Requirement for RAR-mediated gene repression in skeletal progenitor differentiation

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Chondrogenesis is a multistep process culminating in the establishment of a precisely patterned template for bone formation. Previously, we identified a loss in retinoid receptor–mediated signaling as being necessary and sufficient for expression of the chondroblast phenotype (Weston et al., 2000. *J. Cell Biol.* 148:679–690). Here we demonstrate a close association between retinoic acid receptor (RAR) activity and the transcriptional activity of Sox9, a transcription factor required for cartilage formation. Specifically, inhibition of RAR-mediated signaling in primary cultures of mouse limb mesenchyme results in increased Sox9 expression and activity. This induction is attenuated by the histone deacetylase inhibitor, trichostatin A, and by coexpression of a dominant negative nuclear receptor corepressor-1, indicating an unexpected requirement for RAR-mediated repression in skeletal progenitor differentiation.

Inhibition of RAR activity results in activation of the p38 mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) pathways, indicating their potential role in the regulation of chondrogenesis by RAR repression. Accordingly, activation of RAR signaling, which attenuates differentiation, can be rescued by activation of p38 MAPK or PKA. In summary, these findings demonstrate a novel role for active RAR-mediated gene repression in chondrogenesis and establish a hierarchical network whereby RAR-mediated signaling functions upstream of the p38 MAPK and PKA signaling pathways to regulate emergence of the chondroblast phenotype.

Introduction

Almost all skeletal elements form on a cartilage template that is established early during embryogenesis to provide a precisely patterned framework for the future skeleton. During chondrogenesis, numerous factors act together to coordinate commitment and differentiation of skeletal progenitors such that these processes occur in a spatial- and temporal-specific manner. Of these factors, the retinoids have been known for decades to have a considerable influence on cartilage formation. Specifically, an imbalance in vitamin A metabolites, such as retinoic acid (RA),* during development will result in severe skeletal defects among a multitude of other developmental anomalies (Hale, 1935; Warkany and Schraffenberger, 1946; Cohlan, 1953; Wilson et al., 1953; Kalter and Warkany, 1961). Modulation of RA availability during the time period of chondrogenesis has the most profound impact on the skeleton, suggesting that this period of skeletal development is particularly sensitive to the retinoids (Kochhar, 1973; Kwasigroch and Kochhar, 1980). Accordingly, retinoids have been shown by several groups to inhibit chondrogenesis in vivo and in vitro (for review see Underhill and Weston, 1998).

Of the intracellular retinoid binding proteins, nuclear receptors are thought to mediate most of RA's effects on cell behavior. Two subfamilies of nuclear retinoid receptors exist: the RA receptors (RARs) and the retinoid X receptors (RXRs). Within each subfamily there are three subtypes (α, β, and γ), with multiple isoforms of each. These receptors belong to the nuclear hormone receptor superfamily and provide a level at which much of the diversity of retinoid responses is generated (for review see Leid et al., 1992).

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*Abbreviations used in this paper: ATF, activating transcription factor; CRE, cAMP response element; CREB, CRE binding element; EGFP, enhanced green fluorescent protein; HDAC, histone deacetylase; MAPK, mitogen-activated protein kinase; N-CoR, nuclear receptor corepressor; PKA, protein kinase A; RA, retinoic acid; RAR, RA receptor; RXR, retinoid-X receptor; TR, thyroid hormone receptor; TSA, trichostatin A.

Key words: chondrogenesis; Sox9; p38 MAPK; protein kinase A; nuclear corepressors

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Although ligand binding to the RARs followed by recruitment of transcriptional coactivators is the basic mechanism underlying RAR-mediated gene transcription, unliganded receptors are now recognized as having an equally important function by actively repressing target gene expression through the recruitment of nuclear corepressors and associated histone deacetylases (Horlein et al., 1995; Chen et al., 1996; Heinzel et al., 1997; Nagy et al., 1997; Koide et al., 2001).

The RARs and their isoforms exhibit dynamic expression patterns throughout development (Mollard et al., 2000). With respect to skeletal development in the limb, RARα is expressed throughout the limb mesenchyme early in limb development. As cells begin to differentiate into chondrocytes, RARα is downregulated, remaining highly expressed in the perichondrium and in the interdigital region, whereas RARγ expression becomes localized to the cartilaginous elements (Dolle et al., 1989; Mendelsohn et al., 1991; Cash et al., 1997; Mollard et al., 2000). Throughout limb morphogenesis, RARβ is expressed in noncartilage-forming regions such as the interdigital region (Mendelsohn et al., 1991). We demonstrated previously that the continued expression of RARα in prechondrogenic cells prevents their differentiation, resulting in severely malformed skeletal elements in transgenic mice (Cash et al., 1997; Weston et al., 2000). Moreover, inhibition of RARα activity was sufficient to induce chondrogenesis (Weston et al., 2000).

The proper size and shape of the developing skeletal elements relies on the appropriate control of chondroblast differentiation. To date, the Sox genes, L-5, -6, and -9 are the only known transcription factors through which this control is achieved. These genes contain a high mobility group domain and belong to the Sox family of proteins that are homologous to the protein encoded by *Sry* (sex-determining region of Y chromosome). Of this group, Sox9 is known to play an essential role in establishing the precartilaginous condensations and in initiating chondroblast differentiation (Bi et al., 1999; Smits et al., 2001). Specifically, Sox9 binds to a region within the first intron of the *type II* collagen gene (*Col2a1*) to regulate its transcription (Lefebvre et al., 1996). Mutations in *Sox9* underlie the rare congenital dwarfism syndrome, campomelic dysplasia (Foster et al., 1994; Wagner et al., 1994), and Sox9-null mice are embryonic lethal, whereas $Sox9^{-/-}$ cells in chimeric embryos are excluded from all cartilages (Bi et al., 1999). Conversely, if ectopically expressed, Sox9 will induce ectopic *Col2a1* expression and cartilage formation (Bell et al., 1997; Healy et al., 1999). Thus, Sox9 activation of *Col2a1* can be considered to be a hallmark event in cartilage formation.

To date, only a handful of factors have been found to influence Sox9 expression and/or activity, all of which are known modulators of chondrogenesis (Healy et al., 1999; Murakami et al., 2000a,b). Here we demonstrate that Sox9 expression is regulated by the retinoid signaling pathway. Our findings indicate that RAR-mediated repression is required for induction of Sox9. Moreover, expression of a dominant negative RAR leads to an increase in Sox9 reporter activity that is substantially greater than that elicited by any other factor described thus far. In addition, we show that the p38 MAPK and protein kinase A (PKA) signaling cascades are required downstream of retinoid signaling for chondroblast differentiation. Earlier studies have implicated both p38 MAPK and PKA in chondrogenesis (Lee and Chuong, 1997; Nakamura et al., 1999; Huang et al., 2000; Oh et al., 2000; Yoon et al., 2000); however, for the first time our results have enabled us to begin constructing a model for the hierarchical network of events that direct chondroblast differentiation.

Results

Inhibition of RAR activity enhances chondrogenesis through a Sox9-dependent mechanism

Previously, the continued expression of RARα activity in transgenic mice was found to inhibit the chondroprogenitor-to-chondroblast transition. Likewise, inhibition of RARα using the subtype-specific antagonist, AGN194301, induced differentiation in primary limb mesenchymal cultures earlier than normal, resulting in a substantial increase in the number of cartilage nodules that form in these cultures. This induction of cartilage formation was confirmed by the AGN194301-induced increase in expression of cartilage-specific genes such as *Col2a1* (Weston et al., 2000). Given that Sox9 has been shown previously to be important in regulating the expression of *Col2a1*, we analyzed the effects of RARα antagonism on *Sox9* expression and activity in an attempt to further understand the mechanism whereby retinoid signaling regulates chondroblast differentiation.

To follow endogenous Sox9 activity in primary mesenchymal cells, a reporter-based approach was used in which cells were transiently transfected with pGL3(4X48), a reporter containing four repeats of a Sox9 binding site from the first intron of *Col2a1.* The RARα-specific antagonist, AGN194301 (301), induced a concentration-dependent increase in reporter activity, whereas at-RA and the RARα-specific agonist, AGN193836 (836), attenuated reporter activity (Fig. 1 A). Interestingly, when cells were treated with the RAR pan-antagonist, AGN194310 (310), concentrations as low as 10 nM induced Sox9 reporter activity greater than the maximal response elicited by higher doses of 301. The maximal response to the pan antagonist was ~530% induction at 50 nM, whereas the greatest induction of Sox9 reporter activity by the RARα-specific antagonist was ~280% at 1 μM, a concentration at which this antagonist affects ligand binding to other RAR subtypes (Weston et al., 2000). Similar to RAR antagonism, the reduction in reporter activity caused by a pan-agonist such as at-RA was more pronounced than that induced by the RARα-specific agonist, 836. at-RA reduces reporter activity to 53% at 5 nM, whereas in response to a much higher dose of 836 (1 μM) reporter activity is reduced only to 64% of control. Together, these results indicate that a loss in activity of at least two or more RARs is more efficient at inducing cartilage differentiation than inhibition of the RARα subtype alone.

Interestingly, the effects of RAR modulation on Sox9 activity are opposite to that of the effects of each compound on activity of a retinoic acid responsive reporter (pW1-$\beta RARE_3$-Luc) in primary limb mesenchymal cells (Fig. 1 B). For instance, at-RA activates the RARE reporter to a greater extent than 836, whereas reporter activity is attenuated by

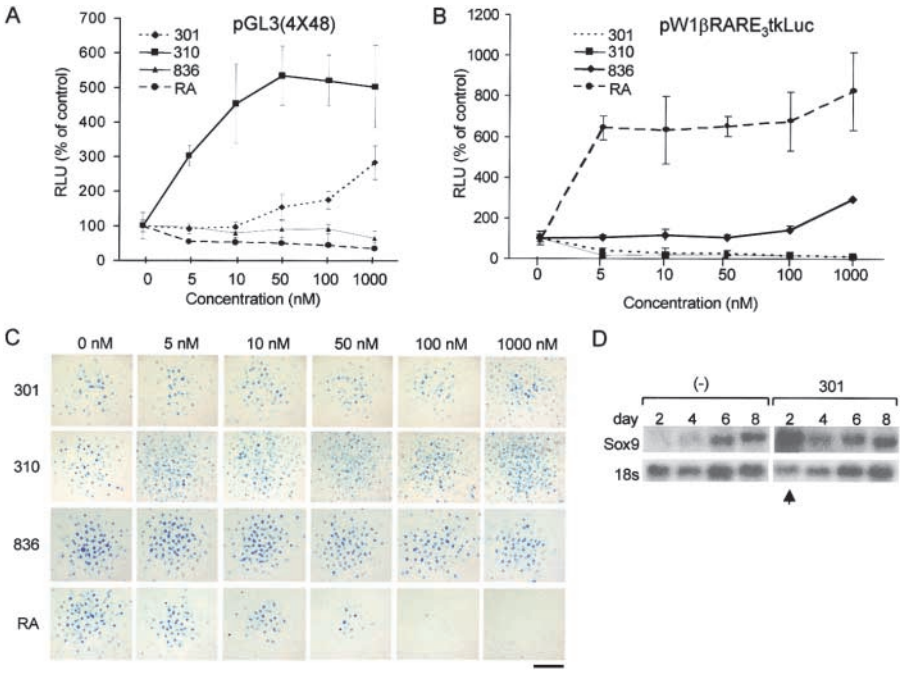


Figure 1. **Inhibition of RAR activity enhances Sox9 activity and expression.** Activation of the retinoid receptors in primary limb mesenchymal cultures with either at-RA or the RARα-specific agonist AGN193836 (836) attenuates activity of the pGL3(4X48) Sox9 reporter (A); however, at-RA appears to be a more effective inhibitor. In contrast, inhibition of RAR activity by the RARα-specific antagonist, AGN194301 (301), or with the RAR pan-antagonist, AGN194310 (310), enhances reporter activity with 310 being more potent than 301. The effects of these compounds on activity of pGL3(4X48) are inversely proportional to their ability to activate a luciferase reporter containing an RA response element (pW1-βRARE$_3$-Luc) (B). The concentration-dependent effects of each compound on Sox9 reporter activity also correspond with the ability of each compound to enhance or inhibit cartilage formation in vitro as indicated by alcian blue staining (C). In response to AGN194301, there is an increase in Sox9 mRNA as early as day 2, but this increase appears to be transient (D). Bar, 1.5 mm. ANOVAs (A and B), $P < .0001$; Bonferroni post-tests indicate significant differences in Sox9 reporter activity at concentrations $\geq 5 \times 10^{-9}$ M for 310, 1×10^{-7} M for 301, 5×10^{-8} M for 836, and 5×10^{-9} M for at-RA. Significant changes in activity of the RARE reporter are induced by concentrations $\geq 5 \times 10^{-9}$ for 310, 1×10^{-7} M for 301, 5×10^{-9} M for at-RA, and 5×10^{-9} M for 836, with all P values at least < 0.05.

310 more effectively than by 301. Thus, activation of the Sox9-responsive region of *Col2a1* appears to be very closely associated with the status of RAR activity. This close association is reflected in the response of primary cultures to treatment with each compound (Fig. 1 C). Treatment with either at-RA, the RARα agonist, or with the antagonists for 4 d affects the formation of cartilage nodules in a manner that would be predicted from their effects on Sox9 reporter activity. More specifically, at-RA is a more potent inhibitor of cartilage nodule formation than 836, whereas the increase in nodule formation can be observed at a lower concentration of the pan-antagonist, (10 nM 310) compared with the RARα-specific antagonist (1 μM 301). Together, these results further validate the utility of the Sox9 reporter assay to indirectly measure the status of chondroblast differentiation, and more importantly they highlight the significant role of RAR-mediated signaling in regulating expression of the chondroblast phenotype.

The enhanced Sox9 reporter activity caused by RAR inhibition is due, in part, to an increase in the expression of *Sox9* mRNA, since treatment of primary cultures with 1 μM 301 results in an precocious increase in *Sox9* expression (Fig. 1 D). There is a noticeable increase in *Sox9* mRNA from 2-d cultures treated with 301, but this increase over control cultures is much less pronounced by days 4 and 6. Thus, inhibition of RAR activity appears to induce an early transient upregulation of *Sox9* mRNA that presumably contributes to the enhanced Sox9 reporter activity seen in response to the same compound.

To confirm the influence of RAR activity on chondroblast differentiation, we introduced modified versions of the RARs or RXRs into primary limb mesenchymal cultures to follow their effect on Sox9 reporter activity. To examine the effect of RAR activation without agonist addition, constitutively active versions of RARα and RXRα were used by fusing the acidic activation domain of VP16 to the COOH terminus of RAR and RXR referred to as RARαVP16 and RXRαVP16, respectively (Underhill et al., 1994). Here we confirmed the ability of these modified receptors to potently activate an RARE reporter in the absence of an exogenous agonist, since cotransfection of RARαVP16 and RXRαVP16 induced RARE reporter activity by 15- and 17-fold, respectively, in the absence of agonist (Fig. 2 A).

In addition to modifying RARE activity with constitutively active versions of the retinoid receptors, dominant negative versions of the receptors (dnRARα and dnRXRα) were also generated and transfected into primary limb mesenchymal cultures. These dominant negative derivatives are COOH-terminal truncations of RARα and RXRα that retain their ability to bind DNA and ligand but lack the AF-2 transactivation function (Damm et al., 1993; Feng et al., 1997). When cotransfected into primary cultures, both dnRARα and dnRXRα are effective at completely blocking activity of the RARE reporter (Fig. 2 B). Cotransfection of the modified receptors affects Sox9 reporter activity in a manner that is inversely proportional to their ability to trans-activate βRARE$_3$-tk-Luc. Both the RARαVP16 and RXRαVP16 inhibit Sox9 reporter activity, whereas the dnRARα and dnRXRα potently activate this reporter (Fig. 2, C and D). Interestingly, the activation induced by cotransfection with dnRARα is more dramatic than that elicited by any other factor studied to date, including those examined by our laboratory and those reported previously. Similar to the results using receptor agonists and antagonists,

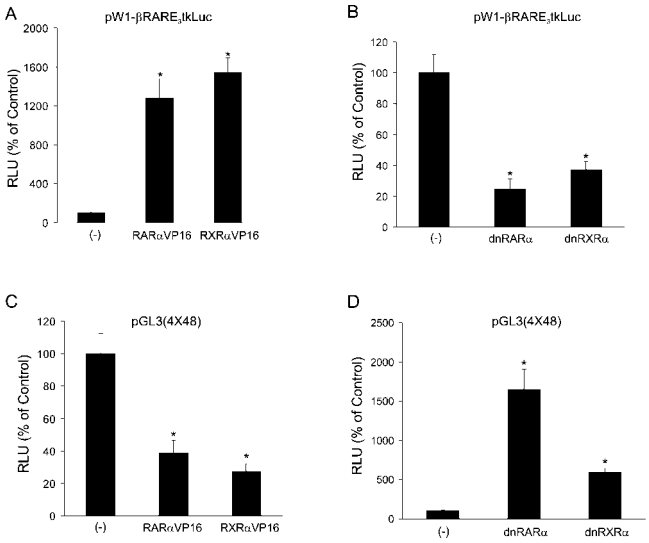


Figure 2. **Sox9 transactivation of *Col2a1* is inversely associated with RAR activity.** To further study the influence of retinoid receptor activity on chondrogenesis, constructs containing constitutively active receptors (RARαVP16 and RXRαVP16) or dominant negative versions of the receptors (dnRARα and dnRXRα) were used. These constructs substantially alter the activity of the pW1-βRARE$_3$-Luc reporter (A and B). RARαVP16 and RXRαVP16, which enhance activity of the RARE reporter (A), attenuate activity of the pGL3(4X48) reporter (C). In contrast, the dnRARα and dnRXRα, which suppress RARE reporter activity (B) substantially activate pGL3(4X48), albeit the dnRARα has a more dramatic effect (D). ANOVAs (A–D), $P < .0001$; Bonferroni post-tests (A–D), $^*P < .001$.

these studies demonstrate a strong influence of retinoid receptor activity on chondrogenesis.

Chondrogenic response to RAR inhibition requires 48 bp enhancer elements within *Col2a1* and is specific to chondrogenic cells

To closely examine the contribution of RAR inhibition to Sox9 activity, reporters with varying sensitivities to Sox9 were used to follow their response to the dnRARα. Of four reporters analyzed, 4X48-p89 and pGL3(4X48), which demonstrate the greatest sensitivity to Sox9 (Fig. 3 A), also exhibit the greatest response to dnRARα (Fig. 3 B). In contrast, pGL3(−89+6), a reporter containing only the minimal *Col2a1* promoter with no 48 bp Sox9 binding sites, exhibits no activity in response to Sox9 and is unaffected by the dnRARα (Fig. 3). A reporter containing two tandem repeats of a larger intron-1 segment of *Col2a1* (including Sox9 binding sites) along with a promoter fragment is only mildly sensitive to Sox9 and is activated to a much lesser extent by dnRARα compared with the 4X48-containing reporters. These results demonstrate a direct relationship between inhibition of RAR signaling and Sox9 activity.

Despite the induction of Sox9 reporter activity elicited by dnRARα in other cells with chondrogenic capacity, such as dedifferentiated rat articular chondrocytes and C5.18 chondroprogenitor cells, activity of Sox9 reporter activity is not noticeably affected in COS P7 cells (Fig. 4). Given that COS P7 cells are nonchondrogenic, these results suggest that the Sox9 reporter induction caused by RAR inhibition may be restricted to cells with chondrogenic capacity.

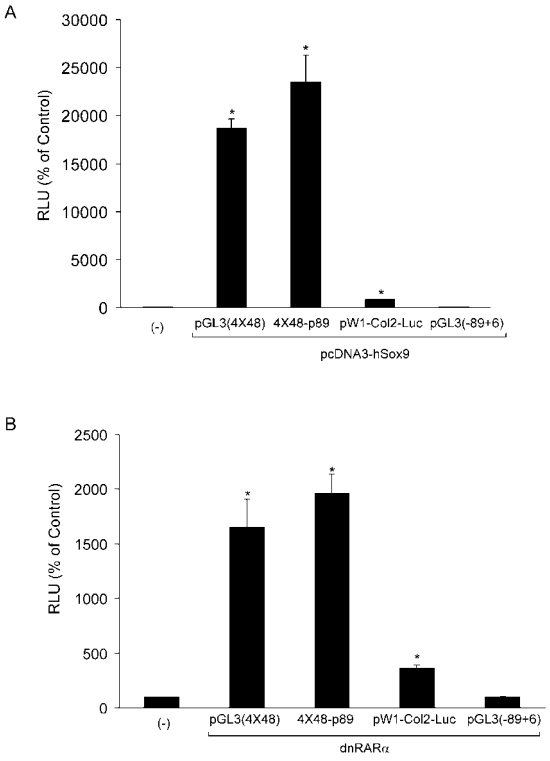


Figure 3. **Sox9 binding sites are essential for dnRARα-induced reporter activity.** To examine the contribution of Sox9 to the effects of RAR inhibition, reporters with varying sensitivities to Sox9 were used to follow their response to the dnRARα. All reporter constructs were cotransfected with pcDNA3-hSox9 (A) or with dnRARα (B). Of four reporters analyzed, 4X48-p89 and pGL3(4X48) are most sensitive to Sox9 (A) and also exhibit the greatest response to dnRARα (B). In contrast, pGL3(−89+6), containing only the minimal *Col2a1* promoter with no Sox9 binding sites, exhibits no activity in response to Sox9 (A) and is unaffected by the dnRARα (B). A reporter (pW1-Col2-Luc) containing two tandem repeats of a larger intron-1 segment of *Col2a1* (including Sox9 binding sites) along with a promoter fragment is only mildly sensitive to Sox9 (A) and is activated to a much weaker extent by dnRARα (B) compared with the 4X48-containing reporters. All reporter inductions by hSox9 or dnRARα were normalized to basal levels of respective reporters. ANOVAs (A and B), $P < .0001$; Bonferroni post-tests, $^*P < .001$.

Chondrogenesis requires histone deacetylase-mediated gene repression

Transcriptional regulation by the retinoid receptors depends for the most part on ligand availability. In the absence of ligand, RAR/RXR heterodimers bind to and repress the transcription of various target genes. Receptor-mediated repression is due to association with nuclear complexes containing corepressors (nuclear receptor corepressor [N-CoR] and SMRT) and histone deacetylases (HDACs) (Nagy et al., 1997). Trichostatin A (TSA) is a *Streptomyces* metabolite that specifically inhibits histone deacetylases leading to hyperacetylation of histones and other proteins (Finnin et al., 1999). To date, TSA has been shown to act as a potent inducer of differentiation in many cell types, some of which are also induced to differentiate by treatment with RA. Interestingly, chondroprogenitors, which in contrast to most cell types do not differentiate in response to RA, also respond uniquely to TSA as indicated

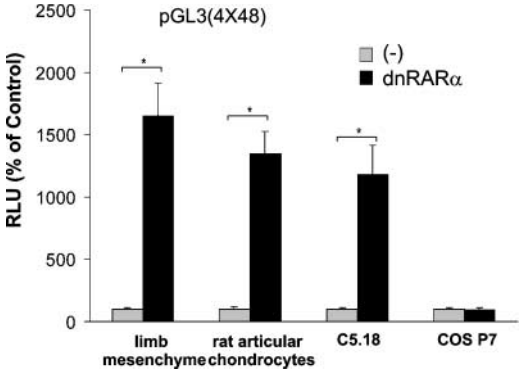


Figure 4. **Induction of Sox9 activity by dnRARα is observed in other chondrogenic cells.** The induction of Sox9 reporter activity by dnRARα in different cells compared with vector-transfected (−) controls is shown. The effect of dnRARα on Sox9 reporter activity is consistent in chondrogenic cells, since considerable increases in pGL3(4X48) are induced not only in limb mesenchymal cells but also in rat articular chondrocytes and in C5.18 cells, which both have chondrogenic capacity. In contrast, no noticeable change in reporter activity is induced in the nonchondrogenic COS P7 cells. Student's *t* tests, $*P < .001$.

by both a dose-dependent decrease in Sox9 reporter activity (Fig. 5 A) and in cartilage nodule formation (Fig. 5 C) in response to a TSA-induced increase in RARE reporter activity (Fig. 5 B). TSA also attenuates the 310-induced increase in Sox9 reporter activity and nodule formation (Fig. 5, A and C). The inhibitory effects of TSA on chondrogenesis are achieved at relatively low concentrations of TSA, since higher concentrations have been used to induce differentiation of many cell types including NIH3T3 cells and acute promyelocytic leukemia blasts (Sugita et al., 1992; Ferrara et al., 2001). Moreover, the well-characterized ability of TSA to inhibit IL-2 gene expression was found to have an IC_{50} of 73 nM (Koyama et al., 2000), which is greater than the highest concentration (10 nM) used here. Thus, these results demonstrate an important requirement for HDAC-mediated gene repression in chondroblast differentiation.

To further examine the importance of nuclear corepressors in chondroblast differentiation, we examined the ability of a dominant negative version of N-CoR, pCMX-G/N-CoR(2174–2453), to modulate Sox9 reporter activity. This construct lacks the HDAC interaction domain and contains the nuclear hormone receptor interaction domain of N-CoR, a region similar to that of SMRT, which was shown recently to disrupt nuclear corepressor function (Koide et al., 2001). Consistent with these activities, the pCMX-G/N-CoR(2174–2453) inhibited the ability of the antagonists and the dnRAR to decrease RARE reporter activity (unpublished data). Expression of pCMX-G/N-CoR(2174–2453) alone led to an ~50% decrease in basal Sox9 reporter activity. Moreover, coexpression of pCMX-G/N-CoR(2174–2453) completely inhibited the stimulatory effects of 301 and 310 and repressed the effect of the dnRAR on the Sox9 reporter (Fig. 5 D). These results suggest that active repression by RARs is required for chondroblast differentiation and that this repression requires deacetylase activity.

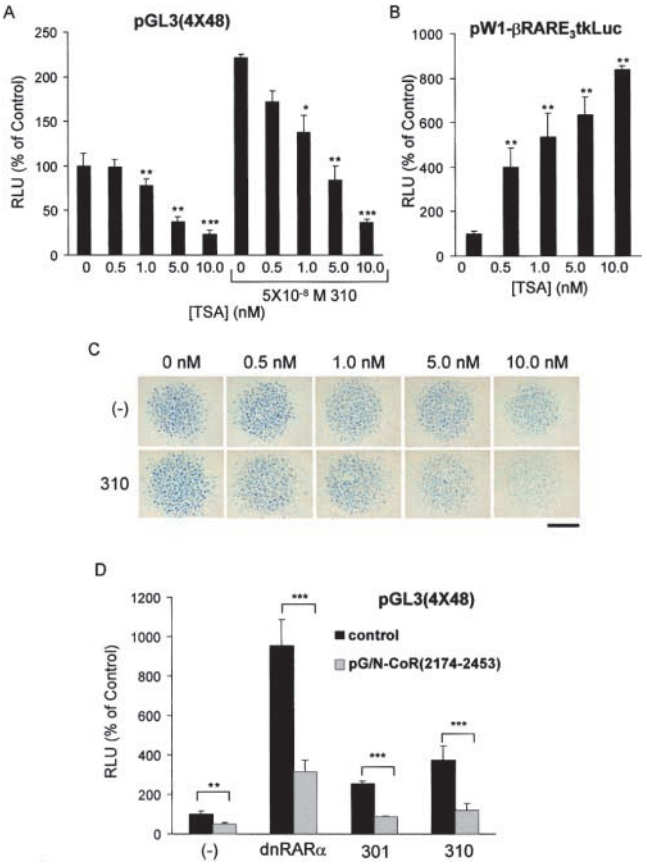


Figure 5. **Histone deacetylase-mediated gene repression is required for chondrogenesis.** The effects of TSA on Sox9 reporter activity in the presence or absence of AGN194310 (A) and on pW1-βRARE$_3$tkLuc (B) were analyzed. TSA attenuated Sox9 reporter activity in a concentration-dependent manner and inhibited the effects of AGN194301 (A). In contrast, TSA enhanced activity of the pW1-βRARE$_3$tkLuc reporter in a concentration-dependent manner (B). The inhibition in Sox9 reporter activity correlates with the decrease in the number of cartilage nodules forming in response to TSA as seen in day 4 alcian blue-stained cultures (C). The increases in Sox9 reporter activity induced by cotransfection with dnRARα or by treatment with AGN194301 or AGN194310 are attenuated by coexpression of pCMX-GAL4/N-CoR (D). Bar, 1.5 mm. ANOVAs (A and B), $P < 0.0001$ for all cases; Bonferroni post-tests (A and B), $*P < .01$, $**P < .001$, and $***P < .0001$, all versus respective control cultures; Student's *t* tests (D), $**P < .001$ and $***P < .0001$.

RAR inhibition activates the p38 MAPK and PKA pathways

To elucidate the mechanism whereby a loss in RAR activity leads to enhanced Sox9 activity, pathway profiling vectors were used to uncover signal transduction pathways that act downstream of retinoid signaling. Various reporters containing reiterated enhancer sequences were transiently cotransfected into primary cultures with a dnRARα. Cotransfection with the dnRARα was used as it is a potent constitutive repressor that consistently induces high Sox9 activity in primary cells. The luciferase-based reporters used contained response elements for activating protein-1 (pAP-1-TA-Luc), cAMP (pCRE-TA-Luc), nuclear factor of κB cells (pNFκB-TA-Luc), nuclear factor of activated T cells (pNFAT-TA-luc), serum, (pSRE-TA-Luc), glucocorticoids (pGRE-TA-Luc), and interferons (pISRE-TA-Luc). Each vector con-

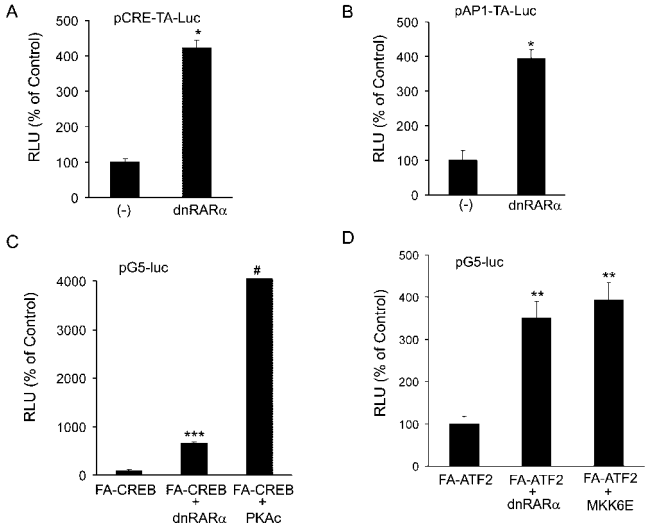


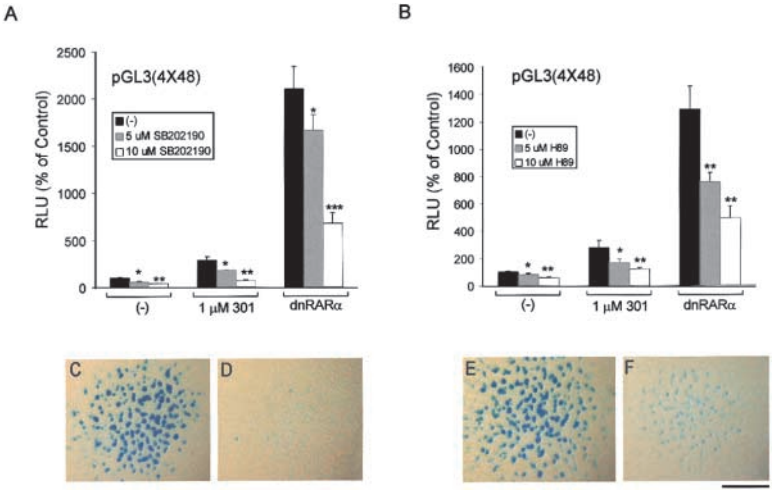
Figure 6. **The p38 MAPK pathway and the PKA pathway are activated in response to RAR inhibition.** Reporters containing a cAMP response element (pCRE-TA-Luc) or activator protein-1 response element (pAP-1-TA-Luc) are both activated in response to cotransfection with dnRARα (A and B). Cotransfection with dnRARα also induces transactivation of a GAL4 reporter (pG5-Luc) by the transcription factors ATF2 and CREB, both of which are fused to the DNA binding domain of GAL4 (FA-ATF2 and FA-CREB) (C and D). The ability of PKAc and MKK6E to activate FA-CREB and FA-ATF2, respectively, was tested for positive control purposes. Student's *t* tests (A and B), $^{*}P < .0005$; ANOVAs (C and D), $P < .0001$; Bonferroni post-tests (C and D), $^{\#}P < .0001$, $^{**}P < .001$, and $^{***}P < .01$.

tained the reiterated response elements upstream of a TATA box and the luciferase gene. Interestingly, when cotransfected with a dnRARα the only reporters appreciably affected (greater than twofold increases) were pCRE-TA-Luc and pAP-1-TA-Luc. Cotransfection with dnRARα enhanced activity of these reporters by greater than fourfold (Fig. 6, A and B), indicating that RAR inhibition may result in activation of pathways upstream of CRE and AP-1 responses.

The PKA pathway is a predominant pathway through which genes containing a cAMP-response element (CRE) are activated. When activated through various stimuli, PKA phosphorylates CRE binding protein (CREB), which binds to and activates genes containing cAMP response elements. Accordingly, cotransfection of pCMV-PKA dramatically enhances activation of pCRE-TA-Luc (unpublished data). Given that a pCRE-TA-Luc is activated in cells transfected with a dnRARα, we tested the ability of this modified receptor to induce activation of CREB. A chimeric trans-activator protein containing CREB fused to the DNA binding domain of the yeast transcriptional activator GAL4 (pFA-CREB) was transiently transfected into cells with a luciferase reporter containing a reiterated GAL4 DNA binding element. Thus, by monitoring the activity of the pG5-Luc reporter the activation of FA-CREB was indirectly followed. Cotransfection of pCMV-PKA into the primary cultures induced an ~40-fold increase in pG5-Luc (Fig. 6 C). Cotransfection with dnRARα enhanced FA-CREB-induced transactivation of pG5-Luc by approximately sixfold.

In addition to the PKA pathway, we investigated potential mechanisms that may underlie the activation of AP-1 by dnRARα. Activating protein-1 collectively refers to dimeric transcription factors composed of Jun, Fos, or activating transcription factor (ATF) subunits. Surprisingly, a dominant negative version of Fos (A-Fos), which substantially diminishes pAP-1-TA-Luc reporter activity, was found to have no noticeable effect on activity of the Sox9 reporter (unpublished), suggesting that the induction of pAP-1-TA-Luc by dnRARα does not involve activation of Jun/Fos dimers. Moreover, constitutively active versions of kinases within the MAPK pathways were tested for their ability to modulate Sox9 transactivation. Of the kinases known to be upstream of AP-1 activation, only a constitutively active version of MKK6 (MKK6E) consistently led to increased Sox9 reporter activity. The predominant targets of MKK6 appear to be the p38 mitogen-activated protein kinase (MAPK) isoforms. When phosphorylated, p38 phosphorylates and activates several targets including the AP-1 component ATF2. As a positive control, MKK6E was cotransfected into cells and found to induce activity of pAP-1-TA-Luc (unpublished data). Given that ATF2 has been shown to bind to AP-1 response elements, we used the pG5-Luc reporter to measure the activity of FA-ATF2, a chimeric of ATF2 and the DNA binding domain of GAL4. Cotransfection of dnRARα induced an increase in FA-

Figure 7. **Inhibition of p38 and PKA prevents chondrogenesis.** In the presence of 5 or 10 μM SB202190, there is a decrease in Sox9 reporter activity compared with untreated controls (A). SB202190 also attenuates the chondrogenic response to AGN194301 and the dnRARα (A). This inhibition is reflected by a lack of cartilage formation in vitro, since almost no cartilage nodules form in response to 10 μM SB202190 (D) in contrast to the presence of numerous nodules in untreated control cultures (C). Similar to SB202190, the PKA inhibitor H89 reduces Sox9 reporter activity both in the presence or absence of AGN194301 or dnRARα (B). In H89-treated cultures (10 μM) (F), fewer nodules are detected compared with untreated cultures (E); these nodules are much smaller and stain only weakly with alcian blue. Bar, 1.5 mm. ANOVAs (A and B), $P < .0001$; Bonferroni post-tests (A and B), $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{\#}P < .005$, all versus respective non-SB202190 or non-H89–treated controls.



ATF2 activation of pG5-Luc that was almost as robust as the induction by MKK6E (Fig. 6 D).

Further support for the role of p38 MAPK and PKA in chondroblast differentiation comes from the reduction in Sox9 reporter activity caused by the p38 MAPK inhibitor SB202190 and the PKA inhibitor H89 (Fig. 7, A and B). These inhibitors also attenuated the induction of Sox9 reporter activity by dnRARα and by 301 (Fig. 7, A and B). Consistent with this, the inhibitors at 10 μM inhibited the formation of cartilage nodules in untreated (Fig. 7, D and F) and 301-treated cultures (unpublished data) compared with untreated cultures (Fig. 7, C and E).

Activation of ATF2 and CREB induces Sox9 transactivation response

The studies described above suggest that the suppression of RAR activity leads to activation of the p38 MAPK and PKA signaling pathways. Phosphorylation of ATF2 and CREB is reflective of activation of p38 MAPK and PKA signaling pathways, respectively. To further investigate a possible role for these signaling pathways in the activation of Sox9, factors involved in these pathways were transiently transfected into the mesenchymal cells along with the Sox9 reporter. Transient transfection of a constitutively active version of MKK6 (MKK6E) induces an approximate threefold activation of FA-ATF2 (Fig. 8 A). When transfected along with p38α or p38β, MKK6E is able to induce FA-ATF2 activity by ~13- and 14-fold, respectively, and even more so with the two isoforms together. However, p38α and p38β alone or in combination have no noticeable effect on Sox9 activity. The ability of each expression plasmid to induce activation of FA-ATF2 is directly proportional to their influence on Sox9 reporter activity (Fig. 8 B), with a >4.5-fold activation by cotransfection with MKK6E along with p38α and p38β. Similarly, Sox9 is activated by the catalytic subunit of PKA, which potently enhances FA-CREB activity. However, the induction of Sox9 activity by PKA is relatively mild given the level of FA-CREB activation elicited by PKA. These results demonstrate the relevance of activation of the p38 and PKA pathways by dnRARα, since each pathway has the potential to induce Sox9 transactivation of *Col2a1*.

Sox9 DNA binding and hence its transcriptional activity has been shown to be induced by PKA-mediated phosphorylation of serines 64 and 181 (Huang et al., 2000). Specifically, PKA phosphorylation of serine 181 in Sox9 was found to occur in chondrocytes of the prehypertrophic zone in response to parathyroid hormone-related peptide (Huang et al., 2000, 2001). To determine if the same phosphorylation event occurs here in response to RAR antagonism, we compared the ability of dnRARα to induce Sox9 reporter activity in the presence of a cotransfected vector containing wtSox9 versus a mutant Sox9 in which serine 181 was replaced with alanine (Sox9-181A). In the absence of exogenous Sox9, Sox9 reporter activity is increased ~4.5 fold by activation of the PKA pathway using pCPT-cAMP (500 μM) in comparison to an ~9-fold increase by coexpression of dnRARα. Cotransfection with wt Sox9 or Sox9-181A increased reporter activity ~100 fold, and this was further increased, albeit slightly in each case (<1.5 fold), by the addition of pCPT-cAMP or by coexpression of PKAc or a

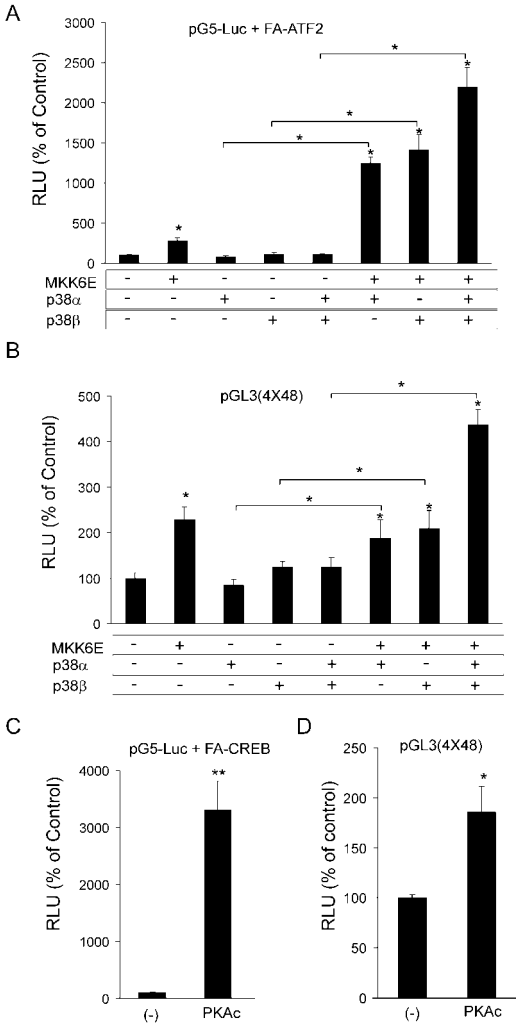


Figure 8. **Activation of ATF2 or CREB induces Sox9 transactivation response.** The effects of different components of the p38 signaling cascade on ATF2 induction were analyzed. Transient transfection with MKK6E induces ATF2 activity greater than twofold; however, transient expression of p38α or p38β either alone or in combination has no appreciable effect on FA-ATF2 activity (A). However, when cotransfected with MKK6E, p38α or p38β can enhance FA-ATF2 activity considerably, and when both isoforms are transfected together along with MKK6E there is an even greater induction of FA-ATF2 (A). The effects of MKK6E, p38α, and p38β on Sox9 reporter activity corresponds with their ability to activate FA-ATF2 (B). MKK6E induces an almost 2.5-fold increase in activity of pGL3(4X48), whereas p38α and p38β alone have no noticeable effect. When cotransfected with MKK6E, p38α and p38β each enhance Sox9 reporter only slightly, but when transfected together along with MKK6E the increase in Sox9 transactivation is greater than fourfold (B). PKAc induces FA-CREB activity by >30-fold (C) but only enhances Sox9 reporter activity slightly (by <2-fold) (D). ANOVAs (A and B), $P < .0001$; Bonferroni post-tests, $^*P < .001$ (A) and $^*P < .001$ (B); Student's *t* test, $^{**}P < .0005$ (C), $^*P < .001$ (D).

dnRARα and decreased by the addition of H89 (Fig. 9 A; unpublished data). There is no significant difference in the activity of Sox9 versus Sox9-181A, suggesting that phosphorylation of serine 181 is not required for Sox9 activity during chondroblast differentiation. As mentioned, immunolocalization studies detected Ser181-phosphorylated Sox9 in prehypertrophic chondrocytes. However, the cells used here are chondroprogenitors, and thus, Sox9 activity may be

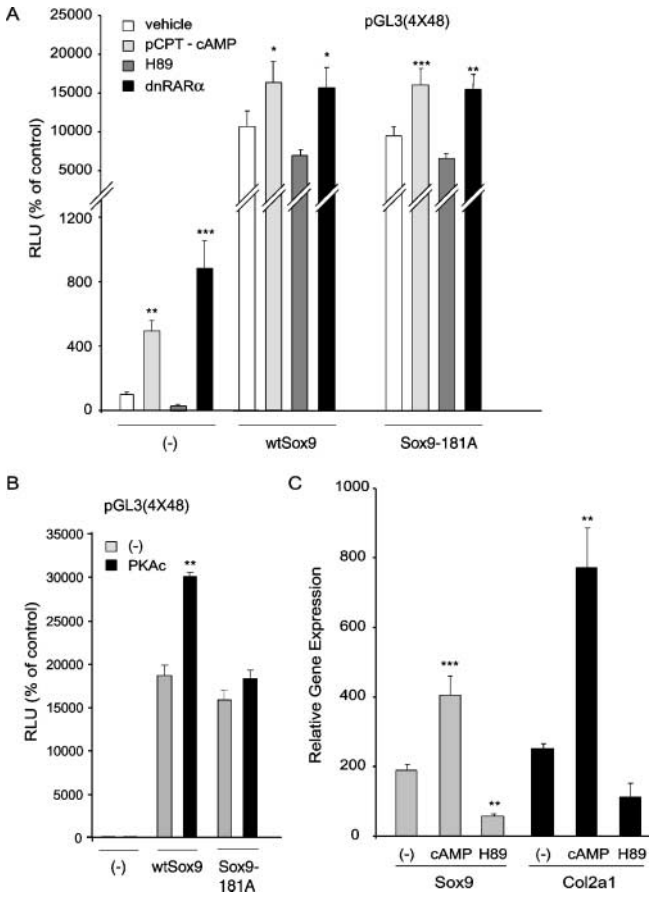


Figure 9. **Regulation of Sox9 expression and transcriptional activity by PKA.** Sox9 expression and activity were measured in response to manipulation of the PKA signaling pathway. In the absence of exogenous Sox9, addition of pCPT-cAMP (500 μM) or cotransfection with dnRARα increases Sox9 reporter activity, whereas H89 (10 μM) represses reporter activity in primary limb mesenchymal cells (A). In the presence of cotransfected Sox9, reporter activity is elevated >100-fold, and the addition of pCPT-cAMP or cotransfection with dnRARα only has a small stimulatory effect (<1.5 fold), whereas the addition of H89 slightly decreases reporter activity. The response of the mutant Sox9-181A is similar to wild-type Sox9 (A). To demonstrate that the mutation functioned as expected, COS P7 cells were transfected with wild-type Sox9 or the mutant Sox9 in the presence or absence of PKA (B). The presence of PKA leads to a >1.5-fold induction in reporter activity, whereas the mutant Sox9 exhibits little increase with cotransfected PKA. Modulation of PKA signaling influences *Sox9* and *Col2a1* expression in primary limb mesenchymal cells (C). Real-time quantitative PCR was used to demonstrate that activation of PKA leads to an increase in *Sox9* and *Col2al* mRNA abundance, whereas treatment with H89 suppresses their expression. ANOVAs (A–C), $P < .0001$; Bonferroni post-tests (A–C), $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$, all versus respective nontreated controls.

regulated through distinct posttranslational modifications within each cell type. To ensure that the mutant Sox9 functions in a manner consistent with that reported previously (Huang et al., 2000), wtSox9 and Sox9-181A were transfected into COS P7 cells in the presence or absence of an expression vector for the catalytic subunit of PKA. COS P7 cells were originally used to identify Ser181 as the PKA phosphorylation site, and as expected the ability of PKAc to activate Sox9 in these cells is almost completely blocked by the Ser181 mutation (Fig. 9 B). Thus, similar to earlier reports Ser181 of Sox9 appears to be required for increased activation of Sox9 by PKA in these cells, but this is clearly not the case in the limb mesenchymal cells used in this study.

To determine if modulation of PKA activity affects the expression of *Sox9* and *Col2a1* transcripts, real-time quantitative PCR was used to measure their relative expression levels in comparison to *rRNA*. *Sox9* and *Col2a1* expression were increased by more than twofold in response to a 2-d treatment with pCPT-cAMP (500 μM) and decreased by more than twofold in response to H89 (10 μM) (Fig. 9 C). A similar increase in *Col2a1* expression by activation of PKA in limb mesenchymal cultures has been reported previously (Kosher et al., 1986). Therefore, our results suggest that PKA regulates Sox9 activity during chondroblast differentiation by influencing *Sox9* expression levels and not through phosphorylation of Ser181. However, a posttranslational role for PKA modulation of Sox9 cannot be entirely excluded, since

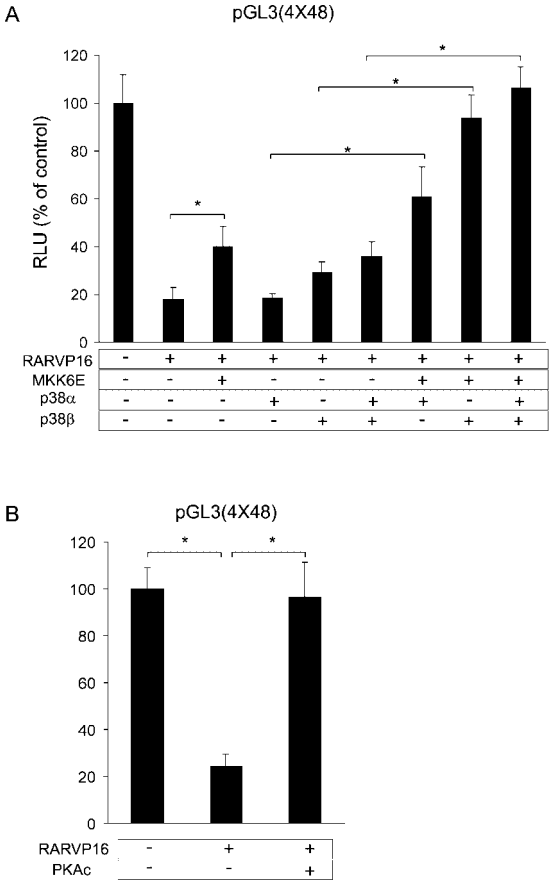


Figure 10. **Activation of ATF2 or CREB can rescue the effects of RARαVP16.** The ability of ATF2 and CREB to reverse the effects of RARαVP16 on Sox9 reporter activity was analyzed. MKK6E can partially prevent the inhibitory response of RARαVP16 whereas p38α and p38β alone or in combination had little if any effect (A). Cotransfection with MKK6E and p38α or p38β was able to partially restore Sox9 activity, whereas cotransfection of MKK6E with both p38α and p38β can completely restore the effects of RARαVP16 (A). Activation of PKA by transient transfection with PKAc was also able to restore Sox9 reporter activity to basal levels in RARαVP16-transfected cultures. ANOVA (A and B), $P < .0001$; Bonferroni post-tests, $^*P < .001$ (A); $^*P < .001$ (B).

cotransfected Sox9s do exhibit slightly increased activity in the presence of cAMP or cotransfected PKA.

The influence of p38 MAPK and PKA signaling pathways on chondrogenesis is best demonstrated by their ability to rescue the decrease in Sox9 activity induced by RARαVP16 (Fig. 10). Although Sox9 activity is only partially restored by cotransfection with RARαVP16 and MKK6E, transfection of each isoform in combination with MKK6E results in levels of Sox9 activity that are almost as high as those obtained in the absence of RARαVP16. Not surprisingly, MKK6E cotransfected with both isoforms of p38 (which causes the most pronounced activation of ATF2) results in a complete rescue of Sox9 activity (Fig. 10 A). Similarly, PKAc can almost completely rescue the effects of RARαVP16 (Fig. 10 B). These results are not due to modulation of RARαVP16, since neither MKK6E or PKAc inhibited RARαVP16 induction of an RARE reporter (unpublished data).

Discussion

The precise timing of differentiation during development is critical for the establishment of a properly patterned embryo. Previously, we identified an important role for RAR activity in the timing of chondroblast differentiation (Weston et al., 2000), prompting us to investigate the mechanism underlying retinoid receptor regulation of the prechondroblast-to-chondroblast transition. Of the three Sox genes identified to date as chondrogenic regulators, *Sox9* has emerged as a central player in regulating the initial stages of the chondrogenic process. Moreover, to reproduce the pattern of *Col2a1* expression in the cartilage elements with a reporter gene only a small (48 bp) enhancer element from the first intron of *Col2a1* containing Sox9 binding sites is required. For these reasons, Sox9 is commonly referred to as a "master regulator" of chondrogenesis, and as such, analysis of Sox9 expression and activity was an obvious starting point for identifying factors downstream of RAR activity that may mediate the effects of RAR activation or inhibition.

Association between retinoid receptor activity and Sox9 transactivation

Using soluble compounds in combination with expression plasmids, we have demonstrated a surprisingly tight correlation between retinoid receptor activity and Sox9 transactivation of *Col2a1*. The extent of RAR inhibition was found to be inversely proportional to the level of Sox9 induction. Moreover, manipulations affecting all three RARs were more influential than those affecting only the RARγ subtype, indicating the importance of at least two RAR subtypes in chondrogenesis. Of particular interest was the dramatic induction of Sox9 activity upon cotransfection with dnRARα. To date, no single factor has been shown to induce Sox9 to a similar extent, including numerous factors known to potently stimulate chondrogenesis such as BMPs-2 and -4 either in soluble form or after cotransfection of expression plasmids containing activated BMP receptors (unpublished data). The ability of the dnRARα to stimulate Sox9 activity to a much greater extent than 301 or 310 is likely due to the constitutive repressive activity of the truncated receptor. Consistent with this, the dnRARα has been shown previously to exhibit enhanced association with nuclear corepressors (Wong and Privalsky, 1998). Interestingly, the ability of the dnRXR to significantly enhance Sox9 activity also suggests that the absence of the RXR AF-2 domain may facilitate formation of an RAR/dnRXR–nuclear corepressor complex.

The more pronounced effects of AGN 194310 and at-RA compared with the more specific compounds, AGN 194301 and AGN 193836, respectively, implicates the involvement of at least two receptor subtypes in regulating chondroblast differentiation. These results are further supported by the effects of the dnRARα, which broadly inhibits RAR-mediated signaling (Damm et al., 1993). Together, these studies suggest that a loss of the ligand-induced activity of multiple RARs can initiate chondroblast differentiation. Accordingly, these findings explain the failure of cells expressing a weak constitutively active RAR in transgenic mice to differentiate into chondroblasts and contribute to cartilage nodules. Continued RAR activity would result in a reduction of Sox9 expression and/or activity, thereby preventing chondroblast differentiation, similar to that observed in *Sox9*-null cells. Interestingly, addition of RA was shown recently to cause a downregulation of *Sox9* in chondrocytes (Sekiya et al., 2001). However, it is unclear from this study whether this reduction is due to a loss of the chondrocyte phenotype or by a transition to hypertrophic chondrocytes, both of which are induced by RA and are associated with a reduction in *Sox9* expression (Horton et al., 1987; Koyama et al., 1999).

Importance of RAR-mediated repression in chondroblast differentiation

Our results clearly demonstrate a critical role for retinoid receptors in chondrogenesis. Surprisingly, homozygous mutant mice lacking individual RARs develop essentially normal skeletons with the exception of RARγ knockout mice in which homeotic transformations of the cervical vertebra and occipital region of the skull are observed along with other minor irregularities such as fusion of the first and second ribs (Li et al., 1993; Lohnes et al., 1993; Lufkin et al., 1993; Mendelsohn et al., 1994; Luo et al., 1995; Kastner et al., 1996; Krezel et al., 1996; Ghyselinck et al., 1997; Iulianella and Lohnes, 1997). The minor phenotypes observed in single RAR KOs have, for the most part, been attributed to the existence of functional redundancy between members of the RAR family. Indeed, embryos in which multiple RARs are deleted exhibit more extensive skeletal defects (Lohnes et al., 1994). However, these effects are less severe than expected based on the dramatic teratogenic effects of RA and the effects observed in mice deficient in enzymes involved in RA synthesis or degradation (Niederreither et al., 1999; Abu-Abed et al., 2001; Sakai et al., 2001).

The discrepancy between our results and the RAR knockout studies may be the result of distinct approaches used in manipulating receptor function. In the present study, we used complimentary approaches to examine the role of RAR repression in skeletal progenitor differentiation. Overexpression of a dnRARα or the addition of soluble RAR antagonists effectively abolishes transcriptional activation while at the same time maintaining or enhancing active repression. However, in the knockout mice the receptors are entirely absent and cannot function as transcriptional activators or re-

pressors. Consequently, genes that would normally be repressed by the receptors in the absence of ligand may exhibit increased expression.

The important role, demonstrated herein, for RAR-mediated repression is consistent with the recently described requirement for RAR-mediated repression in *Xenopus* head formation (Koide et al., 2001). Specifically, activation of the retinoid-signaling pathway was found to adversely affect the development of anterior structures, which could be mimicked by overexpression of a dominant negative SMRT lacking its repression domains. In contrast, repression of RAR-mediated signaling by RAR antagonists was shown to promote the expression of anterior neural markers and to enhance the formation of anterior head structures.

Direct evidence for the importance of unliganded nuclear hormone receptors also comes from studies in which the thyroid hormone receptor (TR) of mice was mutated to abolish the ligand binding function of the receptor (Hashimoto et al., 2001). Despite TR knock-out animals having completely normal structure and function of the central nervous system, mice containing the mutated TR exhibit severe neurological development and dysfunction (Hashimoto et al., 2001). These latter effects are more consistent with the severe CNS dysfunction exhibited in cases of congenital hypothyroidism and thyroid hormone resistance syndrome.

Collectively, these results combined with our findings, which demonstrate a requirement for RAR-mediated repression during skeletal development, form an emerging theme whereby retinoid receptors have an important biological role during development in the absence of ligand.

The mechanisms of retinoid regulation of chondroblast differentiation

In addition to establishing a critical role for RAR-mediated repression in skeletal development, our findings provide a framework to describe the molecular regulation of chondroblast differentiation. Previously, we provided evidence to suggest that BMP signaling operates upstream of the retinoid signaling pathway in chondrogenesis (Weston et al., 2000). Here, we show that repression of RAR-mediated signaling results in the activation of p38 MAPK and PKA signaling pathways and that the effects of RAR antagonism on chondrogenesis can be blocked by inhibition of p38 MAPK and PKA activity. P38 MAPK and PKA have both been implicated previously in cartilage formation (Kosher et al., 1986; Lee and Chuong, 1997; Nakamura et al., 1999; Yoon et al., 2000). Specifically, we have found that inhibition of MAPK p38α and/or β prevents cartilage formation and attenuates Sox9 reporter activity. Conversely, activation of the p38α or p38β pathways is sufficient to promote Sox9 activity. Moreover, activation of p38α/β or PKA signaling are the only means identified to date, with the exception of expression of Sox9, that rescue the negative effects of activation of the RAR-signaling pathway on chondrogenesis. Together, these results suggest that activation of the p38 MAPK and PKA pathways upon RAR-mediated gene repression is necessary for acquisition of the chondroblast phenotype.

The ability of gene repression by the RARs to induce chondroblast differentiation suggests that retinoid signaling directly regulates the expression of a gene(s) whose function is to inhibit chondroblast differentiation. This novel role for gene repression in chondroblast differentiation is opposite to that of myogenic cells in which gene repression prevents differentiation. TSA stimulates differentiation of muscle progenitors, consistent with the recently described requirement for HDAC export from the nucleus before expression of the myoblastic phenotype (McKinsey et al., 2000). Thus, although the status of histone acetylation represents a general mechanism underlying cell differentiation the outcome of such modifications is cell type dependent. In this context, opposite roles for RAR-mediated gene repression in different progenitors may provide a means whereby a common local signal (RA) can differentially regulate cell differentiation. In chondroprogenitors, this repression can clearly direct changes in Sox9 activity; thus, identifying the genes affected directly by RAR repression in the chondrogenic sequence will undoubtedly further enhance our current understanding of the molecular networks underlying chondroblast differentiation.

Materials and methods

Expression plasmids and reporter constructs

The Sox9-responsive reporter (herein referred to as the Sox9 reporter) was generated by subcloning a fragment containing a reiterated (4X48) Sox9 binding sequence coupled to the mouse *Col2a1* minimal promoter (−89 to +6) into pGL3. The fragment containing the 4X48 repeat and minimal promoter was isolated as a BamHI/HindIII fragment from the original 4X48-p89Luc reporter plasmid described previously (Lefebvre et al., 1997) and was subcloned into the BglII and HindIII sites of pGL3-basic (Promega) to generate pGL3(4X48). The reporter pW1-Col2-Luc was generated from the original p309i(182X2)βgeoCol2a1 (Zhou et al., 1995) by subcloning the regulatory region (consisting of a 309-bp promoter region and two tandem repeats of a 182-bp intron-1 fragment) into pW1 (Balkan et al., 1992) as an EcoRI/BamHI fragment. A BglII fragment containing the luciferase gene isolated from pJD205 (de Wet et al., 1987) was subcloned into the BamHI site of pW1-Col2 to generate pW1-Col2-Luc. The pcDNA3-hSox9 expression vector was as described (Lefebvre et al., 1997). To generate a mutated form of hSox9, hSox9 was subcloned into pKSII (Stratagene), and serine 181 was replaced with alanine using the Quick-Change XL system (Stratagene) with the following overlapping primers: 5′-GCCGCGGCGGAGGAAGGCGGTGAAGAACGGGCAGG-3′ and 5′-CCTGCCCGTTCTTCACCGCCTTCCTCCGCCGCGGC-3′. After mutagenesis, the Sox9 mutant and wt Sox9 were subcloned into pcDNA3, and the serine-alanine conversion was confirmed by sequencing.

The dominant negative versions of RARα and RXRα were generated as enhanced green fluorescent protein (EGFP) fusions containing COOH-terminal truncations at amino acid positions 403 and 449, respectively (Damm et al., 1993; Feng et al., 1997). A BglII restriction endonuclease site was incorporated into the primers to facilitate cloning and to allow for an in-frame fusion to pEGFP-N1 (CLONTECH Laboratories, Inc.). Internal primers used for truncation of the receptors were as follows: 5′-AGATCTGGGATCTCCATCTTCAATG-3′ for RARα and 5′-CAGATCTCCGATGAGCTTGAAGAAG-3′ for RXRα. For expression in cells, receptor–EGFP fusion constructs were cloned into the mammalian expression plasmid pSG5 (Stratagene). EGFP-N1 was initially subcloned into the pSG5 vector followed by the corresponding truncated receptor to generate pSG5-dnRARαEGFP and pSG5-dnRXRαEGFP.

Constitutively active versions of RARα and RXRα were subcloned into pSG5HS as Hind III/SpeI fragments isolated from the constructs described (Underhill et al., 1994). These receptors contain COOH terminal fusions to the acidic activation domain of VP16 (Underhill et al., 1994). The constitutively active version of MKK6 used here was the previously described pcDNA3-HA-MKK6E (Han et al., 1996). Expression plasmids, pcDNA3-p38α-Flag, and pcDNA3-p38β2-Flag were used to express p38α and p38β2 in mesenchymal cells (Enslen et al., 1998). To activate the PKA pathway, pCMV-PKA (CLONTECH Laboratories, Inc.), which contains the catalytic subunit of PKA was used. To follow activation of ATF2 and CREB, constructs containing the transactivation domain of these transcription factors fused to the DNA binding domain of GAL4 (pFA-ATF2 and pFA-CREB) were used (Stratagene). The pFA-ATF2 and pFA-CREB plasmids were cotransfected with pG5-Luc, a reporter containing five copies of a GAL4

DNA binding element upstream of a TATA box and the luciferase gene (Stratagene). PCMX-N-CoR and pCMX-GAL4/N-CoR (2174–2453) (referred to as pG/N-CoR[2,174–2,453] herein) consists of the DNA binding domain of GAL4 fused to the 3′ region of N-CoR encompassing amino acids 2,174–2,453 as described (Heinzel et al., 1997).

Reporter vectors from Systems 1 and 2 of CLONTECH Laboratories Inc.'s Mercury Pathway Profiling Systems were used to identify pathways downstream of retinoid signaling. These systems are sets of vectors that contain distinct cis-acting enhancer elements upstream of a TATA box and the luciferase gene.

Establishment and transient transfection of primary limb mesenchymal cultures

Limb mesenchymal cells were harvested from embryonic age 11.25–11.75 mouse embryos as described previously (Weston et al., 2000). The cells were resuspended at a density of $\sim 2.5 \times 10^7$ cells/ml before transfections; otherwise, they were resuspended at $\sim 1.5 \times 10^7$ cells/ml. For transfection purposes, cells were mixed with a DNA/FuGene6 mixture in a 2:1 ratio. FuGene6-DNA mixtures were prepared according to the manufacturer's instructions (Roche Biomolecular). Briefly, 1 μg of reporter, 1 μg of expression vector, and 0.05 μg of pRLSV40 (Promega) were mixed for a total of ~2 μg DNA in 100 μl of media and FuGene6. Fifty microliters of the DNA mixture was transferred into a sterile 1.5 ml Eppendorf tube followed by 100 μl of cells. Cells were gently triturated, and 10 μl was used to seed each single well of a 24-well culture dish. After 1.5 h in a humidified CO_2 incubator, 1 ml of media containing compounds of interest was added to each well and subsequently replaced 24 h after transfection. All-trans RA (at-RA; Sigma-Aldrich), AGN193836 (Teng et al., 1996), AGN194301 (Teng et al., 1997), and AGN194310 (Johnson et al., 1999) (Allergan Inc.) were dissolved in 95% ethanol. SB202190 and SB203580 (Calbiochem) and H89 (Sigma-Aldrich) were dissolved in DMSO (BDH). 8-(4-chlorophenylthio)-cAMP (pCPT-cAMP; Sigma-Aldrich) was dissolved in water just before use.

Analysis of reporter gene activity using the Dual Luciferase Assay System was done following the manufacturer's instructions (Promega). Briefly, ~48 h after the transfections cells were washed once with PBS and lysed in 100 μl of Passive lysis buffer for 20 min. Firefly and renilla luciferase activities were determined using 40 μl of lysate in a 96-well plate-reading luminometer (Molecular Devices). Alcian blue staining of cultures was performed as described previously (Cash et al., 1997).

Cell culture and transient transfection of cell lines

COS P7 cells were maintained in DME containing 10% FBS (GIBCO-BRL) and antibiotics. C5.18 cells, subcloned from the parental chondroblast clone RCJ 3.1C5 (Grigoriadis et al., 1996), were maintained in α-MEM supplemented with 15% FBS and antibiotics. Articular chondrocytes were derived from the knee joints of 1-d-old Sprague-Dawley rats. Briefly, 1–2-mm cartilage fragments from the femoral condyles were isolated, washed three times in sterile PBS, and digested with 0.3% Collagenase P (Worthington Biochemical Corporation) at 37°C for 4 h, adding fresh collagenase P after the first 30 min. After digestion, cells were filtered through a cell strainer (70 μm; Falcon) to obtain a single cell suspension. PBS/Collagenase was removed, cells were resuspended at $\sim 1.5 \times 10^5$ cells/ml, and 6 ml were transferred to a T-25 tissue culture flask. Upon reaching confluence, cells were transferred to a T-75 flask.

For transient transfections, the cells described were plated at 5×10^4 cells/well in 12-well tissue culture plates ~24 h before transfection. FuGene6 transfection reagent was used according to manufacturer's instructions (Roche Biomolecular). Each well of cells was transfected with a FuGene-DNA mixture containing a total of 0.5 μg DNA comprised of 0.3 μg of reporter, 0.2 μg of expression vector, and 0.05 μg of pRLSV40. Media was changed ~24 h after transfection, and luciferase assays were performed ~48 h after transfection. Luciferase assays were done as described above with the exception of using 200 μl/well of Passive lysis buffer (Promega) to obtain cell extracts.

Northern blot analysis and real-time quantitative PCR

Northern blots were performed using total RNA from limb mesenchymal cultures as described previously (Weston et al., 2000). Briefly, total RNA was extracted from cells cultured for 2, 4, 6, or 8 d. Cells were treated with media alone or with AGN194301. Synthesis of the *Col2a1* cDNA fragment used was as described previously (Weston et al., 2000). The *Sox9* cDNA probe was made using an EST clone (GenBank/EMBL/DDBJ under accession no. AI594348 [Research Genetics]). The *Sox9* fragment was released from pT7T3 using EcoRI and NotI.

To monitor changes in transcript levels of *Sox9* and *Col2a1*, quantitative real-time PCR was performed using the 7900HT Sequence Detection System (Applied Biosystems). Primers and TaqMan–minor groove binding probes were designed using PrimerDesigner 2.0 (Applied Biosystems). The following primer/probe sets were used for detection of *Col2a1*: forward primer, 5′-GGCTCCCAACACCGCTAAC, reverse primer, 5′-GATGTTCTGGGAGCCCTCAGT, and probe 6FAM-5′-CAGATGACTTTCCTCCGTC-MGBNFQ. *Sox9* transcripts were detected using the forward primer, 5′-CATCACCCGCTCGCAATAC, reverse primer, 5′-CCGGCTGCGTGACTGTAGTA, and probe, 6FAM-5′-ACCATCAGAACTCCGGCT-MGBNFQ. Primer and probe concentrations were optimized according to the manufacturer's instructions. Total RNA was isolated from primary cultures as described above and treated with amplification-grade DNase I (Invitrogen). Quantification was performed using 4 ng of total RNA, and the expression of *Sox9* or *Col2a1* relative to endogenous *rRNA* was determined using TaqMan Ribosomal Control Reagents (Applied Biosystems) and the comparative C_T method as described in User Bulletin no. 2 (Applied Biosystems).

Statistical analysis

All luciferase assays were performed a minimum of three times using separate preparations of primary cells each time. Each transfection or treatment was performed in quadruplicate for all experiments, with the exception of the COS cell transfections which were performed in triplicate. Real-time PCR analysis was performed using RNA from two separate preparations with treatments done in triplicate for each preparation. All luciferase reporter and expression data was analyzed by analysis of variance (ANOVA) followed by a Bonferroni post-test for multiple comparisons using GraphPad Prism, version 2.0 (GraphPad Software Inc., San Diego, CA). One representative experiment is shown for all luciferase and expression results.

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