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The cellular retinoic acid binding protein I is dispensable

(retinoid signaling pathway/gene targeting/cellular retinoic acid binding protein I mutant/vitamin A)

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ABSTRACT **The cellular retinoic acid binding proteins I and II (CRABPI and CRABPII) bind retinoic acid with high affinity, exhibit distinct patterns of expression during embryonic development, and are thought to play important roles in the RA signaling pathway. We have generated a targeted mutation of the CRABPI gene using the "hit-and-run" strategy and shown that it prevents the production of a functional CRABPI protein. Homozygous mutant mice were normal, indicating that CRABPI does not play a crucial role in the RA signaling pathway.**

Vitamin A is indispensable for vertebrate development and homeostasis. Retinoic acid (RA) administration prevents the appearance of most of the defects induced in vitamin A-deficient animals (for reviews and references, see refs. 1–3). RA excess is teratogenic, which suggests that RA could also play a major role during development (for reviews, see refs. 1 and 3–7). The RA signal is transduced by two families of nuclear receptors, the RARs and RXRs, which act as ligand-inducible transcriptional regulators (3, 8–11). Abnormalities exhibited by fetuses of vitamin A-deprived dams are recapitulated in RAR and/or RXR mouse mutants (ref. 3; unpublished results), thus demonstrating the role of RARs and RXRs in the transduction of the RA signal.

In addition, two cytoplasmic RA binding proteins, the cellular retinoic acid binding proteins I and II (CRABPI and CRABPII) are found in all vertebrates that require vitamin A and appear to be highly conserved (4, 12–14). Both CRABPI and CRABPII transcripts and proteins are expressed in mouse embryos at all stages of development in distinct, often nonoverlapping patterns (for references, see refs. 7 and 15–17). This has suggested that CRABPs have essential roles in the retinoid signaling pathway. It has been proposed that CRABPs could spatiotemporally control the level of "free" intracellular RA available for binding to the nuclear receptors: CRABPs may passively sequester RA, functioning as buffers to maintain tolerable concentrations of intracellular RA, and/or act as modulators of RA catabolism (refs. 18 and 19; for reviews, see refs. 4, 7, 13, and 14). CRABPs may also be involved in the transfer of RA from the cytoplasm to nuclear receptors (20). Thus CRABPs may play essential roles in the retinoid signaling pathway. To determine these roles, we have initiated studies aimed at generating mutant mice devoid of either CRABPI, CRABPII, or both. We report here that CRABPI null mutant mice appear essentially normal, indicating that CRABPI does not play a crucial role during development and homeostasis.

MATERIALS AND METHODS

Gene Targeting of CRABPI. To construct the hit-and-run (H&R) targeting vector (see Fig. 1*A*), a 12-kb *Hin*dIII genomic fragment containing the CRABPI gene (21) was subcloned. A 5-nt insertion between the second and third positions of the alanine codon at amino acid position 5 of the CRABPI exon 1 coding region was performed by site-directed mutagenesis to create a *Not* I restriction site. The thymidine kinase gene (TK) driven by the GTI-II enhancer (22) was added contiguous to the genomic fragment containing the *Not* I mutation, and a GTI-GTII enhancer-driven neomycin-resistance gene (22) was inserted adjacent to the TK gene. The resulting CRABPI H&R targeting construct (p775) was linearized at the *Spe* I site and electroporated into D3 embryonic stem (ES) cells (22, 23). One G418-resistant ES clone was shown to contain a homologous recombination event ("Hit" reaction) and was further selected with ganciclovir. Surviving ES clones having undergone an intrachromosomal homologous recombination event (H&R reaction, ref. 24) were injected into C57BL/6 blastocysts (22). Germline transmission was established by mating with C57BL/6 females.

Western Blot Analysis and Immunohistochemistry. Embryos from a CRABPI(H&R) heterozygote intercrossing were removed at 13.5 days postcoitum (dpc). Cytosolic extracts from embryos, whole cell extracts from transfected COS-1 cells, Western blotting, and immunodetection were as described (25). Rabbit polyclonal antibodies specific for CRABPI and CRABPII were generated (26) using ovalbumin-coupled synthetic peptides as antigens [SPB63 (amino acids 95–108) and SPB64 (amino acids 96–109) for mouse CRABPI and CRABPII, respectively]. Histological analysis and *in situ* hybridization were as described (15, 27). Immunolocalization of CRABPI was performed using rabbit antibodies raised against two synthetic peptides corresponding to residues 69–84 and 95–107 of bovine CRABPI (28).

CRABPI Expression Vectors. A pET-15b-based CRABPI expression vector (pGB26) was constructed as follows: PCR was used to construct unique *Nde* I and *Bam*HI sites at the 5′ and 3′ ends of mouse CRABPI cDNA, respectively. The amplified fragment was ligated into the unique *Nde* I and *Bam*HI sites of pET-15b (Novagen). A *Bgl* II–*Bam*HI fragment from the pET-15b CRABPI was then subcloned between the *Bam*HI–*Bgl* II restriction sites of the pSG5-derived vector pTL-1 (29) to generate pGB26. Site-directed mutagenesis was performed on pGB26 to delete CRABPI amino acids 1–9 [CRABPI(Δ1–9)], yielding pGB27. pGB26 and pGB27 were used to transform the *Escherichia coli* K-12 strain BL21(DE3) (Novagen). Bacterial culture, induction, and preparation of protein extracts and purification of wild-

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Abbreviations: RA, retinoic acid; CRABPI and CRABPII, cellular retinoic acid binding proteins I and II; WT, wild type; RAR, retinoid acid receptor; RXR, retinoid X receptor; ES, embryonic stem; H&R, hit-and-run; dpc, day(s) postcoitum.

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type (WT) CRABPI using a Hitrap chelating column (Pharmacia) will be described elsewhere. Each fraction was analyzed by 15% SDS/PAGE and silver staining. Fractions containing CRABPI were pooled and concentrated (Amicon Centricon 10).

RA Binding Assay. Binding of RA to recombinant CRABPI was measured by overnight incubation at 4°C with 600 nM *all-trans*-[^{3}H]RA in the presence or absence of a 200-fold excess of unlabeled ligand. PAGE/autoradioblotting was employed to separate ligand bound to CRABPI from free ligand (30). After autoradiography analysis, the nitrocellulose filter was cut in square (0.5 cm) pieces along the axis of each sample lane, and radioactivity was counted.

RESULTS

Mice Homozygous for a Disruption of the CRABPI Gene Are Apparently Normal. A 5-nt insertion mutation was introduced into the first exon of the CRABPI gene (ref. 21; see Fig. 1*A*) to generate a unique *Not* I restriction site located at the fifth residue (Fig. 1 *A* and *C*). This frameshift mutation results in a truncated protein of 26 amino acids (Fig. 1*C*), of which only the first 5 are identical to those of the WT 136-amino acid CRABPI (31). The mutant CRABPI H&R gene targeting construct (Fig. 1*A*) was electroporated into D3 ES cells (22, 23). Homologous recombination occurred in 2 of 31 G418-resistant clones. One of these ES clones (CN7, "HIT" allele, Fig. 1 *A* and *B*) was subsequently selected in the presence of ganciclovir. All 47 ES clones surviving ganciclovir selection had resolved the homologous duplicates (H&R alleles, Fig. 1 *A* and *B*). Twenty-six of these ES clones had retained the *Not* I frameshift mutation (CN7.21, Fig. 1 *A* and *B*, probes 1 and 2; data not shown). Two of these ES clones (CN7.21, Fig. 1*A*; CN7.32, data not shown) gave germ-line transmission. All results presented here correspond to offspring of six CN7.21 ES cell-derived chimeric males that were bred with C57BL/6 females. Both male and female heterozygous mice for the H&R mutation [henceforth referred to as CRABPI(H&R)] were healthy and fertile and transmitted the CRABPI(H&R) mutation to their offspring at the expected frequency for a recessive allele (Fig. 2*A*; data not shown). CRABPI(H&R) homozygous animals were indistinguishable from their heterozygous or WT littermates in all respects (growth, fertility, viability) even after several generations of homozygote intercrossing. Histological analysis of serial sections of the entire body of four 14.5- and five 18.5-dpc CRABPI(H&R) homozygous fetuses did not reveal any alteration (data not shown).

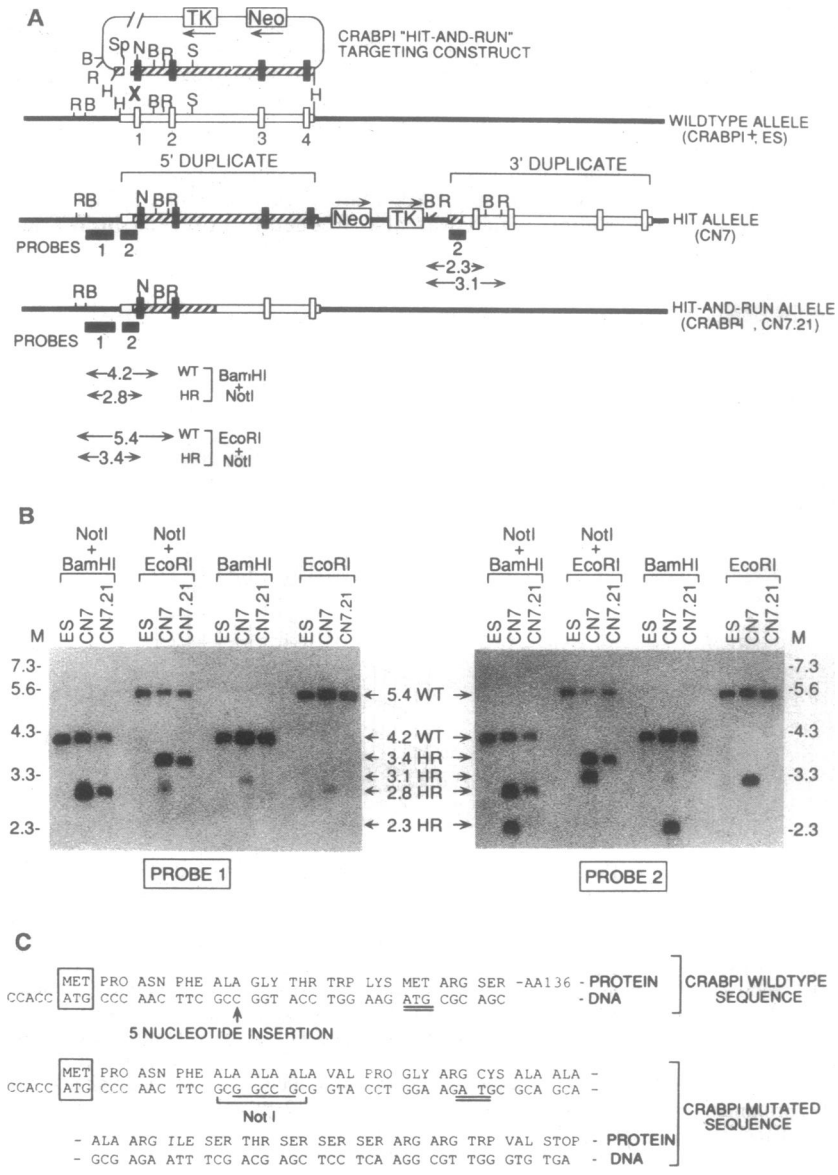

FIG. 1. Generation of the CRABPI mutant allele CRABPI(H&R) in ES cells using the H&R strategy (24). (*A*) The targeting construct is shown above the WT CRABPI allele (CRABP$^+$, ES). The four exons of the CRABPI gene are numbered 1–4. Following a homologous recombination event both a 5′ and 3′ duplicate are formed (HIT ALLELE, CN7). The use of hatching and solids in the targeting construct and mutant alleles indicates contribution of the targeting construct sequences to the mutant allele. TK and Neo correspond to the herpes simplex virus thymidine kinase and neomycin-resistant genes, respectively. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; N, *Not* I; S, *Sal* I; Sp, *Spe* I. Probes 1 and 2 correspond to 1.5-kb *Hin*dIII–*Bam*HI and 1.0-kb *Hin*dIII–*Not* I fragments, respectively. The sizes of the predicted genomic fragments are indicated below. WT, WT allele. HR, homologous recombined or targeted allele. (*B*) The presence of the 5-nt mutation (*Not* I site) in the 5′ duplicate was verified by digestion with *Not* I and *Bam*HI or *Not* I and *Eco*RI using probe 1 (CN7, left panel) used for the targeting construct. The presence of the 3′ duplicate is evidenced with probe 2 (CN7, right panel). The presence of a single resolved "duplicate" is shown by probing probe 2 (CN7.21, right panel). The retention of the *Not* I mutation in the H&R allele is also shown (CN7.21, left panel). (*C*) Nucleotide and predicted protein sequence of the WT and mutated CRABPI (see text). The WT methionine is boxed and the double bar indicates internal methionine at position 10.

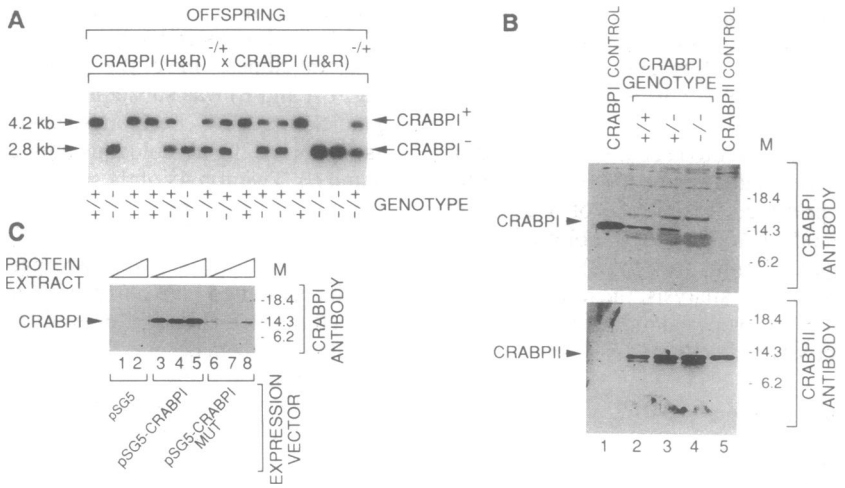

FIG. 2. Analysis of CRABPs WT and CRABPI(H&R) offspring. (*A*) Southern analysis of 18.5-dpc offspring from a cross between CRABPI(H&R) heterozygotes. CRABPI$^+$, WT allele; CRABPI$^-$, mutated allele. Offspring genotypes are indicated: +/+, WT; +/−, heterozygote; −/−, homozygote. (*B*) Western blot analysis of CRABPI and CRABPII in WT and CRABPI(H&R) mutant embryos. Lanes 1 and 5, whole cell extracts prepared from COS-1 cells transfected with CRABPI and CRABPII pSG5 expression vectors, respectively; lane 2, WT embryo; lanes 3 and 4, CRABPI(H&R) heterozygous and homozygous embryos, respectively; ≈1 μg of whole cell protein extract was used per lane for COS-1 transfected controls (lanes 1 and 5), whereas ≈100 μg of embryo cytoplasmic protein was used (lanes 2–4). The top panel was probed with a CRABPI polyclonal antibody (SPB63) and the bottom panel was probed with a CRABPII polyclonal antibody (SPB64). Note the absence of the 15-kDa CRABPI signal in the mutant embryo (lane 4). The hazy band below the CRABPI-specific band is a nonspecific signal and is independent of the CRABPI genotype. M, molecular mass markers in kDa. (*C*) Western blot analysis of WT and mutated CRABPI proteins. Shown are COS-1 cells transfected with control pSG5 vector (lanes 1 and 2), with pSG5-CRABPI (WT CRABPI, lanes 3–5), or with pSG5-CRABPImut (frameshift-mutated CRABPI, lanes 6–8). Whole cell protein extracts were Western blotted and probed with the CRABPI SPB63. Amounts of proteins were as follows: lane 1, 24 μg; lane 2, 48 μg; lane 3, 1.5 μg; lane 4, 3 μg; lane 5, 6 μg; lane 6, 8 μg; lane 7, 16 μg; lane 8, 32 μg.

The CRABPI(H&R) mutants were further examined for skeletal malformations. No malformations were found at rates significantly higher than those found in WT or heterozygous littermates, with the possible exception of a transformation of the sixth lumbar vertebra into a first sacral vertebra, which was also frequently observed in WT and heterozygous littermates (ref. 22; data not shown). We also

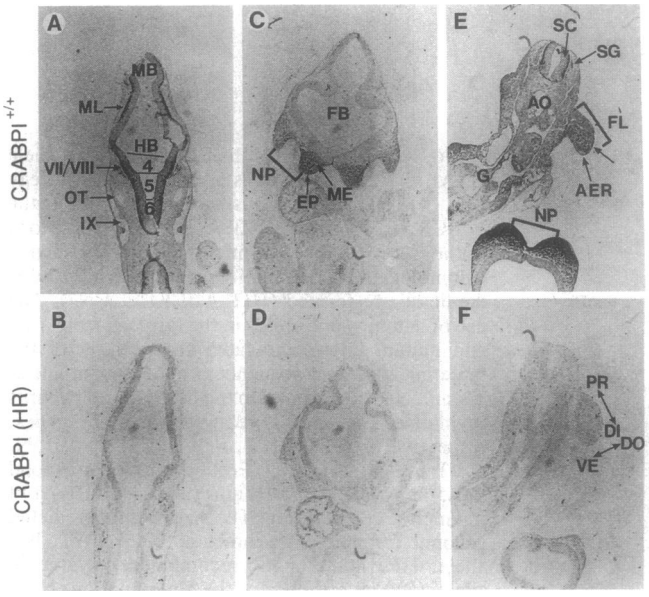

FIG. 3. Immunohistochemical localization of CRABPI in WT and mutant embryos. Shown here are coronal sections through the hindbrain epithelium (*A* and *B*), the fronto-nasal processes (*C* and *D*), and the forelimb level (*E* and *F*) of 10.5-dpc WT (CRABPI+/+) and CRABPI(H&R) homozygous (−/−) embryos. In the WT embryo, CRABPI is detected at high levels in the mantle layer (ML) of the midbrain (MB) and hindbrain (HB), the nasal processes (NP), and the mantle layer of the spinal cord (SC) and more faintly in the distal region of the forelimb bud (FL). AER, apical ectodermal ridge; AO, aorta; DI, distal; DO, dorsal; G, gut; OT, otic vesicle; PR, proximal; SG, spinal ganglia; VE, ventral; 4, 5, and 6, rhombomeres; VII/VIII and IX, cranial ganglia. (×12.)

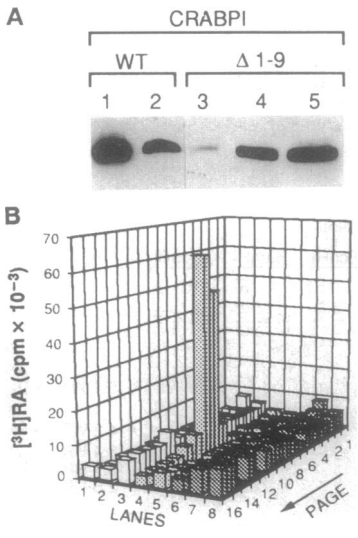

FIG. 4. PAGE/autoradioblotting [^{3}H]RA binding assay with WT CRABPI and mutant CRABPI(Δ1–9) proteins produced in *E. coli*. (*A*) Western blot analysis of mutant protein crude extract (lanes 3–5), and comparison with WT partially purified CRABPI protein (lanes 1 and 2): lane 1, 1 μg; lane 2, 0.5 μg; lane 3, 1.65 μg; lane 4, 8.25 μg; lane 5, 16.5 μg. (*B*) Binding of *all-trans*-[^{3}H]RA. Lane 1, binding buffer; lanes 2 and 3, 10 μg and 100 μg of control *E. coli* BL21 total protein extract, respectively; lane 4, 1 μg of WT purified CRABPI protein diluted in 10 μg of *E. coli* BL21 total protein extract; lane 5, same as lane 4 but incubated with a 200-fold excess of unlabeled RA; lane 6, 18 μg of mutant CRABPI(Δ1–9) protein crude extract; lane 7, 180 μg of mutant CRABPI protein crude extract; lane 8, same as lane 7 with a 200-fold excess of unlabeled RA.

examined RAR mRNA levels in 13.5-dpc, CRABPI(H&R) mutant embryos using probes specific for RARα, RARβ, and RARγ isoforms in RNase protection assays (32). No significant alterations could be detected (data not shown).

The CRABPI(H&R) Disruption Is a Null Mutation. To verify that the engineered frameshift mutation had actually blocked the production of a functional CRABPI, Western blot analyses were performed at 13.5 dpc, when CRABPI is at peak abundance. Although CRABPI could be readily detected in WT and CRABPI(H&R) heterozygotes (CRABPI, Fig. 2*B*, lanes 2 and 3, top panel), no CRABPI was detectable under similar conditions in CRABPI(H&R) homozygotes (CRABPI, Fig. 2*B*, lane 4, top panel; note that the CRABPI polyclonal antibody used here was raised against a C-terminal peptide). In contrast, there was no significant change in CRABPII levels between WT and CRABPI(H&R) heterozygous and homozygous embryos (CRABPII, Fig. 2*B*, compare lanes 2–4, bottom panel). Immunohistochemical analysis was also performed on sections of 10.5-dpc WT and CRABPI(H&R) homozygous embryos using antibodies raised against C-terminal residues of CRABPI (28). No CRABPI signal could be detected in homozygous mutants (Fig. 3 *B*, *D*, and *F*), whereas it was detectable under similar conditions in various tissues of WT littermate embryos (Fig. 3 *A*, *C*, and *E*).

Since the frameshift mutation is positioned at amino acid 5 of the WT CRABPI (see Fig. 1*C*), CRABPI(H&R) mutants might produce undetectable, but phenotypically significant, quantities of an N-terminally truncated CRABPI initiated at methionine position 10 (ref. 33; see Fig. 1*C*). Therefore, we engineered an identical frameshift mutation into the CRABPI cDNA of a CRABPI expression vector, pSG5-CRABPI, to create pSG5-CRABPIMUT (data not shown). Transfection of COS-1 cells with pSG5-CRABPI produced CRABPI at levels (per μg of protein) $\approx$50–100 times greater than observed in WT embryos (Fig. 2*B*, lanes 1–5). pSG5-CRABPIMUT transfected COS-1 cells produced a protein at $\approx$1–2% of the level of WT CRABPI in pSG5-CRABPI transfected COS-1 cells (Fig. 2*C*, compare lanes 5 and 6; data not shown). We conclude that embryos homozygous for the CRABPI (H&R) mutation may produce low amounts ($\approx$1–2%) of an N-terminally truncated CRABPI.

We next investigated whether the truncated CRABPI could efficiently bind RA. pET-15b-based expression vectors for WT CRABPI and CRABPI deleted for the first 9 amino acid residues [CRABPI(Δ1–9)] were constructed and the corresponding proteins were produced in *E. coli*. Western blot analysis showed that 16.5 μg of protein of a crude preparation of CRABPI(Δ1–9) corresponded to $\approx$0.5 μg of partially purified recombinant CRABPI (Fig. 4*A*). Binding of [^{3}H]RA to these two proteins was determined using a PAGE/autoradioblotting assay (30). [^{3}H]RA binding by 1 μg of WT recombinant CRABPI was readily observed (Fig. 4*B*, lane 4), whereas no significant binding above background was detected with equivalent or 10-fold higher amounts (as judged from Western blotting) of recombinant CRABPI(Δ1–9). We conclude that the N-terminally truncated CRABPI(Δ1–9) (Fig. 4*B*, lanes 6 and 7) is at least 50-fold less efficient than WT CRABPI at binding [^{3}H]RA. Taken together, these results indicate that CRABPI(H&R) mutant embryos are

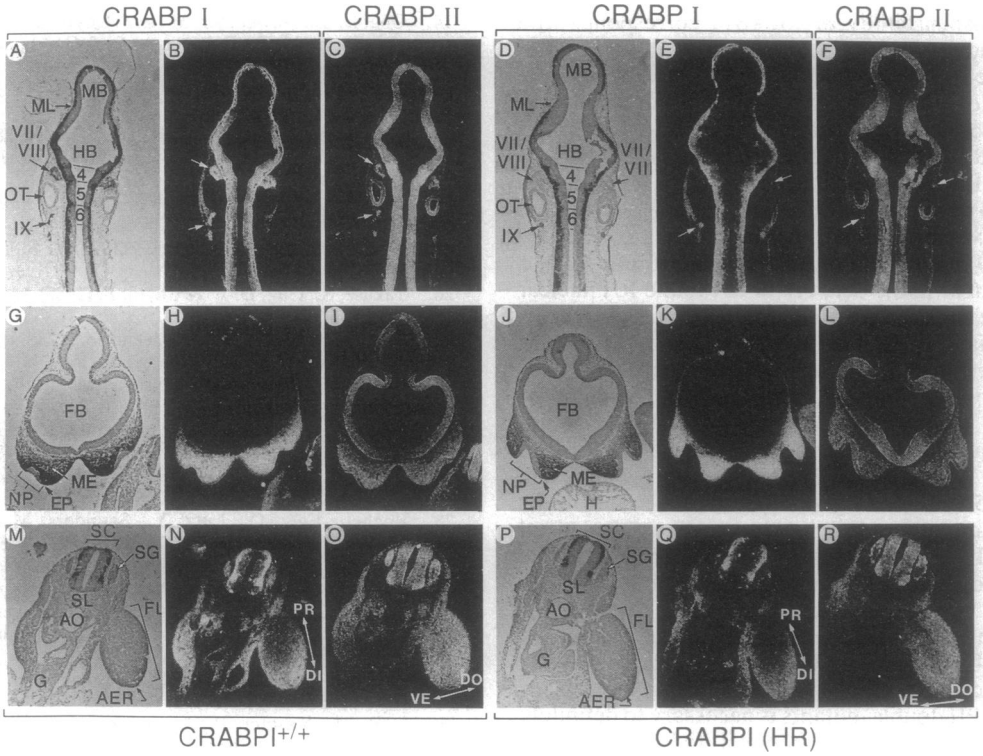

FIG. 5. *In situ* analysis of CRABPI and CRABPII expression. Shown here are coronal sections through the hindbrain epithelium (*A–F*), the fronto-nasal processes (*G–L*), and the forelimb level (*M–R*) of 10.5-dpc WT (CRABPI+/+) and CRABPI(H&R) homozygous (−/−) embryos. (*A*, *D*, *G*, *J*, *M*, and *P*) Bright-field histological views of sections hybridized to the CRABPI probe (regions accumulating highest signal grain appear in black); dark-field views showing the CRABPI and CRABPII hybridization signals (which appear in white) on neighboring sections are beside. There is no significant difference in the distribution of CRABPI signal in mutant versus control embryo—e.g., in the hindbrain and midbrain epithelium (*B* and *E*), the nasal mesenchyme (*H* and *K*), or the forelimb distal mesenchyme (*N* and *Q*). The CRABPII transcript distribution is not altered in the mutant embryo, even in those regions where CRABPI transcripts are maximally expressed (black areas in bright-field views). AER, apical ectodermal ridge; AO, aorta; DI, distal; DO, dorsal; EP, epithelium; FB, forebrain; FL, forelimb; HB, hindbrain; G, gut; ML, mantle layer; MB, midbrain; ME, mesenchyme; NP, nasal process; OT, otic vesicle; PR, proximal; SC, spinal cord; SG, spinal ganglia; SL, sclerotome; VE, ventral; 4, 5, and 6, rhombomeres; VII/VIII and IX, cranial ganglia. (×15.)

unlikely to contain more than 2–4 × 10^{-4} the amount of functional CRABPI present in WT embryos and therefore that the CRABPI(H&R) mutation can be considered as a null mutation.

In situ hybridization was also used to investigate the relative levels of CRABPII transcripts in 10.5-dpc WT and CRABPI(H&R) homozygous embryos (15). There was no alteration in the specific expression pattern of CRABPII in the mutants (Fig. 5, compare *F, L,* and *R* with *C, I,* and *O*; and data not shown). In particular, there was no increase in CRABPII transcripts in the regions where CRABPI is expressed at a high level relative to CRABPII. Thus, the absence of defects in CRABPI(H&R) mutants cannot be attributed to a compensatory up-regulation of CRABPII expression. Moreover, the expression of CRABPI transcripts was not altered in CRABPI(H&R) homozygous embryos (Fig. 5, compare *E, K,* and *Q* with *B, H,* and *N*; and data not shown), indicating that there is no feedback control of the CRABPI promoter by the CRABPI protein.

DISCUSSION

Our present data show that CRABPI null mutants cannot be phenotypically distinguished from WT mice at any stage of development and adult life. In particular, no developmental defects could be found in structures derived from tissues that efficiently express the CRABPI gene during embryogenesis (refs. 7, 15, and 17 and references therein), including the cranio-facial region, the central nervous system, the neural crest derivatives, and the limbs, whose morphogenesis has been postulated to be dependent on a graded expression of CRABPI along the limb antero-posterior axis (7). It is unlikely that the dispensability of CRABPI corresponds to a functional redundancy with CRABPII, since the pattern of expression of the two genes is often nonoverlapping (15, 16), and our present data establish that there was no compensatory expression of CRABPII transcripts in regions that normally express CRABPI at high levels. Moreover, the CRABPII content of CRABPI null mutant embryos was similar to that of WT embryos.

Our results exclude the idea that CRABPI could play important role(s) in the homeostatic control of intracellular RA concentration or in the delivery of RA to the RA nuclear receptors, as previously proposed. However, there remains the possibility that CRABPI could be required under conditions of RA excess to prevent teratogenic effects. To investigate this possibility, CRABPI(H&R) null mutant embryos were exposed to excess RA *in utero*. When RA was administrated to pregnant dams at 8.5 dpc at a low teratogenic dose (10 mg/kg) (34), WT and CRABPI(H&R) heterozygous and homozygous 18.5-dpc mutant fetuses from to the same litters exhibited the same type and incidence of axial skeletal malformations. In addition, there was no increase in the frequency of resorptions of homozygous null embryos (data not shown).

In conclusion, our present study demonstrates that CRABPI cannot be critically involved in any of its previously postulated functions. However, the construction of CRABPI/CRABPII double mutants is necessary to definitely exclude the possibility of a functional redundancy with CRABPII. The high degree of conservation of CRABPI in vertebrates suggests, nevertheless, that it performs an important function(s) that either cannot be easily detected in the protected environment of animal facilities or confers a low viability advantage (3).

P.G., T.L., and A.D. should be considered as equal first authors.

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