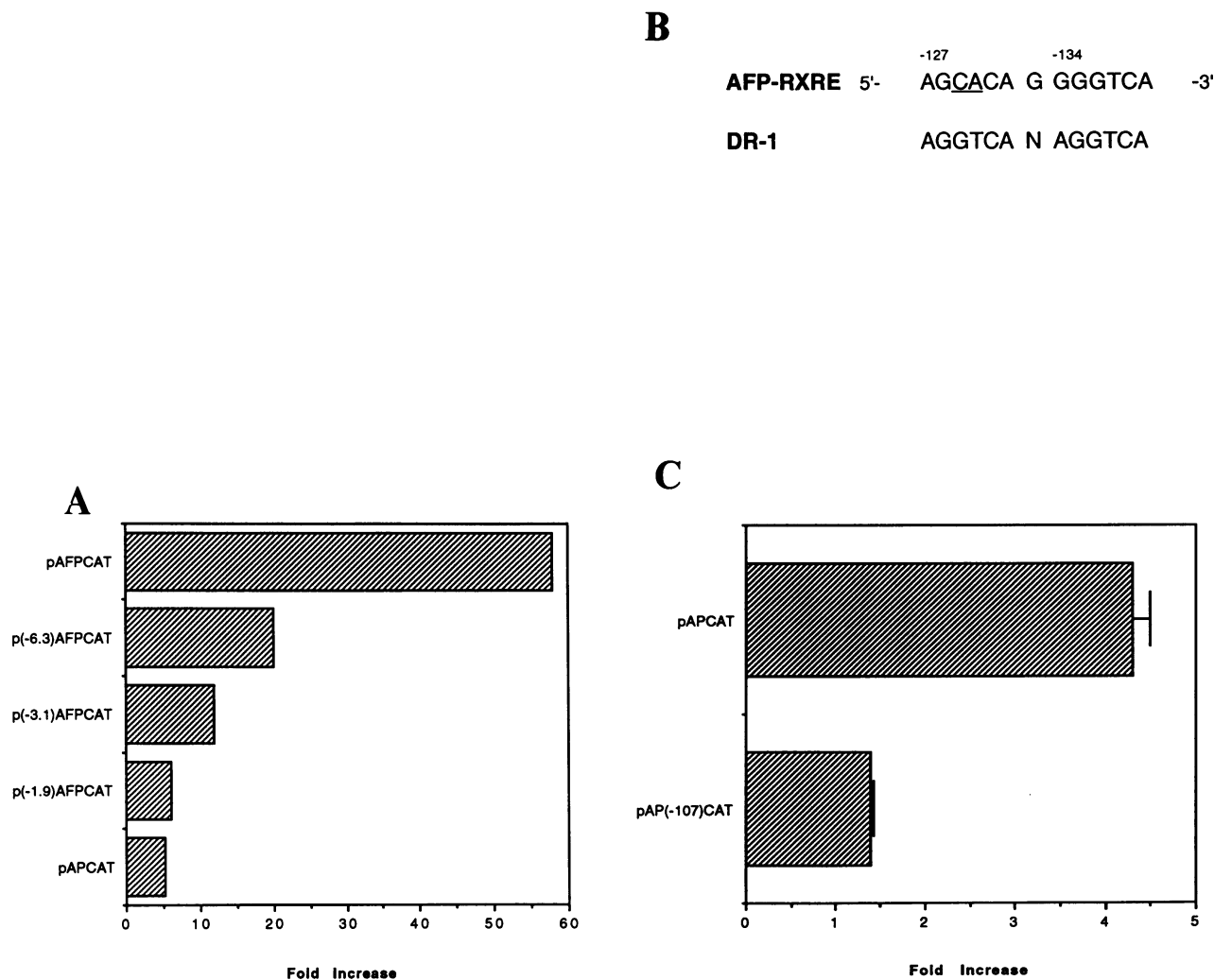
B

	-127	-134	
AFP-RXRE 5'-	AG**C**ACA G	GGGTCA	-3'
DR-1	AGGTCA N	AGGTCA	

A

C

Figure 1. Identification of a RXRE-like sequences in the AFP promoter region. **A.** Transient transfection of hepatoma 7777 cells with pAFPCAT and its deletion mutants. Hepatoma 7777 cells were cotransfected with various mutant constructs, RAR expression vector, and β-galactosidase internal control plasmid pCH110. After transfection, cells were treated with either the vehicle (ethanol) in control or 1 μM RA for 36 hr before harvesting for CAT assays. **B.** Sequence of two direct repeats in the AFP promoter region and direct repeats (DR-1) of AGGTCA motif. **C.** Transient transfections of F9 cells. F9 cells were transfected with reporter constructs, pAPCAT and pAP(−107)CAT, with RXR expression vector and pCH110. After transfection, cells were treated with 10 μM RA. After 36 hr, transfected cells were harvested for CAT assay. The data were plotted as the fold increase of CAT activity (+RA/−RA). Results are from four individual transfections.
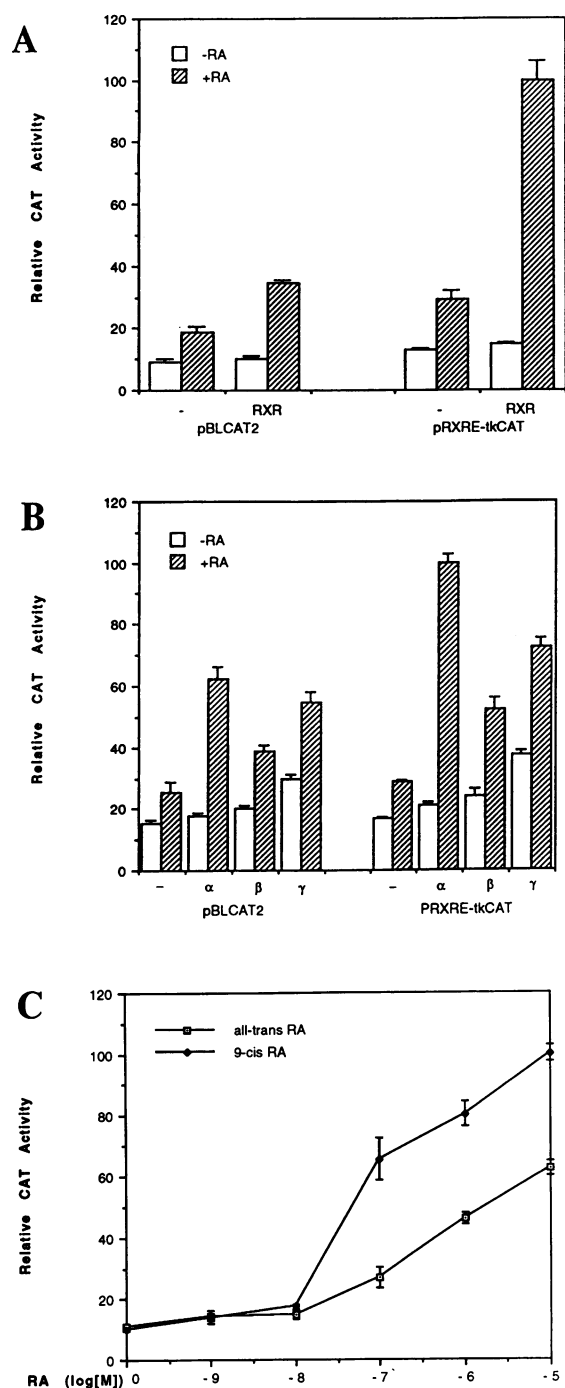
Figure 2. Differential regulation of AFP-RXRE by RXR and RAR. **A.** RXR transactivation of AFP-RXRE. CV1 cells were transfected with reporter construct pBLCAT2 or pRXRE-tkCAT, β-galactosidase internal control plasmid pCH110, and with or without RXRα expression vector pECE-RXRα. After transfection, cells were treated with either the vehicle ethanol in control ($-$RA, white bar) or 10 μM RA ($+$RA, hatched bar). 36 hr later transfected cells were harvested and the CAT activity was measured and plotted as the percent maximal response. **B.** RAR effect on AFP-RXRE. CV1 cells were transfected with reporter pBLCAT2 or pRXRE-tkCAT, β-galactosidase internal control plasmid pCH110, and with or without different RARs expression vectors, pECE-RARα, pECE-RARβ, and pECE-RARγ, respectively. **C.** Dose dependent response of AFP-RXRE. CV1 cells were transfected with pRXRE-tkCAT, pCH110, and pECE-RXR expression vector. After transfection, cells were treated with 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M of either all-*trans*, or 9-*cis* RA respectively. The CAT activity was measured as in A. All results are the average of six individual transfections.
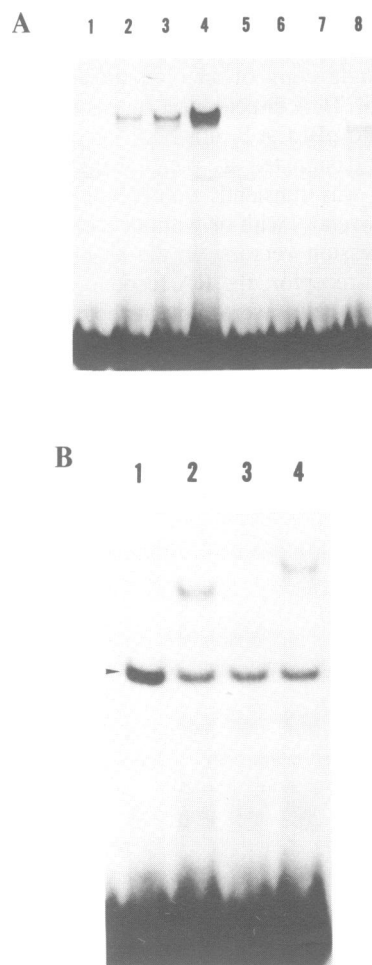

Figure 3. Interaction of bacterial synthesized RXR and RAR with AFP-RXRE. **A.** Dose dependence of binding. Mobility shift assays were performed using labeled AFP-RXRE oligonucleotides and increasing amounts (90 ng, 180 ng, and 360 ng) of bacterial synthesized RXRα (lanes 2–4) or bacterial synthesized RARγ (lanes 6–8). In addition, the probe was also incubated with 180 ng of unprogrammed bacterial lysate as a control (lanes 1 and 5). **B.** Specific binding of RXR to AFP-RXRE. Mobility shift assays were performed using labeled AFP-RXRE oligonucleotides and 180 ng of bacterial synthesized RXRα (lanes 2–4) in the absence (lane 2) or presence of a 25 molar excess of competitor oligonucleotides from chicken ovalbumin promoter (lane 3), or 1 μl anti-flag antibody (lane 4). The unprogrammed bacterial lysate was also incubated with the probe as a control (lane 1). Nonspecific complexes are indicated by the arrowhead.

extent of CAT activity as 9-*cis* RA. For examole, 0.01 μM 9-*cis* RA and 10 μM all-*trans* RA can both induce a 65-fold increase in CAT activity in CV1 cells which were cotransfected with pRXRE-tkCAT and pECE-RXR expression plasmids. Based on these results, we therefore refer to this DNA sequence in the AFP promoter as the AFP gene retinoid X response element (AFP-RXRE). The expression of pRXRE-tkCAT can also be induced by a high concentration of all-*trans* RA because RA can be metabolized to 9-*cis* RA in the cells (14,15). Therefore, 10 μM of all-*trans* RA was used in the rest of the transfection experiments in this report. Although RXR can enhance the transcriptional activity of RAR on a specific RARE (43,53), there was no marked enhancement of CAT activity when RAR and RXR were cotransfected with pRXRE-tkCAT (data not shown).
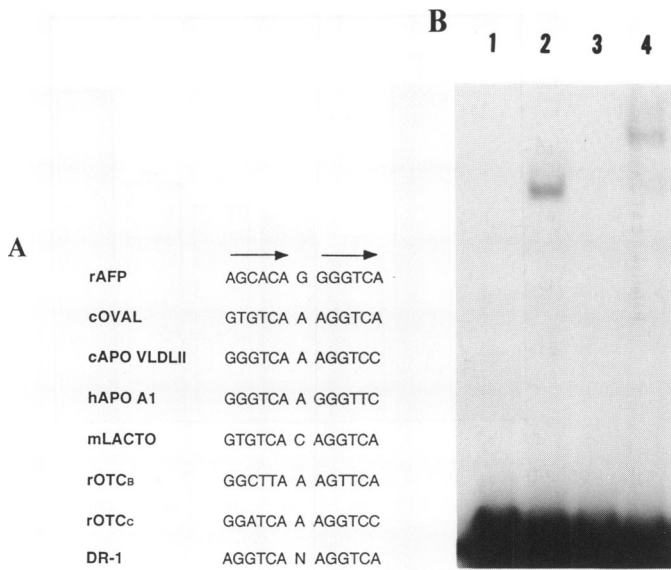
Figure 4. Interaction of COUP-TF with AFP-RXRE. **A.** AFP-RXRE and COUP-TF binding sites in different gene promoters. The sequence of AFP-RXRE was aligned with different natural COUP-TF recognition elements. The arrow indicates the orientation of the A/GGGTCA motif. rAFP, rat α-fetoprotein; cOVAL, chicken ovalbumin; cAPO VLDLII, chicken very low density apolipoprotein II; hAPO A1, human apolipoprotein A1; mLACTO, mouse lactoferrin; rOTC$_B$ or rOTC$_C$, rat ornithine transcarbamylase promoter region B or C. **B.** Specific binding of COUP-TFI to AFP-RXRE. Mobility shift assays were performed using labeled AFP-RXRE oligonucleotides and 1 μl of *in vitro* synthesized COUP-TFI (lanes 2–4) in the absence (lane 2) or presence of a 25 molar excess of COUP-TFI binding element from the chicken ovalbumin promoter (lane 3), or 1 μl COUP-TFI antiserum (lane 4). The unprogrammed rabbit reticulocyte lysate was also incubated with the probe as a control (lane 1).

Figure 5. Repression of COUP-TF on RXR transactivation through AFP-RXRE. **A.** Inhibition of COUP-TF on RXR transactivation. CV1 cells were transfected with 0.6 μg of receptor construct pRXRE-tkCAT and 0.6 μg of β-galactosidase internal control plasmid pCH110 in combination with 0.2 μg of expression vector pECE-RXRα or/and pRShCOUP-TFI. The total amounts of plasmid were 4 μg per 35 mm plate with the supplement of plasmid pBluescript. After transfection, cells were treated with either the vehicle ethanol in control (−RA, white bar) or 10 μM RA (+RA, hatched bar). 36 hr later transfected cells were harvested and the CAT activity was measured and plotted as the percent maximal response. Results were the average of six individual transfections. **B.** Dose dependence of repression. CV1 cells were transfected with 0.6 μg of receptor pRXR-tkCAT, 0.2 μg of pECE-RXRα, and 0.6 μg of pCH110 with increasing amounts (50 ng, 100 ng, 200 ng, 400 ng, 800 ng, and 1600 ng) of pRShCOUP-TFI. Cells were treated with either the vehicle ethanol (−) or 10 μM RA (+). CAT activity is expressed as above. Results are from six individual transfections.

Binding of RXR to AFP-RXRE

Direct interaction between the AFP-RXRE and bacterial expressed RXRα was performed by using the gel retardation assays. A specific retarded band was seen which showed a dose dependent response to increasing amounts of RXRα (Fig. 3A). This specific complex could be eliminated in competition experiments by unlabeled retinoid X receptor binding oligonucleotides (Fig. 3B, lane 3), but not by unlabeled nonspecific oligonucleotides (data not shown). Consistent with the results in competition experiments, RXR specific binding was further confirmed in the upshift experiments. The RXR/AFP-RXRE complex could be recognized by RXR-anti-flag antibody as a decreased mobility of the complex in the presence of antibody (Fig. 3B, lane 4). No shift was observed in the experiment with a nospecific antibody (data not shown). The other nuclear retinoid receptor RAR was also tested for the binding to AFP-RXRE. Bacterial expressed RARγ bound weakly to the AFP-RXRE oligonucleotides, and a weak binding was observed only in the presence of high amount of RAR (Fig. 3A). It has been reported that efficient binding of the RAR to its cognate response element requires heterodimerization with RXR (43,53). However our experiments presented in Fig. 3A demonstrate that RXR can not enhance RAR binding to AFP-RXRE and RAR also could not increase the binding of RXR to this sequence. These results revealed that RXR bound more tightly and more favorably to AFP-RXRE than RAR, consistent with the data from RXR transactivation of pRXRE-tkCAT (Fig. 2A and B).
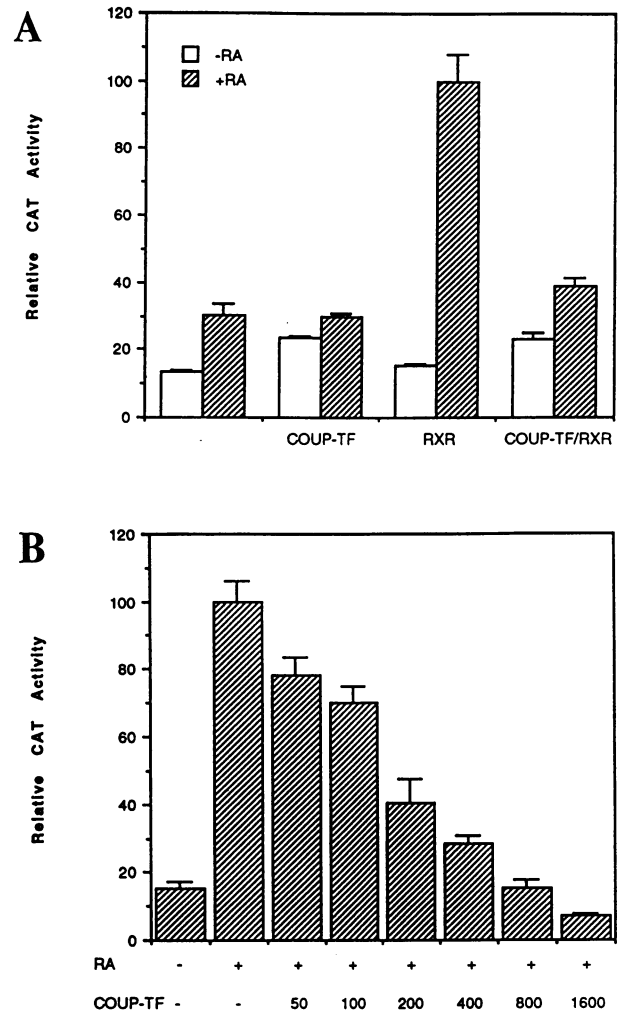
Binding of COUP-TF to AFP-RXRE

COUP-TF is an orphan member of the steroid-thyroid hormone receptor superfamily. On the basis of a three amino acid sequence at the C-terminal base of the first zinc finger, COUP-TF is further classified as a member of the ER-TR subfamily which recognizes A/GGGTCA repeats (42). Recent studies have demonstrated that COUP-TF binds to many A/GGGTCA repeats with different orientation and spacings (24). However, a direct repeat with one nucleotide spacing is the most preferable binding site for COUP-TF (18, 22−24). Natural COUP-TF binding sites also reveal the imperfect direct repeat of the A/GGGTCA motif separated
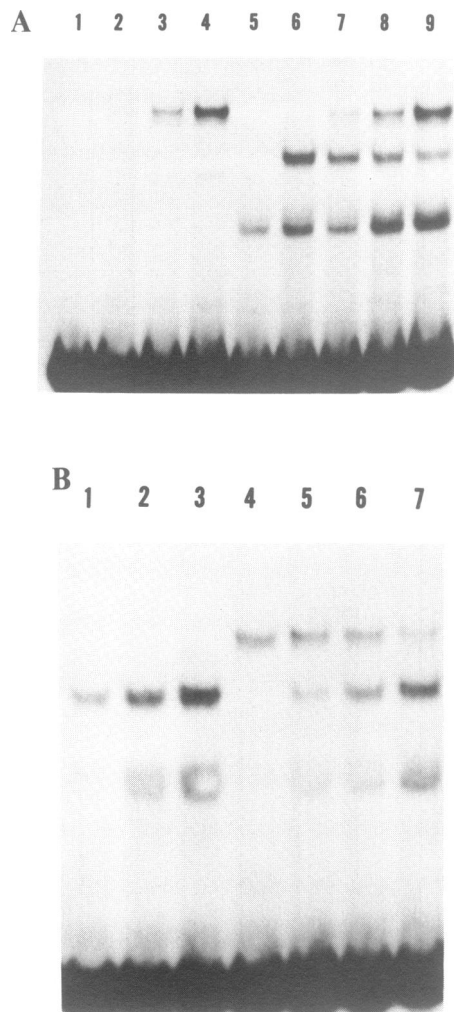
Figure 6. Binding competition between RXR and COUP-TF on AFP-RXRE. A. Inhibition of COUP-TF binding by RXR. Mobility shift assays were performed using labeled AFP-RXRE oligonucleotides and 1 μl of *in vitro* synthesized COUP-TFI (lanes 6−9) with increasing amounts (90 ng, 180 ng, and 360 ng) of bacterial synthesized RXRα (lanes 2−4 and lanes 7−9, respectively). In addition, the probe was also incubated with 180 ng of unprogrammed bacterial lysate (lane 1) and 1 μl of rabbit reticulocyte lysate (lane 5). B. Inhibition of RXR binding by COUP-TF. Mobility shift assays were performed using labeled AFP-RXRE oligonucleotides and 180 ng of bacterial synthesized RXR (lanes 4−7) with increasing amounts (0.5 μl, 1 μl, and 2 μl) of *in vitro* synthesized COUP-TFI (lanes 1−3 and lanes 5−7, respectively).

by one nucleotide (24). In comparison with the sequence of AFP-RXRE in AFP gene promoter, it displays a high sequence similarity to COUP-TF response elements (Fig. 4A). To test whether AFP-RXRE is also capable of directly interacting with COUP-TF, gel retardation assays were performed with *in vitro* synthesized COUP-TF and AFP-RXRE oligonucleotide. Indeed, *in vitro* synthesized COUP-TF bound efficiently to the AFP-RXRE sequence in the AFP gene promoter in dose dependent fashion (Fig. 4B, lanes 1−3). The specific binding of COUP-TF to AFP-RXRE was confirmed in competition experiments. Excess COUP-TF response element from chicken ovalbumin promoter could abolish COUP-TF binding to AFP-RXRE (Fig. 4B, lane 3). In addition, the specific COUP-TF/AFP-RXRE complexes were upshifted by COUP-TFI antiserum (Fig. 4B,
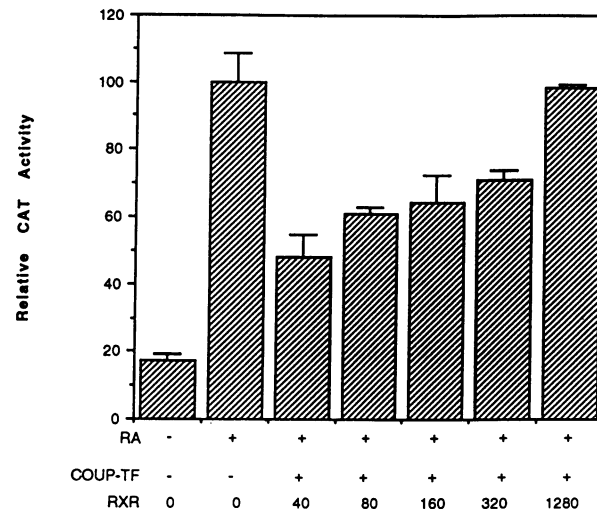

Figure 7. Reversal of COUP-TF repression of RXR transactivation on AFP-RXRE by overexpression of RXR. CV1 cells were transfected with 2 μg of reporter pRXRE-tkCAT and 0.6 μg of β-galactosidase internal control plasmid pCH110 in combination with 80 ng of pRShCOUP-TFI (columns 3−7), and different amounts (40 ng, 40 ng, 40 ng, 80 ng, 160 ng, 320 ng, and 1280 ng) of pECE-RXRα expression vector (columns 1−7, respectively). After transfection, cells were treated with either the vehicle ethanol in control (column 1) or 10 μM RA (columns 2−7). 36 hr later transfected cells were harvested and the CAT activity was measured and plotted as the percent maximal response. Results are the average of three individual transfections.

lane 4). Thus, this direct repeat AFP-RXRE sequence in AFP gene promoter can be recognized by both RXR and COUP-TF transcriptional factors.

Repressing RXR transactivation of AFP by COUP-TF

Previous studies have shown that the consequence of binding of COUP-TF to hormone response elements (HREs) is the repression of the transactivation of steroid/thyroid hormone receptors (18, 21, 26). The observation that COUP-TF can bind to the AFP-RXRE suggested that COUP-TF might down-regulate the RXR induction of AFP gene expression. To assess the repression of COUP-TF on AFP-RXRE, pRXRE-tkCAT was cotransfected into CV1 cells with either RXR expression vector alone or with COUP-TF expression vector. As predicted, the transactivation of RXR was dramatically suppressed by the cotransfection of COUP-TF (Fig. 5A). Furthermore, the inhibition by COUP-TF of RXR transactivation was dose-dependent. With increasing amounts of COUP-TF, CAT activity was progressively decreased and reached the basal CAT expression level when a 4-fold excess of COUP-TFI (800 ng) was cotransfected (Fig. 5B, lane 7). In contrast, COUP-TFI itself had no effect on the regulation of CAT activity with RA treatment (Fig. 5A). Thus, COUP-TFI functions as a potent repressor for RXR on AFP-RXRE activity.

Binding competition between RXR and COUP-TFI on AFP-RXRE

Binding of both RXRα and COUP-TFI to AFP-RXRE and repression of RXR transactivation by COUP-TFI on AFP-RXRE suggests the possibilities of either competing for the binding site for RXR and COUP-TFI or forming inactive heterodimer. In the mobility shift assay, both RXR and COUP-TFI interacted with the AFP-RXRE sequence. In order to maximize the

migratory differences between RXR and COUP-TFI, an N-terminally truncated COUP-TFI (tCOUP-TFI) lacking 54 amino acids which has the same characteristics as full length COUP-TFI on DNA binding and protein dimerization (24), was used in the mobility shift assay. Binding competition between RXR and COUP-TFI was consistently observed in the mobility shift assay (Fig. 6). Addition of increasing amounts of RXR to a constant amount of COUP-TF resulted in the decrease of COUP-TFI binding to AFP-RXRE (Fig. 6A, lane 7, 8, and 9). Similarly, increasing the amount of COUP-TF could also decrease the binding of RXR to AFP-RXRE (Fig. 6B, lane 5, 6, and 7). These results implied that binding of RXR and COUP-TFI to AFP-RXRE are competitive *in vitro*. To further determine if COUP-TF1 can form heterodimers with RXR we analyzed the products of cotranslation of COUP-TF1 and RXR RNAs by gel mobility shift assay as described (26). No intermediate heterodimer complexes were observed (data not shown).

To further confirm the prediction that RXRα and COUP-TFI competitively bind to the AFP-RXRE, increasing amounts of RXR expression vector with a constant amount of COUP-TF I expression vector were cotransfected into CV1 cells (Fig. 7). COUP-TFI (80 ng) repressed RXR (40 ng) transactivation on AFP-RXRE by more than 2-fold (Fig. 7, lane 2, 3). However, the CAT activity is restored gradually by cotransfection of increasing amounts of RXR expression vector, and reached the level observed in the absence of COUP-TFI (Fig. 7, lanes 2 and 7). That repression by COUP-TFI on RA induction can be overcome with increasing amounts of RXR in cells was consistent with data from mobility shift assays that showed both RXRα and COUP-TFI competed for the binding site.

DISCUSSION

In this report, we show that a RXRE has been identified and characterized in the promoter region of the AFP gene. This RXRE is responsible for the RXR transactivation of AFP gene in transfection assay of AFP promoter construct. The AFP-RXRE identified in the AFP promoter region closely resembles the sequence identified in other natural retinoic acid response elements (RARE) (7). The consensus half site of RARE is described as (A/G)G(T/G)TCA. However, each reported RARE has distinct sequence features (7). In the case of AFP-RXRE, there are two tandem direct repeat sequences with one conserved half site GGGTCA, and an imperfect half site AGCACA located at −134 and −127 respectively. There is a one nucleotide spacing between the two direct repeats (DR-1). It has been reported that the spacing between the repeated half sites may contribute to the binding discrimination for different nuclear receptors (17,18). Spacers of 1 (DR-1), 3 (DR-3), 4 (DR-4), or 5 (DR-5) nucleotides may play as a recognition code, and correspond to specific binding elements for RXRs, vitamin D_3 receptor (VDR), thyroid hormone receptor (TRs), and RARs, respectively. Actually, a RXRE in the upstream region of cellular retinol-binding protein type II reveals a sequence feature of direct repeat with single spacer nucleotide which confers specific RXR responsiveness (44). Results from the mobility shift assay demonstrated that the AFP-RXRE preferred binding of RXR to RAR (Fig. 3A). This predominant RXR binding could be dependent on the recognition code of AFP-RXRE.

COUP-TFI is an orphan member of the steroid/thyroid hormone receptor superfamily (20). Based on the molecular size, two classes of COUP-TFs have been characterized, with the low

molecular weight ones in the range of 43−48 kDa and the high molecular weight ones in the range of 66−74 kDa (21, 45, 48). COUP-TFI was originally identified as a transcription activator for the ovalbumin gene promoter *in vitro* (19). However, recent studies demonstrated that, in addition to the positive regulatory roles, COUP-TFI has also showed negative regulatory functions by interfering with the transactivation of other member of the steroid/thyroid hormone receptor superfamily such as the estrogen, vitamin D_3, thyroid hormone, and nuclear retinoid receptors (18, 25, 26). COUP-TF modulates transcription by binding as dimers to the COUP-TF binding sites at specific genes. Until now, several COUP-TF binding sites have been identified in the promoter region of the chicken ovalbumin, rat insulin, apolipoprotein A1 and VLDLII, pro-opiomelanocortin, mouse lactoferrin genes, and ornithine transcarbamylase and HIV-1 long terminal repeat (22, 47−51). Analysis of these natural COUP-TF response elements has revealed that COUP-TF can bind to DNA elements with different spacing and orientation of the imperfect repeat of the A/GGGTCA motif (47−49). Further examination with synthetic oligonucleotides showed that COUP-TF binds predominantly to the A/GGGTCA direct repeat with one nucleotide spacing (18, 23, 24). The RXRE in the AFP promoter region is composed of two tandem direct repeats of the GGGTCA motif and its diverse sequence of AGCACA with 1-bp spacing. This AFP-RXRE displayed high sequence similarity to the COUP-TF binding site (Fig. 4A). Mobility shift assays showed that COUP-TF specifically bound to this AFP-RXRE sequence *in vitro*. Therefore, this unique DNA sequence in the AFP promoter region can be considered as a composite RXRE which is comprised of overlapping RXR and COUP-TF binding elements.

It is considered that the significant activation of a specific hormone response element by a hormone receptor should require its cognate ligand. COUP-TF is an orphan member of steroid/thyroid hormone receptor superfamily. The ligand for COUP-TF has not been identified. In transfection analyses, COUP-TF alone had no obvious transactivation activity on the reporter construct, pRXRE-tkCAT (Fig. 5A). The interpretation of this studies could be explained by the lack of specific ligand for COUP-TF.

In cotransfection analyses, COUP-TF demonstrated a dramatic repression on RXR transactivation on AFP-RXRE. In addition, results from mobility shift assays consistently showed that incubation of a fixed amount of receptor protein with increasing amounts of reciprocal receptor protein decreased the receptor binding activity for both RXR and COUP-TF (Fig. 6). It has been reported that COUP-TFs form stable dimers *in vitro* (26). However, there is no obvious evidence showing the formation of RXR/COUP-TF heterdimers. Thus, these studies demonstrated that the binding of RXR and COUP-TF to AFP-RXRE was in a mutually exclusive manner which may contribute to the inhibition of COUP-TF on RXR transactivation. Recently, it has been demonstrated that COUP-TF was capable of forming heterodimers with RXR (18, 26). However, there was also a study showing that COUP-TF and RXR were not capable of forming a heterodimer (52). The controversial data of heterodimerization may be caused by the difference in the response elements. The heterodimerization between RXR and COUP-TF was found on a direct repeat of the AGGTCA motif separated by a single base pair (DR-1), and not on TREp, the inverted repeat of AGGTCA motif (18, 26, 52). In addition, the heterodimerization studies between COUP-TF and TR were also

different by using two different response elements. The formation of heterodimers between TR and COUP-TF was only found with TREp, but not with DR-1 of AGGTCA motif (26, 52). Structurally, AFP-RXRE is a degenerated DR-1 of AGGTCA motif. RXR and COUP-TF competed for the binding site on AFP-RXRE, instead of forming the heterodimer. Thus, in some cases, the diversity of response elements may also determine the dimerizational potential of receptors from the steroid/thyroid hormone receptor superfamily.

In conclusion, there is a composite RXRE in the AFP promoter region which competitively binds to RXR and COUP-TF. It has been reported that both RXR and COUP-TF exhibit liver-enriched tissue distribution (11,45). Thus, RXR and COUP-TF may play important roles in the regulation of AFP gene expression in liver cells.

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