INTRODUCTION

Vitamin A (retinol), an essential component of the diet, is indispensable for normal growth, vision, maintenance of numerous tissues, reproduction and overall survival (Wolbach and Howe, 1925; for reviews see Sporn et al., 1994, and Blomhoff, 1994). Vitamin A deprivation in the adult is characterized by keratinizing and non-keratinizing squamous metaplasia of numerous epithelia (Wolbach and Howe, 1925; Underwood, 1994, and references therein). Offspring of vitamin A deficient (VAD) dams exhibit a number of congenital abnormalities, indicating that vitamin A is also essential during embryonic development (Wilson et al., 1953, and references therein). Retinoic acid (RA) has been shown to be the active metabolite of vitamin A for post-natal growth and maintenance of adult animals. RA administration can prevent or reverse most of the defects generated by post-natal VAD diet with the exception of the effects on vision (Wald, 1968; Thompson et al., 1964; van Pelt and de Rooij, 1991). The highly teratogenic effects of maternal excess RA administration on mammalian embryos (Kessel, 1992; Morriss-Kay, 1993; Linney and LaMantia, 1994; Nau et al., 1994; Hofmann and Eichele, 1994, and references therein) have suggested that RA could also be the active metabolite of vitamin A during development. This view is supported by the spectacular effects of systemic administration of RA on mouse limb duplication (Rutledge et al., 1994), as well as those of topical application of RA on chick limb morphogenesis and regeneration or generation of amphibian limbs, which led to the controversial proposal that RA could be a morphogen, conferring positional identity during development (Tabin, 1991; Maden, 1993, 1994; Hoffman and Eichele, 1994; Noji et al., 1991; Wanek et al., 1991; Riddle et al., 1993, and references therein).

It is now well established that the biological effects of RA during development and post-natal life are transduced by two families of nuclear receptors, the RARs and the RXRs, which act as all-trans and 9-cis RA-dependent transcriptional regulatory factors (Leid et al., 1992; Chambon, 1994; Kastner et al., 1994b; Mangelsdorf et al., 1994; Giguère, 1994; Petkovich, 1992, and references therein). All abnormalities exhibited by fetuses of VAD dams are recapitulated in RAR and/or RXR single or double mouse mutants (Lohnes et al., 1993, 1994; Lufkin et al., 1993; Mendelsohn et al., 1994a,b; Kastner et al., 1994a; Sucov et al., 1994; Chambon, 1994). In addition to RA nuclear receptors, two cellular cytoplasmic RA binding proteins (CRABP I and CRABP II) have been identified (Ong and Chytil, 1975; Giguère et al., 1990; for reviews see Ross, 1993; Napoli, 1994; Ong et al., 1994; Morriss-Kay, 1993). CRABPs are found in all vertebrates and are conserved across species. CRABP II appears to bind all-trans RA with a significantly lower affinity than CRABP I (Bailey and Siu, 1988; Aström et al., 1991; Siegenthaler et al., 1991; Fiorella et al., 1993), and both proteins bind 9-cis RA with much lower affinity than all-trans RA.

We have disrupted the CRABP II gene using homologous recombination in embryonic stem cells, and shown that this disruption results in a null mutation. CRABP II null mutant mice are essentially indistinguishable from wild-type mice as judged by their normal development, fertility, life span and general behaviour, with the exception of a minor limb malformation. Moreover, CRABP I−/−/CRABP II−/− double mutant mice also appear to be essentially normal, and both CRABP II−/− single mutant and CRABP I−/−/CRABP II−/− double mutant embryos are not more sensitive than wild-type embryos to retinoic acid excess treatment in utero.

Thus, CRABP I and CRABP II are dispensable both during mouse development and adult life. Our present results demonstrate that CRABPs are not critically involved in the retinoic acid signaling pathway, and that none of the functions previously proposed for CRABPs are important enough to account for their evolutionary conservation.

Key words: mouse development, retinoic acid signaling pathway, CRABP I mutant, CRABP I and CRABP II double mutant, vitamin A, gene targeting
RA (Fogh et al., 1993; Allenby et al., 1993; Fiorella and Napoli, 1993). Both CRABPI and II transcripts and proteins are expressed at all stages of mouse embryo development in distinct, often non-overlapping patterns (Ruberte et al., 1992; Lyn and Giguère, 1994; Gustafson et al., 1993; Maden, 1994, and references therein). However, CRABPII, but not CRABPI expression is transcriptionally inducible by RA in cultured cells and human skin in vivo and in vitro (Giguère et al., 1990; Durand et al., 1992; Aström et al., 1991).

It has been shown that CRABPI expression, and to a lesser extent CRABPII expression, is correlated in mouse embryonic tissues with vulnerability of these tissues to RA excess (Dencker et al., 1990; Vaessen et al., 1990; Ruberte et al., 1991, 1992; Maden et al., 1992; Gustafson et al., 1993, and references therein). Furthermore, exogenous radiolabelled RA accumulates in sites expressing CRABPI (Dencker et al., 1990; Gustafson et al., 1993), suggesting that cells that express CRABPI in the embryo are target cells for exogenous and endogenous RA. It has also been shown that RA-induced expression of RARβ2 is reduced by 80-90% in cells expressing elevated levels of CRABPI (Boylan and Gudas, 1991). CRABPs (notably CRABPII) also influence the synthesis and metabolism of RA in vitro (Fiorella and Napoli, 1991; Napoli, 1994; Boylan and Gudas, 1992, and references therein). Taken all together, these studies led to the proposal that CRABPs could act as ‘buffers’ to control spatiotemporally the actual level of ‘free’ intracellular RA available for binding to the nuclear receptors. CRABPs may (i) sequester RA in the cytoplasm, thus controlling its intra-nuclear level and/or (ii) act as modulators of RA catabolism, thus controlling its intracellular concentrations (Ruberte et al., 1992; Morriß-Kay, 1993; Maden, 1994; Napoli, 1994; Ong et al., 1994). In this respect, it has been proposed that the spatial distribution of CRABPI in the chick limb bud may result in a steeper morphogenetic gradient of free RA (Smith et al., 1989; Maden, 1994, and references therein). Alternatively or additionally, CRABPs may shuttle their ligands to the nuclear receptors (Takase et al., 1986). However, the affinity of synthetic retinoids for CRABPI is not well correlated with their biological activity (Darmon et al., 1988; Keidel et al., 1993).

To determine the actual function of CRABPs in the RA signaling pathway in vivo, we have generated mutant mice deficient in either CRABPI, CRABPII or both proteins. We have reported elsewhere that mice deficient in CRABPI are essentially normal (Gorry et al., 1994), which suggested that CRABPI and II could be functionally redundant. We now report that CRABPII null mutant mice do not exhibit any overt phenotypic defects, with the exception of a discrete limb abnormality. Moreover, CRABPII−/−/CRABPII−/− double mutant mice are also essentially normal. Thus, both CRABPI and II appear to be dispensable for mouse development and adult life, indicating that they cannot be critically involved in the basic mechanisms that lead to the transduction of the RA signal by RARs and RXRs. Furthermore mice deficient in CRABPI and II do not seem to be more sensitive than their wild-type littermates to exposure to RA excess during their embryonic life.

**MATERIALS AND METHODS**

**Disruption of the CRABPII locus**
The genomic recombinant phage λEMBL12 (from a mouse Balb/c EMBL phage library) containing the CRABPII gene was obtained as described by Durand et al. (1992). To construct the CIIExt10 targeting vector (Fig. 1A), the 8 kb BamHI-SalI genomic fragment containing exons 1-3 of the CRABPII gene was subcloned in pBluescript SK(+)-vector (Stratagene), after disruption of the HindIII polylinker site. A 0.6 kb BgII fragment containing exon 1 was then subcloned in pD126 vector [a pTZ19U (Pharmacia) modified vector with two BgII sites, a gift of D. Lohnes]. Nucleotides (nt) from position +119 to +164 (as numbered by Durand et al., 1992), containing the initiator ATG codon, were deleted using nuclease Bal31. The resulting product was endblunted with T4 DNA polymerase and Klenow enzyme, and ligated to a HindIII-BgII-HindIII linker to introduce a unique HindIII site. This modified BgII fragment was reintroduced in the BamHI-SalI genomic fragment and the PGK-NEO poly(A)+ cassette (derived from pKJ-I; Adra et al., 1987) was cloned in antisense orientation at the unique HindIII site. The 2.3 kb GTI-II enhancer-driven Herpes simplex virus thymidine kinase gene (HSV TK) fragment (Lufkin et al., 1991) was inserted into the pBluescript SK+ vector to yield the CIIExt10-targeting vector (Fig. 1A). This plasmid was linearized with SalI prior to electroporation into D3 embryonic stem (ES) cells. ES cell culture, G418 and gancyclovir selection, genomic DNA extraction, Southern blotting and generation of chimeras were as described by Lufkin et al. (1991). The 3’ probe corresponds to the 0.6 kb SalI genomic fragment (containing exon 4), derived from the recombinant phage λEMBL12 (see Fig. 1A).

**RNase protection analysis**
Total RNA was prepared from 13.5 dpc (day post-coitum) embryos using the single-step guanidinium-isothiocyanate-phenol technique (Chomczynski and Sacchi, 1987). Approximately 50 µg of RNA was used per hybridization at 55°C. The conditions for the preparation of probes and hybridization reaction were essentially as described by Ausubel et al. (1987). Templates for synthesis of antisense riboprobes were obtained by subcloning the 402 nucleotide-long EcoRI-Apal fragment of CRABPII cDNA, and a 3.8 kb genomic fragment containing exon 1 and exon 2 of CRABPI that generates two protected fragments (166 and 178 nt; nucleotides position: 738-903 and 1417-1595, respectively, as numbered by We et al., 1990). The histone H4 probe was a gift of R. Grosschedl (see Lufkin et al., 1993).

**Protein extraction and immunodetection**
Embryos from CRABPII−/− intercross or CRABPII−/−/CRABPII−/− × CRABPII−/−/CRABPII−/− crosses were collected at 10.5 or 13.5 dpc, immediately frozen in liquid nitrogen, and the yolk sac taken for DNA genotyping. Cytosolic extracts from embryos, whole-cell extracts from transfected Cos-1 cells and western blotting procedures were as described by Rochette-Egly et al. (1991). Monoclonal antibodies specific for CRABPI (3CRA 10F5) and CRABPII (1CRA 4C9), raised against the synthetic peptides SPB63 (amino acids 95-108) and SPB64 (amino acids 96-109), respectively, were used (M.-P. Gaub and P. Chambon, unpublished data). A rabbit polyclonal antibody specific for CRABPI directed against the synthetic peptide SPB63 (Gorry et al., 1994) was also used. Detection of the antigen-antibody complexes was performed using protein A-labeled or anti-mouse immunoglobulins-labeled peroxidase, followed by chemiluminescence detection according to the protocol recommended by the manufacturer (Amersham, UK).

**RA binding assay**
RA binding activity in 10.5 and 13.5 dpc embryos was determined by overnight incubation of 300 µg (protein) embryonic cytosolic extracts at 4°C with 600 nM all-trans [3H]RA (Dupont), in the presence or absence of a 200-fold excess of unlabeled ligand. PAGE/autoradioblotting and exposure were as described (Siegenthaler, 1990).

**RA treatment and skeletal analysis**
For matings, mice were left together for the night and females that
Pregnant females received a single dose of RA, dissolved in sunflower oil, of either 1 mg/kg or 10 mg/kg body weight, that was administered by gastric gavage at various times of gestation. Embryos were delivered by cesarean section at 18.5 dpc, genotyped and stained as in Lufkin et al. (1992). Neonates and adults skeleton were stained following the same procedure except that for the adults the 2% KOH incubation was extended to 3-4 days.

**RESULTS**

**CRABPII gene disruption and germline transmission**

The **CRABPII** gene was disrupted by homologous recombination using an approach based on positive-negative selection (Capecchi, 1989). The construct CIIExt10 (Fig. 1A) has 8.0 kb of mouse genomic DNA and contains a deletion in exon 1.

had a vaginal plug on the next morning were taken as 0.5 dpc at noon. Pregnant females received a single dose of RA, dissolved in sunflower oil, of either 1 mg/kg or 10 mg/kg body weight, that was administered by gastric gavage at various times of gestation. Embryos were delivered by cesarean section at 18.5 dpc, genotyped and stained as in Lufkin et al. (1992). Neonates and adults skeleton were stained following the same procedure except that for the adults the 2% KOH incubation was extended to 3-4 days.
between nucleotides +119 and +164 (included) relative to the CRABPII transcription initiation site, which removes the transcription initiation site and the first 5 amino acids (MacGregor et al., 1992; see Durand et al., 1992). This region was replaced with a PGK-NEO cassette containing a pol(A) addition signal inserted in the opposite direction relative to CRABPII gene transcription. The linearized targeting vector was electroporated into D3 ES cells and 15 independently targeted clones out of 120 colonies resistant to G418 and gancyclovir were identified by Southern blot analysis using a probe located immediately 3' to the targeting construct (Fig. 1A). The structure of the targeted allele was further characterized in three of these clones (BA16, BA131 and BA146). DNAs were digested with either KpnI, HindIII, HindIII and XhoI, Sall or SphI, and hybridized with either the 3’ probe or a NEO probe (Fig. 1B, and data not shown). These three clones were injected into C57BL/6 blastocysts to create chimeras. BA131 and BA146 resulted in germline transmission upon mating with 129/Sv females to bring the mutation onto a pure 129/Sv genetic background. The same phenotype was observed irrespective of the genetic backgrounds.

CRABPII-deficient mice appear essentially normal

Mice heterozygous for the mutation were healthy and fertile and intercrossing of heterozygous mice produced a Mendelian distribution of homozygous offspring (data not shown). Both CRABPII+/− males and females derived from either ES cell line appeared indistinguishable from their wild-type or CRABPII+/+ littermates in all respects (growth, fertility and viability). Histological examination of serial sections of the entire body of two 18.5 dpc CRABPII+/− fetuses did not reveal any abnormalities when compared with wild-type littermates (data not shown). Whole-mount skeletal analysis of 18.5 dpc CRABPII+/− fetuses did not show any skeletal malformation at rates significantly higher than those found in wild-type or heterozygous littermates (Table 1, and data not shown), with the exception of a limb malformation observed in some of the homozygous mutant fetuses (see below).

To verify that CRABPII transcripts were functionally disrupted, RNase protection assays were performed using RNA from 13.5 dpc fetuses. Only the predicted mutant transcript was expressed in the CRABPII+/− embryos (Fig. 1D). There was no apparent increase in CRABPII transcript to eventually compensate for the loss of CRABPII (Fig. 1D). The RNA levels of three RAR isoforms that are RA-inducible, RARβ2 and RARγ2, as well as those of the non-inducible RARα1 and RARγ1 isoforms (Leid et al., 1992) were unchanged, suggesting that the amount of RA that reaches the nucleus was not altered, and also that the CRABPII+/− mutation did not alter RAR expression in general (data not shown). Analysis of CRABPII and II transcript distribution by in situ hybridization on sections of 10.5 dpc CRABPII+/− embryos did not reveal any change in CRABPII expression (data not shown). However, the distribution of CRABPII mutant transcripts was abnormal, since it was ubiquitous (data not shown), in contrast to the tissue-specific pattern of CRABPII expression in wild-type embryos at this stage (Ruberte et al., 1992). This altered expression of mutant transcripts is likely to result from the insertion of PGK promoter sequences into the CRABPII locus. The disruption of the CRABPII gene was also verified by western blot analysis using a specific monoclonal antibody raised against a C-terminal epitope. The CRABPII protein was readily detected in extracts from wild-type and heterozygous 13.5 dpc embryos (Fig. 1E, lanes 2 and 3), whereas no signal was detected in CRABPII homozygote extract (lane 4). When the same extracts were tested for the CRABPII protein using a specific polyclonal antibody (Gorry et al., 1994), no significant change in CRABPII levels were observed between wild-type, heterozygous and homozygous embryos (Fig. 1F, lanes 1-3). We conclude that the present CRABPII gene targeting results in a null mutation.

Mice deficient in both CRABPI and CRABPII are also essentially normal

Outbred (129/Sv/C57BL/6 mixed genetic backgrounds) CRABPII homozygotes were crossed with outbred CRABPII homozygotes (Gorry et al., 1994) to generate fertile CRABPI+/+ /CRABPII+/+ double heterozygous mice. Intercrosses of these double heterozygotes gave rise to CRABPI+/+ /CRABPII+/+ double homozygous animals at the expected Mendelian ratio (data not shown). Both double homozygous mutant males and females grew and bred normally and double homozygote intercrossing gave rise to normal size litters. However, the double homozygous mutants might display a slight viability deficit, since 9% died before 6 weeks of age, whereas only 2% of the control mice (including wild-type, CRABPI+/+ /CRABPII+/+ and CRABPI+/+ /CRABPII+/− littermates) were found dead within

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>Untreated</th>
<th>1 mg/kg, 10.5 dpc</th>
<th>1 mg/kg, 8.5 dpc</th>
<th>10 mg/kg, 8.5 dpc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleft palate</td>
<td>0/41 (17)*</td>
<td>0/41 (12)</td>
<td>0/41 (4/44)</td>
<td>0/41 (13)</td>
</tr>
<tr>
<td>8 Vertebralosternal ribs†</td>
<td>1/6 (16%)</td>
<td>0/41 (0/44)</td>
<td>0/41 (2/28)</td>
<td>0/41 (2/24)</td>
</tr>
<tr>
<td>14 Ribs (anlagen)†</td>
<td>1/6 (16%)</td>
<td>0/41 (0/44)</td>
<td>0/41 (2/28)</td>
<td>0/41 (2/24)</td>
</tr>
<tr>
<td>Extra forelimb bone</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Other abnormalities</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*The number of animals examined.
†Unilateral or bilateral.
NS, not significant.

Table 1. Skeletal abnormalities in 18.5 dpc fetuses of CRABPII+/− or CRABPII−/− intercrosses exposed to RA excess during embryogenesis.
the same period (Table 2). This is in contrast to either CRABPI or CRABPII null mutants, where there was no significant difference in viability compared with wild-type animals (Gorry et al., 1994 and Table 2). Histological examination of serial sections of the entire body from two 17.5 dpc double mutant fetuses and three 1 day post-partum (dpp) neonates did not reveal any abnormalities (data not shown). We also looked for skeletal malformations in double mutant neonates and found no significant differences compared with wild-type animals, except in the limb (see below). No alterations in the expression of the three RAR types could be detected by RNase protection in double homozygous mutants (data not shown).

**Limb malformations in CRABPI−/− mutant and CRABPI−/−/CRABPII−/− double mutant mice**

In approximately 45% of the CRABPI−/− null mutants examined between birth and 6 weeks of age, a limb outgrowth was found, which was always located on the post-axial side of digit five, either uni- or bilaterally, and most often on the forelimbs only (Fig. 2B, white arrow). This anomaly was rarely observed in CRABPII−/− heterozygotes (about 1%) or wild-type (less than 1%) littermates. In CRABPI and II homozygous double mutants, however, 83% of the analyzed animals presented a similar digit outgrowth, which was either bilateral (56%) or unilateral (27%) (Table 3). This outgrowth was also seen on all four limbs at a low frequency in double mutants (Fig. 2C,E and I, white arrows), but only very rarely (0.3%) in CRABPII−/− mutants (Fig. 3, and data not shown). The outgrowth was found to vary greatly in size; in the most severe cases, which were seen only in double mutants, the outgrowth resembled a digit rudiment (Fig. 2E, and data not shown).

Whole-mount skeletal analysis of alcian blue and alizarin red stained CRABPI−/− or CRABPI−/−/CRABPII−/− newborn or adult limbs revealed that most of the conspicuous outgrowths contained a cartilaginous and/or bony element (Fig. 2B,C,E,K,M, black arrow, and data not shown). Outgrowths from the most severely affected double mutant forelimbs contained a recognisable distal phalanx (the one bearing the claw), as well as a second and occasionally a third reduced phalangeal-like bone with the first and third putative phalanges linked together by a piece of bone on the ventral side, each phalanx being separated from its neighbour by a joint (Fig. 2C, and data not shown). Similar alterations were also observed in CRABPI−/−/CRABPII−/− hindlimbs, in which the rudimentary digit consisted of two bones not fully separated by a joint (Fig. 2E).

In addition to post-axial outgrowths, a number of double mutant hindlimbs exhibited a pre-axial outgrowth, which was never seen on forelimbs (Fig. 2G,I, arrowheads). Analysis of skeletal preparations from 1 dpp double mutant hindlimbs revealed that this pre-axial outgrowth contained a bony nodule separated from digit one (Fig. 2K,M, arrowheads). In double mutant adult hindlimbs, a small floating bone with the shape of a distal phalanx was seen next to the most distal phalanx of digit 1 (Fig. 2E, arrowhead). Comparison between 1 dpp wild-type and double mutant hindlimbs indicates that the process of chondrification and ossification occurred normally in the digits of the mutants (Fig. 2J-M).

**The sensitivity of CRABPI−/− single mutants and CRABPI−/−/CRABPII−/− double mutants to RA excess is not increased**

Exposure of mouse embryos to RA excess in utero has been shown to cause a number of teratogenic effects that are both dose- and stage-dependent (Kochhar, 1973; Alles and Sulik, 1989; Mendelsohn et al., 1994b; Kessel and Gruss, 1991; Kessel, 1992; Morriss-Ray, 1993). The CRABPI and CRABPII spatiotemporal patterns of expression during mouse development have been found to correlate with tissue sensitivity to RA excess (see Introduction for references), which suggested that CRABPs may control RA intracellular levels to ensure correct expression of RA target genes. Exposure of CRABPI−/− and CRABPII−/−/CRABPII−/− mutant embryos to low doses of RA that are normally non-teratogenic, at periods of embryonic development particularly susceptible to RA-excess treatment, might therefore cause defects comparable to those found in wild-type embryos exposed to a higher dose of RA at similar stages of development. To test whether this was the case, we studied the effect of RA administration at three different RA-sensitive developmental stages, 7.5, 8.5 and 10.5 dpc, which have been shown to result in malformations of the head, axial skeleton and limbs, respectively.

First, we tested whether CRABPII had a protective function against RA excess. Pregnant females from CRABPI−/+ or CRABPI−/+ intercrosses (129/Sv genetic backgrounds) were given a single 1 mg/kg dose of RA by oral gavage at 8.5 or 10.5 dpc, and the fetuses were examined for skeletal malformations at 18.5 dpc. The resulting skeletons were comparable in all respects to skeletons prepared from wild-type untreated embryos (Table 1). To verify that the mutants were susceptible to RA treatment, pregnant dams were given a 10 mg/kg RA dose at 8.5 dpc. The reported axial skeletal alterations (8 vertebrosternal ribs instead of 7, and 14 thoracic ribs instead of 13; Kessel and Gruss, 1991) were found in fetuses from the three different genotypes analyzed (wild-type, heterozygote and homozygote), although with slightly different frequencies (Table 1). However, we did not observe fusion of the basisphe-

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### Table 2. Viability of CRABPI−/− single mutant and of CRABPI−/−/CRABPII−/− double mutant mice at 6 weeks of age

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Alive</th>
<th>Dead (%)</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Wild-type</td>
<td>81</td>
<td>3 (3)</td>
<td>84†</td>
</tr>
<tr>
<td>CRABPI−/−</td>
<td>92</td>
<td>7 (7)</td>
<td>99†</td>
</tr>
<tr>
<td>CRABPII−/−</td>
<td>105</td>
<td>2 (2)</td>
<td>107†</td>
</tr>
<tr>
<td>CRABPI−/−/CRABPII−/−</td>
<td>81</td>
<td>8 (9)</td>
<td>89†</td>
</tr>
</tbody>
</table>

*‡, compilation from 47, 40 and 43 litters, respectively.
The difference in viability was significant only for the double mutants ($\chi^2$ test, $P<0.025$).

### Table 3. Digit abnormalities in CRABPI−/−/CRABPII−/− double mutant limbs

<table>
<thead>
<tr>
<th>Digit outgrowth</th>
<th>No. found out of total of 52 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-axial</td>
<td></td>
</tr>
<tr>
<td>Present on one forelimb</td>
<td>14 (27)</td>
</tr>
<tr>
<td>Present on both forelimbs</td>
<td>27 (52)</td>
</tr>
<tr>
<td>Present on all four limbs</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Absent</td>
<td>9 (17)</td>
</tr>
<tr>
<td>Pre-axial hindlimb</td>
<td>7 (13)</td>
</tr>
</tbody>
</table>

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The difference in viability was significant only for the double mutants ($\chi^2$,†,‡, compilation from 47, 40 and 43 litters, respectively.

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The sensitivity of CRABPI−/− single mutants and CRABPI−/−/CRABPII−/− double mutants to RA excess is not increased
noid and basioccipital bone reported in 50% of the fetuses analyzed by Kessel and Gruss (1991). This discrepancy is most likely due to the use of different mouse strains in the two experiments. No limb malformations were found in 10.5 dpc CRABPII null mutants at the 1 mg/kg dose, indicating that these mutants do not seem to be more sensitive to RA excess than wild-type embryos, for which 10 mgRA/kg has been shown to induce limb malformations (Satre and Kochhar, 1989; Scott et al., 1994).

Similarly, CRABPII−−/CRABPI−− double mutant embryos (obtained from CRABPIII−−/CRABPI−− or CRABPI−−/CRABPI−− intercrosses) were not more sensitive to RA excess than wild-type embryos. Pregnant dams (from L129/Sv/C57BL/6 mixed genetic backgrounds) were given a 1 mg/kg dosage of RA at 7.5 and 8.5 dpc. None of the RA-treated double mutant skeletons showed deformations of the skull bones or axial alterations, and in these respects they were found to be indistinguishable from the control littermate skeletons (data not shown, see Kessel and Gruss, 1991). Moreover, when a 10 mg/kg dosage of RA was given on these days of gestation or at 10.5 dpc, the teratogenic effects found in the double mutant fetuses were comparable to those obtained in wild-type fetuses (data not shown). Taken altogether, these results clearly show that CRABPIII−−, and CRABPI−−/CRABPIII−− mutant embryos are not more sensitive to RA excess than their wild-type littermates.

Lack of cytoplasmic RA-binding activity in the double mutant embryos

Given the postulated functions of CRABPs (see Introduction), the normal embryonic development of the CRABP double mutants was surprising, and suggested the possible existence of an additional CRABP not yet discovered. Previous attempts to identify a third CRABP have failed (Giguère et al., 1990; and unpublished results from our laboratory). To investigate further the possible existence of a third cytoplasmic RA-binding activity, cytosolic extracts from 13.5 dpc wild-type, CRABPII−− and CRABPI−−/CRABPIII−− embryos were incubated with [3H]RA and protein-[3H]RA complexes were analysed using a PAGE/autoradioblotting binding assay (Siegenthaler, 1990). Fig. 3 shows that, as expected, 13.5 dpc (lanes 3 and 4) and 10.5 dpc (lanes 7 and 8) wild-type cytosolic extracts contain CRABPI and CRABPIII proteins, which bind labelled RA, as determined by immunodetection using specific antibodies (Fig. 3B), and autoradiography (Fig. 3A). The radioactive signals were lost following competition with cold RA. As expected, no radioactive signal was observed for CRABPII in the CRABPII−− protein extract, while binding to CRABPI was readily visible (lanes 5 and 6). Furthermore, there was no significant radioactive signal detectable in the double mutant sample in which the two known RA-binding proteins were absent (lanes 9 and 10). These observations, which argue against the presence of a putative third CRABP at levels similar to those of CRABPI and II, indicate that development proceeds normally in embryos that lack detectable cytoplasmic RA-binding activity.

DISCUSSION

The evolutionary conservation of CRABPI and II and the wide-
spread, partially non-overlapping patterns of distribution of their transcripts in mouse embryos and adult tissues, as well as the frequent specific co-expression of the RA-inducible CRABPII transcripts with those of RARβ and CRABPI, which are also RA-inducible, suggests that each CRABP plays essential and specific functions in vertebrates (see Introduction for references and Eskild and Hansson, 1994). We have recently reported (Gorry et al., 1994) that mutant mice lacking CRABPI are indistinguishable from wild-type animals. We show here that CRABPII null mutant mice also appear normal, with the exception of an additional digit rudiment on the post-axial side of fore- and hindlimbs. These minor malformations which do not exhibit full penetrance are similar to those reported to occur spontaneously at a low frequency in an outbred mouse strain (Cusic and Dagg, 1985). Interestingly, in the latter case the frequency of the abnormality could be increased by RA treatment and was at a maximum (up to approx. 40% of the animals) when RA was administered at 10 dpc. By contrast, RA treatment did not increase the frequency of digit outgrowth in CRABPII−/− mutants, indicating either that the origins of the digit outgrowths are unrelated in these two cases, or alternatively that the CRABPII deficiency in CRABPII null mutants generates a similar situation of RA excess as RA administration to the above outbred mouse strain used by Cusic and Dagg. Extra post-axial digit rudiments were also found in Hoxd-13 null mutant mice (Dollé et al., 1993). In some instances these corresponded to genuine sixth digits fused to the base of neighbouring digits, a feature which was never observed in CRABPII−− or CRABPI and II double mutants in which the digit rudiment bones were always floating. Furthermore, using in situ hybridization, no evident alteration in Hoxd-13 expression could be found in either the limb of 12.5 dpc CRABPII−− fetuses or from 11.5, 12.5 and 13.5 dpc CRABPII−−/CRABPII−− fetuses (data not shown). That CRABPI and II may be partially redundant in preventing the formation of this rudimentary extra-digit is indicated by the increased frequency of the abnormality in the double mutants, when compared with CRABPII−− single mutants. Moreover, since the penetrance of the malformation remains incomplete even in the double mutants, the generation of the digit outgrowths may reflect polygenic defects. The high frequency of unilateral expressivity of the extra forelimb outgrowths indicates that the incomplete penetrance of the limb phenotype cannot be due to variations in the genetic background only. The same considerations apply to the pre-axial outgrowth that was observed in the hindlimbs of some double mutants only. Interestingly, similar forelimb rudimentary digit outgrowths were also observed in Engrailed-1 mutant mice (Wurst et al., 1994).

That CRABPII−−, as well as CRABPII−− single mutant mice were essentially normal could have reflected an extensive functional redundancy between these proteins, similar to that found for the RARs (Lohnes et al., 1994; Mendelsohn et al., 1994a, and references therein). Our present study demonstrates that this is not the case, since the CRABPI and II double mutants were essentially normal (except for the minor limb defects discussed above) in their development, fertility, life span and general behaviour. It is particularly striking that no abnormality could be detected in the many tissues and organs that specifically express either CRABPI or CRABPII, or both CRABPs, during embryogenesis (Ruberte et al., 1992; Lyn and Giguère, 1994). Moreover, CRABP double mutant embryos do not seem to be more sensitive to administration of RA than their wild-type littermates. Importantly, no additional RA binding activity could be detected in the CRABP double mutants, thus excluding a possible functional redundancy with a third, yet unidentified, CRABP.

In conclusion, our present results, which rule out CRABPs as essential components of the RA signaling pathway, suggest that, irrespective of their previously proposed putative functions (see Introduction), CRABPs are dispensable for RA intracellular homeostasis and/or for protection against RA excess. However, the evolutionary conservation of both CRABPI and II in vertebrates indicates that they must perform some specific functions that we have not been able to detect, either because they result in a slight vital advantage that cannot be revealed within a few generations, or because they cannot be revealed in the protected environment of animal facilities (see Chambon, 1994). CRABPs could in fact be important under natural conditions when the animals may have to face malnutrition and hence hypovitaminosis A. We propose that the actual function of CRABPs could be to maintain physiological levels of intracellular RA under conditions of limited supply of vitamin A, and are currently testing this idea using CRABP double mutant mice deprived of vitamin A.

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