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Retinoids Directly Activate the Collagen X Promoter in Prehypertrophic Chondrocytes Through a Distal Retinoic Acid Response Element

Arthur J. Cohen, Luisa Lassová, Eleanor B. Golden, Zeling Niu, and Sherrill L. Adams*

Department of Biochemistry, University of Pennsylvania, School of Dental Medicine, Philadelphia, Pennsylvania 19104

Abstract Retinoids are essential for the terminal differentiation of chondrocytes during endochondral bone formation. This maturation process is characterized by increased cell size, expression of a unique extracellular matrix protein, collagen X, and eventually by mineralization of the matrix. Retinoids stimulate chondrocyte maturation in cultured cells and experimental animals, as well as in clinical studies of synthetic retinoids; furthermore, retinoid antagonists prevent chondrocyte maturation in vivo. However, the mechanisms by which retinoids regulate this process are poorly understood. We and others showed previously that retinoic acid (RA) stimulates expression of genes encoding bone morphogenetic proteins (BMPs), suggesting that retinoid effects on chondrocyte maturation may be indirect. However, we now show that RA also *directly* stimulates transcription of the collagen X gene promoter. We have identified three RA response element (RARE) half-sites in the promoter, located 2,600 nucleotides upstream from the transcription start site. These three half-sites function as two overlapping RAREs that share the middle half-site. Ablation of the middle half-site destroys both elements, abolishing RA receptor (RAR) binding and drastically decreasing RA stimulation of transcription. Ablation of each of the other two half-sites destroys only one RARE, resulting in an intermediate level of RAR binding and transcriptional stimulation. These results, together with our previously published data, indicate that retinoids stimulate collagen X transcription both directly, through activation of RARs, and indirectly, through increased BMP production. J. Cell. Biochem. 99: 269–278, 2006. © 2006 Wiley-Liss, Inc.

Key words: retinoids; chondrocytes; collagen X; retinoic acid receptors

During endochondral bone formation, mesenchymal cells initially differentiate into chondrocytes, forming cartilage elements that serve as templates for most bones of the skeleton [reviewed in Kronenberg, 2003]. The chondrocytes then undergo a process of maturation or terminal differentiation, during which they become hypertrophic, initiate production of collagen X, a protein unique to the hypertrophic cartilage matrix, and increase production of alkaline phosphatase. The hypertrophic cartilage matrix subsequently becomes mineralized and is eventually resorbed and replaced by bone. Several signaling molecules stimulate chondrocyte maturation, including retinoids (the active metabolites of vitamin A) and bone morphogenetic proteins (BMPs).

Retinoid involvement in skeletogenesis was first suggested over 50 years ago by nutritional studies indicating that both vitamin A excess and deficiency lead to disturbances in chondrocyte maturation [Wolbach and Hegsted, 1952, 1953; Wilson et al., 1953]. We showed previously that treatment of cultured chicken sternal chondrocytes with physiological concentrations of retinoic acid (RA) stimulates hypertrophy, production of collagen X and alkaline phosphatase, and mineralization of the extracellular matrix, all hallmarks of terminal differentiation [Oettinger and Pacifici, 1990; Pacifici et al., 1991; Iwamoto et al., 1993a,b, 1994; Koyama et al., 1999]. Furthermore, release of retinoid antagonists into the developing chick limb prevents collagen X gene

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*Correspondence to: Sherrill L. Adams, Department of Biochemistry, University of Pennsylvania School of Dental Medicine, 240 S. 40th St., Philadelphia, PA 19104-6030. E-mail: sherri@biochem.dental.upenn.edu

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expression and chondrocyte hypertrophy [Koyama et al., 1999], providing conclusive evidence that retinoids are essential for chondrocyte maturation.

Despite decades of research regarding the effects of retinoids on endochondral bone formation, the mechanisms by which retinoids induce terminal differentiation of chondrocytes remain largely unknown. We have used expression of the chick collagen X gene as a paradigm for analysis of the signaling pathways by which retinoids activate chondrocyte maturation, since expression of this gene is unique to hypertrophic chondrocytes. The promoter activity of this gene has been analyzed in an essentially homogeneous population of prehypertrophic chondrocytes from the upper sternum of 15-day-old chick embryos [Gibson and Flint, 1985; Volk et al., 1998]. We showed that a collagen X promoter construct containing the 640 bp proximal promoter and the upstream *b2* fragment (nucleotides −2,648 to −2,006) (*b2*/*Prox*; see Fig. 1A) was stimulated about sevenfold after 24 h of treatment with 50 nM RA [Adams et al., 2003], a concentration within the physiological range [Maden et al., 1998]. We showed further that a 130-bp region of the chick collagen X promoter, located over 2.5 kb upstream of the transcription start site, is essential for stimulation by RA [Adams et al., 2003] (Fig. 1A). However, initial computer analysis of this RA responsive promoter region did not identify any binding sites for retinoic acid receptors (RARs) [Volk et al., 1998]. RARs function as ligand-activated transcription factors, binding DNA as heterodimers with RXRs (retinoid X receptors) to an RA response element (RARE) consisting of two direct repeats of a hexameric half-site ($5'\ {}^{A}/_{G}G^{G}/_{T}TCA$ in either orientation) separated by two or five nucleotides (Fig. 1B) [Mangelsdorf et al., 1994]. In the apparent absence of RAR binding sites, we and others have suggested that RA may activate collagen X gene transcription indirectly by stimulation of BMP signaling, since RA stimulates both increased production of BMPs themselves and increased activation of the BMP-regulated transcription factors Smads 1 and 5 [Grimsrud et al., 1998; Adams et al., 2003; Li et al., 2003]. BMPs were previously shown to stimulate collagen X transcription [Volk et al., 1998], and cotransfection of Smads 1 and 5 increased collagen X promoter activity [Drissi et al., 2003; Leboy et al., 2003].

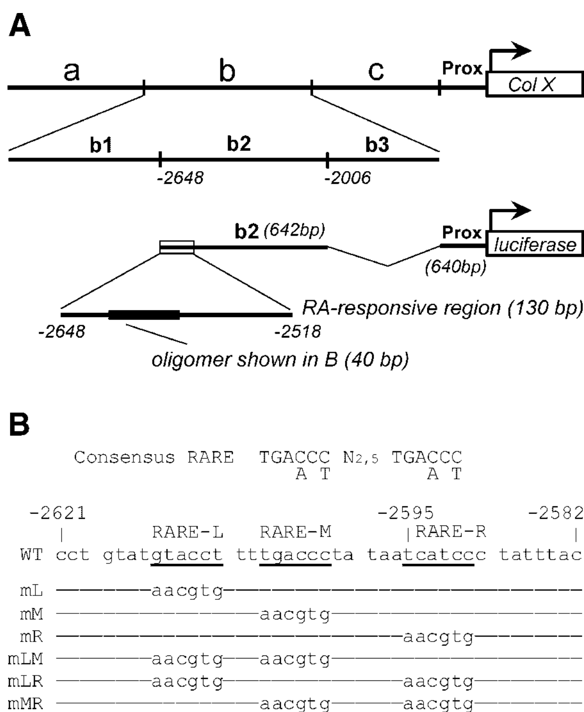

Fig. 1. Retinoic acid responsive region of the chick collagen X promoter. **A**: Diagram of the promoter of the collagen X gene indicating the origin of the *b2* fragment containing the RA-responsive region; diagram adapted from Volk et al. [1998]. **B**: Sequence of the consensus RARE, as well as the WT and mutant oligonucleotides used for electrophoretic mobility shift assays. This portion of the RA-responsive region of the WT Col X promoter contains three potential RARE half-sites, RARE-L, -M, and -R. The identical mutations were introduced into the *b2/Prox* collagen X promoter for transfection assays.

In the experiments shown below, we demonstrate for the first time that retinoids exert a *direct* effect on collagen X transcription through a previously unidentified compound RARE located between −2614 and −2590 nucleotides upstream from the transcription start site. This compound RARE is bound predominantly by RARγ, previously identified as the major RAR in embryonic chick prehypertrophic and hypertrophic chondrocytes [Koyama et al., 1999].

MATERIALS AND METHODS

Chondrocyte Cell Culture

Prehypertrophic chondrocytes were isolated from the upper sterna of 15-day-old chick embryos (BE eggs, Ephrata, PA), as previously described [Leboy et al., 1989, 1997, 2000]. Briefly, after primary culture for 5 days, floating cells were harvested and plated in secondary culture at 5×10^6 cells/10 cm culture plate for

preparation of nuclear extracts or 4×10^5 cells/well in 12-well plates for transfection. Chondrocytes in secondary culture were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) containing 10% NuSerum IV (Collaborative Biomedical, Bedford, MA), penicillin/streptomycin (10 U/ml), hyaluronidase (4 U/ml) to promote attachment (Sigma Chemical, St. Louis, MO), and 2 mM L-glutamine (Sigma). For RA treatment, the medium was replaced with serum-free DMEM containing insulin (60 ng/ml) and cysteine (1 mM) [Leboy et al., 1997]. All-trans RA (50 nM) was added to some cultures for the final 15 h; this RA concentration is within the physiological range [Maden et al., 1998]. For the time course, RA was added 24, 15, or 8 h prior to harvest.

Electrophoretic Mobility Shift Assays

Chondrocytes were grown in secondary culture for 3–4 days and nuclear extracts were prepared using standard procedures [Andrews and Faller, 1991]. Double-stranded oligonucleotide DNA probes (Sigma Genosys, The Woodlands, TX) representing the wild-type (WT) RA-responsive region, as well as several mutations (Fig. 1B), were radiolabeled with $\gamma[^{32}P]ATP$ using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Eight micrograms of nuclear protein were incubated with 10^4 cpm (~0.03 ng) of double-stranded oligonucleotide in the presence of 4 μg of poly [d(I-C)] non-specific competitor (Roche Diagnostics GmbH, Mannheim, Germany) at room temperature for 30 min in 25 mM HEPES, pH 7.5, 12% glycerol, 80 mM KCl, 2 mM DTT, 0.5 mg/ml BSA, with protease inhibitor cocktail (Sigma). Specificity of the binding reactions was determined by preincubation of the nuclear extracts for 5 min with a 150-fold molar excess of the indicated cold competitor oligonucleotides, including a consensus RARE (sc-2559, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), before the addition of radiolabeled probe. RAR components of protein-DNA complexes were identified by addition of the following antibodies (Santa Cruz Biotechnology) to the binding reactions: sc-551X, which recognizes an epitope at the C-terminus of human RARα that is conserved across species; sc-14028X, which recognizes an epitope at the N-terminus of human RARβ that is conserved across species; and sc-773X, which was prepared against full-length human RARγ and recognizes all RAR isoforms. There appear to be no antibodies available that are specific for RARγ which cross-react with the chick protein. DNA-protein complexes were separated on non-denaturing 5% polyacrylamide gels in 35.6 mM Tris, 35.6 mM boric acid, and 10 mM Na_2EDTA. After electrophoresis the gels were dried and placed in cassettes with MS biofilm (Eastman Kodak Co., New Haven, CT) for autoradiography.

Site-Directed Mutagenesis

Substitution mutagenesis of the collagen X promoter was performed using the Genetailor site-directed mutagenesis system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions using *b2/Prox* (Fig. 1A, formerly called *b2/640* [Volk et al., 1998]) as the template for construction of RARE mutations *mL* (mutant Left), *mM* (mutant Middle), and *mR* (mutant Right). For mutant *mLR* the template was *mL* DNA. Primers used for construction of the substitution mutants are listed in Table I.

Real-Time Quantitative PCR

Real-time polymerase chain reaction (PCR) was performed in the Smart Cycler PCR detection system (Cepheid, Sunnyvale, CA) using LightCycler FastStart DNA master SYBR Green I (Roche Diagnostics GmbH, Mannheim,

TABLE I. Primers Used for Site-Directed Mutagenesis and PCR

Mutant	Reverse 3′ primer	Forward 5′ primer
mL	atacaggcaaaactgttgtcctttaatgtg	gtttgcctgtataacgtgtttgaccctataatcatc
mM	aaaggtacatacaggcaaaactgttgtccttt	gtttgcctgtatgtacctttaacgtgtataatcatc
mR	ttatagggtcaaaaggtacatacaggcaaaac	gtacctttgaccctataaaacgtgctatttacaca
mLR	ttatagggtcaaacacgttatacaggcaaa	taacgtgtttgaccctataaaacgtgctatttacac
Target gene (accession #)		
RARα (X73972)	ttgttgggcctcctcttcctcacg	ccgaccagatcacgctgctgcaaag
RARβ (X57341)	ggaacaagttcctcagaactggtgctc	acaaccagcagccgcacatgtc
RARγ (X73973)	gcgtcaccttgttgatctggcagtt	ctcggaggagatggtgcccaattc

All primers are shown in the 5′–3′ direction. RARE half-sites are underlined; mutated half-sites are shaded. The GenBank accession numbers for RAR α, β, and γ sequences are shown.

Germany). Total cellular RNA was prepared from chondrocytes grown in secondary culture for 4 days. The template for PCR was 6 μl of cDNA synthesized with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) using oligo-dT primers. The thermal cycling conditions included an initial heat-denaturing step at 95°C for 10 min, then 45 cycles at 95°C for 15 s, 70°C for 30 s, and 72°C for 30 s. A single fluorescence measure was taken after each annealing step. Following amplification, the melting curves of PCR products were determined to confirm the specificity of the amplification. The cycle threshold (Ct) was defined as the number of cycles at which the fluorescence passed a fixed threshold above baseline. PCR primers for the RAR genes are listed in Table I.

Transient Transfection and Reporter Assays

The promoter mutants were cloned into the *Renilla* luciferase reporter vector pRL-null (Promega Corp., Madison, WI). Chondrocytes were transfected 1 day after plating in monolayer culture using Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) with 100 ng of promoter construct per well plus 200 ng of the plasmid PGL2 (Promega) to control for transfection efficiency. Plasmid DNA was removed from the cells after 5 h and the medium was replaced with fresh serum-free medium; 50 nM RA was added to some cultures 15 h prior to harvest. After 48 h the cells were lysed and luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activity was determined using an Optocomp I luminometer (MGM Instruments, Inc., Hamden, CT). Statistical analysis was performed using an unpaired *t*-test; values of $P \leq 0.05$ were considered significant.

RESULTS

The Collagen X Promoter is Rapidly Activated by RA

We initially determined the kinetics of the RA response by transfecting chick sternal chondrocytes with the RA-responsive promoter construct *b2/Prox* (Fig. 1A); these cells were treated for varying lengths of time with RA and assayed for luciferase activity. The time course shown in Figure 2 indicates that promoter activity increases relatively rapidly in

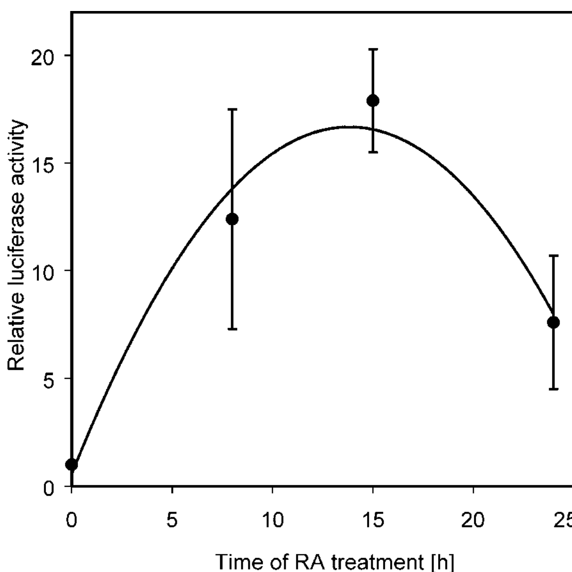

Fig. 2. The collagen X *b2/Prox* promoter responds rapidly to RA treatment. Chondrocytes were transfected with the collagen X reporter plasmid, then cultured in serum-free medium for 48 h; cells were treated with 50 nM RA for 0, 8, 15, or 24 h. Each point represents the average of three independent experiments done in triplicate; error bars equal one standard deviation.

response to RA, reaching about 12-fold stimulation by 8 h and 17-fold by 15 h, then falling to about 8-fold by 24 h. This rapid response is similar to that of other genes that are known to be activated directly by RA. For example, Era-1 (early retinoic acid-1)/Hox 1.6, a gene that is directly activated by RA in F9 cells, reaches peak levels at 12–24 h of RA treatment [LaRosa and Gudas, 1988], similar to collagen X in prehypertrophic chondrocytes. This result led us to consider the possibility that retinoids may act on collagen X not only indirectly, through stimulation of BMP signaling, but also through a direct activation mechanism. Thus we reanalyzed the RA-responsive region of the collagen X promoter for sequences that could function as binding sites for RARs.

RARs Bind to the RA-Responsive Region of the Collagen X Promoter

The previously identified RA-responsive region of the collagen X promoter consists of 130 bp extending from nucleotide −2,648 to −2,518 (Fig. 1A); removal of this region significantly decreased RA-responsiveness of the collagen X *b2/prox* promoter [Adams et al., 2003]. Re-analysis of this sequence with the computer program TESS [Schug and Overton, 1997] did not identify an RARE consisting of two

6-nucleotide half-sites, but did identify a single consensus half-site in reverse orientation, 5′ TGACCC (RARE-M in Fig. 1B). We subsequently identified two additional potential half-sites by visual inspection, one (5′ GTACCT) located 2 bp upstream of RARE-M (RARE-L in Fig. 1B), the other (5′ TCATCC) 5 bp downstream (RARE-R in Fig. 1B). Each of these differs from the consensus by two nucleotides and differs from the RARE half-sites in over 30 RA-responsive genes that we have analyzed. However, since the two and five nucleotide spacing between half-sites is consistent with the sequence of consensus RAREs [Mangelsdorf et al., 1994], it seemed possible that the three potential half-sites could comprise two overlapping RAREs that share the middle half-site.

We performed electrophoretic mobility shift assays to determine whether these sequences contained any functional RARE. A WT 40 bp oligonucleotide that included all three potential half-sites (WT in Figs. 1B and 3A) was incubated with nuclear extracts from cultured chondrocytes isolated from the upper sternum of chick embryos. Several protein-DNA complexes were formed on the WT oligonucleotide (Fig. 3B, lane 2). The complexes of slowest mobility (indicated as "shift" in Fig. 3) were abolished by competition with excess cold WT

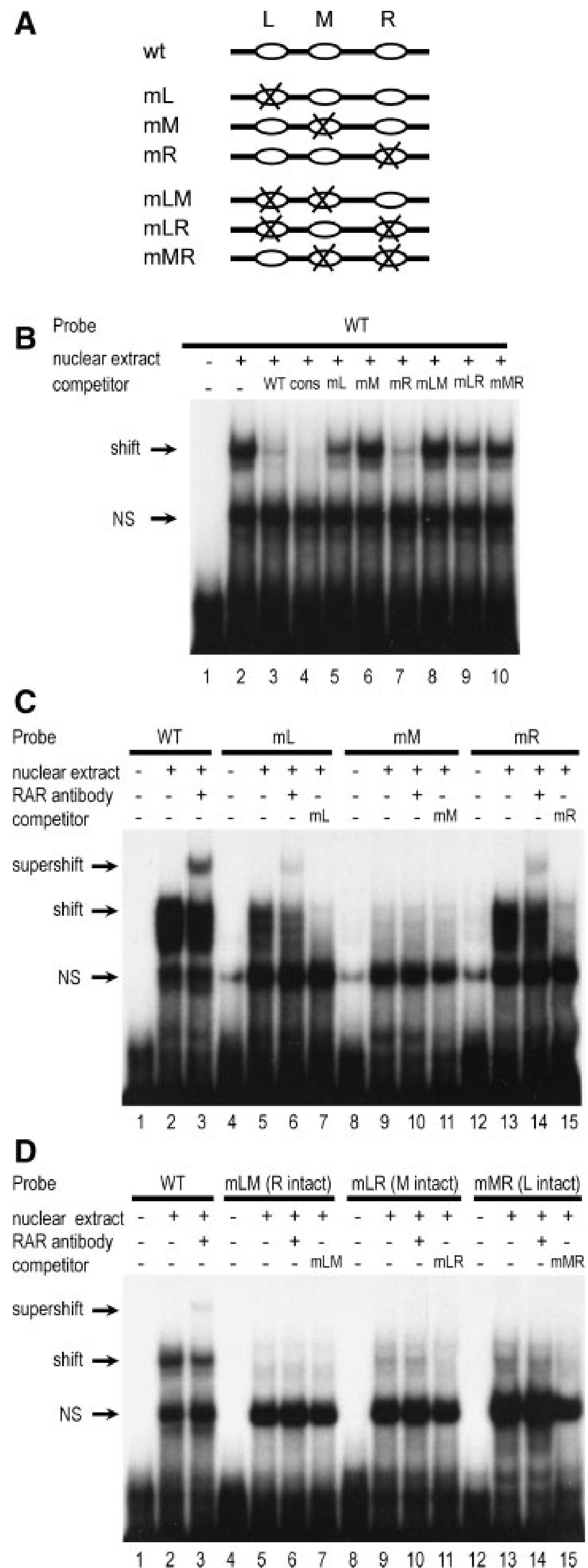

Fig. 3. The RA-responsive region of the collagen X promoter contains two functional RAREs that bind RARγ. **A**: Schematic diagram showing the wild-type (WT) oligonucleotide with three RARE half-sites, L, M, and R, as well as oligonucleotides with mutations in one or more half-sites, shown with an X through the mutant half-site; sequences of these oligonucleotides are shown in Figure 1B. In **panels B–D**, **lane 1** shows oligonucleotide probe incubated in the absence of nuclear extract; "shift" indicates specific protein-DNA complexes; "supershift" indicates complexes supershifted by addition of anti-RARγ; "NS" indicates non-specific complexes. B: The radiolabeled 40-bp WT oligonucleotide was incubated with chondrocyte nuclear extract in the absence (**lane 2**) or presence of a 150-fold molar excess of the following unlabeled cold competitors: *WT* (**lane 3**); *cons*, an oligonucleotide containing a consensus RARE (**lane 4**); or mutant oligonucleotides *mL* (**lane 5**), *mM* (**lane 6**), *mR* (**lane 7**), *mLM* (**lane 8**), *mLR* (**lane 9**), *mMR* (**lane 10**). C: WT and mutant oligonucleotides were incubated with chondrocyte nuclear extract in the absence of antibodies (**lanes 2**, **5**, **9**, **13**) or in the presence of an antibody against RARγ that recognizes all RAR isoforms (**lanes 3**, **6**, **10**, **14**). Specificity of binding was demonstrated by competition with a 150-fold molar excess of unlabeled cold self-competitor (**lanes 7**, **11**, **15**). D: WT and double mutant oligonucleotides *mLM*, *mLR*, and *mMR* were incubated with chondrocyte nuclear extracts in the absence of antibodies (**lanes 2**, **5**, **9**, **13**) or in the presence of the pan-RAR specific antibody used in C (**lanes 3**, **6**, **10**, **14**). Binding specificity was demonstrated by self-competition with a 150-fold molar excess of cold oligonucleotides (**lanes 7**, **11**, **15**).

oligonucleotide (Fig. 3B, lane 3), indicating their specificity. They were also abolished by competition with an oligonucleotide containing a consensus RARE (Fig. 3B, lane 4), suggesting that the WT oligonucleotide contains at least one RARE. The complex of faster mobility was not abolished by either competitor, indicating that it is not specific (NS).

Incubation of protein-DNA complexes with an antibody against human RARγ that recognizes all three RAR isoforms caused a supershift (Fig. 3C, lane 3), indicating that RARs are present in chondrocyte nuclear extracts and bind to the WT oligonucleotide. We showed previously using in situ hybridization that the major RAR isoform in embryonic chick prehypertrophic and hypertrophic cartilages is RARγ [Koyama et al., 1999]; these cells also express a small amount of RARα, but little detectable RARβ. They do not appear to be any antibodies available for use in electrophoretic mobility shift assays that both cross-react with chick RARγ and are specific for RARγ. However, an antibody against an epitope of human RARα that is identical in the chick detected only a small amount of this receptor, while an antibody against an epitope of human RARβ that is identical in the chick detected no RARβ (data not shown). Since the prehypertrophic chick chondrocytes appear to contain little RARα or β, we conclude that the RAR recognized by the antibody in Figure 3C (lane 3) must be predominantly RARγ, consistent with our previously described in situ hybridization results [Koyama et al., 1999]. We have confirmed these results in cultured prehypertrophic chondrocytes using real-time PCR. The Ct values for RARα, β, and γ were 23.0 ± 0.6, 23.0 ± 0.3 and 18.5 ± 0.2, respectively, a cycle threshold difference of 4.5. Thus RARγ is the major isoform in cultured chondrocytes as well as intact embryonic cartilages.

There was no difference in RAR binding comparing nuclear extracts from chondrocytes grown in the presence or absence of RA (data not shown). This result is consistent with previously published reports indicating that RARs bind DNA in the absence of ligand, although they are not transcriptionally active [Poujol et al., 2003].

The RA-Responsive Region of the Collagen X Promoter Contains Two Functional RAREs

To determine which of the three potential RARE half-sites were bound by RARs, we synthesized three mutated oligonucleotides (*mL*, *mM*, and *mR* in Figs. 1B and 3A), and used them as probes for electrophoretic mobility shift assays. Each half-site was mutated to AACGTG, which differs at every nucleotide from the consensus RARE sequence $TGA^{C}/_{A}C^{C}/_{T}$, thus is designed to lack RAR binding capacity. Mutation of the middle half-site, the only consensus half-site, in oligonucleotide *mM* abolished protein binding and the RAR antibody supershift (Fig. 3C, lanes 9 and 10), indicating that this half-site is essential. Mutation of the left and right half-sites, in oligonucleotides *mL* and *mR*, displayed intermediate levels of RAR binding (Fig. 3C, lanes 5, 6, 13, and 14). These results were confirmed using the WT oligonucleotide as a probe with each of the mutated oligonucleotides added as unlabeled competitors. As expected, mutant oligonucleotide *mM* did not compete at all for protein binding (Fig. 3B, lane 6), confirming that the middle half-site is essential for RAR binding. In contrast, oligonucleotides *mL* and *mR* (Fig. 3B, lanes 5 and 7) partially competed for protein binding, consistent with the fact that, when used as probes, they displayed intermediate binding affinity.

Since there are reports of nuclear receptors binding to single half-sites [reviewed in Privalsky, 2004], we asked whether any of the observed protein binding could be due to RAR binding to single RARE half-sites. We synthesized three more oligonucleotides that each retained one intact half-site, with the other two half-sites mutated (Figs. 1B and 3A, oligonucleotides *mLM*, *mLR*, and *mMR*). None of these oligonucleotides displayed a significant amount of protein binding (Fig. 3D), and none competed effectively for binding to the WT oligonucleotide (Fig. 3B), indicating that there is little RAR binding to single half-sites. There was also no supershift when antibodies were added to the reactions with any of the double mutants (Fig. 3D, lanes 6, 10, and 14).

Taken together, these data indicate that the three half-sites comprise two functional RAREs which share the middle half-site. However, RARs appear to have higher affinity for oligonucleotide *mR* than for *mL* (compare lane 14 with lane 6 in Fig. 3C), suggesting that the RARE comprised of the left and middle half-sites (a direct repeat separated by two nucleotides) functions better than the RARE

comprised of the right and middle half-sites (a direct repeat separated by five nucleotides).

Both RAREs Contribute to RA Responsiveness of the Collagen X *b2/Prox* Promoter

In the experiments described above, we identified two overlapping RAREs that share a middle half-site and demonstrated that both RAREs bind RARγ in vitro. To determine whether these sites play a role in stimulation of the collagen X promoter by RA in cultured cells, we created four mutants of the Col X *b2/Prox* promoter for use in transfection experiments (*mL*, *mM*, *mR*, and *mLR* in Figs. 1B and 3A). Each mutant had one or more half-sites replaced by AACGTG, the same sequence used to mutate the oligonucleotides in the gel shift assays described above. The plasmids were transfected into prehypertrophic chondrocytes and some were treated with 50 nM RA for the last 15 h of culture, the optimal time determined in Figure 2. Data are displayed as fold stimulation by RA (relative luciferase activity of RA-treated vs. untreated cells) (Fig. 4). The WT *b2/Prox* promoter was stimulated an average of 15-fold. Mutants *mL* and *mR*, each with one intact RARE, displayed a sevenfold RA stimulation, about half that of the WT promoter. In contrast, mutants *mM* and *mLR*, each with no intact bipartite RARE, displayed only a threefold RA stimulation, significantly less than either *mL* or *mR*. The decrease in RA stimulation was statistically significant for all mutants compared to the WT *b2/Prox* promoter ($P \leq 0.00003$). These results confirm that this region of DNA contains two functional RAREs that share a half-site. Mutagenesis of the middle half-site is the most deleterious, because it destroys both RAREs, while mutagenesis of the left or right half-site only destroys one RARE.

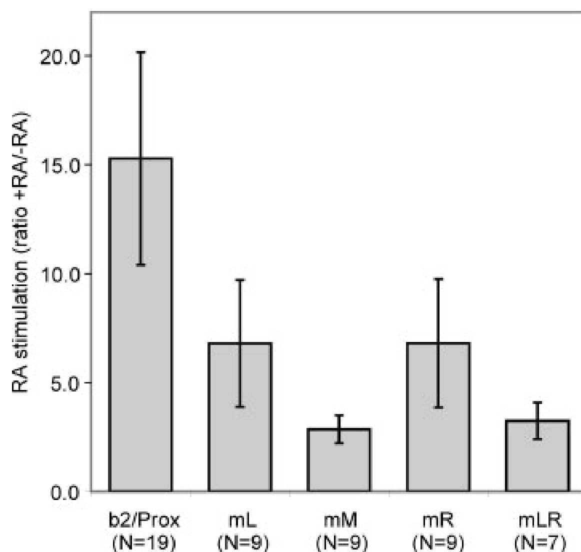

Fig. 4. Both RAREs play a role in RA stimulation of the collagen X *b2/Prox* promoter. The same RARE mutations analyzed in EMSAs (shown schematically in Fig. 3A) were introduced into the Col X b2/Prox promoter and analyzed by transfection into prehypertrophic chondrocytes; 50 nM RA was added for the last 15 h of culture. Data are expressed as fold RA stimulation over control for each promoter construct. Each point represents the average of 7–19 independent experiments done in triplicate; error bars show one standard deviation.

The fact that mutants *mM* and *mLR* of the Col X *b2/prox* promoter are still stimulated threefold by RA is consistent with our previously published data analyzing 5′-end deletion mutants of *b2/Prox* [Adams et al., 2003]. Deletion of 53 nucleotides from the 5′ end of the *b2* fragment, removing nucleotides −2,648 to −2,595 (indicated in Fig. 1B), removed the left and middle RARE half-sites, leaving no functional RARE. However, this promoter mutant also retained a threefold stimulation by RA. Only further deletion to nucleotide −2,518 completely abolished the RA stimulation, suggesting that there may be an additional, less active RA-responsive region between nucleotides −2,595 and −2,518.

DISCUSSION

Growth of endochondral bones requires a precise balance between chondrocyte proliferation and maturation or terminal differentiation; disruption of either process decreases bone growth [reviewed in Kronenberg, 2003]. This process is conserved among vertebrates, and is regulated by a wide array of signaling molecules. Indian hedgehog (Ihh) stimulates chondrocyte proliferation and also stimulates production of parathyroid hormone-related protein (PTHrP) in the perichondrium. In the absence of Ihh, chondrocyte proliferation is decreased, while in the absence of PTHrP, chondrocytes become hypertrophic prematurely, suggesting the presence of endogenous signals that promote maturation. Several such positive regulators of chondrocyte maturation have been identified, including retinoids and BMPs.

Retinoids are present at low levels in cartilage of both chick and mouse embryos and at high

levels in perichondrium of chick embryos [von Schroeder and Heersche, 1998; Koyama et al., 1999]. In fact, retinoid concentrations in the perichondrium surrounding embryonic chick cartilages appear comparable to those in liver, a major reservoir of retinoids [Blaner and Olsen, 1994]. In addition, mRNA encoding RARγ is found at high levels in prehypertrophic and hypertrophic chondrocytes of chick embryonic cartilages [Koyama et al., 1999], indicating that these cells should be competent to respond to retinoids.

In experimental animals, hypervitaminosis A causes a widened hypertrophic zone, early mineralization and premature growth plate closure, while hypovitaminosis A results in an underdeveloped hypertrophic zone [Wolbach and Hegsted, 1952, 1953; Wilson et al., 1953]. Chronic high levels of retinoids have also been shown to cause premature growth plate closure in humans [Pease, 1962; Prendiville et al., 1986]. We and others have shown that RA stimulates maturation of cultured chondrocytes, including expression of the collagen X gene, alkaline phosphatase activity, increase in cell size and mineralization of the extracellular matrix [Iwamoto et al., 1993a,b, 1994; Yoshida et al., 2001; Li et al., 2003]. Furthermore, release of retinoid antagonists near developing chick limbs results in failure of chondrocytes to undergo maturation, as evidenced by lack of collagen X expression; this leads to drastically reduced bone growth [Koyama et al., 1999]. These data demonstrate that retinoids are essential for chondrocyte maturation.

Similarly, BMPs and their receptors are present in cartilages of both chick and mouse embryos [Zou et al., 1997; Zhang et al., 2003; Yoon et al., 2005], and BMPs stimulate maturation of cultured chondrocytes [Leboy et al., 1997; Volk et al., 1998, 2000; Grimsrud et al., 1999, 2001; Valcourt et al., 2002]. Furthermore, overexpression of Noggin, an antagonist of BMP signaling, in developing chick limbs results in an absence of hypertrophic cells [Pathi et al., 1999], indicating that BMP signaling is also essential for chondrocyte maturation.

We and others have shown that retinoids stimulate production of BMPs, as well as activation of Smads, the transcription factors that mediate BMP signaling, in prehypertrophic chondrocytes [Grimsrud et al., 1998; Adams et al., 2003; Li et al., 2003]. These data, together with the fact that consensus RAREs were not initially identified in the chick collagen X promoter, previously led to the conclusion that the effects of retinoids on chondrocyte maturation may be entirely indirect. The results shown here present a more complex picture, in which both retinoids and BMPs can act directly to activate chick collagen X transcription. Therefore, we present an expansion of our earlier model [Koyama et al., 1999] in which we proposed that abundant retinoids present in the perichondrium diffuse into the prehypertrophic cartilage and activate RARs to stimulate chondrocyte maturation. At the time that model was proposed we had not identified the direct targets of the ligand-activated RARs. In the data presented above, we have shown for the first time that retinoids directly stimulate transcription of a portion of the chick collagen X promoter by activating RARs bound to a compound RARE located 2,600 bp upstream of the transcription start site (see model in Fig. 5). These results may provide an explanation for some of the profound effects retinoids exert on endochondral bone formation, both in animal models of vitamin A function and in human

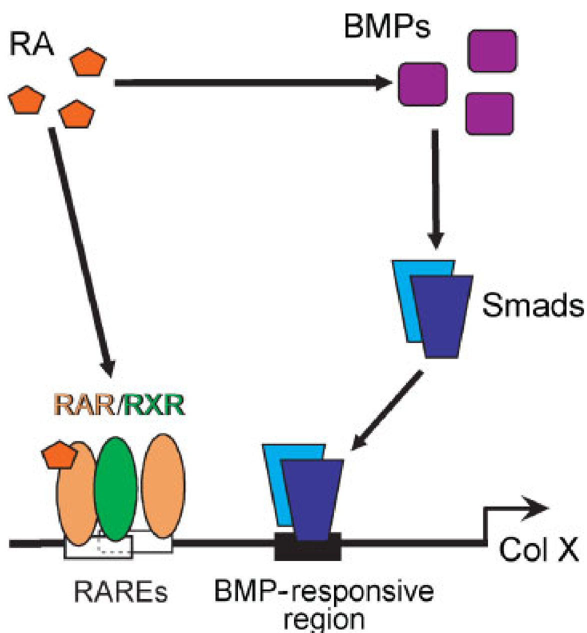

Fig. 5. A model of retinoid action on the collagen X promoter. RA stimulates collagen X transcription directly by activating an RARγ/RXR heterodimer already bound to the distal compound RARE. RA also stimulates BMP production, resulting in activation of BMP-activated Smads which presumably bind to a BMP-responsive region downstream of the RARE. There is potential for another nuclear receptor bound to the remaining half-site to be involved in regulation of transcription of this gene, depicted here as a second RAR.

clinical applications of synthetic retinoid analogs. It remains to be confirmed whether the mechanism we observe for this promoter fragment in the chick is conserved across species and is active in the endogenous promoter in vivo.

The compound RARE that we have described in the collagen X promoter appears to be unique among retinoid-regulated genes. The presence of the dual RAREs is clearly beneficial in terms of RA stimulation, since the WT promoter has twice the activity of the mutant promoters with only a single RARE (Fig. 4). A model that could explain these results is one in which an RXR receptor monomer (the obligatory heterodimeric partner of RARs) occupies the middle half-site and alternately interacts with RAR partners on either side (see model in Fig. 5). For example, as one RAR is degraded after activation by ligand binding [Bastien and Rochette-Egly, 2004], another RAR that is already bound to the third half-site could immediately be available for dimerization, thereby increasing the rate of transcriptional activation. Furthermore, the presence of three half-sites presents the possibility of more complex regulation in which the alternative partner could be a different nuclear receptor, for example, a thyroid hormone receptor, which might further stimulate transcription, or an inhibitor of transcription such as COUP (chicken ovalbumin upstream regulator) [reviewed in Zhang and Dufau, 2004].

Retinoids also stimulate a 30- to 35-fold increase in mRNAs encoding BMPs 2 and 6 as well as a lesser increase in BMP 4 and 7 mRNAs [Grimsrud et al., 1998; Adams et al., 2003]. Since BMPs also act on chondrocytes to stimulate collagen X production [Volk et al., 1998; Drissi et al., 2003], this is likely to result in a significant amplification of the retinoid signal (see model in Fig. 5). It is important to note that direct evidence of Smad binding to any site within the collagen X promoter has not yet been provided. However, we showed previously that deletion of 130 nucleotides downstream of the compound RARE (−2,518 to −2,365) abolished BMP responsiveness of the promoter [Adams et al., 2003]; this region contains several potential Smad binding sites. Furthermore, replacement of nucleotides −2,572 to −2,552, a region containing one potential Smad binding site, resulted in decreased response of the collagen X promoter to cotransfected Smad-expressing cDNAs [Drissi et al., 2003]. We thus propose that both RARγ (with its RXR heterodimer partner) and Smads may be required to achieve maximal stimulation of the collagen X promoter.

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