Postaxial polydactyly in forelimbs of CRABP-II mutant mice

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SUMMARY

The cytoplasmic retinoic acid (RA)-binding protein CRABP-II is expressed widely throughout early morphogenesis in mouse embryo, but its expression becomes more restricted as organogenesis progresses. CRABP-II expression remains strong in the developing limb bud suggesting a role for this protein in limb patterning. Here, we show that the CRABP-II promoter can direct expression of a lacZ transgene in a specific posterior domain during limb bud development. In order to investigate in more detail the role played by CRABP-II in RA signal transduction, we have also generated mice homozygous for a null mutation of this gene. CRABP-II−/− mice are viable and fertile but show a developmental defect of the forelimb, specifically an additional, postaxial digit. This digit is generally, but not exclusively, limited to a single forepaw of an individual animal. The penetrance of the phenotype varies according to the genetic background, occurring most frequently on the inbred 129Sv background (50%), less frequently on the C57Bl/6 background (30%) and rarely on the outbred CD1 background (10%). This developmental abnormality implies a role for CRABP-II in normal patterning of the limb.

Key words: vitamin A, retinoic acid, limb, mouse, CRABP-II, polydactyly

INTRODUCTION

Normal development of vertebrate embryos requires retinoic acid (RA). Regulation of physiological levels is vital, as RA is a potent teratogen. In both deficiency and excess, RA can cause a syndrome of developmental defects known as retinoid embryopathy (RAE) (Lammer et al., 1985). These traits include craniofacial and limb abnormalities, heart defects and malformations of the central nervous system. Although the role of RA in both normal and teratogenic development has been the subject of much investigation, its fundamental role in the process of morphogenesis is still uncertain. However, the mechanism of transduction of the RA signal is becoming more clear.

RA affects expression of responsive genes via the action of two distinct families of nuclear receptors, known as the RAR and RXR families. Belonging to the steroid/thyroid hormone superfamily of nuclear receptors, these RA receptors are ligand-dependent transcriptional regulators and act by binding to specific DNA sequences (RA response elements (RAREs)) contained in the regulatory regions of responsive genes (reviewed in Giguère, 1994). At least three genes have been identified within each receptor family, and each gene gives rise to multiple isoforms, some of which are inducible by RA. Differential expression of these isoforms (which act as RAR/RXR heterodimers) could give rise to a wide variety of isoform combinations which may possess specific regulatory functions.

A second class of proteins involved in RA signal transduction are the cytoplasmic RA-binding proteins, of which two have so far been identified, named CRABP-I (Chytil and Ong, 1984) and CRABP-II (Giguère et al., 1990). These are small, closely related peptides of approximately 16×10³ M_r which bind RA with high affinity, although the affinity of CRABP-II for all-trans RA is 15-fold lower than that of CRABP-I (Fiorella et al., 1993; Fogh et al., 1993). Both CRABP genes are expressed by embryonic day 7.5, as detected by in situ hybridization (Ruberte et al., 1992; Lyn and Giguère, 1994). Throughout development there is some overlap in the patterns of expression, but also regions where expression of one gene is unique. CRABP-I is expressed in regions of the developing embryo which are particularly sensitive to exogenous RA (Ruberte et al., 1992) and is thought to play an important role in regulation of intracellular RA levels. The binding protein may facilitate metabolism of RA to polar metabolites, or may simply sequester RA making it unavailable to the nuclear receptors (Fiorella and Napoli, 1991). Further evidence in support of this role is provided by the ability of CRABP-I to reduce RA-induced activation of transcription by RARs in F9 teratocarcinoma cells (Boylan and Gudas, 1991). The role of CRABP-II is still unclear. By analogy, it is possible that CRABP-II plays a similar role to CRABP-I as it is also expressed in RA-sensitive tissues, but it is also expressed in tissues not known to be sensitive to RA (Ruberte et al., 1992). Unlike CRABP-I, CRABP-II expression is inducible by RA (Giguère et al., 1990). The promoter region of the gene contains two RAREs (Durand et al., 1992), and an additional
level of control is provided by a post-transcriptional mechanism (MacGregor et al., 1992). CRABP-II is largely co-expressed with another autoregulated member of the RA signal transduction pathway, namely RARβ2 (de Thé et al., 1990; Hoffman et al., 1990; Sucov et al., 1990).

Further insight into gene function can be gained by studying the effects of specific mutations. To this end the technique of gene targeting by homologous recombination in embryonic stem cells has proved highly useful. Recently, several different RAR and RXR isoform-specific mutations have been reported, as well as total null mutations (Li et al., 1993; Lohnes et al., 1993; Lufkin et al., 1993; Sucov et al., 1994) Surprisingly, individual isoforms of each receptor seem to be dispensable for normal development. At least two isoforms must be eliminated before any obvious defect is produced, an effect seen when isoforms from either the same or different genes within the family are targeted (Lohnes et al., 1993; Lufkin et al., 1993; Luo and Giguère, unpublished results).

To investigate the role of CRABP-II in RA signal transduction, we have disrupted the CRABP-II gene to give a null mutation. Mice homozygous for the mutation are viable and fertile. Defects are limited to the forelimb in which there is an additional, postaxial digit, which is generally, but not exclusively, unilateral. This limb abnormality can be related to the expression domains of a CRABP-II promoter-lacZ transgene within the developing limb. The possibility that loss of CRABP-II may result in an alteration of RA levels on the posterior side of the limb bud is discussed.

MATERIALS AND METHODS

Transgenic mice: production and expression analysis

The vector p2.7ClIIlacZ was made by inserting a 2.7 kb BglII fragment containing the CRABP-II promoter and 121 bp of 5' untranslated sequence (MacGregor et al., 1992; see also Fig. 2A) upstream of the E. coli lacZ reporter gene in pSDKIacZpA. Transgenic mice were generated on an outbred CD-1 background by standard procedure (Hogan et al., 1986), and transgenic offspring were identified by Southern blots of tail DNA with a HindIII probe derived from the shorter vector arm (probe A in Fig. 2). Positive clones contained a 4.3 kb mutant band as well as a 2.7 kb wild-type band. These clones were verified by a second genomic digest (XhoI), probed with an external 3' probe (probe B, Fig. 2) which gave a 9 kb wild-type and 11 kb mutant band. Cells from 3 targeted lines were introduced into C57Bl/6 blastocysts by microinjection as described (Papaioannou and Johnson, 1993).

Immunohistochemistry

10.5 dpc embryos were fixed in 4% paraformaldehyde, infiltrated with 30% sucrose and prepared for immunohistochemistry as described (LaMantia et al., 1993). The affinity purified anti-CRABP-II polyclonal antibody was diluted to 1-2 µg/ml and the monoclonal anti-NCAM (Chemicon) antibody was used at 1 µg/ml for immunohistochemical staining. Antibody binding was detected using DTAF-conjugated species-specific secondary antibodies (Chemicon or Accurate chemicals) and photographed using a Zeiss fluorescence microscope (Axioscope).

RNA isolation, skeletal staining and RA treatment of pregnant females

Total RNA was prepared from embryos using TRIZOL reagent (BRL). Newborn pups and adult limbs were prepared for skeletal analysis by alcin blue/alizarin red staining as described (Lufkin et al., 1992). RA treatment was performed as described (Lyn and Giguère, 1994).

RESULTS

A CRABP-II promoter lacZ transgene is expressed in the posterior domain of the developing limb bud

Previous northern blot and in situ hybridization studies of the pattern of expression of CRABP-II have demonstrated that CRABP-II mRNA is abundantly expressed in the mesenchymal cells of the developing limb bud (Giguère et al., 1990; Ruberte et al., 1992; Lyn and Giguère, 1994). In order to study the transcriptional regulation of the CRABP-II gene during limb bud development, we have generated transgenic mice expressing a 2.7 kb CRABP-II promoter-lacZ fusion gene. Two transgenic males produced litters in which the expected 50% showed essentially identical and very specific patterns of expression. Fetuses derived from Tg15-3 show the strongest staining, with blue color being apparent within 15 minutes of immersing in substrate (see below), and the analysis of expression patterns was performed on litters sired by mouse Tg15-3. During early development, the CRABP-II promoter-lacZ fusion gene exhibits a pattern of β-galactosidase activity reminiscent of the sites that have been demonstrated to express CRABP-II mRNA (Fraser, Lyn and Giguère, unpublished data). At a later stage, intense β-galactosidase activity is restricted to the developing limb buds (Fig. 1A) while weak lacZ expression could still be detected in the fronsonasal and pharyngeal arch mesenchyme (data not shown). Interestingly, strong lacZ expression is detected in the posterior mesenchyme of the developing limb buds as staining was apparent in the posterior domain between 15 and 30 minutes after initiation of staining (Fig. 1A-C). Longer incubation with X-gal (Fig. 1D, 1.5 hours) revealed a graded expression of the lacZ transgene both in the anteroposterior and dorsoventral axes. Overnight staining showed an homogenous distribution of lacZ expression throughout the mesenchyme (data not shown), with
the exception of anterior, ventral mesenchyme of the limb. This latter observation is consistent with previously reported pattern of CRABP-II expression in the limb bud (Ruberte et al., 1992). These data reveal that transcriptional activity of the CRABP-II promoter is higher in the posterior mesenchyme of the developing limbs, and further suggest that, if CRABP-II plays a role in limb development, disruption of the CRABP-II gene may lead to patterning defects preferentially affecting the posterior region.

**Generation of homozygous CRABP-II null mutants**

The vector pNTC2.KO (Fig. 2A) was electroporated into ES cells and five independent lines containing a mutated CRABP-II allele were detected by Southern blotting (Materials and Methods). A male chimera derived from one of these lines transmitted the ES genotype when mated with either C57Bl/6, CD1 or 129Sv females. As expected, 50% of the ES-derived progeny were heterozygous (CRABP-II+/−) for the mutant allele, and these animals appeared phenotypically indistinguishable from their wild-type (CRABP-II+/+) littermates. Heterozygotes were crossed to generate homozygous mutant mice. Table 1 shows the number of each genotype obtained (Fig. 2B shows a sample litter). No under-representation of homozygote (CRABP-II−/−) offspring was found, indicating that the CRABP-II−/− mutation is not lethal at an embryonic stage and that the pups are viable until at least 3 weeks. No adult CRABP-II−/− animals have died unexpectedly and the oldest mice have lived at least 10 months. Homozygote males and females were crossed with heterozygous mice to assess fertility. In both types of cross, several litters of average size were obtained showing that homozygotes are fully fertile.

**CRABP-II−/− mutant mice are true nulls**

To ensure that the targeting event produced a truly null phenotype, CRABP-II−/− embryos were examined for both residual protein and mRNA. CRABP-II protein was evaluated by immunocytochemical staining with a polyclonal antibody specific for CRABP-II (M. Colbert, unpublished). Transverse sections through the spinal cord were taken at the level of the forelimbs from 10.5 dpc embryos produced by heterozygote crosses. At this gestational stage the expression of the CRABP-II gene is high within this region (Ruberte et al., 1992, 1993). As shown in Fig. 3A, the cells within the developing motor regions in the basal plate of the spinal cord as well as the cells in the dorsal root ganglia are the most intensely stained by the antibody against CRABP-II in the wild-type embryos. The staining pattern of similar sections from heterozygotes is identical although it appears quantitatively less than the wild-type embryos (compare Fig. 3C with A). No

<table>
<thead>
<tr>
<th>Genotype (3 weeks)</th>
<th>+/+</th>
<th>+/−</th>
<th>−/−</th>
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<tr>
<td>Number of mice</td>
<td>72</td>
<td>121</td>
<td>55</td>
</tr>
<tr>
<td>Expected (if 1:2:1)</td>
<td>62</td>
<td>124</td>
<td>62</td>
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Numbers indicate offspring from heterozygous intercross. DNA was made from tail snips for determination of genotype.
CRABP-II protein, however, could be detected by the antibody in the CRABP-II−/− embryos (Fig. 3E). Serial sections from each genotype were also stained with antibodies against NCAM as control. In these sections, the patterns were indistinguishable (Fig. 3B,D,F). As additional verification, total RNA was isolated from 12.5 d.p.c embryos of each genotype (littermates from heterozygote intercross) and compared by band intensity in a northern blot. No transcripts could be detected in CRABP-II−/− embryos (Fig. 4). Again, the heterozygote shows a weaker signal than the wild type.

Expression of CRABP-I, RARβ2 and a CRABP-II promoter/lacZ transgene are unaffected in CRABP-II−/− mice

We investigated the possibility that in CRABP-II−/− mice the loss of CRABP-II may be compensated for by an increase in CRABP-I. Total RNA isolated from 12.5 d.p.c. embryos was again compared by band intensity on a northern blot. The level of CRABP-I RNA was the same in CRABP-II−/− embryos as in wild-type or heterozygous littermates (Fig. 4). We also used the same RNA to investigate expression of RARβ2, a RA-responsive gene, which may show an elevated level of expression in the absence of CRABP-II if this results in more free RA (or conversely a decrease in expression if CRABP-II has the opposite function). Again we could detect no major change in the level of expression of the RARβ2 gene (Fig. 4). To study whether CRABP-II participates in an autoregulatory mechanism, we transmitted onto the CRABP-II−/− mutant background the CRABP-II promoter/lacZ transgene that expresses lacZ in the posterior mesenchyme of the limb bud (see above). Expression of lacZ was not affected by the loss of CRABP-II (data not shown).

CRABP-II−/− mice have an additional postaxial digit

A superficial examination of CRABP-II−/− adult mice revealed the presence of an additional postaxial digit (Fig. 5C,D). This digit is small, containing a single phalangeal bone, and is not therefore a true duplication of digit 5. The defect is apparently restricted to the forelimbs as no alteration of the hindlimbs has been observed. In general only one forelimb per individual is affected, although we have found several animals in which both forelimbs carry the defect (on both C57Bl/6 and 129Sv backgrounds). Interestingly, the penetrance of this phenotype varies according to the genetic background (Table 2). It occurs most frequently in the 129Sv background (approximately 50%), less frequently in the C57Bl/6 background (approximately 30%) and rarely occurs in the CD1 background (approximately 10%). No other defect in the limb was found, for example in the length of the digits or the long bones. Other regions of the skeleton were examined but no defects were detected. No limb defects were observed in heterozygous or wild-type mice on any genetic background.

Fig. 2. Schematic representation of the CRABP-II targeting vector and recombination at the CRABP-II locus. (A) Map of the CRABP-II locus from the 129Sv mouse strain. Top, the wild-type locus. Coding regions of exons are shown as shaded boxes, non-coding regions as open boxes. Fragments used to probe Southern blots are indicated. Centre, the targeting vector pNTC2.KO. Bottom, the structure of the disrupted CRABP-II locus, showing replacement of genomic sequences by the neo cassette. The genomic fragments detected by probe A (BglII digest) and probe B (XhoI digest) are illustrated. (B) Southern blot of a typical litter from a heterozygote intercross. Genomic DNA was digested with BglII and probe A was used to distinguish wild-type and mutant alleles.
CRABP-II is expressed widely throughout embryonic development (Ruberte et al., 1992; Lyn and Giguère, 1994). We performed a histological examination of several adult tissues including brain, liver and kidney, all sites of CRABP-II expression in the embryo, and also adult skin, the predominant site of expression in the adult (Giguère et al., 1990). In no case could we detect any change in morphology when comparing CRABP-II+/+ and CRABP-II−/− individuals (data not shown).

**CRABP-II−/− mice show no change in sensitivity to exogenously administered RA during early embryogenesis**

If CRABP-II is involved in catabolism and/or sequestration of RA, CRABP-II−/− embryos may be more sensitive to exogenously administered RA as their ability to catabolize and/or sequester RA is reduced. Conversely, if CRABP-II has the opposite effect CRABP-II+/+ embryos may be less sensitive to exogenously administered RA. To test this, heterozygous matings were set up. At day 7.5 p.c. pregnant females were treated with RA at a range of concentrations from 5 mg/kg to 50 mg/kg, and the embryos removed at day 10.5 p.c. By this method a gross response to RA is readily observable by examination of size and morphology of the embryos, although a more detailed analysis will be required to monitor subtle morphological differences (R. Conlon, personal communication). At high doses (50 mg/kg and 20 mg/kg) the embryos were greatly reduced in size, the morphology was grossly abnormal and there were several resorptions. All embryos analyzed (17 and 26 embryos from mothers treated with RA at concentrations of 50 mg/kg and 20 mg/kg, respectively), irrespective of genotype, responded to the same degree. At the lowest dose, the affect was much milder. The embryos (12 embryos from mothers treated with RA at a concentration of 5 mg/kg) were of fairly normal size and morphology with only slight defects in the head region, and all embryos were affected to the same extent, irrespective of genotype.

**DISCUSSION**

CRABP-II is presumed to play a role in transduction of the RA signal, but the nature of that role is unclear. We have generated a null mutation of the CRABP-II gene in order to gain an insight into function of this protein. The results of this study indicate that CRABP-II is involved in the patterning of the forelimb, as loss of this protein causes postaxial polydactyly. It is surprising, given the widespread expression of the CRABP-II gene throughout development, that the limb defect is the only obvious consequence of this mutation. This may reflect compensation for the loss by other RA-binding proteins, such as CRABP-I and possibly others which have yet to be identified (Giguère et al., 1990). Expression of CRABP-I appears to be unchanged in CRABP-II+/− mice, but as the affinity of CRABP-I for RA is relatively much higher than that of CRABP-II there may be no requirement for more CRABP-I if the protein is not normally saturated. If other binding proteins can compensate for loss of CRABP-II, then it might be expected that where CRABP-II is uniquely expressed there would be structural abnormalities. However, we found no such defects at the level of gross morphology. It is possible there are changes affecting the cytoarchitecture, which could have more subtle consequences. For example, such a defect within the telencephalon could lead to alterations in sensory functions, or behavior.

It is interesting that the one region where a defect is seen is one in which both CRABPs are expressed, namely the forelimb. In the developing limb bud there is a complex pattern of CRABP expression. Although both genes are expressed in the developing limb buds, their spatial restrictions are quite different (Ruberte et al., 1992), leading perhaps to localized regions of higher or lower RA concentration. At the anterior side of the limb bud, CRABP-I forms a proximodistal gradient, the highest levels being found at the distal side (Dollé et al., 1989b). Expression is abundant and more uniform on the posterior side. In situ hybridization studies have shown that CRABP-II is apparently expressed more uniformly in these dimensions, although a dorsoventral gradient was also observed, the highest levels being found on the dorsal side. In this study, we have demonstrated that a CRABP-II promoter directs strong expression of a lacZ transgene in the posterior mesenchyme of the limb bud. At the posterior side therefore, where the extra digit is found, there is a high level of both binding proteins. This is also the location of the ZPA, where the level of RA is high (Thaller and Eichele, 1987). If the function of both CRABP-I and II is to bind RA and prevent access to the receptors, then in CRABP-II−/− embryos there may be an excess of RA on the posterior side of the limb bud where there is a lack of CRABP-II. This would assume that the level of RA is high enough that CRABP-I is saturated.

Until fairly recently, RA itself was considered as a likely candidate for a limb morphogen, possibly by diffusing across the limb bud from the ZPA to form a gradient which could give positional information to the mesenchymal cells (Eichele, 1989). More recent evidence suggests that the product of the Sonic hedgehog (Shh) gene may act as the limb morphogen, as Shh is expressed in the putative ZPA and ectopic expression of Shh leads to limb duplication (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993). RA has been shown to induce expression of Shh concomitantly with production of the ZPA, while sonic hedgehog itself exhibits ZPA-like activity (Riddle et al., 1993). Thus RA probably acts at an earlier stage in pattern formation, perhaps by determining the formation of the ZPA. It is interesting to note that expression of the CRABP-II promoter-lacZ transgene is preferentially localized to a posterior domain in the limb bud (Fig. 1).

Both RA and sonic hedgehog act to repattern the limb by

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**Table 2. Frequency of occurrence of polydactyly in CRABP-II−/− adult mice, according to the genetic background of the mutation**

<table>
<thead>
<tr>
<th>Genetic background*</th>
<th>Forelimbs affected</th>
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<tr>
<td></td>
<td>One limb</td>
</tr>
<tr>
<td>129Sv</td>
<td>3</td>
</tr>
<tr>
<td>C57Bl/6</td>
<td>4</td>
</tr>
<tr>
<td>CD1</td>
<td>1</td>
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*Embryos were obtained by mating a male chimera to 129Sv, C57Bl/6 or CD1 females.
Fig. 3. Immunohistochemical staining of sections through the trunk region of 10.5 d.p.c. embryos from a heterozygote intercross. (A,C,E) Stained with a polyclonal anti-CRABP-II antibody, the genotypes are as indicated. (B,D,F) Consecutive sections from the same embryos as A, C and E respectively, stained with a control anti-NCAM antibody.
Polydactyly in *CRABP-II* mutant mice

These genes are activated in a sequential manner to give a pattern of nested expression across the limb bud; the first gene to be activated showing the most anterior expression boundary and each successive gene showing a more posterior restriction until five distinct regions are specified (Dollé et al., 1989a; Izpisúa-Belmonte et al., 1991; Nohno et al., 1991). Ectopic administration of RA or ectopic expression of *ssh* causes Hoxd genes to be expressed to more anterior positions than normal (Izpisúa-Belmonte et al., 1991; Riddle et al., 1993). If there is an excess of RA in the limb bud of the *CRABP-II*−/− mice then, the limb defect could be due to interference with normal *Shh* function, possibly resulting in mis-expression of Hoxd genes.

How this results in an additional, postaxial digit is not clear, but could be a result of an excess of mesenchymal cells on the posterior side of the limb bud. This is seen in the *Hoxd-13* mutant mice (Dollé et al., 1993) where a disruption of the Hoxd pattern resulted in limb defects, including an additional postaxial digit. In these mice, a large field of uncondensed mesenchymal cells was observed on the posterior side of the limb bud. The mechanism suggested by Dollé et al. (1993) for digit formation depends on a ratio between condensed and uncondensed mesenchymal cells; too many uncondensed cells in one area could spontaneously condense to form an extra digit. We

![Fig. 4. Northern blot analysis of total RNA isolated from 12.5 d.p.c. embryos. CRABP-II mRNA was detected using a mouse CRABP-II cDNA probe containing exon 2, 3 and 4 sequences but lacking exon 1 sequence 5′ of the neo insertion site. CRABP-I was detected using the entire mouse CRABP-I cDNA. Both CRAB signals were detected after overnight exposure to X-ray film at −70°C. RARβ2 mRNA was detected using a probe derived from RARβ2-specific exon 1. The signal was weak but detectable after 7 day exposure. A mouse β-actin cDNA probe was used as a loading control.](image)

![Fig. 5. Forelimbs and skeletal preparation of littermates from heterozygous mating. (A,C) Photograph of wild-type (A) and mutant (C) forelimb of newborn littermates (129Sv background) taken at the same magnification (12×). (C) Mutant forelimb shows an extra sixth digit. (B,D) Photograph taken at the same magnification showing alcin blue/alizarin red staining for cartilage and bone, respectively. The forelimbs are taken from newborn wild-type (B) and mutant (D) littermates. An additional postaxial sixth digit (VI) is present with one phalange.](image)
do not know if a similar effect is seen in the CRABP-II$^{-/-}$ mice, but if so it is likely to be less obvious than in the Hoxd-13$^{-/-}$ mice where the limb defects are more severe. In CRABP-II$^{-/-}$ mice, the extra digit tends to be more rudimentary than in Hoxd-13$^{-/-}$ mice and never occurs in the hindlimb.

Recently the disruption of the mouse En-1 gene has been described (Wurst et al., 1994). One of the defects seen is a postaxial, mostly unilateral, extra digit. This similarity with the CRABP-II phenotype is produced despite the fact that En-1 expression in the limbs is initially restricted to the ventral ectoderm and later to the AER, where CRABP-II and the Hoxd genes are not expressed. This illustrates a possible interaction between the ectoderm and the underlying mesenchyme which is important not just for proximodistal axis specification but also for the anteroposterior axis. It is known that to be maintained the AER requires posterior mesenchyme and that conversely the AER produces mitogenic factors (possibly FGF-4) that are needed for maintenance of the ZPA as well as for limb outgrowth (Laufer, 1993; Vogel and Tickle, 1993). This interaction between mesenchyme and ectoderm may be mediated by ssh (Riddle et al., 1993). These complex interactions between mesoderm and ectoderm provide the underlying reasons for observing similar phenotypes in mice carrying mutations in two very different genes.

It is interesting that the extra digit is never found on the hindlimbs, in which the same mechanism of digit specification is proposed to exist. This could be a reflection of the relative timing of forelimb and hindlimb development, as hindlimb development is delayed by approximately one day. It does illustrate, however, that forelimb and hindlimb development, despite similarities in gene expression within the limb buds, are not equivalent.

It has already been noted that more than one RAR or RXR isoform must be mutated before a phenotype is observed. It is possible that targeting of two different components of the RA signal transduction pathway, for example a receptor isoform and a binding protein such as CRABP-II, will produce a more severe phenotype than either single mutation. It is clear that the mechanism of RA signal transduction is more complex than was initially visualized, with much functional compensation being revealed amongst the receptors and now the binding proteins.

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