

Cell-Type-Specific Regulation of the Retinoic Acid Receptor Mediated by the Orphan Nuclear Receptor TLX†

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Malformations in the eye can be caused by either an excess or deficiency of retinoids. An early target gene of the retinoid metabolite, retinoic acid (RA), is that encoding one of its own receptors, the retinoic acid receptor β (RAR β). To better understand the mechanisms underlying this autologous regulation, we characterized the chick RAR β 2 promoter. The region surrounding the transcription start site of the avian RAR β 2 promoter is over 90% conserved with the corresponding region in mammals and confers strong RA-dependent transactivation in primary cultured embryonic retina cells. This response is selective for RAR but not retinoid X receptor-specific agonists, demonstrating a principal role for RAR(s) in retina cells. Retina cells exhibit a far higher sensitivity to RA than do fibroblasts or osteoblasts, a property we found likely due to expression of the orphan nuclear receptor TLX. Ectopic expression of TLX in fibroblasts resulted in increased sensitivity to RA induction, an effect that is conserved between chick and mammals. We have identified a *cis* element, the silencing element relieved by TLX (SET), within the RAR β 2 promoter region which confers TLX- and RA-dependent transactivation. These results indicate an important role for TLX in autologous regulation of the RAR β gene in the eye.

The vitamin A derivative retinoic acid (RA) has been suggested to play important roles in vertebrate embryonic development and cell differentiation. Vitamin A deficiency and/or excessive doses of RA are known to result in a spectrum of distinct malformations during organogenesis and pattern formation (reviewed in references 8, 19, and 49). Two classes of receptors, RA receptors (RARs) and retinoid X receptors (RXRs), which belong to a large family of nuclear hormone receptors, mediate RA signaling. These receptors are capable of binding specific target DNA sequences in the regulatory regions of responsive genes, termed RA response elements (RAREs), to activate or repress transcription (5, 29).

Among vitamin A metabolites, all-*trans*-RA (at-RA) has been shown to bind the RARs, whereas a stereoisomer of at-RA, 9-*cis*-RA, acts as a high-affinity bipotential ligand for both RARs and RXRs. A RAR and RXR preferentially form a heterodimer to bind RAREs consisting of a direct repeat of canonical AGGTCA sequences separated by two or five nucleotides (28).

Three subtypes of the gene for RAR, α , β , and γ , have been identified in chick (33), mouse (58), and human (22) and are expressed in distinct spatial and temporal patterns during embryogenesis. In the chick embryo, RAR β has been shown to be prominently expressed in the developing central nervous system and RAR γ expression is mainly restricted to the skin, whereas RAR α expression is rather ubiquitous (32, 44, 48, 50). Several isoforms exist for each subtype. In mammals, the genes for all three subtypes can be transcribed from at least two different promoters, resulting in variation at the amino termini between isoforms. For RAR β , four distinct mRNAs result from use of alternative promoters (β 1 and β 2) and splicing

variations (59). In chick, distinct forms of RAR β mRNAs display individual patterns of expression during embryogenesis (45, 48).

One of the parameters modulating the differential expression of RARs and RXRs is RA itself. The human and mouse RAR β 2 promoters have a well-conserved RARE (the so-called β RARE) just upstream of the TATA box. As a result, the RAR β 2 promoters are highly inducible by RA and are considered to be one of the earliest targets of RA action (11, 51).

In transgenic mice with the RAR β 2 promoter fused to the *Escherichia coli* β -galactosidase (β -gal) gene, β -gal activity was present in the pigmented retina (31, 41). β -gal staining was also observed in the eye of a RARE- β -gal transgenic embryo. Staining was increased upon maternal treatment with RA, suggesting that *in vivo*, some of the morphogenetic effects of RA could be mediated through localized transcriptional activity controlled by the various RARs (2, 43).

In chick embryos, RAR β transcripts can be upregulated by exogenously added RA. Implantation of RA-soaked beads in limb buds causes rapid (within 4 h) accumulation of RAR β 2 mRNA (39, 54). This induction was also observed by Northern analysis using mRNA isolated from facial primordia (45). Together these data strongly suggest that the mechanisms underlying the autologous regulation of RAR β gene expression are well conserved between mammals and avians.

TLX is an orphan nuclear receptor originally identified on the basis of its similarity to RXR; it is structurally and functionally (biochemically) homologous to the *Drosophila* terminal-gap gene *tailless*. Expression of TLX is restricted to the fore- and midbrain, neuroepithelium, retina, and nasal epithelium (18, 36, 57). We and others have shown that the function of TLX is necessary for the proper formation of specific eye and brain structures, but the precise molecular mechanisms of its action are still being unraveled (17, 35, 56).

Here we show that the RAR β 2 promoters and TLX proteins

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† This paper is dedicated to K. Umesono.

are highly conserved during evolution and that TLX can modulate the autoregulation of the RAR β promoter.

MATERIALS AND METHODS

Isolation of chick RAR β promoter. A chick 3-day embryonic cDNA library was screened with a fragment of mouse RXR β as the probe (57). Multiple cDNA clones were found to encode the chick RAR β , including clones corresponding to mRNAs utilizing both β 1 and β 2 promoters. Sequence information from the RAR β 2 cDNA clone containing the longest 5' noncoding region was used to design primers for PCR. The primer sequences used for genomic PCR analyses were as follows: NMO1 (5'-GCTCTGCAGGGCTGCTGGGAGTTT-3'), NMO2 (5'-AATCTCTCTAGAACCAGTCCCGTTCCTCAG-3'), NMO8 (5'-CCCTCAGCCATGAATAGATCCTT-3'), and NMO9 (5'-CCTGCCTCTCTGGCTGCTGCTT-3'). First, for the NMO9-NMO2 primer combination, PCR amplification was carried out for 33 cycles (30 s at 94°C, 1 min at 50°C, 2 min at 72°C) with 50 ng of chick genomic DNA, 200 ng of each primer, and 2.5 U of *Taq* DNA polymerase (Life Technologies). A second round of amplification was carried out for 25 cycles, using 1/50 of the first PCR mixture as the template with the NMO1-NMO8 primer combination. Products from the second PCR were separated on agarose gel and purified, and a 220-bp fragment was ligated into the TA cloning vector pMOSBlue (Amersham). The DNA sequence was determined with an AutoCycle sequencing kit on an A.L.F. II DNA sequencer (Pharmacia). Sequences of at least two clones from each of three independent PCR products were determined.

Cloning of human TLX cDNA. The National Center for Biotechnology Information expressed sequence tag database was searched for sequences related to the chick TLX, using the program BLASTN (1). Two expressed sequence tags with similarity to chick TLX, those with the GenBank accession numbers R18964 and R43976, were identified from a single human infant brain cDNA clone, am156j01. The plasmid encompassing am156j01 was used to design oligonucleotide probe NMO63 (5'-GACAACCTCCGGTTAGATGC-3'). The full-length human TLX cDNA clone in the mammalian expression vector was selected using the GENETRAPPER cDNA Positive Selection System (Life Technologies) from among 4×10^{11} clones of a human fetal brain cDNA pCMV-SPORT2 library (Life Technologies).

Cell culture and transfection assay. CV-1 and MC3T3-E1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and α -MEM medium (Life Technologies), respectively, supplemented with 10% fetal bovine serum (FBS). Retina cells were isolated from day-4.5 chick embryos according to the method described previously (40). Cells were washed with phosphate-buffered saline-EDTA, treated with 0.125% trypsin, and plated on 24-well dishes (Costar). Retina cells were kept in 10% FBS-DMEM for 4 days. Then 2 h prior to transfection, medium was replaced with 10% charcoal-resin double-treated FBS-DMEM. Transfections were performed by the calcium phosphate precipitation method as previously described (53). Cells were transfected for 6 h in 24-well dishes with a total of 750 ng of DNA/well adjusted by pGEM4 plasmid together with 250 ng (or 150 ng for the thymidine kinase [tk]-driven reporter) of reporter plasmid, 350 ng of reference plasmid (pCMX- β GAL), and 50 ng of receptor plasmid. After washing out of DNA precipitates, cells were incubated with added ligand for 36 h. Cell extracts were subsequently prepared and assayed for luciferase and β -gal activities. All data points were determined in triplicate and normalized for transfection efficiency with β -gal as an internal control. at-RA (Nacalai, Kyoto, Japan) and 9-*cis*-RA (Wako Pure Chemical, Osaka, Japan) stock solutions were prepared in 20% dimethyl sulfoxide-80% ethanol. Synthetic retinoids LG69 and TTNPB, kindly provided by R. Heyman, were solubilized in dimethyl sulfoxide and added at the indicated concentrations.

Serum stripping. FBS (Life Technologies) was equilibrated twice with equal volumes of *n*-heptane for 2 h at room temperature. After the second separation, the recovered serum was stirred twice with 50 g of resin (AG 1-X8, analytical grade, 200/400 mesh; Bio-Rad)/liter and 20 g of charcoal/liter. After centrifugation to remove particulates, the serum was passed through a 0.22- μ m-pore-size filter for sterilization.

Reverse transcription-PCR analyses. Total RNA was isolated from primary cultured chick retina cells with ISOGEN (Nippon Gene, Toyama, Japan), and cDNA template was prepared with a RNA PCR kit (Takara Shuzo, Kyoto, Japan) according to the manufacturer's instructions. Using 200 ng of each primer, PCR amplification was performed for 30 cycles (30 s at 94°C, 60 s at 68°C, 90 s at 72°C) with 2.5 U of *Ex-Taq* DNA polymerase (Takara Shuzo). The sequences of the 5' primers for RAR β transcripts are as follows: NMO46 (5'-ACTGAATGGTGGTCTGAGACACGGACTAAG-3') for β 1 and NMO3 (5'-CTGAGGAACCGGGACTGGTCTAGAGAGATT-3') for β 2. NMO21 (5'-CTTGAACAAGTCTCAGACTGGTCTC-3') was used as the common 3' RAR β primer. The NMO53 (5'-GATGGTCAGGTCATCACCATTGG-3') and NMO54 (5'-CATCGTACTCTGCTGTGATCC-3') primer set was used for β -actin. The identity of each RAR β promoter-derived PCR product was confirmed by sequencing of at least four independent clones.

Plasmid constructions. The expression vectors pCMX-hRXR α (55), pCMX-cTLX, pCMX-mTLX (57), pCMX-hRAR α , and pCMX- β GAL and reporter plasmids tk-LUC and m β RARE-tk-LUC (53), which were used in the transfection assay, have been previously described. The coding region of human COUP-TFII/ARP-1 (24) was cloned into the *Eco*RI site of the pCMX vector (53) to

create the pCMX-hCOUP-TFII expression vector. The c β RARE-tk-LUC, SET-tk-LUC, SET-m β RARE-tk-LUC, m β RARE-SET-tk-LUC, SETm1-m β RARE-tk-LUC, and SETm2-m β RARE-tk-LUC reporter plasmids were prepared by inserting the synthetic oligonucleotides c β RARE (agctTGGGTTACACGAAA GTTCACTCAGct), SET (agctTGGGTCATTGAAGGTTAGct), SETm1 (agctTGAACCATTGAAGGTTAGct), SETm2 (agctTGGGTCATTG AAAACTAGct), and m β RARE (agctTAAGGGTTCACCGAAAGTTC ACTCGCATct) into the *Hind*III site of a tk-LUC basal luciferase reporter or m β RARE-tk-LUC reporter. The mouse RAR β 2- Δ 2.2k-LUC promoter reporter plasmid was made by inserting a 2.2-kb fragment (including the transcription start site of mouse RAR β 2) into the *Sal*I/*Xho*I site to replace the tk promoter of the tk-LUC reporter plasmid. For the chick RAR β 2- Δ 119-LUC, first, the PCR-amplified fragment with NMO2 and NMO7 (5'-AAGCTCTG TGAGAATCTCTGGGAG-3'), which encodes the -119 to +68 region of the chick RAR β 2 promoter, was ligated into the TA cloning vector pMOSBlue (Amersham). The *Hind*III/*Bam*HI fragment was excised for ligation into the *Hind*III/*Bgl*II site to replace the tk promoter of the tk-LUC reporter. For the chick RAR β 2- Δ 85-LUC and the mouse RAR β 2- Δ 66-LUC, first, the PCR fragment-amplified with KMO45 (5'-GTACGTCGACTGGGTCATTGAAGGTTA GCAG-3') and NMO2 or with NMO5 (5'-GGTGGATCCAGCCCGGGGA AGGGTTCACCGAA-3') and NMO6 (5'-GTACTCGAGGCAGCGGAACT CTGGTCCCCCTT-3') was excised with *Bam*HI and *Sal*I or *Xho*I. The fragment was ligated into the *Sal*I/*Bgl*II or *Bam*HI/*Xho*I site, respectively, to replace the tk promoter of the tk-LUC reporter. Sequences of the PCR-amplified region were confirmed by standard methods.

Gel retardation assays. Proteins were synthesized by an in vitro transcription-translation system using rabbit reticulocyte lysate (Promega) with plasmid pCMX-hRAR α , pCMX-hRXR α , pCMX-cTLX, or pCMX-hCOUP-TFII. For binding, 4 μ l of lysate was incubated first in 10 mM Tris-HCl (pH 8.0), 80 mM KCl, 1 mM dithiothreitol, 0.1% NP-40, 1 μ g of poly(dI-dC), and 7.5% glycerol on ice for 20 min. Excess unlabeled competitor oligonucleotides, when included, were added during this preincubation period. Then 0.2 to 0.6 pmol of 32 P-labeled oligonucleotide (3×10^5 cpm, prepared by filling in with Klenow polymerase in the presence of [α - 32 P]dCTP) probe was added to the reaction, followed by incubation on ice for 30 min. The protein-DNA complexes were resolved on a 5% polyacrylamide gel in 0.5 \times Tris-borate-EDTA buffer. Gels were subsequently dried and subjected to autoradiography. The same oligonucleotides described in plasmid constructions were used for gel retardation assays for SET, m β RARE, and SET-m β RARE. The *Kr*üppel oligonucleotide was described previously (57). The sequence of the nonspecific competitor is agctACAAGGTTCCAGAGGTTACAGTCct.

Nucleotide sequence accession numbers. The sequences reported in this paper have been deposited in the GenBank database (accession no. AF220160, AF220161, AF220162, and AF220163 for the chick RAR β 2 promoter region and RAR β 4', RAR β 4M, and RAR β 4M' cDNA and AF220532 for human TLX cDNA).

RESULTS

Induction of RAR β expression in chick retina cells. The expression of RAR β s in chick embryonic retina cells was examined by reverse transcription-PCR. Three distinct bands were observed by agarose gel analysis (Fig. 1). Subcloning and subsequent DNA sequencing of the PCR products revealed the presence of five RAR β isoforms; in addition to the previously reported β 2 and β 4 (38, 48), three were novel (Fig. 1). These isoforms are apparently generated by alternative splicing and differ only in their amino termini (Fig. 1). The chick RAR β 4M (corresponding to mouse RAR β 4) isoform lacks an apparent acceptor sequence (AG) at nucleotide positions 617 and 618, suggesting the presence of an intron between nucleotides 618 and 619; an intron is present at the analogous position in mouse RAR β (37). In cultured retina cells, the expression of all RAR β 2 isoforms is induced within 12 h of addition of at-RA (Fig. 1). In contrast, the RAR β 1 isoform appears not to be affected by exogenous at-RA, indicating a specific role for the RAR β 2 promoter in retina cells (Fig. 1).

Determination of chick RAR β 2 promoter sequence. To compare the mammalian and avian RAR β 2 promoters, we isolated the chick RAR β 2 promoter region by genomic PCR. To accomplish this, 5' primers were designed based upon the well-conserved human and/or mouse sequences, while 3' primers were designed based on chick-specific sequence from within the cDNA. Over 90% sequence identity was observed among human, mouse, and chick genes in the 150-bp region surround-

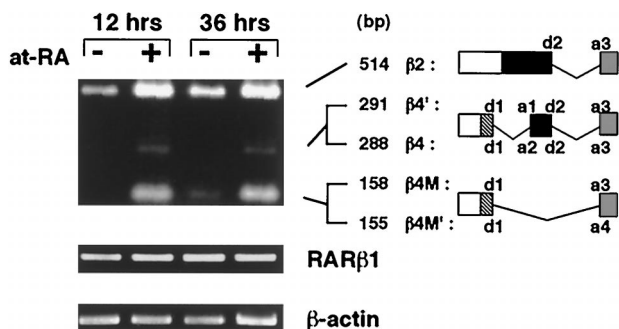


FIG. 1. Induction of RARβ2 isoforms by RA in chick retina cells. RNAs were reverse transcribed and amplified with chick RARβ2-, β1-, or β-actin-specific primers. Three major bands were resolved by 1.2% agarose gel in Tris-borate-EDTA buffer for RARβ2 isoforms with apparent estimated sizes of 514, 288 to 291, and 155 to 158 bp. The expression of RARβ2 isoforms is induced by 1 μM at-RA within 12 h in chick retina cells. No induction was observed 36 h after medium change in the absence of exogenous at-RA. Note that the expression of RARβ1 or β-actin transcripts is not affected by at-RA. Donor and acceptor sites for splicing are indicated in the schematic representation of chick RARβ isoforms (see Fig. 2 for details). The nomenclature of RARβ isoforms is based on correlation to mouse and human RARβ isoforms.

ing the transcription start site. The high conservation in sequence also allowed prediction of the putative transcription start site for the chick gene (11). We confirmed the presence of a cyclic AMP RE, AP-1 binding site (TRE), RARE (βRARE), RNA polymerase initiator site (INR), and TATA box (12) that are conserved in mouse and human. Some short upstream open reading frames (uORFs) which have been reported to regulate the translational level of mouse RARβ2 were also found (Fig. 2) (42).

Induction of RARβ2 promoter by at-RA is cell type specific. As an initial step to characterize the function of the chicken RARβ2 promoter, we generated a luciferase reporter construct and confirmed the ability of this region to direct RARβ2 transcription by transient transfection in primary cultured chick embryonic retina cells. Promoter activity could be induced by at-RA concentrations as low as 0.1 μM (Fig. 3A). Activation by 9-*cis*-RA or a RAR-specific agonist (TTNPB) but not by a RXR-specific agonist (LG69) was also observed. These results suggest that the induction of RARβ2 expression is mediated primarily by RAR, not RXR.

Using this promoter construct, we examined the at-RA sensitivity of the RARβ2 promoter in the monkey fibroblast cell line CV-1 and mouse osteoblast cell line MC3T3-E1. Although promoter activity can also be induced by at-RA in these cell lines, the level of induction is much lower than that observed in the retina (Fig. 3B). These results, consistent with other reports, indicate that there is cell type-specific modulation of promoter activity and/or at-RA sensitivity (10, 15, 26, 46).

Orphan receptor TLX potentiates at-RA induction of RARβ2 promoter. To investigate whether the above cell type specificity is a result of the amount of RARs and/or RXRs expressed in the cells, we tested the effect of overexpression of RARs or RXRs in CV-1 cells. RARα or RXRα only weakly potentiated at-RA induction of the RARβ2 promoter (Fig. 4A). RARβ, RARγ, RXRβ, RXRγ, or combinations of RARs and RXRs were also unable to significantly potentiate at-RA induction (data not shown). We examined the effect of various orphan nuclear receptors and found that TLX, which is expressed in retina, fore- and midbrain, and nasal epithelium, potentiated at-RA response in CV-1 cells (Fig. 4A). This effect was observed using chick, mouse, and human TLX constructs on both chick and mouse RARβ2 promoter reporter constructs, indi-

cating that conservation of TLX-mediated at-RA signaling has occurred through evolution (Fig. 4B to D). These results, along with the fact that primary chick retina cells but not CV-1 or MC3T3-E1 cells (R. T. Yu, unpublished results) express high endogenous levels of TLX, suggested the involvement of TLX in activation of the RARβ2 promoter in retina cells.

Identification of a TLX-responsive element. To investigate the mechanism by which TLX modulates RA activation of the RARβ2 promoter, we made a series of deletion constructs. In chick retina cells, these constructs were equally induced by at-RA, thus posing difficulty for identification of the TLX-responsive element using this system. CV-1 cells, on the other hand, lack endogenous TLX and thus served as a good system for this analysis. Transient transfection of the above deletion constructs revealed that fragments as short as -85 relative to the transcription start site were sufficient and that the region between -85 and -66 was necessary to observe the TLX effect on at-RA response (Fig. 4D). Examination of this region revealed the presence of a putative RARE. Based on its function as described below, this element is referred to as the silencing element relieved by TLX (SET) and is conserved between mammalian and avian RARβ2 promoters (Fig. 4D and 5A)

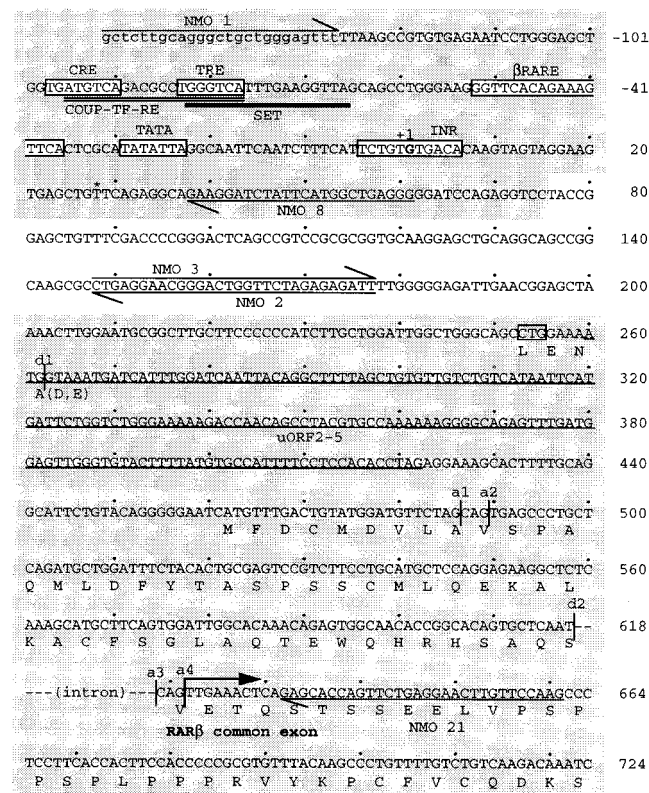


FIG. 2. Nucleotide sequence and predicted amino acid sequence of chick RARβ. PCR primers used are indicated by arrows. The putative transcription start site is designated +1. The cAMP RE (CRE), AP-1 binding site (TRE), βRARE, TATA box, and RNA polymerase initiator site (INR) sequences are indicated by boxes. An asterisk (*) indicates the 5' end of the cDNA confirmed by cDNA library screening. Splicing donor and acceptor sites are indicated by "d" and "a", respectively. The boxed CTG indicates the non-AUG initiation codon proposed for the mRARβ4 transcript (37). The large arrow indicates the common region for all RARβ transcripts. The regions of short uORFs (uORF2 to -5) reported for mouse RARβ2 (42) are underlined, and regions highly conserved between mammals and avians are indicated by shaded boxes. COUP-TF-RE (26) and SET sequences are indicated by double and bold underlines, respectively.

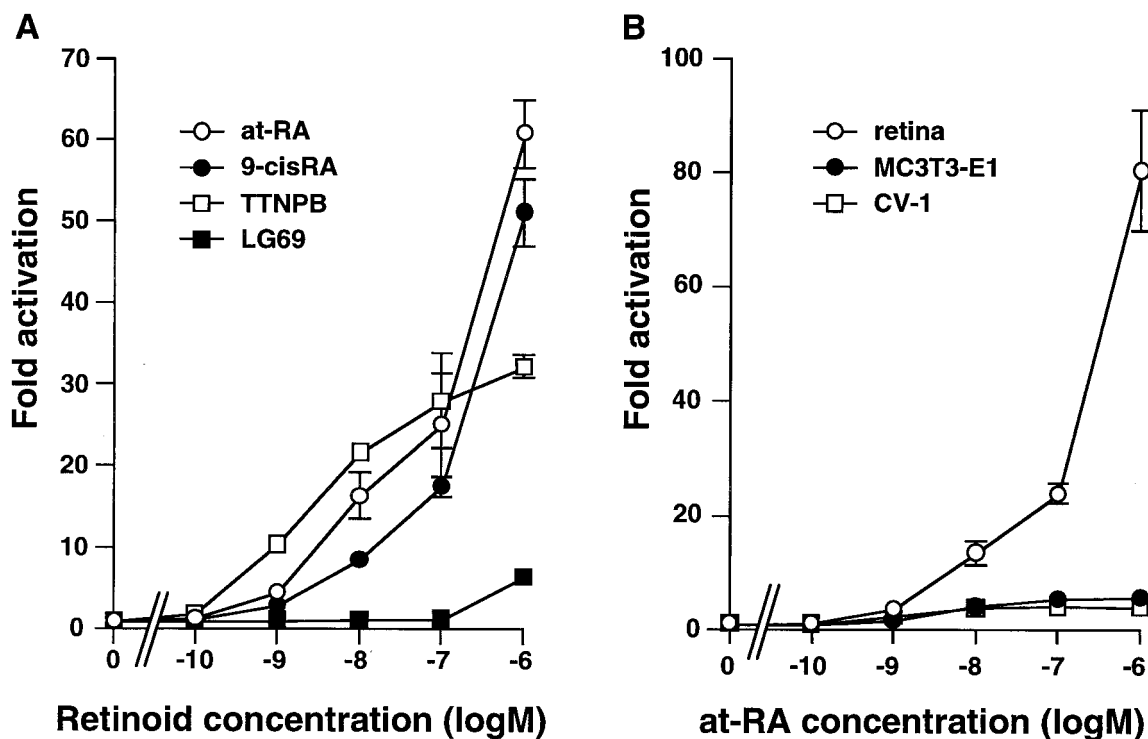


FIG. 3. (A) RAR β 2 promoter activity is induced by RAR agonists but not by RXR-specific agonists. cRAR β 2 promoter-driven luciferase reporter plasmids (cRAR β 2- Δ 119-LUC) were transfected into primary cultured chick retina cells. Final concentrations of retinoids are indicated. (B) Induction of cRAR β 2 promoter activity by at-RA is cell type specific. cRAR β 2 promoter-driven luciferase reporter plasmids were transfected as described for Fig. 3A. at-RA was added to the indicated final concentrations. Promoter activity is induced by at-RA in all cell types, but the magnitude of induction differs.

(10, 11, 47). To test the at-RA and TLX responsiveness of SET, we subcloned each element into a luciferase reporter driven by a tk minimal promoter. Transient transfection analysis revealed that SET does not function as a RARE in chick retina cells that express a high endogenous level of TLX (Fig. 5B). Consistent with the transfection results, gel retardation assays revealed that RAR-RXR heterodimers cannot bind to SET (Fig. 5C). TLX binds to neither SET nor β RARE (Fig. 5D) and does not appear to affect the binding activity of RAR-RXR to a composite β RARE-SET (Fig. 5E). Likewise, COUP-TFII, another orphan nuclear receptor shown to be important for the at-RA dependent activation of the RAR β 2 promoter (26), did not affect the binding of RAR-RXR to β RARE-SET (Fig. 5E).

Reconstitution of a TLX-sensitive promoter with SET and β RARE. Similar to the situation observed with the deletion constructs, tk-luciferase constructs containing β RARE with or without SET showed the same level of induction by at-RA in chick retina cells (Fig. 6A). Using CV-1 cells, we could examine the effect of TLX more clearly. SET does not function as a RARE in CV-1 cells, and separately, neither SET nor β RARE confers an effect in response to TLX in CV-1 cells (Fig. 6B and C). However, when SET and β RARE are introduced together in a tk-luciferase reporter, the at-RA induction conferred by β RARE is suppressed (Fig. 6C and D) and expression of TLX relieves this silencing effect (Fig. 6C and D). This effect of SET on β RARE appears position independent; similar results were observed when the relative positions of SET and β RARE were flipped (Fig. 7B). The ability of TLX to restore the promoter activity of SET- β RARE to the same level obtained with β RARE alone but no further and the inability of TLX to directly bind either SET or β RARE suggest that SET binds a putative repressor which can be sequestered by TLX.

The silencing effect of SET is independent of that of TRE or COUP-TF-RE. To see if the silencing effect of SET is related to TRE or COUP-TF-RE (26), we introduced mutations in the region of SET overlapping with or outside those elements (Fig. 2 and 7). Mutations introduced within the overlapping region retain the silencing effect to some extent, making interpretation difficult. However, mutations introduced outside the overlapping region clearly resulted in loss of the suppression effect of SET in CV-1 cells (Fig. 7), showing that elements in the SET region independent of TRE or COUP-TF-RE are indispensable for the SET silencing effect.

DISCUSSION

Evolutionary conservation of promoter region and splicing variations of RAR β 2. Autoregulation of RAR β transcripts by at-RA has been reported in human, mouse, and chick (11, 39, 45, 51, 54). We demonstrated that in chick the RAR β 2 promoter is responsible for this autoregulation and that RAR(s), not RXR(s), appears to be the primary target of RA. Comparison of the chick RAR β 2 promoter region with that of human and mouse revealed a strong overall conservation of structural organization, 90% in the 150-bp region surrounding the transcription start site. As for mouse, chick embryonic retina cells also express several isoforms of RAR β mRNAs. Some of the short uORFs (uORF2 to -5) which are hypothesized to be important for regulating translation of the transcripts (42), as well as the CTG start codon proposed for mouse RAR β 4, are conserved in chick (Fig. 2) (37). Together with the report that the RAR β 4 is functionally tumorigenic compared to the β 2 isoform (3), our results suggest a conserved indispensable role for the autologous regulation and splicing variation of the RAR β gene.

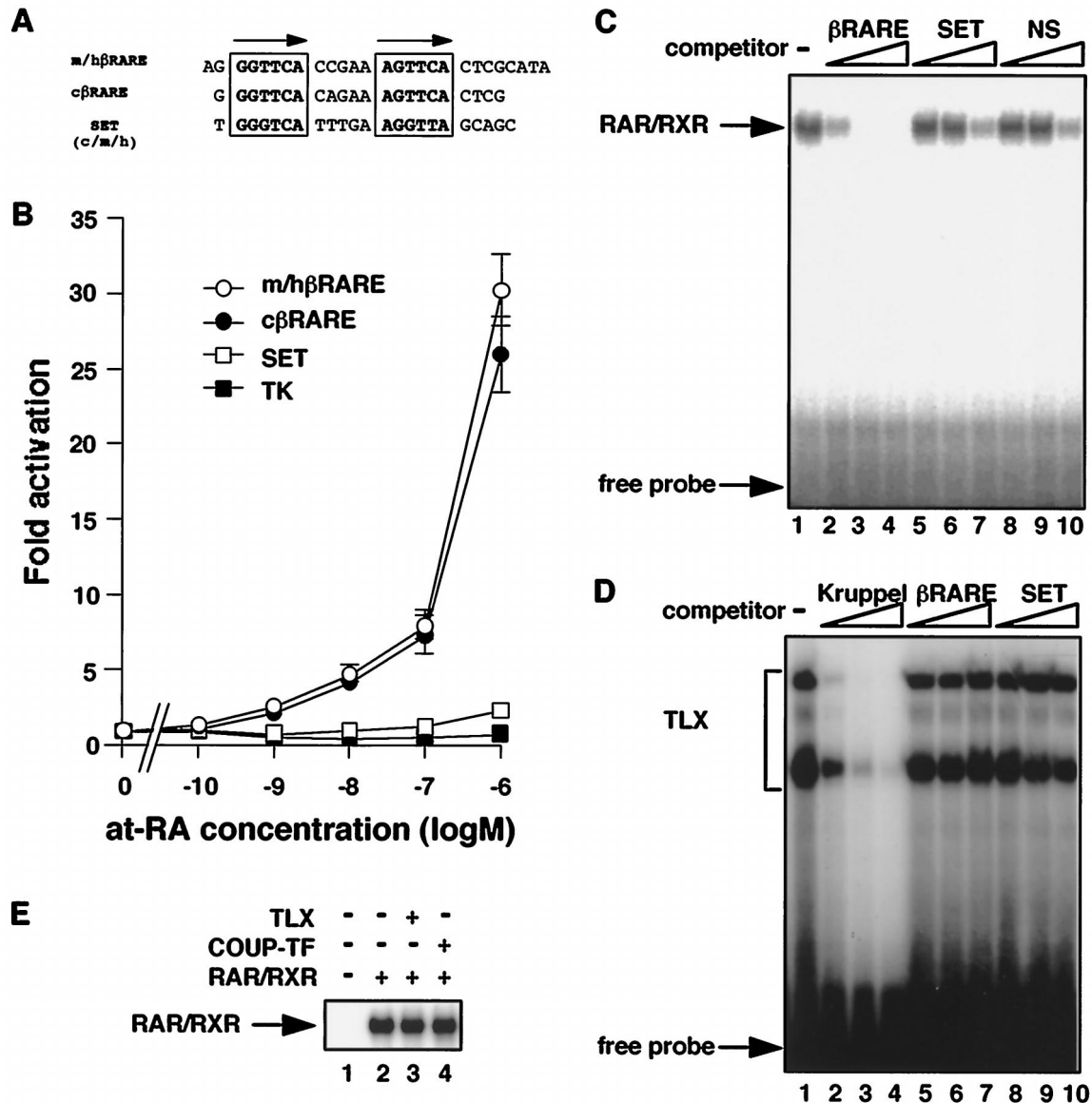


FIG. 5. SET does not function as a RARE. (A) Three DR-5-like elements were tested for RA responsiveness. Synthetic oligonucleotides with *Hind*III linkers were ligated in the tk-driven luciferase reporter plasmid. (B) Only authentic βRAREs in the RARβ2 promoter region confer the effect of at-RA induction. (C) DNA binding assays. RAR-RXR heterodimers do not bind to SET. RAR-RXR bound to labeled βRARE probe is blocked by competition from the addition of excess βRARE unlabeled probes (lanes 2 to 4) but not by SET or nonspecific (NS) probes (lanes 5 to 10). Competitor probes are indicated above, with 2-fold excess added in lanes 2, 5, and 8; 20-fold excess in lanes 3, 6, and 9; and 200-fold excess in lanes 4, 7, and 10. (D) TLX does not bind directly to SET. TLX bound to labeled *Krüppel* probe is blocked by competition from the addition of excess *Krüppel* unlabeled probes (lanes 2 to 4) but not by βRARE or SET probes (lanes 5 to 10). Competitor probes are indicated above, with 4-fold excess added in lanes 2, 5, and 8; 20-fold excess in lanes 3, 6, and 9; and 40-fold excess in lanes 4, 7, and 10. (E) TLX does not affect binding of RAR-RXR to SET-βRARE. RAR-RXR bound to labeled SET-βRARE probe (lane 2) is not affected by the addition of TLX (lane 3) or COUP-TF (lane 4). -, absence of substance; +, presence of substance.

element, SET, as a cell type-specific enhancer by deletion analysis of the RARβ2 promoter. SET acts as a cell type-specific repressor in fibroblasts, and TLX is able to relieve this silencing effect of SET.

TLX is a member of the nuclear receptor subfamily II, which consists of TLX, photoreceptor-specific nuclear receptor (PNR), COUP-TFs, RXRs, hepatocyte nuclear factor 4 (HNF-4), and TR2 (6, 14, 21). We have shown that TLX can down-regulate Pax2 expression by direct binding to its promoter region (56). COUP-TFs are also known to repress the transcription of various genes through the COUP-TF binding element (52), and overexpression of COUP-TFs in CV-1 cells can

repress RARβ2 promoter activity (M. Kobayashi and K. Umesono, unpublished results).

However, the expression level of COUP-TFs has been reported to be positively correlated with the at-RA responsiveness of the RARβ2 promoter in human cancer cell lines (26). COUP-TFs have also been reported to act as auxiliary cofactors for HNF-4 in the activation of the liver-specific HNF-1 promoter (23). COUP-TFs do not directly bind to the promoter but influence promoter activity through protein-protein interaction with HNF-4. TLX may similarly interact with a repressor protein that binds to SET and through this interaction relieve its repression. It is also possible that TLX and the

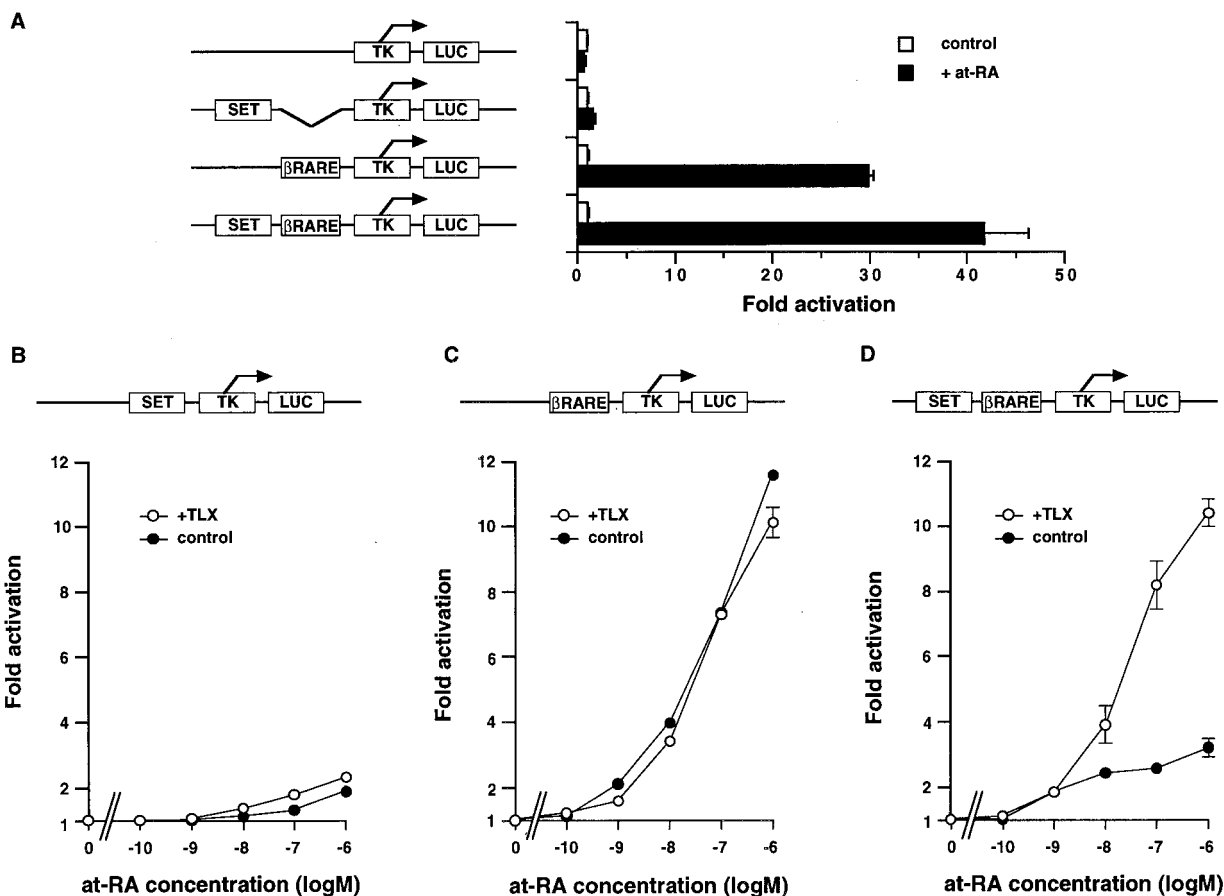


FIG. 6. Reconstitution of TLX responsiveness of the RAR β 2 promoter. (A) tk-luciferase reporter constructs with synthetic oligonucleotides depicted in Fig. 5A were transfected into chick retina cells. SET does not have any effect in retina cells. at-RA was added to a final concentration of 1 μ M. (B to D) The same plasmids were transfected into CV-1 cells. The introduction of SET and β RARE in the same construct restores the effect of at-RA- and TLX-dependent transactivation. Final concentrations of at-RA are indicated.

SET binding protein may compete for a cofactor. The intact structure of TLX appears to be required, as overexpression of the TLX DNA binding domain or ligand binding domain alone does not potentiate activation (data not shown). Overexpression of the TLX DNA binding domain fused to the VP16 activation domain or engrailed repressor domain (56) likewise did not potentiate activation (data not shown). These results suggest that potentiation likely does not occur through the direct DNA binding activity of TLX. COUP-TFs as well as RAR-RXR heterodimers have been reported to enhance the activation of the estrogen receptor (ER) promoter together with ER upon the addition of estrogen (25). This interaction of COUP-TF and ER on the ER promoter resembles to some extent the relationship between TLX and RAR on the RAR β 2 promoter.

Together, these observations appear to suggest that enhancement or repression by TLX and/or COUP-TFs is highly dependent on other factors specific to individual cell lines and/or each promoter context. Given that we were able to reconstruct TLX-responsive ability with SET and β RARE in the context of the tk promoter, our finding provides an important step towards revealing some of the mechanisms behind transcriptional regulation by cell-specific factors.

To further explore the transcriptional mechanisms involving TLX, the identification of its interacting protein is necessary. Recently, Lin et al. found that at-RA-dependent RAR β 2 transcription requires direct binding of COUP-TFs to the element

overlapping SET on the RAR β 2 promoter and that COUP-TFs enhance the interaction of RAR with its coactivator, CREB-binding protein (Fig. 2) (26). However, we found that introducing mutations in the SET region outside the COUP-TF-RE resulted in loss of the suppression effect of SET in CV-1 cells (Fig. 7). Therefore, it seems that the effect of SET that we observed occurs through distinct mechanisms.

Based on analogy with other nuclear receptors, corepressor proteins, such as SMRT and Nco-R, are good candidates for TLX-interacting protein (reviewed in reference 30). Whether the effect of SET and TLX might also be applicable to different REs for other RXR heterodimers is an intriguing future question too, and such studies may reveal other aspects of TLX function.

The function of TLX and RAR β in eye development. The importance of TLX function in vertebrate eye development has been implicated by gene knockout experiments in mice and the introduction of dominant-negative and dominant-active TLX constructs in chick and *Xenopus* (17, 56). These observations suggest that TLX is involved in the signaling pathways regulating various steps in retina and optic nerve formation and maintenance. Northern and in situ hybridization analyses of early chick embryos confirmed its restricted expression in fore- and midbrain and retina (57; Yu and Kobayashi, unpublished results). In this report, we showed that the signal transduction pathways controlled by RAR β can also be influenced by TLX. This finding is significant because although gene knock-

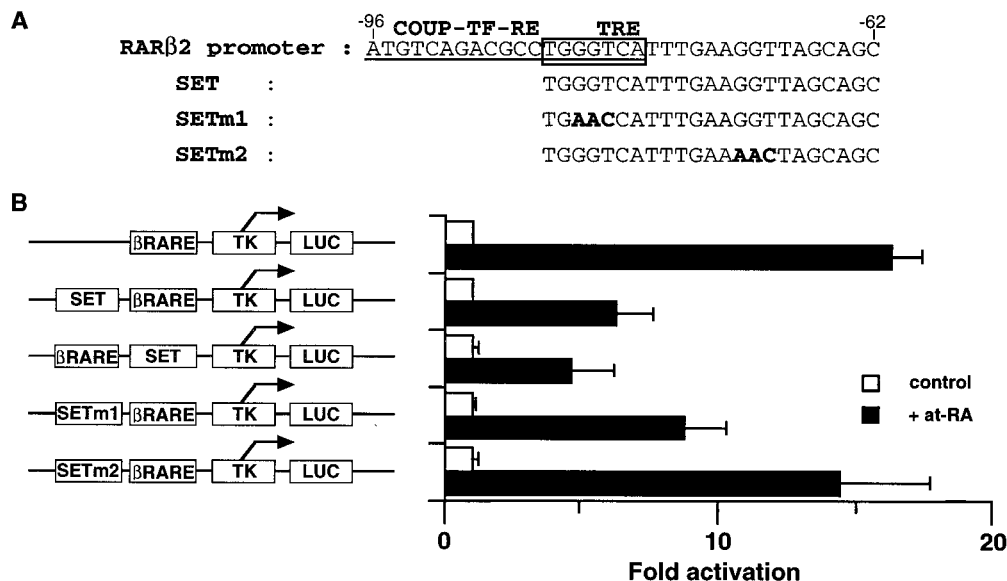


FIG. 7. The silencing effect of SET is independent of that of TRE or COUP-TF-RE. (A) Sequences of mutated SET oligonucleotides are aligned with that of the RAR β promoter. TRE, COUP-TF-RE (26), and mutated residues are boxed, underlined, and boldfaced, respectively. (B) tk-luciferase reporter constructs with synthetic oligonucleotides were transfected into CV-1 cells. The silencing effect of SET is position independent. Introducing mutations in the SET region outside TRE or COUP-TF-RE resulted in loss of the suppression effect of SET. at-RA was added to a final concentration of 1 μ M.

out and transgenic experiments in mice have suggested the importance of RAR β in eye development (16, 19, 20, 27), little is known about its upstream regulation.

Considering the high degree of structural and functional conservation of TLX and the RAR β promoters, it is reasonable to predict a fundamental role for TLX in the regulation of RAR in eye development. Our findings should provide a new contribution to better understanding of the complexity underlying the mechanisms of eye development.

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