Retinoic Acid Receptor α Function in Vertebrate Limb Skeletogenesis: a Modulator of Chondrogenesis

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Abstract. Retinoic acid is a signaling molecule involved in the regulation of growth and morphogenesis during development. There are three types of nuclear receptors for all-*trans* retinoic acid in mammals, RARα, RARβ, and RARγ, which transduce the retinoic acid signal by inducing or repressing the transcription of target genes (Leid, M., P. Kastner, and P. Chambon. 1992. *Trends Biochem. Sci.* 17:427–433). While RARα, RARβ, and RARγ are expressed in distinct but overlapping patterns in the developing mouse limb, their exact role in limb development remains unclear. To better understand the role of retinoic acid receptors in mammalian limb development, we have ectopically expressed a modified RARα with constitutive activity (Balkan, W., G.K. Klintworth, C.B. Bock, and E. Linney. 1992. *Dev. Biol.* 151:622–625) in the limbs of transgenic mice. Overexpression of the transgene was associated with marked pre- and postaxial limb defects, particularly in the hind limb, where expression of the transgene was consistently seen across the whole anteroposterior axis. The defects displayed in these mice recapitulate, to a large degree, many of the congenital limb malformations observed in the fetuses of dams administered high doses of retinoic acid (Kochhar, D.M. 1973. *Teratology.* 7:289–295). Further analysis of these transgenic animals showed that the defect in skeletogenesis resided at the level of chondrogenesis. Comparison of the expression of the transgene relative to that of endogenous RARα revealed that downregulation of RARα is important in allowing the chondrogenic phenotype to be expressed. These results demonstrate a specific function for RARα in limb development and the regulation of chondroblast differentiation.

RETINOIC acid (RA)[1] is an important signaling molecule involved in the regulation of growth during embryonic development and cell differentiation. In the developing mammalian limb, RA affects the differentiation of many cell lineages, including those of mesenchymal and chondrogenic origin (Solursh and Meier, 1973; Lewis et al., 1978; Zimmerman and Tsambos, 1985). RA is important in normal limb ontogeny and in excess is a potent teratogen, causing characteristic perturbations of normal limb development in a stage- and dose-dependent manner (Shenefelt, 1972; Kochhar, 1973; Kwasigroch and Kochhar, 1980). The timing of RA treatment and the resultant limb defects appear to coincide with the timing of mesenchyme condensation and differentiation into chondrocytes between embryonic day (E) 11 and E14 (Kwasigroch and Kochhar, 1980). RA treatment at earlier stages (i.e., E9 to E10) has limited effects that are primarily restricted to the digits and that have been attributed to changes in the apical ectodermal ridge and the associated underlying mesenchyme (Sulik and Dehart, 1988; Tickle et al., 1989). At later stages (i.e., >E14), RA treatment has little or no effect on limb patterning and development. Consistent with its role in mesenchyme growth and differentiation, RA has dramatic effects on chondrogenesis of limb mesenchyme in vitro and in vivo. Most of these observed effects arise from changes in gene transcription mediated, in part, by the nuclear hormone receptors for RA. There are two subfamilies of nuclear retinoid receptors known to modulate the actions of RA. The RA receptors (RARs) α, β, and γ, and the retinoid X receptors (RXRs) α, β, and γ, both of which act as ligand-dependent transcription factors through the formation of heterodimers bound to specific RA response elements (RAREs) (for review see Leid et al., 1992*a*; Linney, 1992; Giguère, 1994 and references therein). Both the RARs and RXRs are

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1. *Abbreviations used in this paper*: E, embryonic day; RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; tgRARα, transgenic RARα.

widely expressed in a number of fetal and adult tissues in specific spatial and temporal patterns (Dollé et al., 1989*b*; Krust et al., 1989; Zelent et al., 1989; Dollé, 1990; Ruberte et al., 1990, 1991, 1993; Dollé et al., 1994).

In the developing murine limb, the RARs are expressed in distinct and sometimes overlapping spatiotemporal patterns. The RARs α and γ are expressed in overlapping regions during fore limb development from E9.5 to E11.5 (Dollé et al., 1989*b*; Ruberte et al., 1990). RARγ then becomes preferentially localized to the precartilage condensation and cartilage. In contrast, RARα expression appears to progressively decrease during this chondrogenic sequence and becomes primarily restricted to the surrounding mesenchyme and interdigital zone, where it is comparatively highly expressed (Dollé et al., 1989*b*). RARβ is expressed in the proximal mesenchyme early in limb outgrowth and later is found in the interdigital zone and in the interior, anterior, and posterior necrotic zones (Dollé 1989*b*; Mendelsohn et al., 1991). Two of the presumptive heterodimeric partners for the RARs, RXRα, and RXRβ, are expressed ubiquitously throughout limb development up to E16.5 (Dollé et al., 1994). In the developing chick limb, RARβ1 and RARβ2 have been shown to exhibit distinct expression patterns during the differentiation events associated with chondrogenesis. Initially, RARβ1 expression is ubiquitous and not specifically located to any region, while RARβ2 is restricted to the early mesenchymal condensate in the limb core (Smith et al., 1995). At later stages, RARβ2 is no longer detected in the limb core, where chondrocytes have formed, but is restricted to a thin cell layer lateral to the maturing cartilage.

Given the distinct spatiotemporal expression patterns observed for each of the RARs during mouse embryogenesis and in adult tissues, it has been proposed that each receptor may perform unique functions during development and homeostasis (for review see Chambon, 1994; Underhill et al., 1995 and references therein). Boylan et al. (1993, 1995), for example, have recently shown that targeted disruption of RARα and RARγ in F9 embryonal carcinoma cells results in receptor-specific alterations in RA-mediated differentiation and RA metabolism. In the whole animal, however, the absence of any obvious abnormalities in homozygous null fetuses for a number of receptors and their isoforms suggests that there may be a high degree of functional redundancy among members of the RAR family (Li et al., 1993; Lohnes et al., 1993, 1994; Lufkin et al., 1993; Kastner et al., 1994; Mendelsohn, 1994*a*,*b*; Sucov et al., 1994). Their importance in limb development was demonstrated by the observation that compound homozygotes of null alleles of RARα and RARγ exhibited a range of limb abnormalities from reductions to duplications (Lohnes et al., 1994). Hence, it appears that in early limb development RARα is able to substitute for RARγ and vice versa, but at least one of the receptors is minimally required for proper limb development.

To evaluate the function of RARα in limb development, we have generated transgenic mice that ectopically express a constitutively active form of human RARα1 in the developing limb under the control of a Hoxb-6 promoter fragment. We have previously shown that mice expressing this modified receptor in the eye under the control of the αA-crystallin promoter develop microphthalmia and cataracts, both of which are observed in mice treated with RA in utero (Balkan et al., 1992*b*). Analysis of transgene expression in Hoxb-6 animals revealed two levels of expression in the developing limb. High levels of transgene expression throughout the limb bud produced a range of phenotypic abnormalities that recapitulate many of the congenital limb malformations observed in the fetuses of dams administered high levels of RA, whereas animals with low levels of transgene expression appeared normal. Further analysis of these animals as described herein has revealed that RARα is important in regulating chondrogenesis in vivo.

Materials and Methods

Gel Mobility Shift Assay

A RARβ2 RARE double-stranded oligonucleotide probe was prepared by annealing the following oligos: 5′ TCGAGGGTAGGGTTCACCGAAAGTTCAC 3′ and 3′ CCCATCCCAAGTGGCTTTCAAGTGAGCT 5′. The probe was filled in using [^{32}P]dCTP and Klenow DNA polymerase. All proteins were purified using the pGEX-2T bacterial expression system (Pharmacia LKB Biotechnology, Piscataway, NJ) according to the manufacturer's instructions. Protein–DNA binding reactions were carried out in 20 μl of binding buffer (10% glycerol, 10 mM Hepes, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 mM DTT) containing 1 μg poly dI-dC and 0.15 μg of hRARα or hRARα-CB with or without 0.15 μg of mRXRβ on ice for 10 min. After this preincubation, 0.1 ng of labeled probe (67,000 cpm) was added to each tube, and the reactions were allowed to proceed for an additional 20 min at room temperature. Unlabeled probe (50 ng, 500×) was included in some reactions as a specific competitor. The samples were resolved on a prerun 4% nondenaturing polyacrylamide gel at 4°C.

Generation of Transgenic Mice

The transgene was constructed in the pGEM9zf(−) derivative, pW1, which contains a BamHI restriction site juxtaposed between the HindIII and SpeI restriction sites and the polyadenylation sequence from SV-40 in the NsiI site (Balkan et al., 1992*a*). A 3.3-kb fragment of the Hoxb-6 promoter that contains sequences immediately upstream of the transcriptional start site was liberated from pKSIIHoxb-6 (Schughart et al., 1991) by restriction endonuclease digestion with EcoRI and BamHI and directionally subcloned into pW1, thereby generating pW1Hoxb-6. pW1Hoxb-6tgRARα1 was made by subcloning the constitutively active RARα1 (Balkan et al., 1992*b*) into the BamHI restriction site of pW1Hoxb-6. Hoxb-6tgRARα1 DNA for microinjection was released from the vector backbone using NotI and SfiI, separated on agarose gel, and gel purified using geneclean (BIO 101, La Jolla, CA) according to the manufacturer's instructions. Transgenic mice were made by microinjection of C57Bl6F_1/J- (Jax Laboratories, Bar Harbor, ME) fertilized mouse eggs with DNA at a concentration of 2–3 ng/μl. Mice that carried the transgene were identified by slot-blot hybridization of tail DNA with an *Escherichia coli* β-galactosidase DNA probe.

Analysis of Transgenic Mice

Embryos were stained for β-galactosidase activity using a protocol described by Balkan et al. (1992*a*). The morning of the day of the copulation plug was considered E0.5.

Differential skeletal staining of embryos with alcian blue 8GS and alizarin red S was performed as described by McLeod (1980). Postterm mice were stained using a similar protocol with extended periods of incubation.

Western analysis was performed using standard procedures. Hind limb bud protein extracts were prepared from E11.5 animals. A cut parallel to the body wall was made to release the limb buds, and hind limb bud pairs from individual animals were transferred to 100 μl of treatment buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiled for 5 min with intermittent mixing. Protein extracts (10 μl for J limbs, 20 μl for I limbs) were separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with an anti–β-galactosidase antibody (Promega Corp., Madison, WI) for 2 h and washed, and an anti–

mouse secondary antibody conjugated to alkaline phosphatase was added for 1 h. Alkaline phosphatase activity was detected using a mixture of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Promega Corp.) according to the manufacturer's instructions.

Preparation of Micromass Cultures

Hind limbs of E11.5 embryos were collected aseptically and teased apart into small pieces with forceps in Puck's saline G buffer. Cells were dissociated by incubation in the presence of dispase (1 U/ml) (GIBCO BRL, Gaithersburg, MD) with shaking at 37°C in Puck's saline A buffer containing 10% chicken serum. Digestion was terminated by resuspension in SCM (60% Ham's F12, 40% DME supplemented with antibiotics and 10% FBS) followed by gentle trituration to obtain a fine cell suspension. Cells were passed through a No. 20 Nitex (Tetko Co., Elmsford, NY) mesh to remove cell clumps and to obtain a single cell suspension. Cell number was adjusted to 1×10^7 cells/ml and micromass cultures were initiated by carefully placing 10 μl of the cell suspension into the center of a well in a 24-well plate. Cultures were placed in a 37°C incubator containing a humidified atmosphere and 5% CO_2. After attachment (~1.5 h), 1 ml of SCM was added to each culture, and cultures were returned to the incubator. Media changes were performed at least once a day. Cultures were incubated for a period of 1 to 6 d, at which time they were processed for β-galactosidase activity, and duplicate cultures were stained for the presence of sulfated glycosaminoglycans found in cartilage matrix using alcian blue at acidic pH (Lev and Spicer, 1964). Alcian blue staining was carried out as follows. Cells were washed twice with PBS, fixed for 15 min in Kahle's fixative, washed once with 0.1 N HCl, and stained overnight with 0.1% alcian blue in 0.1 N HCl. After staining, the cultures were washed twice with 70% ethanol and stored in the same. Nodule numbers were determined by counting the number of alcian blue–stained nodules within a fixed area (3 mm^2). Statistics were determined using the Student's *t*-test.

Synthesis of Hybridization Probes

Single-stranded RNA probes with incorporated digoxigenin were transcribed from linear DNA templates according to manufacturer's instructions (Boehringer Mannheim Corp., Indianapolis, IN). Probes were quantified by spotting various dilutions of probe in 10× SSC (1.5 M NaCl, 150 mM sodium citrate) and a control digoxigenin-labeled RNA on a nylon membrane; detection of probe using a sheep anti–digoxigenin conjugated alkaline phosphatase antibody (Boehringer Mannheim Corp.) was as described by the manufacturer.

Probes for mRARα and mRARγ were produced from templates that included part of the 3′ coding and 3′ untranslated region of mRARα and the 3′ DEF region of RARγ. Hybridization probes were synthesized from linearized plasmid DNA templates using in vitro transcription. An ~600-bp riboprobe for mRARα was made using T3 RNA polymerase and a pKSII (Stratagene, La Jolla, CA) plasmid cleaved with EcoRV (cleaves internally) that carried a 2.2-kb mRARα insert. mRARγ riboprobe was synthesized from a pBS (Stratagene) template containing a 1.0-kb mRARγ insert that had been linearized with EcoRI.

Whole-Mount In Situ Hybridization of Micromass Cultures

Whole-mount in situ hybridizations of micromass cultures were performed as described by Conlan and Rossant (1992) with modifications. Micromass cultures were washed twice with PBS and fixed in 4% paraformaldehyde overnight at 4°C. After fixation, cultures were washed three times with cold PBS and dehydrated through a PBS/methanol series to 100% methanol and stored in 75% methanol at 4°C until use. Before hybridization, cultures were rehydrated in PBT (PBS, 0.05% Tween-20) and treated with proteinase-K for 6 min, washed twice with 2 mg/ml glycine in PBT, and fixed for 20 min in 4% paraformaldehyde. Cultures were washed three times with PBT and twice in hybridization buffer (50% formamide, 0.75 M NaCl, 10 mM Pipes, pH 6.8, 1 mM EDTA, 100 μg/ml tRNA, 0.05% heparin, 0.01% BSA, 1% SDS) and prehybridized for at least 1 h at 55°C. Hybridizations were carried out overnight at 55°C in a programmable dry oven (Bellco Glass Inc., Vineland, NJ) with 1 μg/ml riboprobe. Conditions for washing and antibody incubation were similar to that previously described (Conlon and Rossant, 1992). Color development was carried out overnight in the dark in 10% polyvinyl alcohol (Aldrich, average mol wt 13,000–23,000) (Barth and Ivarie, 1994). Addition of polyvinyl alcohol was found to increase signal to noise ratio.

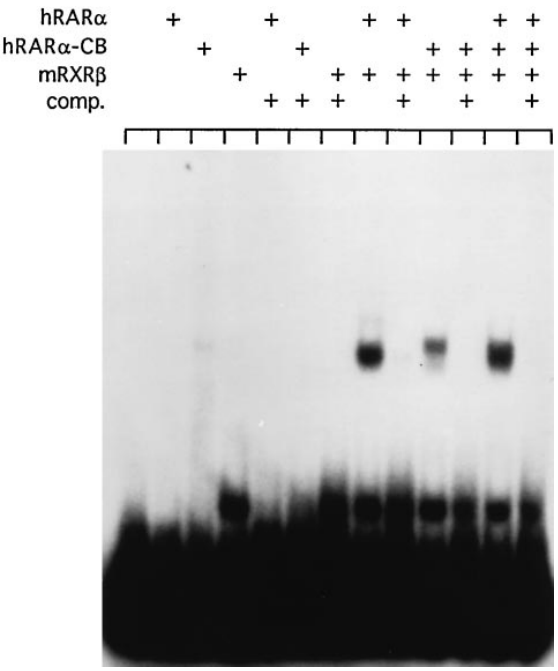


Figure 1. hRARα and tgRARα exhibit similar DNA-binding properties. The ability of equivalent amounts of hRARα- and tgRARα (hRARα-CB)-purified protein to shift a βRARE oligonucleotide probe in the absence or presence of a RXRβ partner are shown. Note that on a molar basis, there is ~2/3 less tgRARα than hRARα protein and that the larger tgRARα protein results in reduced band mobility relative to hRARα.

Results

Construction of Hoxb-6hRARα-LacZ Transgenic Mice

Transgenic mice were generated that expressed a constitutively active human RARα1 (a chimeric receptor with β-galactosidase fused to the carboxy terminus, denoted tgRARα herein) (Balkan et al., 1992*b*) to high levels in the developing limb. In vitro analysis of tgRARα binding to a DNA probe containing the βRARE showed that efficient binding was dependent upon the presence of an RXR heterodimer partner (Fig. 1), consistent with that observed for wild-type RARs (Yu et al., 1991; Leid et al., 1992*b*). The murine Hoxb-6 (Hox 2.2) promoter fragment, which has been shown previously to direct expression of a LacZ reporter gene to the developing limbs (Schughart et al., 1991), was used to target expression of the fusion receptor in the transgenic animals.

Transgenic mice carrying tgRARα under the control of the murine Hoxb-6 promoter fragment were generated by injecting one-cell embryos with the construct shown in Fig. 2 *A*. Owing to the presence of RAREs in several Hox promoters (Langston and Gudas, 1992; Popperl and Featherstone, 1993; Marshall et al., 1994), we checked the ability of both hRARα and tgRARα to *trans*-activate the Hoxb-6 promoter fragment in transient expression assays. Neither hRARα nor tgRARα was able to *trans*-activate the Hoxb-6 promoter fragment in the absence or presence of 500 nM all-*trans* RA (data not shown). Screening of transgenic mice was done by slot-blot hybridization of DNA isolated

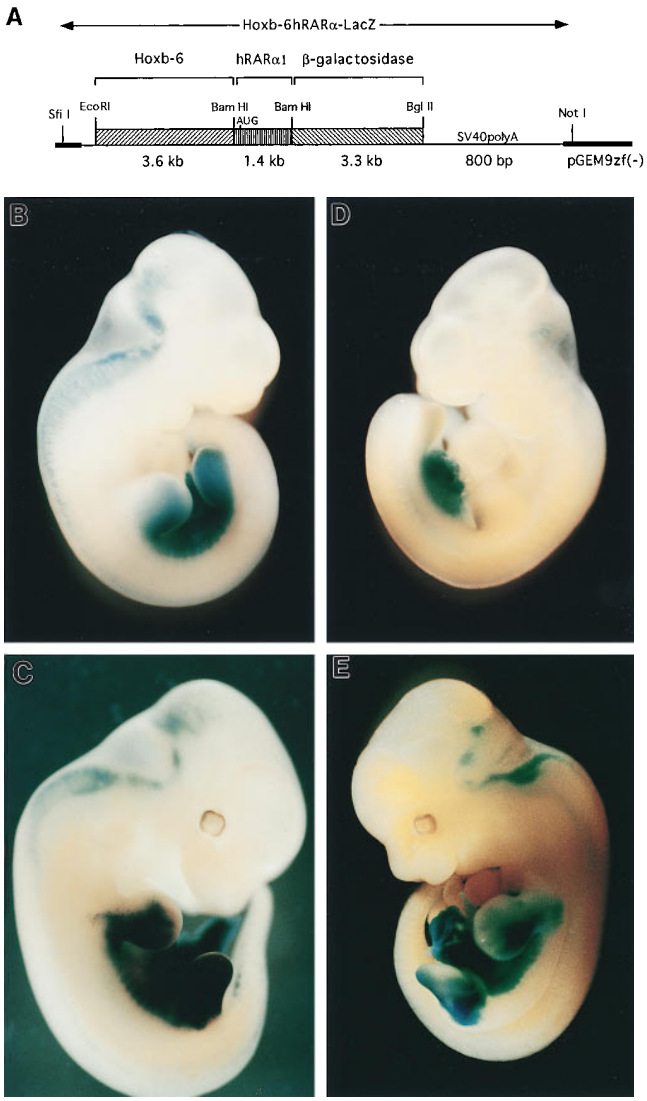


Figure 2. Structure and expression of the Hoxb-6tgRARα fusion gene construct. (*A*) A 9-kb linear DNA fragment containing the Hoxb-6tgRARα fusion gene was excised from a pGEM9zf(−) parent plasmid with SfiI and NotI and used for microinjection into fertilized eggs. (*B–E*) Expression patterns of the transgene in E10.5 and E11.5 mouse embryos. (*B* and *C*) tgRARα is expressed at high levels within the developing limbs at E10.5 (*B*) and E11.5 (*C*) in the three transgenic lines in which limb malformations were seen. A representative progeny from founder male J is shown. (*D* and *E*) The chimeric receptor is expressed at a lower level in the developing limbs at E10.5 (*D*) and E11.5 (*E*) in the five transgenic lines in which no limb malformations were observed. A representative progeny from founder male I is shown.

from mouse tails. 12 founders were generated and used to establish eight lines of transgenic mice. Of the 12 founders generated, four exhibited similar congenital limb malformations (A–D, see below), while eight (E–L) appeared phenotypically normal. Original founders as well as male and female progeny from all eight lines have been studied extensively, and a range of phenotypic abnormalities have been observed, depending on the level of transgene expression in the developing limbs.

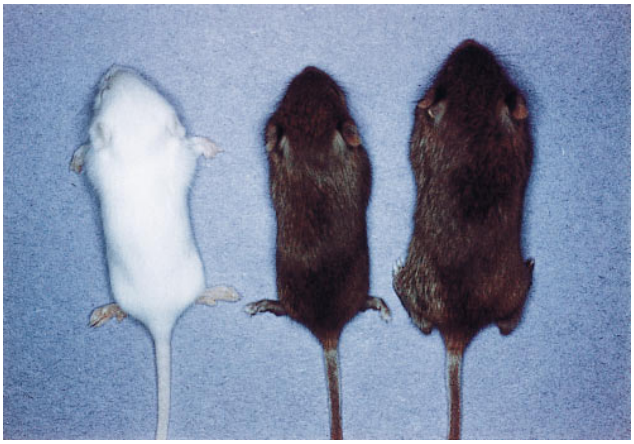


Figure 3. Limb malformations seen in Hoxb-6tgRARα transgenic mice. Hemizygous animals from independent transgenic lines showed strong skeletal malformations affecting both the fore and hind limbs, with variability in the severity of the malformations seen both between and within transgenic lines. Three 2-wk-old animals from a litter derived from founder male J are shown to illustrate the range of limb malformations seen. The animal on the left exhibits strong skeletal malformations affecting both the fore and hind limbs, while only the hind limbs of a littermate (*center*) are grossly malformed. A nontransgenic littermate (*right*) is shown for comparison. Note the overall difference in size between the normal and transgenic animals.

The expression of tgRARα in the developing limbs of embryos derived from the eight phenotypically normal transgenic founders was analyzed in detail. At E10.5, the transgene was expressed in the mesenchyme of the emerging limb buds and in the ventrolateral mesenchyme between the limb buds. In the fore limb bud, β-galactosidase activity was restricted to the posterior region whereas the entire hind limb bud expressed tgRARα (Fig. 2, *B* and *D*). While no differences in the transgene expression pattern were seen between the eight lines, significant differences in tgRARα expression levels were observed. In three of the eight lines (J, K, and L), tgRARα was expressed at high levels in the posterior half of the fore limb and across the entire hind limb (Fig. 2 *B*). In the other five lines (E–I), however, low levels of transgene expression were seen in both the fore and hind limbs (Fig. 2 *D*). At E11.5, differences between the two levels of transgene expression persisted, which was further confirmed by Western analysis with an anti–β-galactosidase antibody (data not shown). Specifically, in the hind limbs of embryos derived from high expressing founders, transgene expression was seen across the whole anteroposterior axis and extended distally into the progress zone (Fig. 2 *C*). In embryos derived from low expressors, tgRARα expression was restricted to anterior and posterior stripes in the mesenchyme of the developing hind limb with no significant expression of the transgene in the progress zone of the fore or hind limb (Fig. 2 *E*). Tissue sections from E12.5 high expressor hind limbs confirmed that transgene expression was not present in the ectoderm but was present in chondrogenic condensations that will become the tibia and fibula and strongly expressed in the underlying mesenchyme, including the progress zone (data not shown). These results are summa-

Table I. Summary of Transgenic Lines Generated

Founder	Limb phenotype	Transgene expression level	Affected progeny
A (M)	yes	ND	ND
B (ND)	yes	ND	ND
C (F)	yes	ND	ND
D (F)	yes	ND	ND
E (M)	no	L	no
F (M)	no	L	no
G (M)	no	L	no
H (M)	no	L	no
I (M)	no	L	no
J (M)	no	H	yes
K (M)	no	H	no*
L (F)	no	H	yes

M, male; *F*, female; *L*, low; *H*, high; *, low germline transmission.

rized in Table I. Furthermore, a transgenic line that expressed a potent dominant-negative RAR to a level similar to that of the high transgene-expressing lines described herein exhibited no perceptible phenotype suggesting that the tgRARα is not functioning in a dominant-negative manner (our unpublished observations).

While founders J, K, and L were phenotypically normal, they did pass the transgene in a Mendelian fashion to their progeny, and all transgene positive offspring exhibited limb malformations like those seen in the four affected founders (Fig. 3). Because some founder males with limb malformations had difficulty mating, lines established from male J and female L were relied upon for most of the analysis of the effects of ectopic tgRARα expression on limb development. Interestingly, no limb malformations were observed in the offspring of the five phenotypically normal founders (E–I) with the lower levels of tgRARα expression, suggesting that high level expression of the fusion receptor is required for alterations in normal limb development.

Consequences of Hoxb-6tgRARα Transgene Expression

The limb malformations associated with expression of the transgene were analyzed in detail in embryos and animals derived from a representative line (J) (Figs. 4 and 5); similar malformations were seen in four founders and two other independent transgenic lines. Pronounced bilateral defects were always present in the hind limb, whereas de-

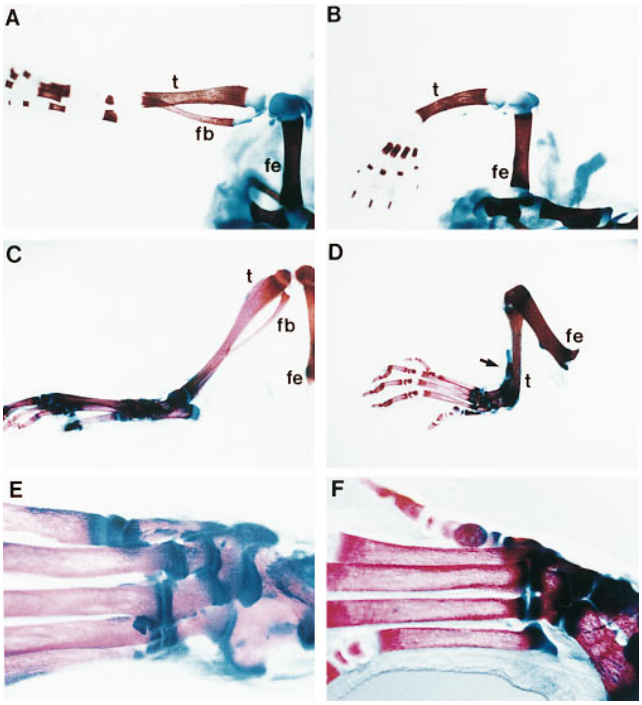


Figure 4. Alizarin red S– and alcian blue–stained preparations of left fore limb skeletons from normal and transgenic 2-wk-old mice. All skeletons were stained with alizarin red S (a bone-specific stain) and alcian blue (a cartilage-specific stain). (*A*) Dorsal view of a normal fore limb. (*B*) Close-up of the carpal region shown in *A*. (*C*) Dorsal view of a mildly affected transgenic fore limb. The ulna is slightly thickened and foreshortened. (*D*) Close-up of the carpals shown in *C*; note that the fifth metacarpal is reduced to a small nodule with no corresponding phalanx (*arrow*). (*E*) Dorsal view of a severely affected transgenic fore limb. The ulna is dramatically thickened, foreshortened, and postaxially deflected, and the fourth distal phalanx is duplicated (*arrowheads*). (*F*) Close-up of the carpal area in *E*. *r*, radius; *u*, ulna; *h*, humerus.

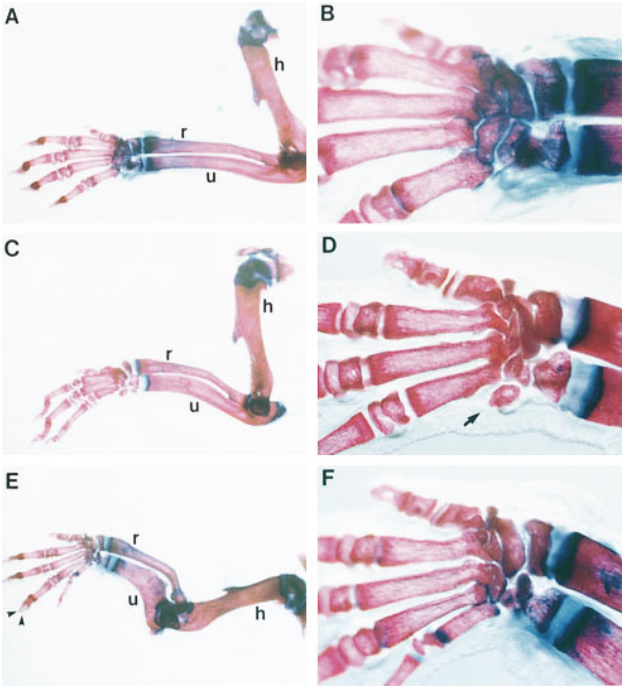


Figure 5. Alizarin red S– and alcian blue–stained preparations of left hind limb skeletons from normal and transgenic newborn animals and 2-wk-old mice. All skeletons were stained as in Fig. 3. (*A*) Lateral view of the hind limb of a normal newborn. (*B*) Lateral view of the hind limb of a transgenic newborn showing the missing fibula and rotated ankle and foot. (*C*) Lateral view of the hind limb of a normal 2-wk-old animal. (*D*) Ventral view of the hind limb of a transgenic 2-wk-old animal showing the cartilaginous remnant of the fibula and the mildly shortened and misshapen tibia. The foot is rotated ~180° postaxially out of its normal plane. (*E*) Close-up of the ankle shown in *C*, dorsal view. (*F*) Close-up of the ankle shown in *D*, dorsal view. *t*, tibia; *fb*, fibula; *fe*, femur.

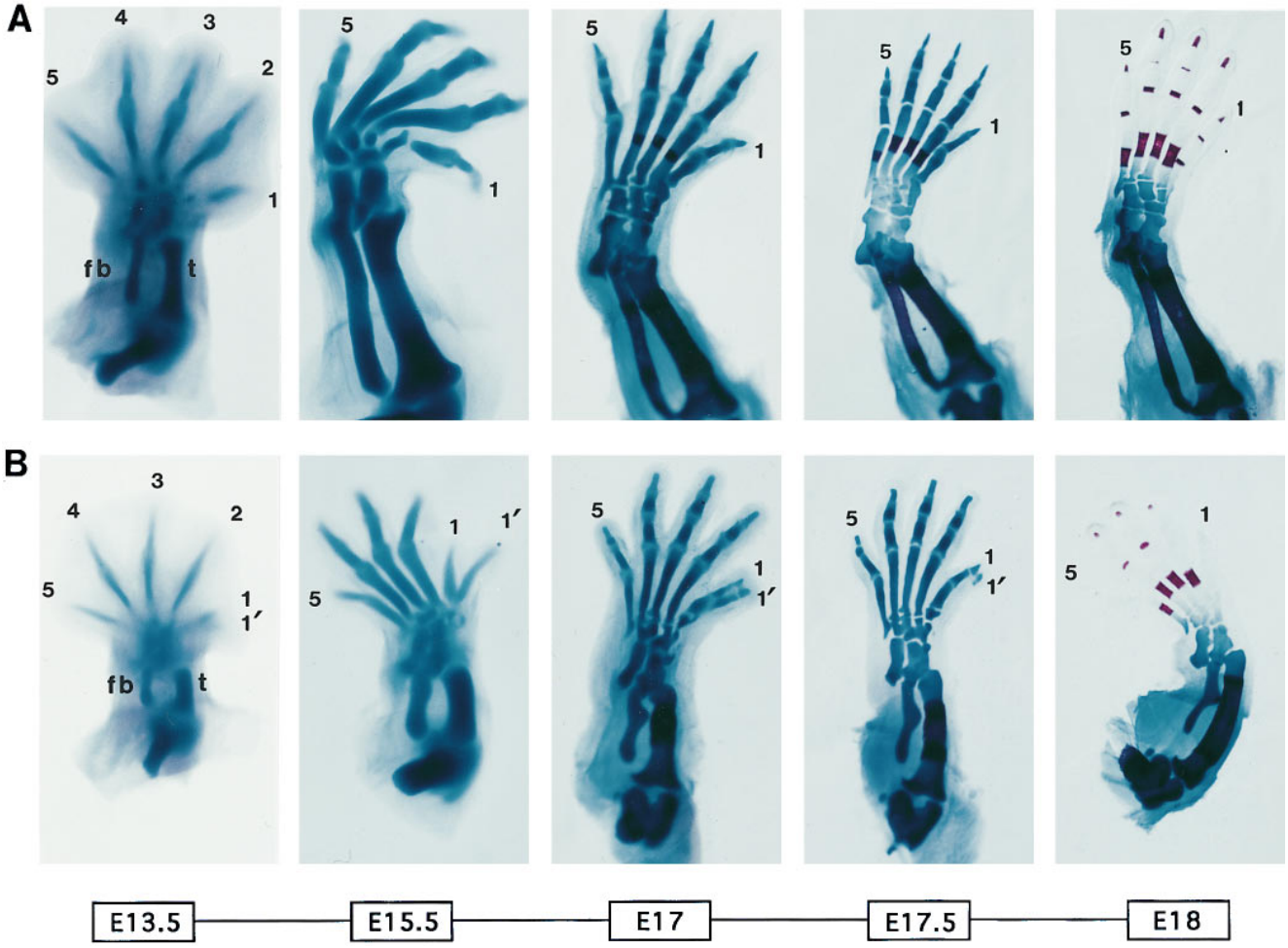


Figure 6. Analysis of the chondrogenic sequence in hind limbs of control and transgenic fetuses. Control fetuses are shown in *A*, and Hoxb-6tgRARα fetuses are shown in *B*. Whole-mount alcian blue and alizarin red stainings of hind limbs are shown at five successive developmental stages: E13.5, E15.5, E17, E17.5, and E18. *1–5*, digit numbers; *1′*, extra digit 1.

fects in the fore limb were variable. In the affected animals, there was a predilection towards the loss of postaxial structures, and, in general, the degree of postaxial deflection in the fore limb appeared to be a good indicator of the severity of the defect.

Malformations in the fore limb (Fig. 4) were primarily restricted to the field of transgene expression that included the ulna, ulna carpal bone, and their respective articulating bones. Although the transgenic animals exhibited a marked variability in the severity of the defect in the fore limbs, all the transgenic animals contained a deformed ulna, which varied from mild with a slight postaxial curvature to severe. The ulna in the severely affected animals was dramatically thickened (in some cases to twice that of the size of the radius), postaxially deflected, and foreshortened (compare Fig. 4, *A* and *E*). In comparison, the radius and humerus appeared to be only mildly affected. Furthermore, the radius was always found to be longer than the ulna, and the degree of this disparity appeared to correlate well with the amount of postaxial bending, suggesting that the disproportionate growth of the ulna and the radius in the proximodistal axis most likely contributed to the postaxial deflection. Malformations in the wrist consisted of a reduction in the size of the ulnar carpal and fourth carpal bone with the loss of the fourth carpal–fifth metacarpal articulating surface (Fig. 4, *D* and *E*). The pisiform bone also appeared to be sometimes absent or not mineralized. Most of the metacarpals were shortened, and in some of the mildly affected animals, metacarpal 5 was reduced to a mineralized nodule with no corresponding phalanx (Fig. 4 *D*). Additionally, in some of the more severely affected animals we periodically observed a very broad fourth medial phalanx with a complete duplication of the fourth distal phalanx (Fig. 4 *E*).

Founders A, B, C, and D, as well as those transgenic animals derived from founder J, all exhibited bilateral hind limb malformations (Fig. 5). In all animals examined, the fibula was malformed; this ranged from complete absence, except for a small distal nodule, to thickened partial fibulas that extended more proximally (Fig. 5, *B* and *D*). In addition, the extent of the fibular defect was found to be nearly perfectly symmetrical and to be 100% penetrant, with variability in the degree of severity of the effect on the foot and ankle. No proximal fibula–associated structures were observed using alizarin red or alcian blue stain-

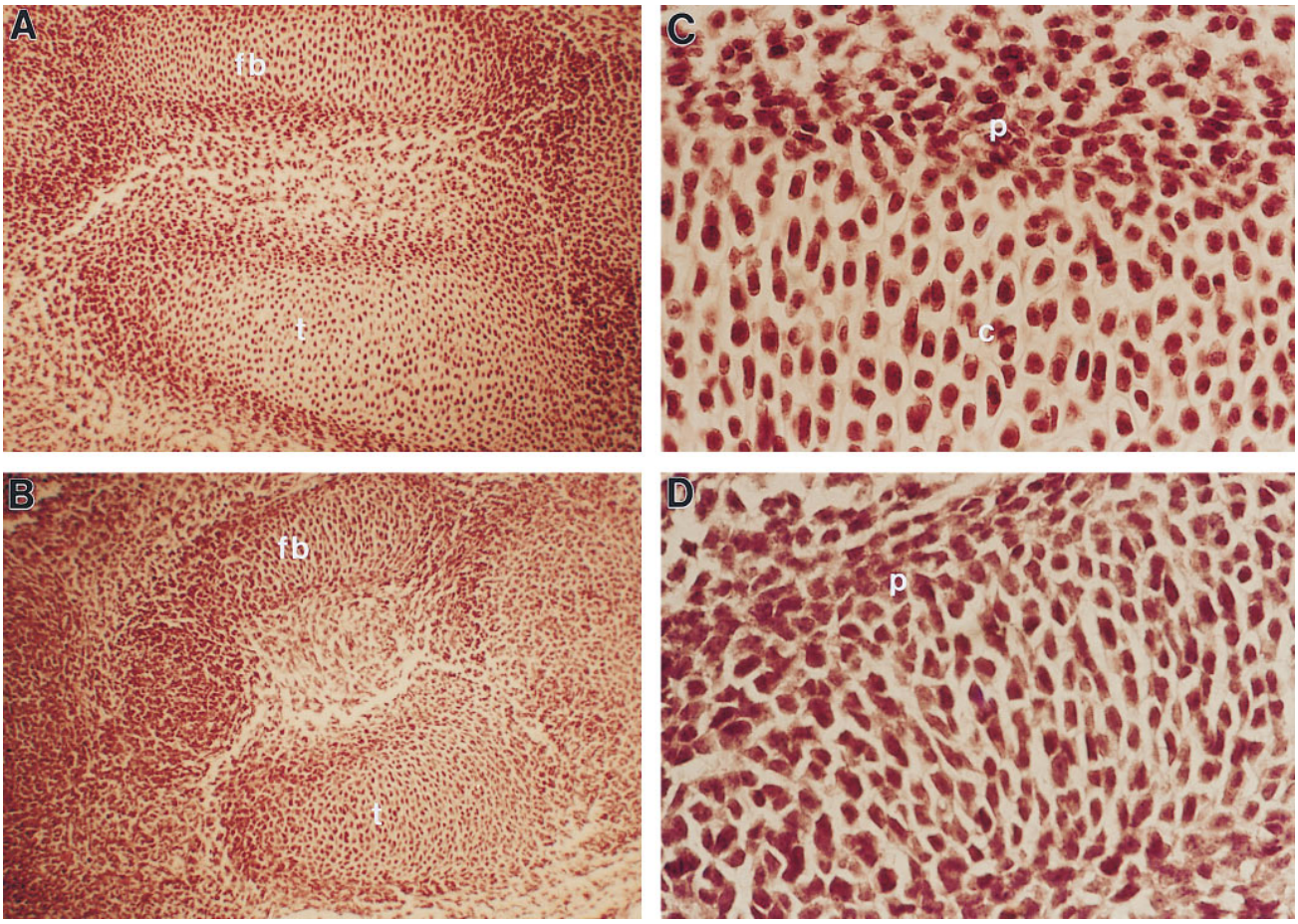
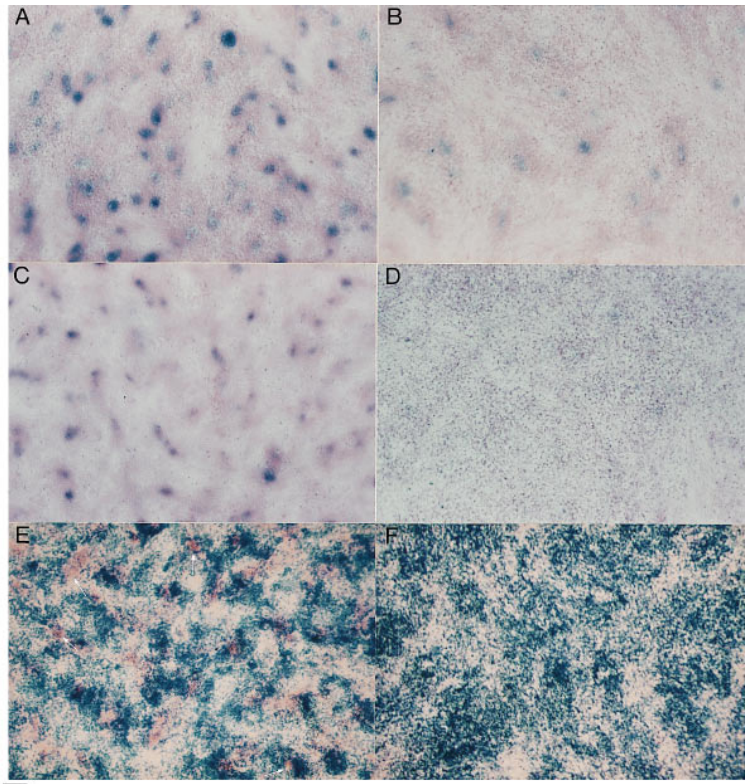


Figure 7. Chondrogenesis of the tibia and fibula is delayed in Hoxb-6tgRARα mice. (*A–D*) Histological sections prepared from E13.5 control (*A* and *C*) and transgenic (*B* and *D*) fetuses. (*A*) In control animals, both the tibia and fibula are well defined by the perichondrium, and a close-up of the fibula (*C*) shows an abundance of oval-shaped maturing chondrocytes. (*B*) In contrast, the tibia and fibula from transgenic animals are not as well organized, and a close-up of the fibula (*D*) shows that it is primarily composed of tightly packed mesenchymal cells. *t*, tibia; *fb*, fibula; *p*, perichondrium; *c*, maturing chondrocytes.

ing. In contrast, the tibia was found to be only marginally affected, exhibiting a dorsal curvature and slight shortening and thickening. The calcaneus was found to be reduced in size, displaced laterally to the outside of the fibula, and missing an articulation surface for the fifth metatarsal. In some animals, the navicular and the cuboidum were found to be fused to the second and third tarsals, respectively. Occasionally, the fifth proximal and medial phalanges were missing, with the presence of the distal phalanx (Fig. 5 *F*). As observed in the fore limb, the defects in the hind limb appear to be primarily restricted to the posterior half of the limb and to involve the distal long bones and proximal foot bones.

To establish that the transgene does not have nonautonomous effects on chondrogenesis in regions where it is not expressed, the axial bones of transgenic and control animals were stained with alizarin red and alcian blue. No significant differences were observed in these bones between control and transgenic animals of similar embryonic age (data not shown).

To understand what changes have occurred during development of the limb to give rise to the aforementioned malformations, a series of E13.5 through E18 fetuses were collected and stained with alizarin red and alcian blue (Fig. 6). Transgenic animals were readily distinguishable from their nontransgenic littermates as early as E11.5. The hind limbs of the transgenic animals were somewhat smaller and contained a disproportionately large outgrowth in the region of the anterior margin, which will give rise to digit 1 (data not shown). Similar outgrowths are also observed in the chick limb bud after placement of an RA-soaked bead in the anterior margin (Summerbell, 1983). At later times in development, digit 1 was found to be duplicated, thereby generating a 1, 1, 2, 3, 4, 5 digit pattern (Fig. 6 *B*, E15.5, E17, and E17.5). This duplication was seen in ~40% of the animals, and the extent of the duplication correlated well with the size of the outgrowth. In most instances, this duplicated digit was not maintained, as an analysis of many ($n > 200$) adult animals revealed no such digit duplication. As can be seen from the embryonic panel, the digit duplication appears to be lost during the course of development with the duplicated digit 1′ becoming fused with digit 1 (Fig. 6 *B*, E17 and E18). This was evident in the hind limbs of the adult animals, where digit 1 appeared to be composed of several individual and overlapping bones (Fig. 5 *F*).


A
B
C
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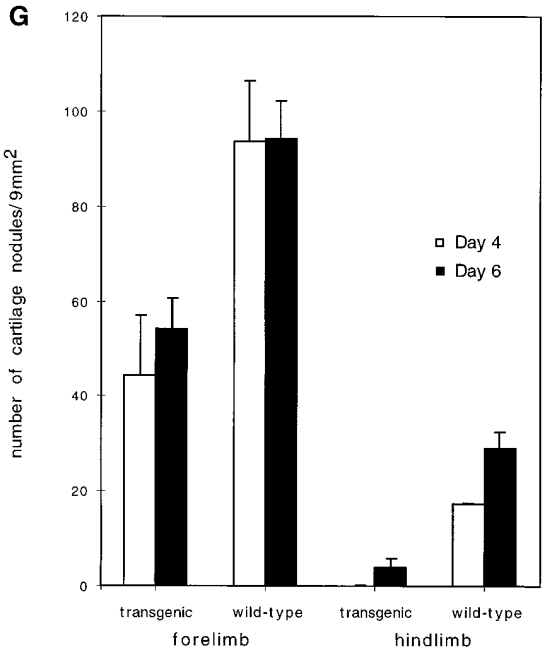
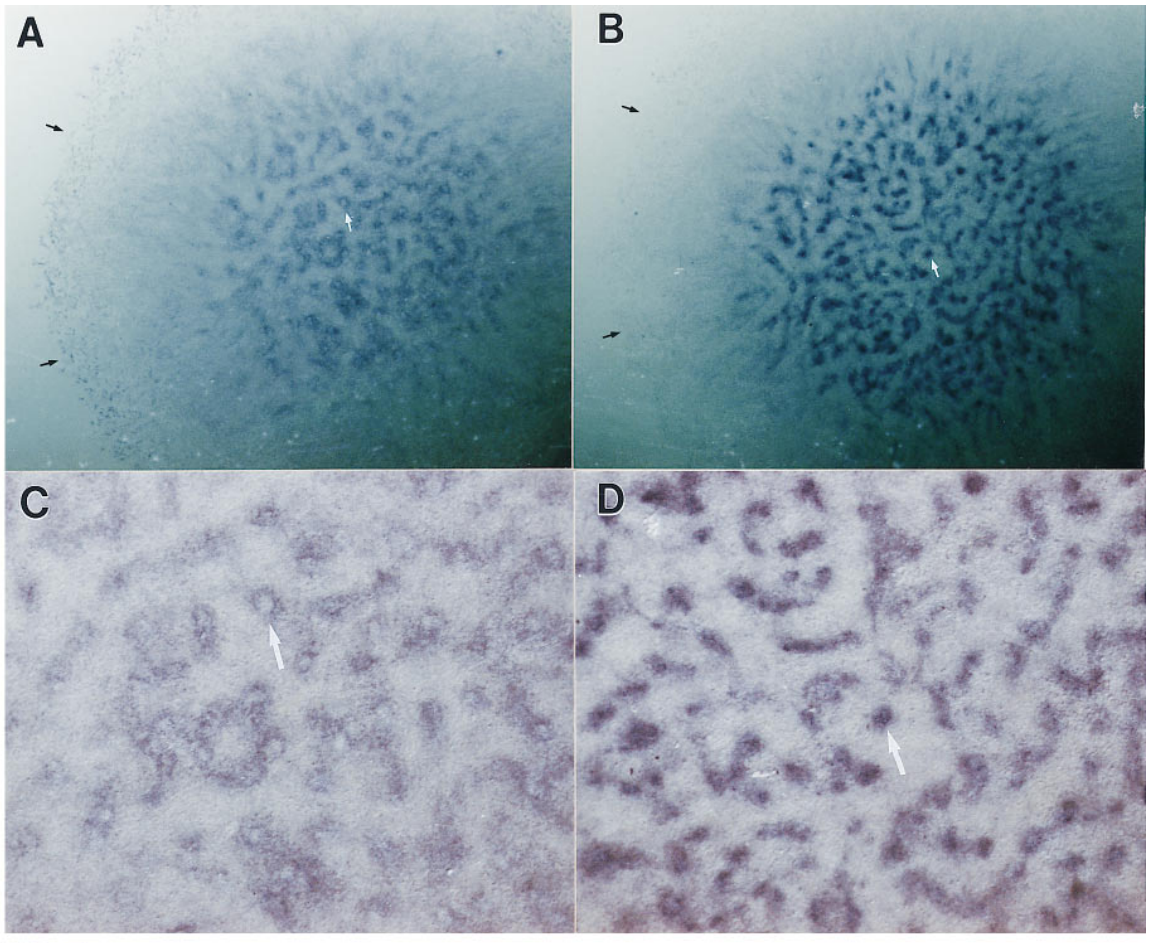
E
F


G
number of cartilage nodules/9mm2
120
100
80
60
40
20
0
Day 4
Day 6
transgenic
wild-type
transgenic
wild-type
forelimb
hindlimb


A
B
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Development of the various elements of the hind limb of transgenic animals was significantly delayed in comparison to wild-type animals. This was most apparent at later embryonic stages, where ossification of the proximal phalanges of the hind limbs from transgenic animals was observed to be at least 0.75–1 d behind that of wild-type animals. However, earlier in development the disparity in the size of the limbs was also evident; in this case, the bone primordia were shortened and slightly misshapen. Analysis of hind limb histological sections prepared from E13.5 fetuses revealed that chondrogenesis of the tibia and fibula was delayed in transgenic animals (Fig. 7). The primitive fibula of the wild-type animals contained maturing chondrocytes circumscribed by a well-defined perichondrium. In contrast, the fibula from transgenic animals was not as well organized. The fibula was primarily composed of closely packed chondroprogenitor cells with a lower cytoplasmic/nuclear ratio with a morphology consistent with condensed mesenchymal cells. Chondrogenesis of the digital rays were also found to be delayed in the transgenic animals (data not shown). Hence, overexpression of tgRARα appeared to inhibit or interfere with chondrogenesis in the hind limb by delaying chondroprogenitor differentiation.

Assessment of Chondrogenic Potential of Transgene-expressing Mesenchymal Cells

To examine more thoroughly the defect in transgenic animals that interferes with chondrogenesis in endochondral bone formation, the ability of the mesenchyme from transgenic limbs to form cartilage nodules was assayed in vitro using micromass culturing methods. Limb mesenchyme cultured under micromass conditions closely follows the progression of limb mesenchyme in vivo, precartilaginous condensations are first evident after 2 d in culture with cartilage nodules appearing ~1 d later (Ahrens et al., 1977). Cultures were seeded at high density (1×10^5 cells/10 μl) in a 24-well plate with dissociated limb mesenchyme pooled from either fore or hind limbs of E11–11.25 transgenic or non-transgenic embryos. These cultures were allowed to grow for up to 3, 4, or 6 d. Cartilage matrix production was assayed for by fixing and staining the cultures with alcian blue at low pH , while both the extent and distribution of transgene-expressing cells were determined by staining fixed cultures with X-gal.

Within 3 d, cultures initiated from wild-type fore or hind limb buds showed numerous well-defined alcian blue–stained cartilage nodules (Fig 8, *A* and *B*). No alcian blue nodules were observed in cultures after 2 d; instead, both fore limb and hind limb cultures contained numerous condensations (data not shown). Similar to wild-type cultures, transgenic fore and hind limb micromass cultures after 2 d displayed many condensations (data not shown). Transgene-expressing cells were found to be distributed throughout the cultures and within condensations. In the hind limb cultures, the transgene-expressing cell was the predominant cell type found within the condensations, while in the fore limb there was a mixture of cells, with some condensations being devoid of transgene-expressing cells. The number of transgene-expressing cells from fore limb–derived cultures were much lower than those of the hind limb cultures, which is consistent with the differences observed in transgene expression between the fore and hind limb buds in vivo.

In contrast to wild-type cultures, cultures initiated from the hind limb buds of transgenic animals exhibited few if any alcian blue–stained nodules at 4 d (Fig. 8 *C*) and only slightly more nodules (<5) at 6 d postinitiation (Fig. 8 *G*). Examination of these cultures with dark-field microscopy confirmed the absence of any apparent nodules or discrete condensations (data not shown). In contrast, cultures initiated from the fore limbs of transgenic animals displayed numerous well-defined alcian blue–stained cartilage nodules after 4 d (Fig. 8 *A*) and slightly more after 6 d (Fig. 8 *G*). However, their appearance was delayed and their number was significantly reduced in comparison to wild-type cells (Fig. 8 *G*). In addition, the nodules were mostly confined to those regions containing nontransgene-expressing cells (Fig. 8 *E*). Furthermore, the differences in chondrogenesis observed between the cultures of fore and hind limb buds from transgenic animals correlated well with the observed differences in the severity of fore and hind limb malformations seen in these animals. It should be noted that the transgene continued to be abundantly expressed throughout the culture period in both fore- and hind limb–derived cultures (Fig. 8, *E* and *F*), and that there was no

Figure 8. Analysis of chondrogenic capacity of limb mesenchyme from the fore and hind limb of E11.25 transgenic embryos in comparison to E11.25 wild-type embryos in micromass culture. In control fore (*A*) and hind limb (*B*) cultures, there are numerous well-defined alcian blue–stained cartilage nodules, whereas in fore (*C*) and hind limb (*D*) cultures initiated from limb mesenchyme of transgenic animals, there are much fewer alcian blue–stained cartilage nodules relative to that of the control cultures. Cultures were stained with alcian blue after 3 d and counterstained with eosin/phloxine. Transgenic fore (*E*) and hind limb (*F*) cultures were fixed and stained with X-gal after 4 d and counterstained with eosin/phloxine. The cartilage nodules appear pink, while the transgene-expressing cells appear blue. Note that the nodules (*white arrows*) in the fore limb cultures contain very few if any transgene-expressing cells and that after 4 d in culture, there are numerous transgene-expressing cells, which were especially apparent in the hind limb cultures. All pictures taken at the same magnification of 50×. (*G*) Quantification of the number of cartilage nodules seen in each group after 4 and 6 d in culture. The number of cartilage nodules observed in the transgenic cultures is significantly less ($P < 0.0001$) than the number of nodules observed in the corresponding wild-type culture both after 4 and 6 d of culture. Bar: (*A–F*) 0.2 mm.

Figure 9. Examination of RARα and RARγ gene expression in 4-d wild-type micromass cultures with whole-mount in situ hybridization. RARα (*A* and *C*) expression is found throughout the culture circumscribing the individual nodules (*white arrow*), but it is not found within the core of the nodules that contain chondroblasts. In contrast, RARγ (*B* and *D*) is strongly expressed within the core of the nodules (*white arrow*) and to a lesser extent in the surrounding mesenchyme. The black arrows denote the edge of the culture. Upper panel, 15× magnification; lower panel, 75× magnification. Bar: (*A* and *B*) 1 mm; (*C* and *D*) 0.2 mm.

significant growth disparity between wild-type and transgenic cultures. Consistent with the above in vivo observations, these results suggest that expression of tgRARα delayed or inhibited chondroblast differentiation subsequent to condensation.

Analysis of RARα and RARγ Gene Expression in Micromass Cultures

Previous studies have shown that RARα and RARγ are expressed throughout the fore limb mesenchyme between E9.5–E11.5, at which time RARγ expression becomes restricted to precartilage and cartilage (Dollé et al., 1989*b*; Ruberte et al., 1990). Whole-mount in situ hybridization methodologies were adapted to the analysis of gene expression in micromass cultures so that the expression of endogenous RARα could be compared to tgRARα. In agreement with previous studies, by day 4 of culture, RARγ was found to be expressed in the core of the nodule, where chondroblasts surrounded by cartilaginous extracellular matrix were observed (Fig. 9, *B* and *D*). RARα, on the other hand, was found to be expressed in the peripheral region of the nodule in condensed mesenchymal cells that had not yet differentiated into chondroblasts (Fig. 9, *A* and *C*). RARα expression was either absent or expressed at very low levels within the core of the nodule, in chondroblasts (Fig. 9, *A* and *C*).

Discussion

Transgenic animals that ectopically express a constitutively active hRARα1 in the developing murine limb exhibited a spectrum of defects that included polydactyly, syndactyly, ectrodactyly, fibular deficiencies, and tarsal and carpal fusions. The phenotypes observed in these animals are similar to those that have been observed in a number of vertebrate systems in which embryos were administered systemic or localized doses of RA (Shenefelt, 1972; Kochhar and Aydelotte, 1973; Summerbell, 1983; Tickle et al., 1985, 1989). A number of different roles have been proposed for the function of the RARs during limb development (Mendelsohn et al., 1991, 1992), some of which include precartilage and cartilage formation, apoptosis, and patterning of the limb. We have found that RARα is an important regulator of chondrogenesis during limb development.

Transgene Expression and Chondrogenesis

The RARs are ligand-activated receptors that function as heterodimers. Hence, transgene activity would rely on the presence of a suitable heterodimeric partner. This condition is satisfied during the period of transgene expression before overt cytodifferentiation (E13–13.5) within the limb. RXRα and RXRβ are expressed throughout the limb mesenchyme at these stages and as such could provide a suitable heterodimeric partner for the transgene.

Postnatal transgenic lines that expressed the transgene to high levels during limb development (E9.5–E15) presented with a number of paraxial skeleton deficits, while no apparent phenotype was observed in transgenic lines expressing the transgene to a lower level. Although the expression patterns for the transgene in the two lines were similar, the transgene in the high expressing lines is expressed to a much greater level relative to that of the low expressing lines in the core mesenchyme (along the median axis). This mesoderm will contribute to the future skeletal elements, suggesting that transgene expression in this region may intefere with skeletogenesis and contribute to the observed defects. Consistent with these observations, the limb defects became first evident during embryogenesis within the early stages of skeletogenesis (E13.5) during formation of the cartilaginous template. Furthermore, histological sections prepared from E13.5 limbs from transgenic and a nontransgenic littermate confirmed that development of the limb cartilage was significantly delayed in the transgenic animals. Taken together, these observations suggested that expression of the transgene interfered with chondrogenesis.

Micromass cultures have been previously shown to closely follow those events of chondrogenesis in vivo and have been used to study the role of various molecules in chondrogenesis during endochondral bone formation. Cartilage nodules are first evident as a coalescence of mesoderm cells that form a condensation. These aggregates continue to expand both by cell division and selective recruitment of neighboring cells. After a period of cellular growth, cells within the interior of the condensation begin to differentiate and elaborate cartilage matrix that can be specifically stained with alcian blue. Micromass cultures derived from wild-type and transgenic hind limbs appear similar up to ~2–2.5 d. Before this time, there is a similar number of well-defined condensations in both cultures. It is at the onset of chondroblast differentiation when differences between the two cultures become readily visible. In the wild-type cultures, chondroblasts begin to appear within the core of the condensations after 3 d in culture as shown by alcian blue staining for cartilage matrix. Whereas, in transgene-expressing cultures, especially those from hind limbs, little or no alcian blue staining is observed after 4 or 6 d in culture, which is indicative of an absence of chondroblast differentiation and elaboration of extracellular cartilage matrix. Hence, transgene expression appears to interfere with the transition from a committed chondroprogenitor cell, found within condensing mesoderm, to a chondroblast located within the core of the nodule.

Analysis of RARα gene expression in micromass cultures after 4 d has shown that RARα mRNA is absent in the core of the nodules but is abundantly expressed in the condensing mesoderm. Therefore, RARα mRNA is downregulated during chondroblast differentiation. In contrast, RARγ appears to be upregulated in the core of the nodules in concert with chondroblast differentiation. In cultures initiated from transgene-expressing limb mesenchyme, RARα is found to be abundantly expressed in condensing mesoderm throughout the culture period and continues to be expressed within the core of condensations well after endogenous RARα expression is downregulated. Together these results suggest that continued expression of RARα during mesoderm-chondroblast differentiation interferes with the appearance of chondroblasts. Hence, downregulation of RARα appears to be a necessary step in chondroblast differentiation (Fig. 10). This model has consequences for both understanding the role

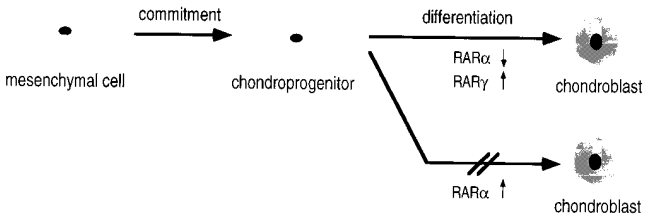

Figure 10. Loss of RARα expression is required for the chondroblast phenotype to be expressed. Mesenchymal cells are pluripotent and contribute to a number of cell lineages in the developing limb (e.g., muscle, cartilage, loose connective tissue, etc.). Cells that become committed to a chondrogenic fate express RARα and RARγ before chondroblast differentiation RARα expression is decreased. Transgenic mice in which RARα is placed under the control of the Hoxb-6 promoter continue to express RARα during chondroprogenitor-chondroblast differentiation. These animals display a number of limb malformations because of an inhibition of chondrogenesis, specifically chondroblast differentiation.

of the receptors in teratogenesis as well as in the phenotypes observed in RAR null mutants.

Transgenic Mice Exhibit Phenotypes Consistent with RA Treatment

RA has been shown to modify limb development in a number of phylogenetically distinct species (Tabin, 1991; White et al., 1994). Many of the phenotypes associated with overexpression of tgRARα in the developing limb are similar to those observed in the fetuses of dams treated with teratogenic doses of RA. RA teratogenesis causes a spectrum of defects within the limbs that are most commonly reductionist in nature and are thought to be, in part, a consequence of a delay in chondrogenesis (Kwasigroch and Kochhar, 1980; Zimmerman and Tsambos, 1985; Kwasigroch et al., 1986). These malformations include, within the hind limb, fibular agenesis, brachypodism, syndactyly, ectrodactyly, bowing of the tibia, and syntosis of the zeugopods (Shenefelt, 1972; Kochhar, 1973; Kochhar and Aydelotte, 1974). All of these defects are found to varying extents in the hind limbs of the tgRARα transgenic mice described herein.

Several studies have shown that RA inhibits chondrogenesis and that this inhibition is most likely RAR-mediated (Eckhardt and Schmitt, 1994; Von Schroeder et al., 1994). Further studies with receptor-specific agonists have shown that an RARα-specific agonist is significantly more potent in inducing limb malformations than either RARβ- or RARγ-specific agonists (Elmazar et al., 1996). RARγ is expressed during chondrocyte maturation, whereas RARα and RARβ are not; RARα appears to be expressed in most other tissues of the developing limb. Therefore, under conditions of retinoid excess where expression of RARβ (Mendelsohn et al., 1991; Jiang et al., 1994) and possibly RARα (both genes are RA responsive) are elevated within the limb mesenchyme, a delay or inhibition of chondroprogenitor differentiation could ensue, thereby interfering with normal skeletogenesis. No differences in the expression of RARβ were observed between the limbs of tgRARα transgenic mice and wild-type animals (our unpublished observations), and expression was restricted to the anterior and posterior necrotic zones and in the interdigital spaces, suggesting that RARβ is not contributing to the observed phenotype in the transgenic mice.

Application of RA to the anterior margin of the developing chick limb has been shown to induce digit and skeletal element duplications (Tickle et al., 1982). Minor digit duplications are apparent in the transgenic animals described herein. Duplications were observed in the fourth distal phalanx within the forelimb and digit 1 in the hind limb. Both of these duplications occurred at the anterior boundary of transgene expression: digit 4 in the fore limb and digit 1 in the hind limb. Numerous genes have been shown to be important in outgrowth and patterning of the vertebrate limb, and some of these include sonic hedgehog (Riddle et al., 1993), HoxD cluster (Dollé et al., 1989*a*), and fibroblast growth factors (Niswander et al., 1993, 1994). Analysis of the expression patterns of *sonic hedgehog*, *fibroblast growth factor 4*, and *Hoxd9* through *d12* mRNAs with whole-mount in situ hybridization showed no dramatic changes between transgenic and control animals; staining patterns were found to be consistent with those reported by others (our unpublished observations). These results suggest that the change in patterning observed in the transgenic animals may not involve changes in the expression of one of these gene products, or because the duplications are relatively minor (fourth distal phalanx in the fore limb and periodic partial digit 1 duplication in the hind limb), any changes in expression of these genes might be fairly subtle and therefore difficult to detect.

Recently, mice have been made with null alleles of RARα, RARβ, RARγ, and RXRα (Lohnes et al., 1993; Lufkin et al., 1993; Sucov et al., 1994; Luo et al., 1995). Mice homozygous for either mutant allele do not show any phenotype in the paraxial skeleton, whereas mice homozygous for both, (RARα−/−, RARγ−/−), have severe limb malformations (Lohnes et al., 1994). The limbs of these animals display proper anteroposterior symmetry, suggesting that the primary defect is not at the level of pattern formation (Lohnes et al., 1994). With the exception of the RXRα null mice, treatment of RAR null embryos with teratogenic doses of RA results in limb defects comparable to that seen in heterozygous or wild-type embryos. The observation that RXRα null mutants are resistant to RA-induced limb defects underscores the importance of RXRα as an integral component of the RA signaling cascade in vivo (Sucov et al., 1995). It is interesting to note that in both RARα/RARβ2 and RARα/RARγ null mice, the mice display ectopic cartilages in a number of sites throughout the animal. Furthermore, ectopic cartilages were sometimes present in the interdigital region, and the synovial joints were poorly defined in RARα/RARγ null mice. Given that the presence of RARα and possibly the other RARs may be important in regulating cartilage formation, this suggests that their absence creates permissive conditions and could be expected to give rise to ectopic cartilages, as observed in these animals.

We have provided evidence that the loss of RARα expression appears to be an important regulatory component of mesoderm differentiation into chondroblasts in that chondroprogenitor cells that express RARα failed to differentiate into chondroblasts. Continued expression of

RARα in cells destined to become chondroblasts contributes to a different cellular fate, which is currently under investigation. Overall, downregulation of the RARs may be an important determinant in regulating cellular differentiation which has practical implications in understanding their role in normal and abnormal development.

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References

Ahrens, P.B., M. Solursh, and R.S. Reiter. 1977. Stage related capacity for limb chondrogenesis in cell culture. *Dev. Biol.* 60:69–82.

Balkan, W., M. Colbert, C. Bock, and E. Linney. 1992*a.* Transgenic indicator mice for studying activated retinoic acid receptors during development. *Proc. Natl. Acad. Sci. USA.* 89:3347–3351.

Balkan, W., G.K. Klintworth, C.B. Bock, and E. Linney. 1992*b*. Transgenic mice expressing a constitutively active retinoic acid receptor in the lens exhibit ocular defects. *Dev. Biol.* 151:622–625.

Barth, J., and R. Ivarie. 1994. Enhanced detection of low abundance transcripts in early stage quail embryos by a modified nonradioactive whole mount *in situ* hybridization technique. *Biotechniques.* 17:326–327.

Boylan, J.F., D. Lohnes, R. Taneja, P. Chambon, and L. Gudas. 1993. Loss of retinoic acid receptor γ function in F9 cells by gene disruption results in aberrant *Hoxa-1* expression and differentiation upon retinoic acid treatment. *Proc. Natl. Acad. Sci. USA.* 90:9601–9605.

Boylan, J.F., T. Lufkin, C.C. Achkar, R. Taneja, P. Chambon, and L.J. Gudas. 1995. Targeted disruption of retinoic acid receptor α (RARα) and retinoic acid receptor γ (RARγ) results in receptor-specific alterations in retinoic acid-mediated differentiation and retinoic acid metabolism. *Mol. Cell. Biol.* 15:843–851.

Chambon, P. 1994. The retinoid signaling pathway: molecular and genetic analyses. *Semin. Cell Biol.* 5:115–125.

Conlon, R.A., and J. Rossant. 1992. Exogenous retinoic acid rapidly induces anterior ectopic expression of murine *Hox-2* genes in vivo. *Development (Camb.).* 116:357–368.

Dollé, P., J.-C. Izpisua-Belmonte, H. Falkenstein, A. Renucci, and D. Duboule. 1989*a.* Coordinate expression of the murine Hox-5 complex homeobox-containing genes during limb pattern formation. *Nature (Lond.).* 342:767–772.

Dollé, P., E. Ruberte, P. Kastner, M. Petkovich, C.M. Stoner, L.J. Gudas, and P. Chambon. 1989*b*. Differential expression of genes encoding α, β, and γ retinoic acid receptors and CRABP in the developing limbs of the mouse. *Nature (Lond.).* 342:702–705.

Dollé, P., E. Ruberte, P. Leroy, G. Morriss-Kay, and P. Chambon. 1990. Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development (Camb.).* 110:1133–1151.

Dollé, P., V. Fraulob, P. Kastner, and P. Chambon. 1994. Developmental expression of murine retinoid X receptor (RXR) genes. *Mech. Dev.* 45:91–104.

Eckhardt, K., and G. Schmitt. 1994. A retinoic receptor α antagonist counteracts retinoid teratogenicity in vitro and reduced incidence and/or severity of malformations in vivo. *Toxicol. Lett. (Amst.).* 70:299–308.

Elmazar, M.M.A., U. Reichert, B. Shroot, and H. Nau. 1996. Pattern of retinoid-induced teratogenic effects: possible relationship with relative selectivity for nuclear retinoid receptors RARα, RARβ, and RARγ. *Teratology.* 53: 158–167.

Giguère, V. 1994. Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. *Endo. Rev.* 15:61–79.

Jiang, H., M. Gyda III, D.C. Harnish, R.A. Chandraratna, K.J. Soprano, D.M. Kochhar, and D.R. Soprano. 1994. Teratogenesis by retinoic acid analogs positively correlates with elevation of retinoic acid receptor-β2 mRNA levels in treated embyros. *Teratology.* 50:38–43.

Kastner, P., J.M. Grondona, M. Mark, A. Gansmuller, M. LeMeur, D. Decimo, J.-L. Vonesch, P. Dollé, and P. Chambon. 1994. Genetic analysis of RXRα developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell.* 78:987–1003.

Kochhar, D.M. 1973. Limb development in mouse embyros. I. Analysis of teratogenic effects of retinoic acid. *Teratology.* 7:289–295.

Kochhar, D.M., and M.B. Aydelotte. 1974. Susceptible stages and abnormal morphogenesis in the developing mouse limb, analysed in organ culture after transplacental exposure to vitamin A (retinoic acid). *J. Embryol. Exp. Morph.* 31:721–734.

Krust, A., P. Kastner, M. Petkovich, A. Zelent, and P. Chambon. 1989. A third human retinoic acid receptor, hRAR-γ. *Proc. Natl. Acad. Sci. USA.* 86:5310–5314.

Kwasigroch, T.E., and D.M. Kochhar. 1980. Production of congenital limb defects with retinoic acid: phenomenological evidence of progressive differentiation during limb morphogenesis. *Anat. Embryol.* 161:105–113.

Kwasigroch, T.E., J.F. Vannoy, J.K. Church, and R.G. Skalko. 1986. Retinoic acid enhances and depresses in vitro development of cartilaginous bone anlagen in embryonic mouse limbs. *In Vitro Cell. Dev. Biol.* 22:150–156.

Langston, A.W., and L.J. Gudas. 1992. Identification of a retinoic acid responsive element 3′ of the murine homeobox gene Hox-1.6. *Mech. Dev.* 38:217–228.

Leid, M., P. Kastner, and P. Chambon. 1992*a.* Multiplicity generates diversity in the retinoic acid signalling pathways. *Trends Biochem. Sci.* 17:427–433.

Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J.-Y. Chen, A. Staub, J.-M. Garnier, S. Mader, and P. Chambon. 1992*b.* Purification, cloning, and RXR identity of the HeLa Cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell.* 68: 377–395.

Lev, R., and S.S. Spicer. 1964. Specific staining of sulphate groups with alcian blue at low pH. *J. Histochem. Cytochem.* 12:309.

Lewis, C.A., R.M. Pratt, J.P. Pennypacker, and J.R. Hassell. 1978. Inhibition of limb chondrogenesis in vitro by vitamin A. *Dev. Biol.* 64:31–47.

Li, E., H.M. Sucov, K.-F. Lee, R.M. Evans, and R. Jaenisch. 1993. Normal development and growth of mice carrying a targeted disruption of the α1 retinoic acid receptor gene. *Proc. Natl. Acad. Sci. USA.* 90:1590–1594.

Linney, E. 1992. Retinoic acid receptors: transcription factors modulating gene regulation, development and differentiation. *In* Current Topics in Developmental Biology. R.A. Pedersen, editor. Academic Press, San Diego. 309–350.

Lohnes, D., P. Kastner, A. Dierich, M. Mark, M. LeMeur, and P. Chambon. 1993. Function of retinoic acid receptor γ in the mouse. *Cell.* 73:643–658.

Lohnes, D., M. Mark, C. Mendelsohn, P. Dollé, A. Dierich, P. Gorry, A. Gansmuller, and P. Chambon. 1994. Function of the retinoic acid receptors (RARs) during development. (I) Craniofacial and skeletal abnormalities in RAR double mutants. *Development (Camb.).* 120:2723–2748.

Lufkin, T., D. Lohnes, M. Mark, A. Dierich, P. Gorry, M.-P. Gaub, M. LeMeur, and P. Chambon. 1993. High postnatal lethality and testis degeneration in retinoic acid receptor α mutant mice. *Proc. Natl. Acad. Sci. USA.* 90:7225–7229.

Luo, J., P. Pasceri, R.A. Conlon, J. Rossant, and V. Giguère. 1995. Mice lacking all isoforms of retinoic acid receptor β develop normally and are susceptible to the teratogenic effects of retinoic acid. *Mech. Dev.* 53:61–71.

Marshall, H., M. Studer, H. Popperl, S. Aparicio, A. Kuroiwa, S. Brenner, and R. Krumlauf. 1994. A conserved retinoic acid reponse element required for early expression of the homeobox gene Hoxb-1. *Nature (Lond.).* 370:567–571.

McLeod, M.J. 1980. Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red s. *Teratology.* 22:299–301.

Mendelsohn, C., E. Ruberte, M. LeMeur, G. Morriss-Kay, and P. Chambon. 1991. Developmental analysis of the retinoic acid-inducible RAR-β2 promoter in transgenic animals. *Development (Camb.).* 113:723–734.

Mendelsohn, C., E. Ruberte, and P. Chambon. 1992. Retinoid receptors in vertebrate limb development. *Dev. Biol.* 152:50–61.

Mendelsohn, C., D. Lohnes, D. Decimo, T. Lufkin, M. LeMeur, P. Chambon, and M. Mark. 1994*a.* Function of the retinoic acid receptors (RARs) during development. (II) Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development (Camb.).* 120:2749–2771.

Mendelsohn, C., M. Mark, P. Dollé, A. Dierich, M.P. Gaub, A. Krust, C. Lampron, and P. Chambon. 1994*b.* Retinoic acid receptor β2 (RARβ2) null mutant mice appear normal. *Dev. Biol.* 166:246–258.

Niswander, L., C. Tickle, A. Vogel, I. Booth, and G.R. Martin. 1993. FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell.* 75:579–587.

Niswander, L., S. Jeffery, G.R. Martin, and C. Tickle. 1994. A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature (Lond.).* 371:609–612.

Popperl, H., and M.S. Featherstone. 1993. Identification of a retinoic acid response element upstream of the murine *H0X-4.2* gene. *Mol. Cell. Biol.* 13: 257–265.

Riddle, R.D., R.L. Johnson, E. Laufer, and C. Tabin. 1993. *Sonic hedgehog* mediates the polarizing activity of the ZPA. *Cell.* 75:1401–1416.

Ruberte, E., P. Dollé, A. Krust, A. Zelent, G. Morriss-Kay, and P. Chambon. 1990. Specific spatial and temporal distribution of retinoic acid receptor γ transcripts during mouse embryogenesis. *Development (Camb.).* 108:213–222.

Ruberte, E., P. Dollé, P. Chambon, and G. Morriss-Kay. 1991. Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development (Camb.).* 111:45–60.

Ruberte, E., V. Friederich, P. Chambon, and G. Morriss-Kay. 1993. Retinoic acid receptors and cellular retinoid binding proteins. III. Their differential transcript distribution during mouse nervous system development. *Development (Camb.).* 118:267–282.

Schughart, K., C.J. Bieberich, R. Eid, and F.H. Ruddle. 1991. A regulatory region from the mouse Hox-2.2 promoter directs gene expression into devel-

oping limbs. *Development (Camb.).* 112:807–811.
Shenefelt, R.E. 1972. Morphogenesis of malformations in hamsters caused by retinoic acid: relation to dose and stage at treatment. *Teratology.* 5:103–118.
Smith, S.M., I.J. Kirstein, Z.-S. Wang, J.F. Fallon, J. Kelley, and J. Bradshaw-Rouse. 1995. Differential expression of retinoic acid receptor-β isoforms during chick limb ontogeny. *Dev. Dyn.* 202:54–66.
Solursh, M., and S. Meier. 1973. The selective inhibition of mucopolysaccharide synthesis by vitamin A treatment of cultured chick embryo chondrocytes. *Calcif. Tissue Res.* 13:131–142.
Sucov, H.M., E. Dyson, C.L. Gumeringer, J. Price, K.R. Chien, and R.M. Evans. 1994. RXR α mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes Dev.* 8:1007–1018.
Sucov, H.M., J.C. Izpisua Belamonte, Y. Ganan, and R.M. Evans. 1995. Mouse embyros lacking RXR-α are resistant to retinoic-acid-induced limb defects. *Development (Camb.).* 121:3997–4003.
Sulik, K.K., and D.B. Dehart. 1988. Retinoic-acid-induced limb malformations resulting from apical ectodermal ridge cell death. *Teratology.* 37:527–537.
Summerbell, D. 1983. The effect of local application of retinoic acid to the anterior margin of the developing chick limb. *J. Embryol. Exp. Morph.* 78:269–289.
Tabin, C.J. 1991. Retinoids, homeoboxes, and growth factors: toward molecular models for limb development. *Cell.* 66:199–217.
Tickle, C., B.M. Alberts, L. Wolpert, and J. Lee. 1982. Local application of retinoic acid to the limb bud mimics the pattern of chick wing development. *Nature (Lond.).* 296:564–565.
Tickle, C., J. Lee, and G. Eichele. 1985. A quantitative analysis of the effect of all-trans-retinoic acid on the pattern of chick wing development. *Dev. Biol.* 109:82–95.
Tickle, C., A. Crawley, and J. Farrar. 1989. Retinoic acid application to chick wing buds leads to a dose-dependent reorganization of the apical ectodermal ridge that is mediated by the mesenchyme. *Development (Camb.).* 106:691–705.
Underhill, T.M., L.E. Kotch, and E. Linney. 1995. Retinoids and mouse embryonic development. *Vitam. Horm.* 51:403–457.
Von Schroeder, H.P., Y. Hasimoto, and J.N.M. Heersche. 1994. The effects of natural and synthetic retinoids on the differentiation of RCJ C5.18 chondrogenic cells. *Teratology.* 50:54–62.
White, J.A., M.B. Boffa, B. Jones, and M. Petkovich. 1994. A zebrafish retinoic acid receptor expressed in the regenerating caudal fin. *Development (Camb.).* 120:1861–1872.
Yu, V.C., C. Delsert, B. Andersen, J.M. Holloway, O.V. Devary, A.M. Naar, S.Y. Kim, J.-M. Boutin, C.K. Glass, and M.G. Rosenfeld. 1991. RXRβ: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell.* 67:1251–1266.
Zelent, A., M. Krust, M. Petkovich, P. Kastner, and P. Chambon. 1989. Cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin. *Nature (Lond.).* 339:714–717.
Zimmerman, B., and D. Tsambos. 1985. Evaluation of the sensitive step of inhibition of chondrogenesis by retinoids in limb mesenchymal cells in vitro. *Cell Diff.* 17:95–103.