online commentary

A novel retinoic acid-response element requires an enhancer element mediator for transcriptional activation

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The *Col11a2* gene codes for α2(XI), a subunit of type XI collagen that is a critical component of the cartilage extracellular matrix. The 5′ regulatory region of *Col11a2* was subjected to deletional analysis to detect any regulatory element in addition to the two known chondrocyte-specific enhancer elements B/C and D/E. Deletion of the region from – 342 to – 242 bp reduced transcriptional activity to less than 50 % of wild-type, but the sequence showed no independent ability to increase transcription from a minimal promoter. When cloned downstream of the D/E enhancer, however, a subsection of the sequence nearly doubled transcriptional activity and produced an additional 3-fold activation in response to RA (retinoic acid). A 6-bp direct repeat, separated by 4 bp (a DR-4 element) near the 5′-end of this region, was found to be essential for its activity, and was further shown to bind the RA X receptor β in electrophoretic mobility-shift assays. The present study has revealed a novel RA-response element in *Col11a2* that does not interact directly with the promoter, but instead requires the D/E enhancer to mediate transcriptional activation. Proteins bound at the enhancer, therefore, would be expected to affect the transcriptional response to RA. Such a system of regulation, particularly if found to be operating in other cartilage genes, could explain the conflicting responses RA produces in chondrocytes under different experimental conditions.

Key words: chondrocyte-specific enhancer element, *Col11a2*, DR-4 element, retinoic acid-response element, transcriptional activation.

INTRODUCTION

Type XI collagen is a critical component of the cartilage extracellular matrix. It is composed of three distinct protein subunits, α1(XI), α2(XI) and α3(XI), wound into a right-handed triple helix. Type XI collagen triple helices bundle together with type II and type IX collagen triple helices to form collagen fibrils, which are deposited as a meshwork in the cartilage extracellular matrix and provide cartilage with its impressive resistance to shear forces. Evidence suggests that type XI collagen functions to limit the diameter of cartilage collagen fibrils during fibrillogenesis, and that correct fibril diameter is required for proper cartilage formation [1]. Consistent with this model, mutant mice that have no type XI collagen in the extracellular matrix exhibit unusually large fibril diameters and severe chondrodysplasia [2–4].

The three subunits of the type XI collagen triple helix are encoded by the *Col11a1*, *Col11a2* and *Col2a1* genes, and mutations in any of these genes can cause abnormal cartilage development. For example, mice that are homozygous for the *cho* mutation, a 1 bp deletion in the *Col11a1* gene, exhibit severe chondrodysplasia, dwarfism and cleft palate [5]. Mice that are heterozygous for the mutation are predisposed to develop osteoarthritis [6,7]. Mutations in *COL11A1* cause Marshall syndrome and Stickler syndrome in humans, and mutations in *COL11A2* cause Stickler syndrome and otospondylomegaepiphyseal dysplasia disorders, which are all marked by chondrodysplasia and osteoarthritis [8–15]. Mutations in the *COL2A1* gene, which encodes the α1(II) subunit of type II collagen as well as the α3(XI) subunit of type XI collagen, lead to chondrodysplasias with a range of severity extending from mild dwarfism to neonatal lethality [16–23].

Although all three type XI collagen genes are clearly important for chondrogenesis, our recent work [24,25] has focused on transcriptional regulation of the *Col11a2* gene. *Col11a2* is expressed almost exclusively in chondrocytes, and it may therefore provide important insights into general mechanisms of chondrocyte-specific gene regulation that drive chondrocyte differentiation. Previous research identified the regions from -742 to $+380$ at the 5′-end of the *Col11a2* gene and from $+1468$ to $+2151$ in the first intron as together sufficient to produce chondrocyte-specific gene expression in transgenic mice [24]. Within these regions, three chondrocyte-specific enhancer elements of approx. 50 bp each have been identified and confirmed in transgenic mice [25,26]. Two are located in the 5′ region (named B/C and D/E) and one is in the first intron (F/G) [24].

The goal of the present study was to scan the other sequences within the *Col11a2* 5′ regulatory region to identify any other important regulatory element besides the B/C and D/E enhancers. The present study has revealed a novel RARE (retinoic acid-response element) that does not interact directly with the promoter, but instead requires interaction with the D/E enhancer to increase transcription. This new role of the D/E enhancer as a mediator of RA (retinoic acid) responsiveness implies that transcriptional activation in response to RA would be modulated not only by receptors and cofactors bound at the RARE, but also by proteins bound at the D/E enhancer element. This complex system of transcriptional activation, especially if found to be functioning in other cartilage genes that respond to RA, could explain the different and sometimes conflicting effects that RA has been reported to have on chondrocytes under different experimental conditions.

Abbreviations used: EMSA, electrophoretic mobility-shift assay; RA, retinoic acid; RAR, RA receptor; RARE, RA-response element; RCS cells, rat chondrosarcoma cells; RXR, retinoic acid X receptor; T_3, 3,3′,5-tri-iodo-L-thyronine; TR, thyroid hormone receptor.

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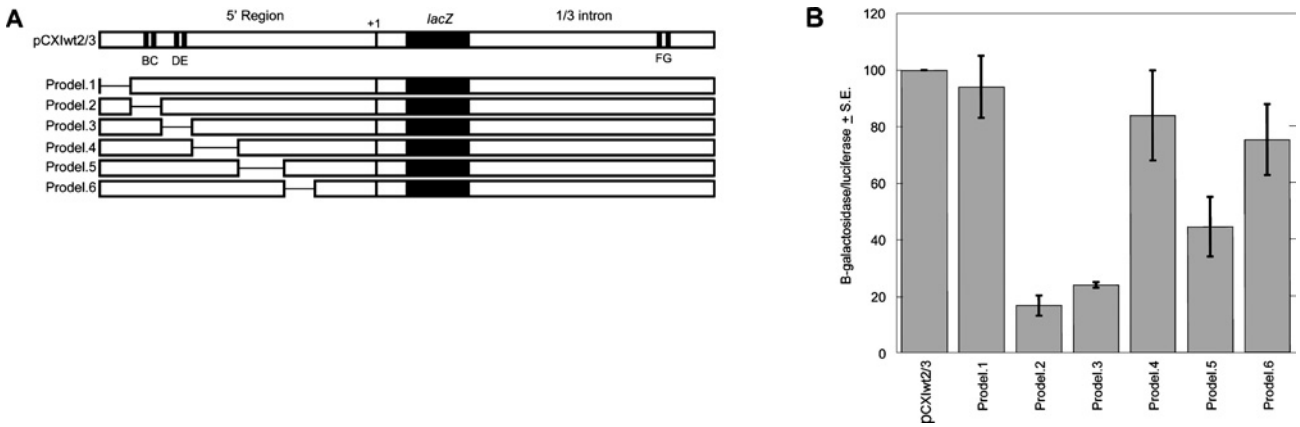

Figure 1 The *Col11a2* 5′ region contains at least three subsections with positive regulatory activity

(**A**) Six different deletions of approx. 100 bp each were introduced into the 5′ region of *Col11a2* in the context of the pCXIwt2/3 plasmid, which has previously been shown to direct chondrocyte-specific expression in transgenic mice [24]. The bars marked B–G represent six heptameric consensus sequences that each bind the transcriptional activator Sox9. (**B**) The effect of each deletion was tested by transient transfection in RCS cells. Each experiment included 1.5 μg of experimental plasmid and 0.5 μg of pGL-3 (Promega), a luciferase-expressing plasmid that was included as an internal control for transfection efficiency. Results are normalized to the activity of the pCXIwt2/3 plasmid, and each bar reflects data from at least three independent experiments, each performed in duplicate or triplicate.

MATERIALS AND METHODS

Deletion series plasmids

The plasmid pCXIwt2/3 contains the regulatory elements necessary to direct chondrocyte-specific gene expression in a developing mouse embryo [24]. Briefly, pCXIwt2/3 includes a 1122 bp fragment from -742 to $+380$ of the mouse *Col11a2* gene cloned upstream of a β-galactosidase reporter and a 684 bp fragment from $+1468$ to $+2151$ of the *Col11a2* first intron cloned downstream of the reporter. Deletions of approx. 100 bp each were introduced by PCR into the *Col11a2* 5′ region to identify sequences that participate in the regulation of transcription (see schematic representation of deletion series in Figure 1). Correct construction of plasmids was verified by sequencing at the Brigham Young University DNA Sequencing Center.

Luciferase reporter plasmids

The luciferase reporter plasmids (4xB/C)p95Luc and (4xD/E)-p95Luc have been described previously [24,25]. Each plasmid contains four tandem copies of the B/C or D/E chondrocyte-specific enhancer element cloned upstream of a 95 bp minimal promoter and a luciferase reporter. In the present study, one, two, four, eight and 16 tandem copies of the Prodel.5 element were cloned in place of the enhancers to test Prodel.5 for independent enhancer activity. To test the ability of the novel 5A, 5B and 5C elements to affect transcription from the B/C and D/E enhancers, the novel elements were cloned into (4xB/C)p95Luc and (4xD/E)p95Luc between the multimerized enhancers and the minimal promoter. Constructions were verified by sequencing at the Brigham Young University DNA Sequencing Center.

Cell culture

RCS (rat chondrosarcoma) cells (a gift from B. de Crombrugghe of the M. D. Anderson Cancer Center, Houston, TX, U.S.A.) were used in all transfection experiments [27]. Cells were cultured at 37 °C under 5 % CO_2 in Dulbecco's modified Eagle's medium, supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), L-glutamine (2 mM) and 10 % (v/v) fetal calf serum.

Transfections

Transient transfections were performed in 6-well plates using LIPOFECTAMINE™ reagent (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. A total of 2 μg of DNA was used in each reaction mixture. Cells were incubated for 48 h before lysis and preparation of cellular extracts. In transfections with T_3 (3,3′,5-tri-iodo-L-thyronine) and/or *all-trans*-RA, cells were grown in the absence of serum throughout the entire transfection. T_3 (1 μM) and/or *all-trans*-RA (5 μM) were added 24 h before harvesting. Internal controls for transfection efficiency were included in each reaction; the pGL-3 control luciferase-expressing plasmid (Promega, Madison, WI, U.S.A.) was included in experiments where β-galactosidase was the experimental reporter, and the plasmid pSV-β-galactosidase (Promega) was included in experiments where luciferase was the experimental reporter. Cellular extracts were prepared, and enzyme activity assays were performed to measure luciferase (Luciferase Assay System, Promega) and β-galactosidase (Galacto-Light Plus System; Tropix, Bedford, MA, U.S.A.) expression levels using a Turner Designs TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, U.S.A.). Data were analysed and *P* values determined using the Student's *t* test.

EMSAs (electrophoretic mobility-shift assays)

Double-stranded DNA probes were radiolabelled by 5′ overhang end-filling with Klenow fragment. Nuclear extract was prepared as described previously [28]. Radiolabelled probe was incubated with 9.5 μg of nuclear extract in the presence of 1.5 μg of poly(dI-dC) · (dI-dC) non-specific competitor at room temperature (23 °C) for 30 min in DNA-binding buffer [20 mM Hepes, pH 7.9, 10 % (v/v) glycerol, 50 mM KCl, 0.05 % Nonidet P40, 0.5 mM EDTA, 0.5 mM dithiothreitol and 1 mM PMSF]. The specific competitor was included in each experiment at a concentration of 25-, 50- and 100-fold molar excess over the concentration of the radiolabelled probe. For antibody supershift experiments, antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was preincubated with nuclear extract and non-specific competitor for 20 min in DNA-binding buffer before the addition of the radiolabelled probe. DNA–protein complexes were separated on

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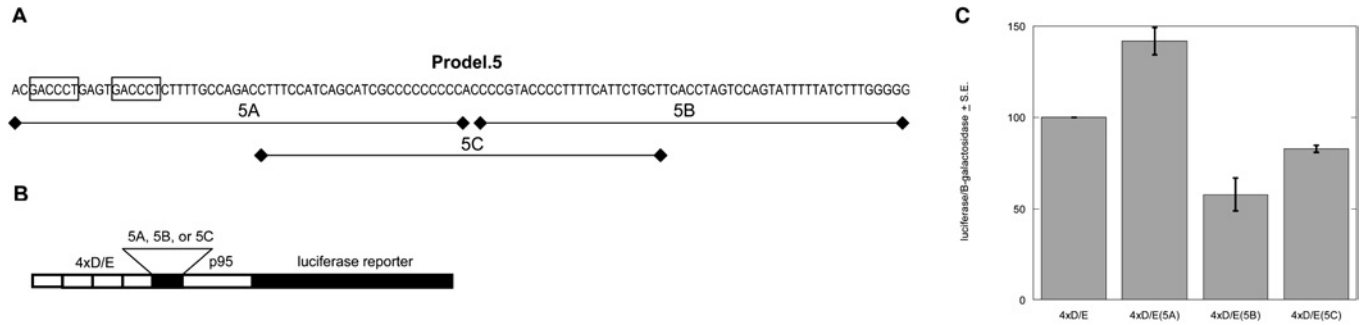

Figure 2 The 5A subsection of Prodel.5 contains a positive regulatory element

(**A**) Sequence of the Prodel.5 region indicating subsections 5A, 5B and 5C. The 6 bp direct repeat (DR-4 element) is marked by boxes. (**B**) Schematic representation of the plasmids used for testing the effect of 5A, 5B and 5C on B/C and D/E enhancer activity. The 5A, 5B or 5C fragment was inserted between the multimerized enhancer and the minimal 95 bp promoter in the (4xB/C)p95Luc or (4xD/E)p95Luc plasmids. (**C**) The effects of the 5A, 5B and 5C insertions on transcriptional activity were tested in transient transfections of RCS cells. Each experiment included 1.5 μg of experimental plasmid and 0.5 μg of pSV-β-galactosidase plasmid (Promega) as an internal control for transfection efficiency. Results are normalized to the activity of the (4xD/E)p95Luc plasmid with no insertion and include data from at least three independent experiments, each performed in duplicate or triplicate.

4 % (w/v) polyacrylamide gels for 2–3 h at 100 V, and complexes were visualized by autoradiography.

RESULTS

The 5′ region of the *Col11a2* gene contains an additional positive regulatory element

A deletion series was constructed to determine whether the 5′ region of the *Col11a2* gene contains any other regulatory element in addition to the two known enhancers. The deletion series was constructed in a plasmid called pCXIwt2/3, which contains 1122 bp (from -742 to $+380$) of the 5′ region and 684 bp (from $+1468$ to $+2151$) of the first intron of *Col11a2* regulating a *lacZ* reporter gene. This plasmid contains all the elements necessary to produce chondrocyte-specific gene expression in a developing mouse embryo [24]. Six deletions of approx. 100 bp each were introduced into the 5′ region (Figure 1A). The region from -142 to $+1$ bp was not deleted, since it contains the proximal promoter, which is essential for transcriptional initiation.

Deletion plasmids were transiently transfected into RCS cells, and expression levels of the β-galactosidase reporter gene were measured as an indicator of transcriptional activity. The deletions that eliminated the B/C and D/E enhancers (designated Prodel.2 and Prodel.3) reduced transcriptional activity significantly ($P < 0.001$ and $P = 0.008$ respectively), verifying that positive regulatory elements can be detected using this approach (Figure 1B). Another deletion called Prodel.5 (spanning the region from -342 to -242 bp) reduced transcriptional activity to less than 50 % of wild-type ($P = 0.013$). This result implied the presence of a previously unknown positive regulatory element in that region (Figure 1B). Transcriptional activities of plasmids Prodel.1, Prodel.4 and Prodel.6 were not significantly different from that of the pCXIwt2/3 plasmid.

The Prodel.5 region has no independent ability to increase transcription from a minimal promoter

Because the Prodel.5 deletion in the 5′ region eliminated a positive regulatory element, the possibility that the element might contain an independent enhancer was examined. A 111 bp fragment containing the entire Prodel.5 region was cloned into the same p95Luc plasmid that was previously used to test the B/C and D/E enhancers, with the Prodel.5 region being cloned in place of the enhancer elements upstream of a 95 bp minimal promoter and a luciferase reporter. Four copies of the B/C or D/E enhancer, cloned upstream of the minimal promoter, were sufficient to produce easily detectable transcriptional activity. One, two, four, eight and 16 copies of the Prodel.5 fragment, however, failed to produce transcriptional activation. None of the Prodel.5 constructs displayed more than 0.1 % of the activity of the (4xD/E)p95Luc plasmid, demonstrating that the Prodel.5 region does not contain an element that is independently capable of driving transcription in RCS cells (results not shown).

The Prodel.5 region contains an element that co-operatively increases the activity of the D/E enhancer element

The absence of independent transcriptional activation by the Prodel.5 region suggested the possibility that the region may instead affect transcription levels by interacting with the nearby 5′ enhancer elements B/C and D/E. The Prodel.5 region was divided into three fragments for testing: the first 56 bp called 5A, the second 55 bp called 5B and a 50 bp fragment overlapping parts of both 5A and 5B, which was designated 5C (Figure 2A). These fragments were cloned into the (4xB/C)p95Luc and (4xD/E)p95Luc plasmids between the B/C or D/E enhancer elements and the 95 bp minimal promoter (Figure 2B). When the fragments were cloned with the B/C enhancer, none significantly increased the transcriptional activity of the enhancer (results not shown). When the Prodel.5 fragments were cloned with the D/E enhancer element, however, the 5A fragment increased transcriptional activity from the enhancer approx. 1.4-fold ($P = 0.002$; Figure 2C). The 5B and 5C fragments both decreased enhancer activity, suggesting the presence of a negative regulatory element that may deserve study in the future. The 5B and 5C fragments clearly do not contain the element responsible for the positive activity of the Prodel.5 region.

The 5C fragment did not increase the activity of the D/E enhancer, even though the 5A and 5C fragments have 26 bp in common (Figure 2A). This result suggested that the first 30 bp of the 5A fragment were responsible for its activity. To test this hypothesis, the 30 bp fragment (called 5Ashort) was cloned between the D/E enhancer and the minimal promoter in the (4xD/E)p95Luc plasmid (Figure 3A). This construct produced reporter gene expression that was almost 2-fold higher than with the enhancer alone ($P < 0.001$), demonstrating that the first 30 bp segment of the Prodel.5 region does, in fact, include an element

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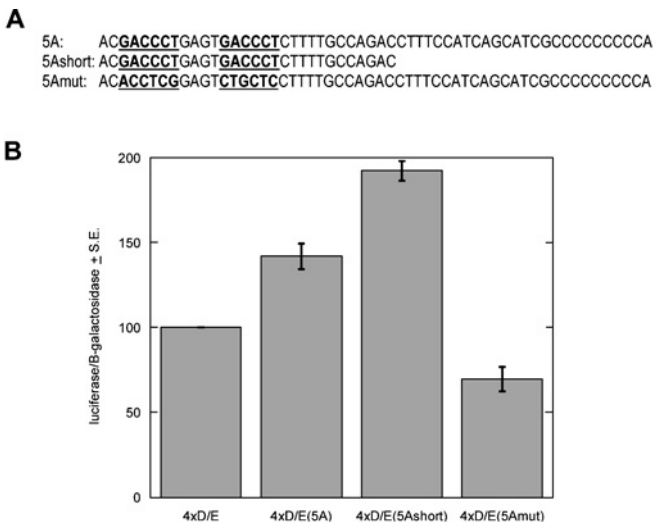

Figure 3 The activity of 5A requires a DR-4 element in the first 30 bp

(**A**) Sequence of the 5A element in comparison with the 5Ashort fragment, which includes only the first 30 bp of the Prodel.5 region, and the 5Amut fragment, which contains substitution mutations within the DR-4 element (underlined). (**B**) The 5A, 5Ashort and 5Amut fragments were inserted into the (4xD/E)p95Luc plasmid after the pattern shown in Figure 2(B), and transient transfections of each plasmid were performed in RCS cells. Each experiment included 1.5 μg of experimental plasmid and 0.5 μg of pSV-β-galactosidase plasmid (Promega) as an internal control for transfection efficiency. Results are normalized to the activity of the (4xD/E)p95Luc plasmid with no insertion and include data from at least three independent experiments, each performed in duplicate or triplicate.

that increases the transcriptional activity of the D/E chondrocyte-specific enhancer element (Figure 3B).

A DR-4 element in 5A is responsible for its effect on transcriptional activity

The first 30 bp of the 5A region were examined to identify possible transcription factor binding sites, and a 6 bp direct repeat separated by 4 bp (a DR-4 element) with homology with the hormone response element was identified (Figure 3A). To determine whether the DR-4 element participated in the activation of transcription, a mutated version of the 5A element (called 5Amut) containing multiple substitutions within the direct repeat was cloned into the (4xD/E)p95Luc plasmid between the enhancer and the minimal promoter (Figure 3A). The plasmid was transiently transfected into RCS cells. Mutation of the DR-4 sequence was found to inactivate the 5A element completely (Figure 3B).

The DR-4 element forms specific complexes with proteins found in chondrocytic cell nuclear extracts

Five distinct DNA–protein complexes were observed in EMSAs performed using the 5A element as probe with RCS cell nuclear extract as a protein source (Figure 4A, arrows). When excess unlabelled probe was included in the experiment, complexes I and V were competed for, indicating that they contain proteins that bind specifically to sites in the 5A element (Figure 4A). Complex III was also competed for, although not as strongly as I and V. Mutation of the DR-4 element (5Amut probe) prevented formation of complexes I and V, demonstrating that these specific complexes contain proteins bound at the DR-4 element (Figure 4B, arrows). When included as excess unlabelled competitor, the 5Amut probe failed to compete with wild-type radiolabelled probe for the formation of complexes I and V (Figure 4C, arrows),

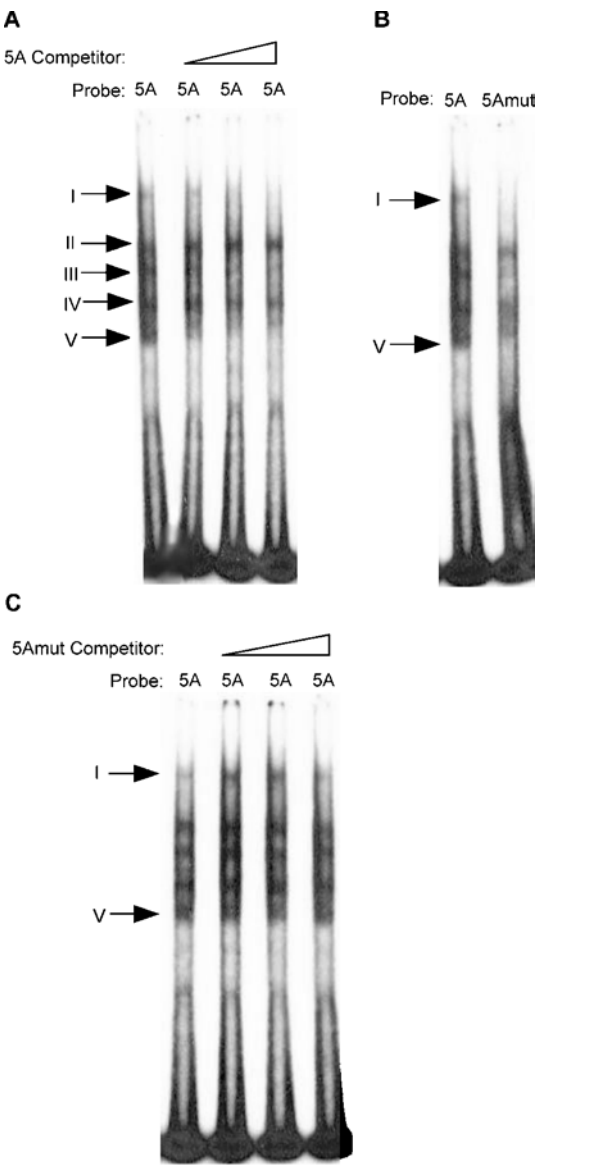

Figure 4 5A forms specific DNA–protein complexes in EMSA with proteins that bind at the DR-4 element

EMSAs were performed using 5A and 5Amut DNA as probe and RCS cell nuclear extract as the source of proteins. (**A**) The 5A probe formed five distinct complexes with proteins from the RCS cell nuclear extract. Two of these, designated I and V, were competed for by excess unlabelled 5A probe. (**B**) The 5Amut probe, which contains substitution mutations in the DR-4 element (see Figure 3A for sequence), does not form complexes I or V. (**C**) Unlabelled 5Amut probe does not compete with 5A for formation of complexes I and V.

indicating once again that the two complexes contain proteins bound specifically to the DR-4 element.

RA increases transcriptional activity through the DR-4 element

The DR-4 element has homology with the paired 'half-sites' that are known to bind the α, β and γ subtypes of TRs (thyroid hormone receptors), of RARs (RA receptors) and of RXRs (retinoic acid X receptors) [29]. To test whether any of these receptors activate 5A through the DR-4 element, serum-depleted RCS cells transfected with the 4xD/E(5A) reporter plasmid were cultured in the presence of T_3 and/or *all-trans*-RA. T_3 serves as a ligand for the α, β and γ subtypes of TR. *all-trans*-RA serves as a direct

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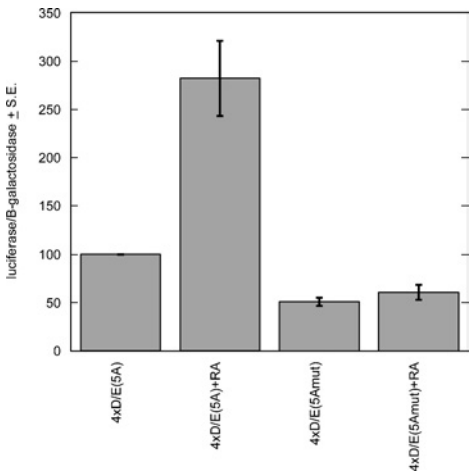

Figure 5 5A increases transcription in response to RA and requires the DR-4 element for that responsiveness

Plasmids 4xD/E(5A) and 4xD/E(5Amut), previously used for experiments shown in Figure 3, were transiently transfected into RCS cells either with or without addition of 5 μM *all-trans*-RA 24 h before harvesting. Results are normalized to the activity of the 4xD/E(5A) plasmid without *all-trans*-RA and include data from at least two independent experiments, each performed in duplicate or triplicate.

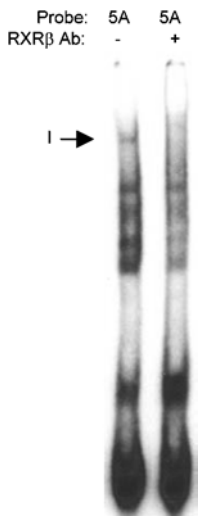

Figure 6 EMSA complex I contains RXRβ

EMSAs were performed using 5A DNA as probe and RCS cell nuclear extract as the source of proteins. Antibodies against the RXRβ were included and were found to block-shift complex I, which contains proteins bound at the DR-4 element. Parallel experiments were performed using antibodies against RXRα, RXRγ, RARα, RARβ and RARγ, but none of these other antibodies changed the banding pattern of the 5A probe (results not shown).

ligand for the α, β, and γ receptor subtypes of RAR, and also undergoes protein-mediated isomerization to 9-*cis*-RA, which is the ligand for the α, β and γ receptor subtypes of RXR [30–32].

Addition of T_3 had no significant effect on the transcriptional activity of the 4xD/E(5A) reporter plasmid (results not shown). *all-trans*-RA, however, increased transcriptional activity by approx. 2.75-fold ($P = 0.001$; (Figure 5). T_3 in combination with *all-trans*-RA did not produce significantly more transcriptional activity compared with *all-trans*-RA alone (results not shown). Mutating the DR-4 element (5Amut) made 5A non-responsive to *all-trans*-RA, verifying that the direct repeat is, in fact, a RARE and it is the sequence within 5A through which RA produces transcriptional activation (Figure 5).

RXRβ binds to the DR-4 element

Cultured rat chondrocytes express mRNA for the RA receptor subtypes RXRα (high levels), RARγ (moderate levels), and RARα and RXRβ (low levels) [32]. Antibodies against these four RA receptor subtypes as well as the other two receptor subtypes (RARβ and RXRγ) were utilized in EMSAs to determine which RA receptors bind to the 5A DR-4 element. Experiments presented in Figure 4 had previously demonstrated that complexes I and V contain proteins bound at the DR-4 element. Inclusion of antibodies against RXRβ resulted in the block-shift of complex I (Figure 6). The other five types of RAR and RXR antibodies did not change the EMSA banding pattern (results not shown). These results demonstrate that RXRβ is present in RCS cells and that it binds to the critical DR-4 element in the *Col11a2* 5A region.

DISCUSSION

In the present study, we have demonstrated the existence of a novel positive regulatory element in the 5′ region of the *Col11a2* gene. This new element, called 5A, is distinct from the two chondrocyte-specific enhancer elements we have previously identified in the 5′ region. The new element has no independent enhancer activity, but instead works in co-operation with the D/E enhancer element to increase its activity nearly 2-fold. The portion of the 5A element responsible for this activity was found to be a 6 bp direct repeat separated by 4 bp (a DR-4 element), which is similar to, but not identical with, the prototypical RARE.

The sequence of the DR-4 element identified within 5A is different from the prototypical RARE in two ways. First, a 4 bp separation between the direct repeats is typical of thyroid hormone response elements, whereas the consensus RARE sequence consists of two direct repeats separated by 5 bp (DR-5) [29]. The present experiments demonstrated, however, that the 5A element is not responsive to T_3 and therefore does not probably bind the TR. Secondly, the 5A element direct repeat is homologous with the RARE consensus sequence (AGGTCA) on the antisense strand, so that the repeated sequence in the sense strand of 5A reads GACCCT-(GAGT)-GACCCT. Despite these differences, the DR-4 element in 5A binds RXRβ *in vitro* and is transcriptionally responsive to RA.

The observation that RXRβ binds the 5A element is particularly interesting because of the proximity of the RXRβ gene to the *Col11a2* gene. The putative end of the RXRβ gene sequence is 742 bp upstream of the transcriptional start site of the *Col11a2* gene in mouse and 1.1 kb upstream of the *COL11A2* gene in humans [33,34]. Reverse transcriptase-PCR experiments in mouse revealed that some transcripts from the RXRβ gene extend into the *Col11a2* gene, prompting the authors to suggest that there may be some co-ordinate regulation of the two genes [35]. The results presented here suggest that the two genes may not only be co-ordinately regulated, but that the product of one may, in turn, regulate the expression of the next.

RXRβ forms heterodimers with other members of the type II nuclear receptor subfamily when binding at direct repeat elements. Members of this subfamily include TR, RAR and vitamin D_3 receptor [29]. The evidence presented here that the TR ligand T_3 does not activate the 5A element suggests that TR is not the heterodimeric binding partner of RXRβ in 5A. In addition,

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the EMSA results presented here suggest that none of the RAR receptors binds the 5A element direct repeat, even though others have shown that both RARγ and RARα are transcribed in chondrocytes [32]. RXRβ has other known heterodimeric partners, but most are probably not expressed in chondrocytes. These include liver X receptor, peroxisome proliferator-activated receptor, farnesoid X receptor, nerve growth factor-inducible B and others [29]. Additional research, therefore, is needed to identify the heterodimeric binding partner of RXRβ in the *Col11a2* 5A element.

Various studies have produced conflicting reports of RA's effects on chondrocytic cells. In a cartilage-derived cell line called TC6, RA was shown to increase expression of *Sox9*, which activates *Col11a2* and other cartilage-specific genes [24,25,36,37]. RA has also been shown to stimulate proliferation of cultured rabbit costal chondrocytes and chick embryonal growth plate chondrocytes and to stimulate chondrogenesis of chick limb bud mesenchymal cells [38–40]. In contrast, however, RA has been shown to suppress *Sox9* expression in primary chondrocytes, reduce transcription of cartilage matrix genes in chondrocytes and inhibit proliferation of chondrosarcoma cells in culture [41–43]. These contradictory observations are probably due to differences in the tissues, cells or cell lines examined, and in the transcriptional regulatory proteins they contain.

The present study has demonstrated that, in the specific case of RCS cells, a DR-4 sequence in the *Col11a2* 5A element cooperates with the D/E chondrocyte-specific enhancer to approximately double its transcriptional activity. The addition of 5 μM *all-trans*-RA (which undergoes protein-mediated isomerization to 9-*cis*-RA) to the cell culture medium produced an additional 3-fold activation of transcription from the 4xD/E(5A) reporter construct [30–32]. Mutation of the DR-4 repeat completely inactivated the 5A element and eliminated its responsiveness to RA, confirming that the DR-4 element, which binds RXRβ in EMSA, is the site through which RA affects transcription.

The results of the present study suggest an unconventional mechanism of *Col11a2* transcriptional activation by RXRβ. Nuclear receptors have traditionally been seen as proteins that bind DNA and then interact with proteins at the promoter to activate transcription. In one study, however, a RARE was identified that produced only 50% of its activity by interacting directly with the promoter, but required a separate element containing binding sites for three additional transcription factors to achieve full RA responsiveness [44]. Here, we demonstrate a novel RARE that does not directly interact with the promoter to activate transcription. Instead, the RARE in the *Col11a2* 5A element apparently binds proteins that must interact with proteins bound at the D/E enhancer to achieve RA responsiveness. The interaction between the RARE- and D/E enhancer-bound proteins appears to be highly specific, as the RARE failed to co-operate with the B/C chondrocyte-specific enhancer from the *Col11a2* gene.

This requirement for interactions between RARE- and D/E enhancer-bound proteins adds a new level of complexity to RA-mediated transcriptional activation. The presence or absence of transcription factors that bind to the D/E enhancer would be expected to alter the responsiveness of *Col11a2* to RA, even in the face of unchanged protein binding at the RARE. This additional level of complexity in transcriptional activation may help explain the varied and sometimes opposing effects that RA has been reported to exert on chondrocytes, particularly if such a mechanism of enhancer-mediated RA responsiveness is found to operate in the transcription of other cartilage genes.

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