RXRα mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis

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We have established a targeted loss-of-function mutation in the RXRα gene in the mouse germ line that results in embryonic lethality between E13.5 and E16.5 when bred to homozygosity. The major defect responsible for lethality is hypoplastic development of the ventricular chambers of the heart, which is manifest as a grossly thinned ventricular wall with concurrent defects in ventricular septation. This phenotype is identical to a subset of the effects of embryonic vitamin A deficiency and, therefore, establishes RXRα as a genetic component of the vitamin A signaling pathway in cardiac morphogenesis. The cardiac outflow tracts and associated vessels, which are populated by derivatives of the neural crest and which are also sensitive to vitamin A deficiency, are normal in homozygous embryos, indicating the genetic independence of ventricular chamber development. Hepatic differentiation was dramatically but transiently retarded yet is histologically and morphologically normal. These results ascribe an essential function for the RXRα gene in embryonic development and provide the first evidence of a requirement for RXR in one of its predicted hormone response pathways.

[*Key Words*: Gene targeting; cardiogenesis; retinoic acid]

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Retinoic acid (RA) and related vitamin A derivatives (retinoids) comprise a collection of molecules that serve as signals to trigger and modulate complex morphogenic events during vertebrate development and to maintain homeostasis in the adult. Retinoids display profound effects on cell differentiation and proliferation and have been used extensively to influence differentiation in organ and cell culture systems (for review, see Brockes 1989; Tabin 1991). Retinoids can block the effects of tumor promoters in cell culture and have been used in the chemoprevention, as well as the primary treatment, of certain solid tumors and leukemias in man (Warrell et al. 1991; Hong and Itri 1994). Exposure of vertebrate embryos to RA leads to a variety of teratogenic effects depending on the time and dose of the exposure (Morriss-Kay 1992; Linney and LaMantia 1994). The most prominent target tissues include the heart, the axial skeleton, cranial and cardiac neural crest-derived tissues, and the limbs. Paradoxically, and perhaps more importantly, vitamin A deficiency leads to an overlapping spectrum of defects (Wilson and Warkany 1949; Wilson et al. 1953), indicating a requirement for retinoids during normal development as well as a putative common target whose proper action is essential for the execution of developmental programs. A central question arising from these observations is how a simple molecule such as RA can lead to such diverse biological effects.

A great deal of this complexity can be explained by the observation that retinoid receptors are members of the nuclear receptor superfamily of ligand-dependent transcription factors (Evans 1988; Green and Chambon 1988). The receptors comprise two distinct subfamilies composed of three retinoic acid receptors (RARs) and three evolutionarily distinct retinoid X receptors (RXRs) (Mangelsdorf et al. 1992 and references therein). The RARs and RXRs share overlapping ligand specificity—both receptors bind 9-*cis* RA with high affinity, whereas only the RARs bind all-*trans* RA (Heyman et al. 1992; Levin et al. 1992). It has been shown in vitro that RXRs are able to bind DNA as homodimers, whereas RARs, as well as receptors for thyroid hormones (TRs), vitamin D (VDR), and peroxisome proliferators (PPARs), form heterodimers with RXRs that bind DNA in a highly cooperative fashion (Yu et al. 1991; Kliewer et al. 1992a,b; Leid et al. 1992; Marks et al. 1992; Zhang et al. 1992). The RXRs, therefore, are proposed to play a central role in mediating multiple hormonal signaling pathways.

Each RXR (and RAR) subtype is differentially ex-

pressed in a characteristic spectrum of tissues during normal embryonic development and beyond (Mangelsdorf et al. 1992). The RXRα gene, for example, is abundant in the intestine, heart, muscle, liver, kidney, and skin of the adult, whereas the RXRβ gene is expressed at a low level in nearly all tissues. RXRγ shows the most restricted pattern of expression in both the embryo and adult with highest levels in mesoderm and its derivatives and in parts of the nervous system.

To allow a more complete understanding of the diverse role of retinoid receptors in development, it will be necessary to link the known defects associated with retinoid excess or deficiency to individual receptor gene products. Toward this end, we and others have undertaken a functional analysis of individual receptor genes in vivo, through the introduction of specific mutations into the germ line of mice. Mutations of the RARα and RARγ genes have recently been reported (Li et al. 1993; Lohnes et al. 1993; Lufkin et al. 1993). Surprisingly, these individual mutations are not embryonic lethal, and actually display fairly subtle phenotypes. In contrast, we find that mutation of the RXRα gene results in embryonic lethality due to dysmorphic hypoplastic development of the ventricular chambers of the embryonic heart. The identical phenotype was recognized over 40 years ago as associated with embryonic vitamin A deficiency (Wilson and Warkany 1949), and therefore establishes the molecular basis for this physiological defect as a requirement for RXRα. The mutation also results in a strikingly delayed development of the embryonic liver, although this is unlikely to be causal to the embryonic lethality. These results provide the first genetic evidence for a role of RXRs in retinoid signaling and establish an essential role for this receptor in embryogenesis.

Results

Targeted disruption of the RXRα gene

The organization of the 5′ end of the RXRα gene is shown in Figure 1A. The third exon (B/C1 exon) of the gene encodes the first part of the DNA-binding domain, a domain that is required for receptor function. A targeting construct was prepared in which a PGK–*neo* cassette was introduced in an antisense orientation between an *Eco*RV site in the third exon and an *Xba*I site in the intron immediately downstream, so that part of the coding sequence, the splice donor, and the 5′ part of the third intron are deleted. Homologous sequence of 15 kb at the 5′ side and 1 kb 3′ drive homologous recombination of the targeting construct; a herpes simplex virus–thymidine kinase (HSV–TK) cassette was included to allow for negative selection against random integration events.

The targeting construct was introduced by electroporation into the J1 line of embryonic stem (ES) cells, and colonies isolated after selection in G418 and FIAU. Of a total of 77 colonies screened, 3 were identified as homologous recombinants, and one of these colonized the germ line when introduced into chimeric animals. Cell lines and animals were genotyped with two different probes on Southern blots, and by PCR analysis (Fig. 1A). Probe A lies inside the targeting construct, whereas probe B lies ~0.5 kb outside; both probes gave identical patterns on blots with *Bam*HI (Fig. 1B) or *Hin*dIII digests (data not shown) of genomic DNA. This indicates correct single-copy integration of the targeting construct by homologous recombination into the RXRα locus. PCR amplification using a *neo*-specific primer and a primer outside of the targeting construct confirmed this result (data not shown).

Heterozygous offspring derived from germ-line transmission of the targeted allele were normal in all regards and were crossed. From 18 litters, 83 pups were born, none of which were homozygous for the mutated allele, predicting an embryonic lethal phenotype (Table 1). Isolation of embryos in utero revealed a Mendelian frequency of live homozygotes through embryonic day 14.5 (E14.5) but a decreasing recovery at stages beyond (Table 1). Concurrently, an increasing number of dead embryos were seen at these later stages and, where tissue could be recovered for genotyping, often proved to be homozygotes, although a small number of nonhomozygous embryos were also seen which died in utero. The RXRα mutation therefore results in a period of embryonic lethality between E13.5 and E16.5, with most embryos viable at E14.5 and not by E15.5. It should be noted that these animals and embryos are not on a uniform genetic background, which may account for part of the observed variability.

RNA was isolated from embryos of normal appearance at E13.5 and analyzed for expression of the RXRα gene. Using a full-length RXRα cDNA probe on Northern blots (data not shown), we were surprised to find a readily detectable transcript even in RXRα homozygous knockout embryos. Analysis by reverse transcription–polymerase chain reaction (RT–PCR) (Fig. 1C) indicated that although no bona fide RXRα transcript is made in homozygous embryos, an aberrant transcript was produced that originates from within the *neo* gene cassette and reads on the antisense strand of the *neo*-coding sequence and the PGK promoter, through the third intron and into the fourth exon (C2) and beyond. Three novel bands are seen in homozygotes (and heterozygotes; data not shown) but not in wild-type embryos, upon amplification with a *neo*-specific primer and a primer derived from the E domain of the RXRα gene (Fig. 1C, lane 8). As established by cloning and sequencing, the upper band represents complete read through of the third intron, and the lower two represent cryptic splice donor sites in the third intron, which are spliced to the fourth exon. In no case could an open reading frame be established contiguous with the RXRα-coding sequence. Other reports have also mentioned an aberrant transcript derived from the *neo* gene cassette in other gene knockout experiments (e.g., Hasty et al. 1993). This analysis also detects a very low level of products of varying sizes in homozygotes when a primer from the second (A2) exon is used (Fig. 1C, lane 5). The origin of these rare transcripts is not clear, but note that the second and fourth exons of the RXRα gene are out of frame with respect to each other, suggesting

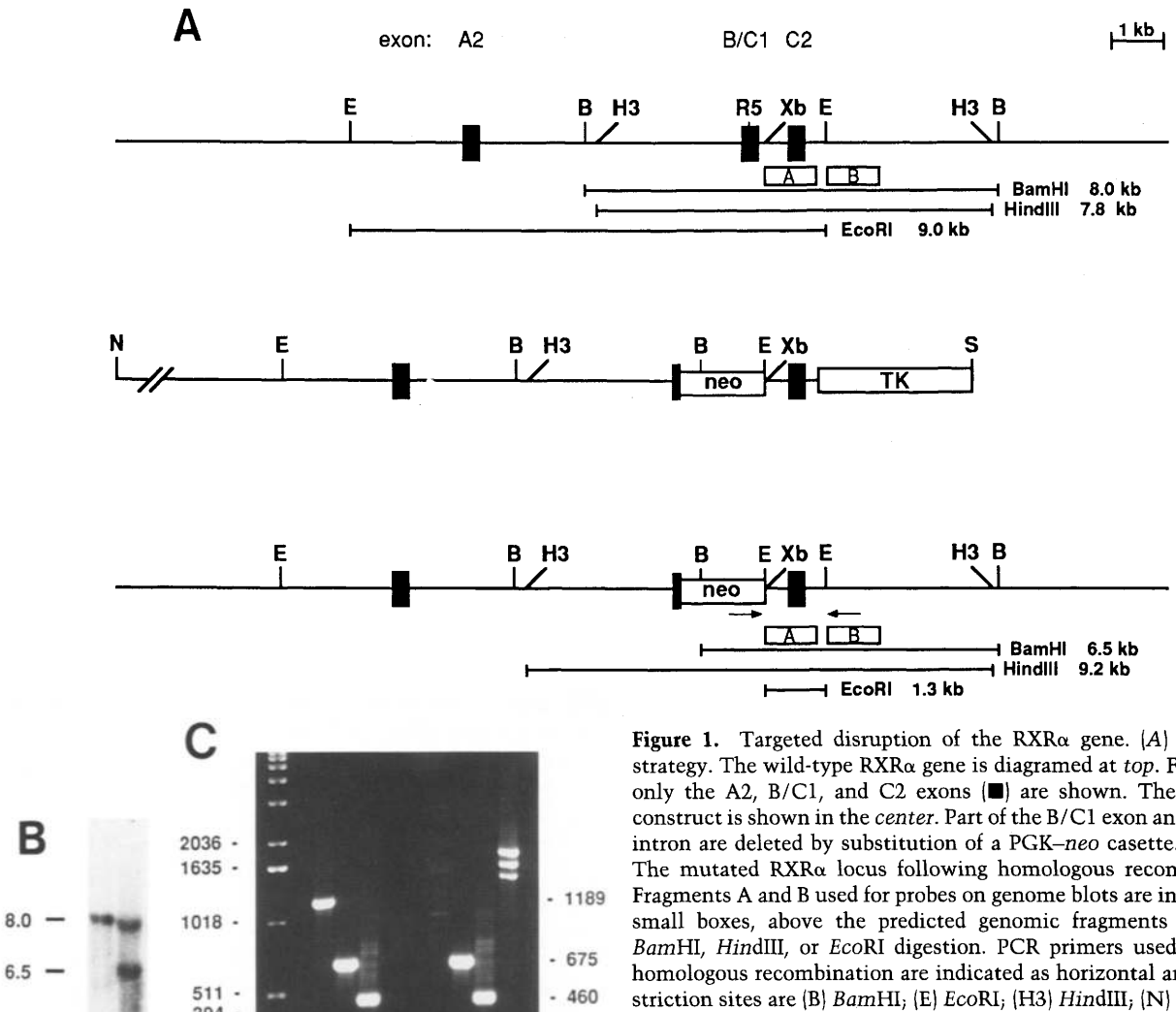

Figure 1. Targeted disruption of the RXRα gene. (*A*) Targeting strategy. The wild-type RXRα gene is diagramed at *top*. For clarity, only the A2, B/C1, and C2 exons (■) are shown. The targeting construct is shown in the *center*. Part of the B/C1 exon and adjacent intron are deleted by substitution of a PGK–*neo* casette. (*Bottom*) The mutated RXRα locus following homologous recombination. Fragments A and B used for probes on genome blots are indicated in small boxes, above the predicted genomic fragments following *Bam*HI, *Hin*dIII, or *Eco*RI digestion. PCR primers used to verify homologous recombination are indicated as horizontal arrows. Restriction sites are (B) *Bam*HI; (E) *Eco*RI; (H3) *Hin*dIII; (N) *Not*I; (R5) *Eco*RV; (S) *Sal*I; (Xb) *Xba*I. (*B*) Genome blot. *Bam*HI-digested genomic DNAs from a wild-type mouse (lane *1*) or a RXRα heterozygote (lane *2*) were blotted and hybridized with probe B. The 8.0-kb wild-type allele and the 6.5-kb targeted allele are indicated. (*C*) RT–PCR analysis. Whole-embryo RNA from wild-type (lanes *1–4*) or RXRα homozygous (lanes *5–8*) littermates at E13.5 were reverse transcribed and PCR amplified. All PCR reactions utilized a common 3′ primer from the RXR E domain; the 5′ primers were from the RXRα gene A2 exon (lanes *1,5*), D domain (lanes *2,6*), the RXRβ gene E domain (lanes *3,7*) as a control, or the *neo* gene (lanes *4,8*). Lane M is the BRL 1-kb ladder; sizes of relevant marker bands are shown at *left*. Predicted bands of 1189, 675, and 460 bp are seen in lanes *1–3* and *6–7*; three novel bands of 1.8, 1.6, and 1.5 kb are amplified with the *neo* 5′ primer (lane *8*).

that even if a splice variant transcript was produced by skipping over the mutated third exon,the resultant transcript would also not encode a functional protein.

Analysis of RXRα homozygous embryos

Homozygous embryos recovered at various stages of embryonic development were analyzed microscopically and histologically. Homozygous embryos from E9.5 and E11.5 were normal and identical to wild-type and heterozygous littermates in size and appearance (Fig. 2A,B) and in histological sections (data not shown). The first manifestation of the RXRα mutation is evident at E12.5 in the embryonic liver and heart. The liver originates from the cardiac–hepatic eminence as an endodermal condensation below the emerging heart tube at E9.5 and is one of the first endodermal tissues to become morphologically recognizable. The liver increases in volume through mid-gestation and by E12.5 is sufficiently developed in wild-type embryos to allow visualization through the skin by surface examination (Fig. 2C). In contrast, homozygous RXRα embryos are greatly deficient in the amount of liver present (Fig. 2D; see also Fig. 6B, below); this is seen in every embryo examined and is not a consequence of the amount of vascularization or blood flow present. By visual inspection, reconstruction of liver volume from serial sections, and by dissection and dry weight, we estimate that E12.5 homozygous em-

Table 1. *Offspring of RXRα heterozygous crosses*

	Litters	+/+	−/+	−/−
E9.5	1	3	2	3
E10.5	6	12	20	13
E11.5	4	7	14	7
E12.5	9	18	38	9
E13.5	9	9 (1)	33	10 (3)
E14.5	16	25	38	20 (1)
E15.5	5	10	18	7 (5)
E16.5	4	5 (1)	14	4 (3)
E17.5	4	5	10	1 (1)
$P > 1$	18	20	63	0

Offspring of RXRα heterozygous parents, either postparturition ($P > 1$) or taken mid-gestation at E9.5–E17.5, were genotyped. Numbers in parentheses indicate embryos of the given genotypes that were found dead upon isolation, determined either by gross observation or by absence of blood flow when the umbilical cord was cut.

bryos have a liver mass of ~30% of wild-type littermates. As revealed by histological analysis, the hepatic tissue of RXRα homozygotes is of normal morphology (see, e.g., Fig. 6B, below). Despite the dramatic suppression of hepatic proliferation at E12.5, there is a substantial recovery, to ~60% of wild type, by E14.5 (Fig. 2E,F; see also Fig. 3A,B). This suggests that mid-gestation failure of liver proliferation is not likely to be a major contributor to the lethality seen in homozygous embryos. There was no indication that hematopoiesis, which occurs in the liver in early to mid-gestation embryos, was compromised in homozygotes.

Embryos at E14.5 appeared near normal in terms of size, shape, and overall developmental progress (Fig. 2E,F). Histological sections through homozygous and wild-type littermates indicated that most major organ systems were present and of normal appearance in homozygotes, including tissues such as intestine, skin, and kidney in which RXRα is abundantly expressed (Fig. 3E–J). However, these embryos showed a swelling of the skin, sometimes quite dramatic (Fig. 3B), which upon histological analysis proved to be an edematous accumulation of fluid under the dermal layer of the skin (Fig. 3D). This edema was usually substantially pronounced in the folds of skin around the eye, causing a translucent appearance (Fig. 2F); the eye tissue itself was normal in these embryos.

Cardiac defects in RXRα homozygous embryos

Generalized edema is often associated with defects in cardiac structure or function, and suggested the possibility of heart failure in RXRα homozygous embryos leading to accumulation of interstitial fluid. Normal heart development (for review, see Litvin et al. 1992) involves the concerted differentiation of cardiogenic mesoderm, which gives rise to the myocardium and the endocardium, and the cardiac neural crest, from which migratory progeny populate the aorta, pulmonary artery, and associated structures. The myocardial component is comprised of ventricular trabeculae, which form a contractile lattice inside the ventricular chambers, and of the atrial and ventricular cardiac walls. The four chambers of the heart become distinct during mid-gestation by the formation of the atrioventricular valves and the interatrial and interventricular septa. Proliferation of myocytes at the ventricular epicardial surface (known as the compact zone) from E12.5 onward generates the thickened outer wall and contributes to the muscular interventricular septum that separates these two chambers.

As revealed in Figure 4, the major defect in RXRα homozygous embryos was in the heart. Homozygous embryos at E12.5 appear comparable to wild-type littermates in the extent of ventricular wall thickness and total ventricular mass (Fig. 4A,B; see also Fig. 6A,B, below), but display other abnormalities. The contour of the ventricular surface is uneven as is also manifest later (Fig. 4D,F). The visceral pericardial lining appears irregular (Figs. 4B and 6B, below), rather than tightly apposed to the ventricular wall, although later at E14.5 the pericardium appears relatively normal (Fig. 4F). Pericardial space was also very prominent in most embryos at this stage (Figs. 4B and 6B, below; see also Fig. 2D). An uneven ventricular surface and loosely apposed pericardium are features typical of E11.5 embryos. Homozygous embryos examined at E11.5 and earlier were identical to wild-type littermates (data not shown), suggesting that the onset of developmental defects may occur around this time.

By E14.5, cardiac development in wild-type mouse embryos has proceeded to near maturity, in that all four chambers are present and separate, mitral and tricuspid valves are fully developed, and the aortic and pulmonary outflow tracts are separated and originate from opposite ventricles. The ventricles at this point are dense and muscular, both in terms of the thickness of the outer ventricular wall and in the extensive and well-organized trabeculation inside the ventricular chambers (Fig. 4E). In contrast, homozygous RXRα embryos display ventricular chamber hypoplasia, in which there is absence of proliferation of the compact muscular layer of the ventricular wall (Figs. 4F and 5). Trabeculation inside the ventricle chamber is present but diminished, and frequently displayed disorganization at the muscular septum. This results in a ventricular septal defect in which a passage between ventricles could be observed, both between the ventricular septum and the endocardial cushion and through fenestrations in the septal tissue itself (Fig. 4F). All homozygous RXRα embryos displayed hypoplasia of the ventricular wall and (with one exception) obvious ventricular septal defects (Table 2). The aorta and pulmonary artery outflow tracts and valves, which depend on the influence of cardiac neural crest progeny for proper differentiation, appeared normal and were properly septated (Fig. 6C–F) in all but one embryo. In some cases, the right ventricle appeared dilated, and enlarged right atria were frequently seen, while mitral atresia was seen in one embryo. It is likely that the primary defect in RXRα mutant embryos is in the differentiation or maturation of the ventricular cardiac myo-

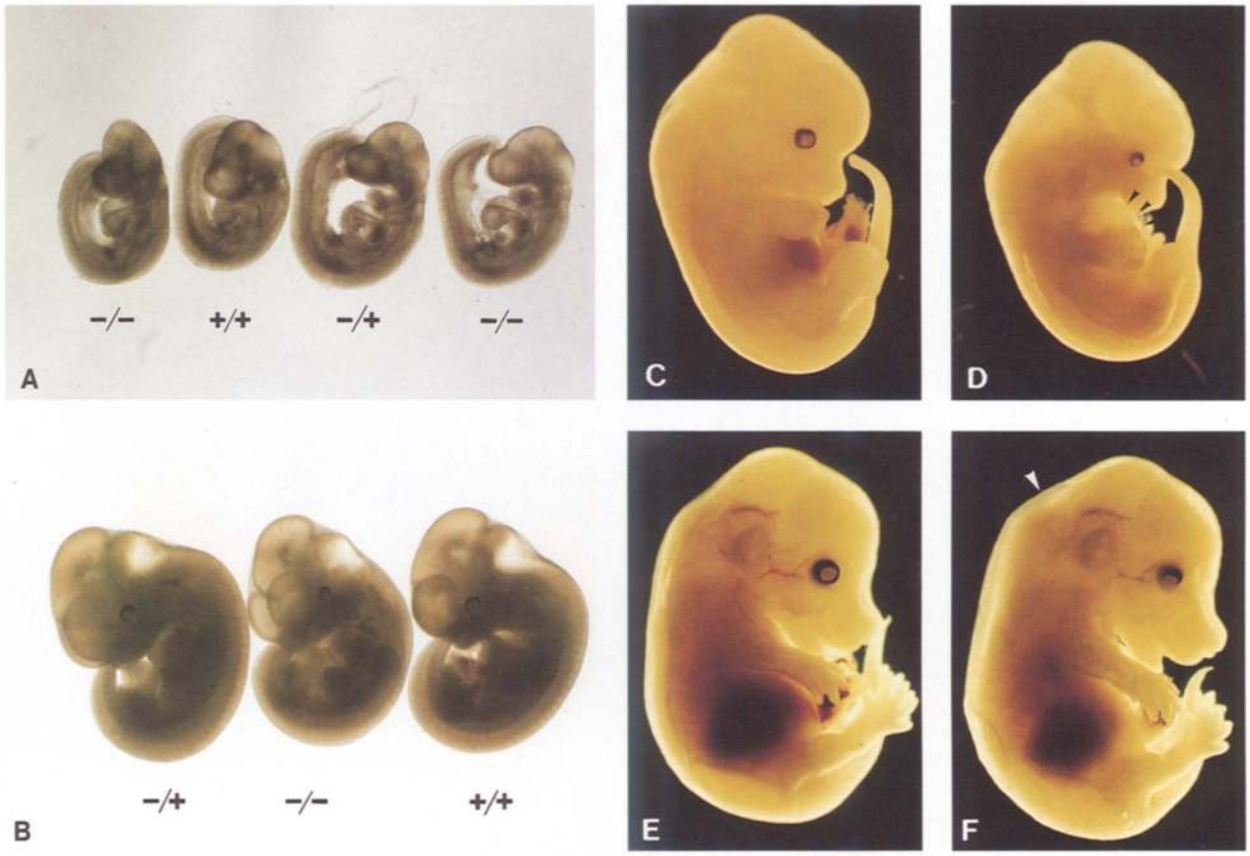

Figure 2. Littermate embryos derived from RXRα heterozygous crosses. Bright-field illumination of embryos at E9.5 (*A*) and E11.5 (*B*); genotypes are shown below each embryo. (*C*) Dark-field illumination of a wild-type embryo at E12.5. A substantial amount of liver is visible through the skin. (*D*) A homozygous littermate of the embryo shown in *C*. No liver is visible; arrowheads point to the swelling in the pericardial space. (*E*) Dark-field illumination of a wild-type embryo at E14.5. (*F*) A homozygous littermate of the embryo shown in *E*. A substantial amount of liver is visible; the arrowhead points to edematous swelling along the back, which is fairly mild in this embryo. Swelling of the skin around the eye is also apparent.

cytes, which is compromised in all homozygous embryos. Defects seen only sporadically are presumably secondary to hemodynamic disturbances caused by decreased cardiac performance.

The cardiac dysmorphogenesis of homozygous embryos did not completely abolish all organized cardiac contractions, as the ventricular chambers could be observed to pump and blood flowed from the umbilical cord when it was severed. However, cardiac function in the affected embryos is likely to be severely compromised by the hypoplasia of the ventricular chamber wall, which is responsible for generating the systolic force of embryonic blood flow. At E14.5, the embryo is beginning a phase of exponential growth, increasing in weight over 50% per day (Kaufman 1992). It is almost certain that homozygous embryos die in utero from an inability of the defective hearts to provide a sufficient flow of blood to sustain this rapid growth, resulting in a form of embryonic congestive heart failure.

Discussion

A paradox to come out of the original discovery of the RXRs is their dual ability to serve as homodimeric receptors in response to 9-*cis* RA as well as to serve as heterodimeric partners for hormonal signaling mediated by the RAR, TR, and VDR. Thus, in principle, the RXRs can mediate retinoid signaling via two different pathways as well as participate in nonretinoid signaling via other receptor partnerships. Whereas previous studies have been suggestive of the potential importance of RXR in hormonal signaling, there is no direct evidence for its essentiality in any of these responses. Accordingly, the data presented in this manuscript provide unequivocal evidence as to the necessary role of RXRα in normal embryogenesis. An important question arising out of these observations is whether this defect is a consequence of a failure to transmit a hormonal signal and, if so, which pathway is affected. Suggestive evidence comes from classical studies of nutritional deficiency, which indicate that vitamin A deficiency results in the same type of embryonic cardiac defects observed in RXRα mutant embryos. In one study (Wilson and Warkany 1949), approximately half of all affected embryos displayed hypoplastic ventricular chambers, with ventricular septal defects, identical to the phenotype de-

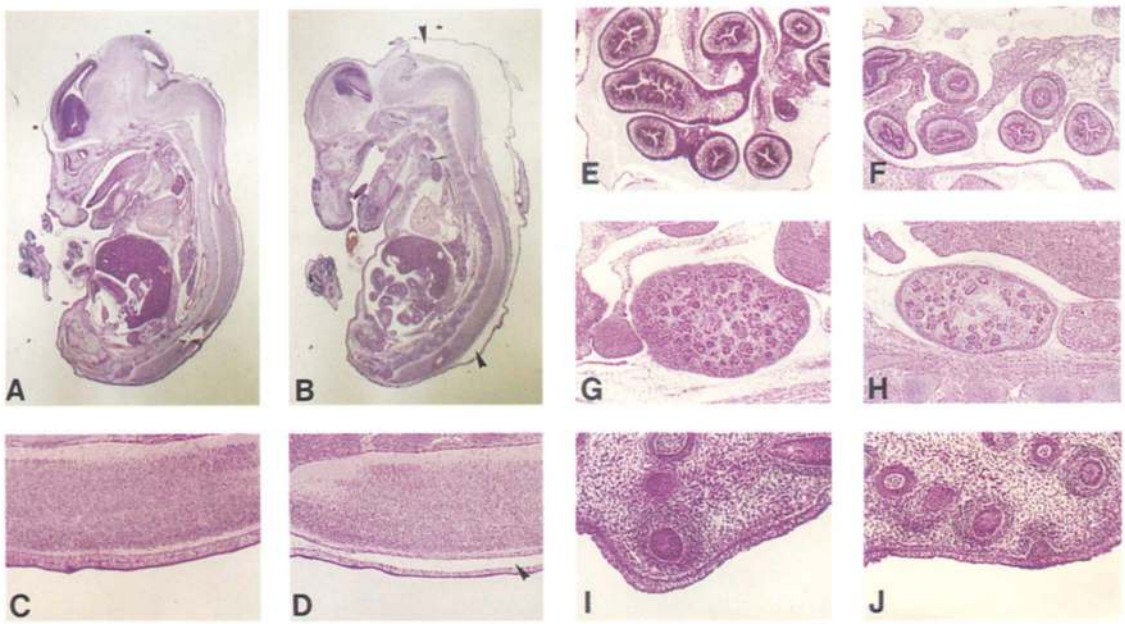

Figure 3. Histological analysis of E14.5 embryos. *A, C, E, G,* and *I* are from a wild-type embryo; *B, D, F, H,* and *J* are from a homozygous littermate. (*A,B*) Whole embryo saggital sections. Edema is prominent along the back of the homozygous embryo (indicated by arrowheads). (*C,D*) Skin adjacent to the spinal cord. The subdermal edema that separates the skin from the underlying spinal cord is evident in the homozygote (indicated by the arrowhead). (*E,F*) Intestine; (*G,H*) kidney; (*I,J*) skin from the snout. Round hair follicles (in some cases sectioned transversely) are apparent.

scribed here. In contrast, fetal thyroid hormone deficiency produces minimal effects on growth or maturation with replacement required only postnatally (Letarte et al. 1980; Fisher 1986). Similarly, inability to respond to vitamin D_3 because of a mutation in the VDR produces a normally developed embryo with rickets seen as a postnatal effect (Hughes et al. 1988). The concordance of the RXRα phenotype with vitamin A deficiency suggests that a retinoid-dependent pathway is likely to be compromised in the mutant background and implicates either an RXR homodimer or an RXR–RAR heterodimer process. These observations indicate an essential role for RXRα in vitamin A signaling and provide the first evidence of a requirement for RXR in one of its predicted hormone response pathway. Because delayed development of the liver is not a phenotype associated with vitamin A deficiency (nor T_3 or D_3 deficiency) this defect is likely attributable to a failed heterodimer formation with another RXR partner, possibly PPAR, which is abundantly expressed in the liver (Issemann and Green 1990).

Vitamin A deficiency, excess, and development

Vitamin A deprivation of pregnant female rodents (Warkany and Schraffenberger 1946; Wilson and Warkany 1948; Wilson and Warkany 1949; Wilson et al. 1953) results in a broad spectrum of embryonic defects in the eye, kidney, genital accessory organs, epithelium of the genitourinary tract, skeleton, diaphragm, and lung, as well as in the heart. The heart defects (Wilson and Warkany 1949) include dysmorphic development of the cardiac mesoderm, as described above, and defects in derivatives of the cardiac neural crest, including the truncus arteriosus (the common precursor of the pulmonary artery and the aorta) and the aortic arch system. With the exception of the ventricular phenotype, and to the extent that the above structures are present at E14.5, no obvious defects or malformations were observed in these additional tissues in homozygous RXRα embryos. There is also a syndrome of defects observed in adults maintained on a vitamin A-deficient diet, primarily involving epithelial keratinization in a variety of organs (Sporn et al. 1994 and references therein). It has been reported that mutations of the RARα (Lufkin et al. 1993) and RARγ (Lohnes et al. 1993) genes result in epithelial defects in the testes (RARα) and in seminal vesicle and prostate glands (RARγ); that is, defects seen in adult vitamin A deficiency. Together, these results support the proposal that the action of RA and its metabolites in development and in the adult operate through the products of individual retinoid receptor genes.

In contrast to vitamin A deficiency, retinoid excess, as experimentally administered in embryonic teratogenic studies, causes the inappropriate activation of all retinoid-dependent pathways in sensitive tissues. Paradoxically, different but overlapping phenotypes emerge from these two treatments. Teratogenic doses of RA have not been reported to cause any ventricular phenotype. However, retinoid treatment, as with retinoid deficiency, leads to truncus arteriosus and aortic arch abnormalities. Lineage tracings have documented that these teratogenic

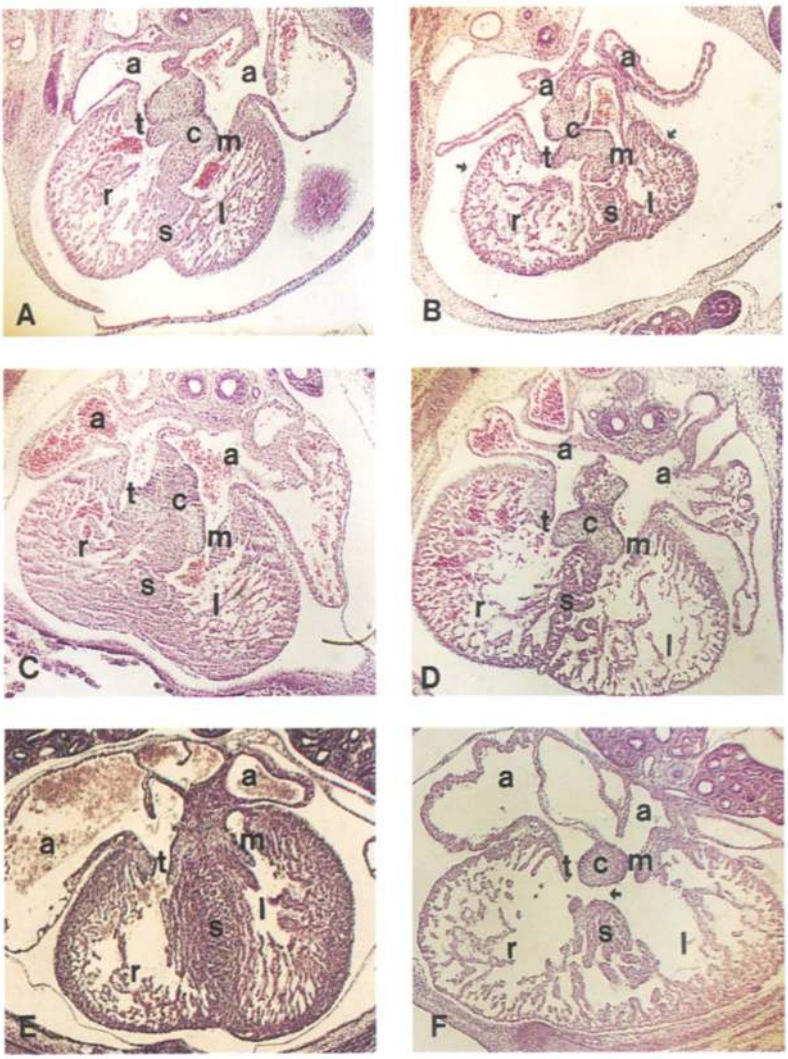

Figure 4. Transverse sections at the level of ventricle inflow valves. *A*, *C*, and *E* are from a wild-type embryo; *B*, *D*, and *F* are from a homozygous littermate. Embryos were isolated at E12.5 (*A*,*B*), E13.5 (*C*,*D*), and E14.5 (*E*,*F*) of development. Abbreviations are (a) atria, (c) endocardial cushion, (l) left ventricle, (m) mitral valve, (r) right ventricle, (s) interventricular septum, and (t) tricuspid valve. Arrows in *B* point to the loosely attached pericardial layer; arrow in *F* indicates a ventricular septal defect at the septal–cushion fusion.

cardiac defects occur in structures derived from the cardiac neural crest, which populates the outflow tracts and associated vessels in the aortic arch system (Kirby et al. 1983; Kirby and Waldo 1990; Kirby 1993). In this study the differentiation of the cardiac neural crest was not obviously compromised in the RXRα mutant background. The aorta and pulmonary artery outflow tracts were remarkably well formed in homozygous embryos, even when derived from deformed ventricles. No morphological defects were apparent in the thymus or thyroid gland, which are also derived from the cardiac neural crest and are also sensitive to teratogenic retinoid exposure. This finding indicates that although the normal differentiation of the cardiac neural crest is under retinoid control, there is no obvious requirement for RXRα in these processes. Furthermore, no defects in craniofacial derivatives of the cranial neural crest or in the limbs (both known teratogenic targets of retinoic acid) were evident. Accordingly, these results unlink the role of RA in neural crest and ventricular chamber differentiation and indicate these to be genetically separable processes.

Specificity and redundancy in retinoid signaling

Previous studies (Mangelsdorf et al. 1992) have documented the expression of RXRα mRNA in the adult heart, and, although in situ hybridization studies have so far not detected expression in the embryonic heart, we have confirmed the expression of RXRα in E8.5 heart tubes and E12.5 and E13.5 ventricles by RT–PCR (data not shown). Furthermore, there is indirect functional evidence for the expression of RXR in the embryonic heart: A RA-responsive transgene is both basally and inducibly expressed in ventricular myocytes at E12.5 and earlier (H.M. Sucov and R.M. Evans, unpubl.), and a similar reporter gene is RA-inducible in microinjected cultured E14.5 ventricular myocytes (V. LaMorte, H.M. Sucov, and R.M. Evans, unpubl.). These observations strongly support the proposal that the ventricular defect is a prob-

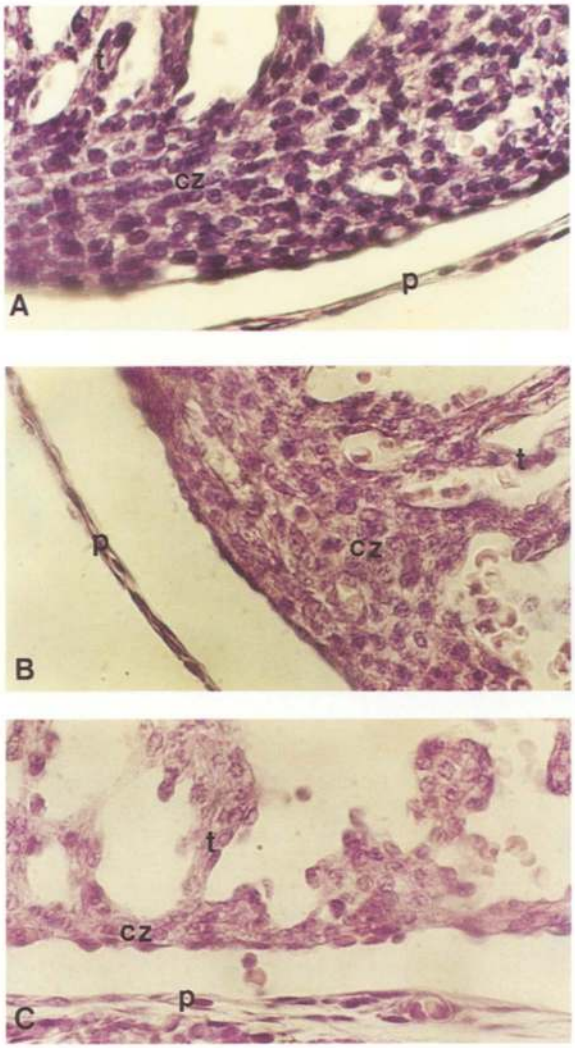

Figure 5. The compact zone of E14.5 embryos. Higher magnification views of the compact zone of wild-type (*A*), heterozygous (*B*), and homozygous (*C*) embryos. Abbreviations are (cz) compact zone, (p) pericardium, and (t) trabeculae.

able consequence of the absence of RXRα in the differentiating heart itself, as opposed to a secondary effect. It also should be noted that whereas normal cardiac development proceeds from E7.5 to birth, the mutant hearts become dysmorphic by E12.5, indicating a putative temporal boundary for the requirement for RXRα expression.

The RXRα gene is abundantly expressed in other tissues of the embryo. Histological analysis indicates that these are normal in appearance in homozygous embryos (up to the time of death), with the only defect being the delayed growth of the liver. Although they are histologically normal, we have not yet determined whether any of these tissues display normal function. The RXRα gene may be required for certain gene regulatory processes that do not relate to morphogenesis but, rather, to the physiology of the organ. It should be possible to address this issue by monitoring the expression of specific markers for these tissues, some of which are known to be under retinoid control (e.g., Kliewer et al. 1992b).

The spectrum of tissues that utilize retinoid signaling, as delimited by vitamin A deficiency and retinoid teratology experiments, is far larger than can be accounted for by the known defects in the already established retinoid receptor gene mutations. The inference from the gene knockout experiments already available (this paper; Li et al. 1993; Lohnes et al. 1993; Lufkin et al. 1993) is that the defects observed tend to be far less widespread than the expression patterns might indicate. The most likely explanation is that there is considerable redundancy in expression and function between the receptor genes, so that mutation of a single gene does not compromise most tissues. The defects that are observed may represent either unique spatial expression of a given receptor gene or a requirement for a unique functional property of a given receptor protein. We are taking two experimental approaches to address the issue of redundancy as it pertains to the RXRα phenotype. One is to determine whether expression of RXRβ or RXRγ from transgenic promoters can rescue the RXRα phenotype, as presumably RXRα itself will be able to do. A second approach is to identify the pathways of ventricular muscle development that are compromised in the mutant background, identify the genes that are misexpressed as a consequence, and determine the biochemical nature of the requirement for RXRα in their expression.

RXRα and the pathogenesis of ventricular chamber defects

In RXRα homozygotes, the central defect in cardiogenesis appears to be a failure of normal ventricular muscle to develop sufficiently to meet the demands of the growing embryo. Formation of the mature ventricular chamber requires the development of extensive ventricular trabeculation and of the dense compact muscle layer in the ventricular free wall. The increased thickness of the ventricular wall allows the myocardium to generate sufficient mechanical force to maintain blood flow through the growing embryo. As noted in this study, lack of RXRα permits formation of a trabeculated, yet dysmorphic ventricular chamber, with the thickness of the ventricular wall resembling the relatively thin-walled atrial chamber. We suggest that the development of the compact muscular layer of the ventricles is dependent on the RXRα signaling pathway, whereas the formation of the inner trabecular layer may continue through a compensating or alternative pathway. Although the RXRα phenotype may simply be the result of a defect in the proliferative capacity of fully differentiated ventricular muscle cells, the primary defect may instead reflect an arrest or delay in the sequential maturation of ventricular muscle cell lineages (Kubalak et al. 1994).

The poor development of the muscular ventricular septum seen in homozygous embryos is most likely accounted for by the lack of ventricular wall enlargement. The septum is formed by two processes. The trabeculae condense at the interventricular groove, which denotes

Table 2. *Cardiac defects in E14.5 RXRα homozygous embryos*

	RXRα genotype (+/+)	(−/+)	(−/−)
	6 embryos	5 embryos	10 embryos
Ventricular chambers			
wall mass decreased	0/6	0/5	10/10
two chambers	6/6	5/5	10/10
disorganized trabeculi	0/6	0/5	10/10
ventricular septal defects	0/6	0/5	9/10
Atrial chambers enlarged			
left	0/6	0/5	0/10
right	0/6	1/5	5/10
Atrial and ventricular valves			
mitral atresia	0/6	0/5	1/10
tricuspid atresia	0/6	0/5	0/10
Outflow tract abnormalities			
aortic valve abnormal	0/6	0/5	0/10
pulmonary valve abnormal	0/6	0/5	0/10
aortic pulmonary septum abnormal	0/6	0/5	1/9

Serial 10-μm tissue sections were taken through the heart, stained with hematoxylin and eosin, and photographed. Photos were scored independently three or four times for each of the listed features. In cases where the denominator does not equal the total number of fetuses examined, the sections were damaged such that an accurate assessment could not be made.

the future boundary of the developing right and left ventricular chambers. In addition, the medial walls of the expanding ventricles fuse together and grow inward, forming the major muscular portion of the septum. Most likely, it is this latter process that is disturbed in the RXRα embryos. Although the septum is the thickest portion of the ventricle in the normal E14.5 embryo, in RXRα homozygotes it appears to be simply a fenestrated sheet of trabeculae with little contribution of the muscular tissue normally derived from proliferation of the compact zone of the ventricle wall. A consequential lack of fusion of the endocardial cushions to the basal portion of this incomplete septum results in the observed ventricular septal defects.

Congenital cardiovascular malformations represent the single largest group of congenital defects in newborn human infants, affecting 1/200 live births on an annual basis. Of these, ventricular septal defects comprise almost one-third of these cases and represent the most frequent cardiac malformation in man (Friedman 1988). As noted in a recent review (Chien 1993), our current understanding of cardiovascular developmental defects is at a primitive stage, and there are few currently recognized candidate genes or molecular insights for the pathogenesis of the wide variety of well-defined phenotypes. However, recent advances have revealed an early patterning of the ventricular segment of the primitive murine heart tube at day E8.0 (O'Brien et al. 1993), and ventricular chamber specification can occur independent of heart tube formation in ES cell models of cardiogenesis (Miller-Hance et al. 1993). Utilizing both positive and negative chamber-specific markers, sequential stages of maturation of ventricular muscle cell lineages have been elucidated (Kubalak et al. 1994) and candidate regulatory factors and pathways have recently been identified (Pollock and Treisman 1991; Yu et al. 1992; Chien et al. 1993; Komuro and Izumo 1993; Lints et al. 1993; Zhu et al. 1993). The question arises as to where RA and RXRα intersect with these other pathways and whether the cardiac phenotype reflects maturational arrest in ventricular muscle cell lineages. RXRα-deficient mice should provide a unique and valuable experimental model system to further dissect normal and abnormal ventricular chamber development at a molecular level.

Materials and methods

Targeting construct

RXRα genomic clones were isolated from a mouse 129/Sv genomic library. The targeting construct represents 15 kb to the 5′ side and 1.0 kb to the 3′ side of the third exon, which encodes the B and the first part of the C (DNA-binding) domains. A PGK–*neo* cassette was introduced in the opposite orientation with respect to the RXRα gene between an *Eco*RV site in the exon and an *Xba*I site in the intron ~0.3 kb downstream.

Cells

ES cells of the J1 line (Li et al. 1992) were grown on a feeder layer of G418-resistant embryonic fibroblasts (derived from E13.5 embryos heterozygous for a viable mutation in the RARα1 gene (Li et al. 1993)). ES cells were electroporated with *Not*I-linearized targeting construct under conditions as described previously (Li et al. 1992). Cells were selected with G418 and FIAU at concentrations of 175 μg/ml (active form) and 0.2 μM, respectively; individual colonies were picked after 8 days of selection and expanded for analysis. Correctly targeted cells were injected into C57Bl/6 blastocysts to create chimeric male founders, which were mated to C57Bl/6 females to achieve germ-line transmission of the targeted allele.

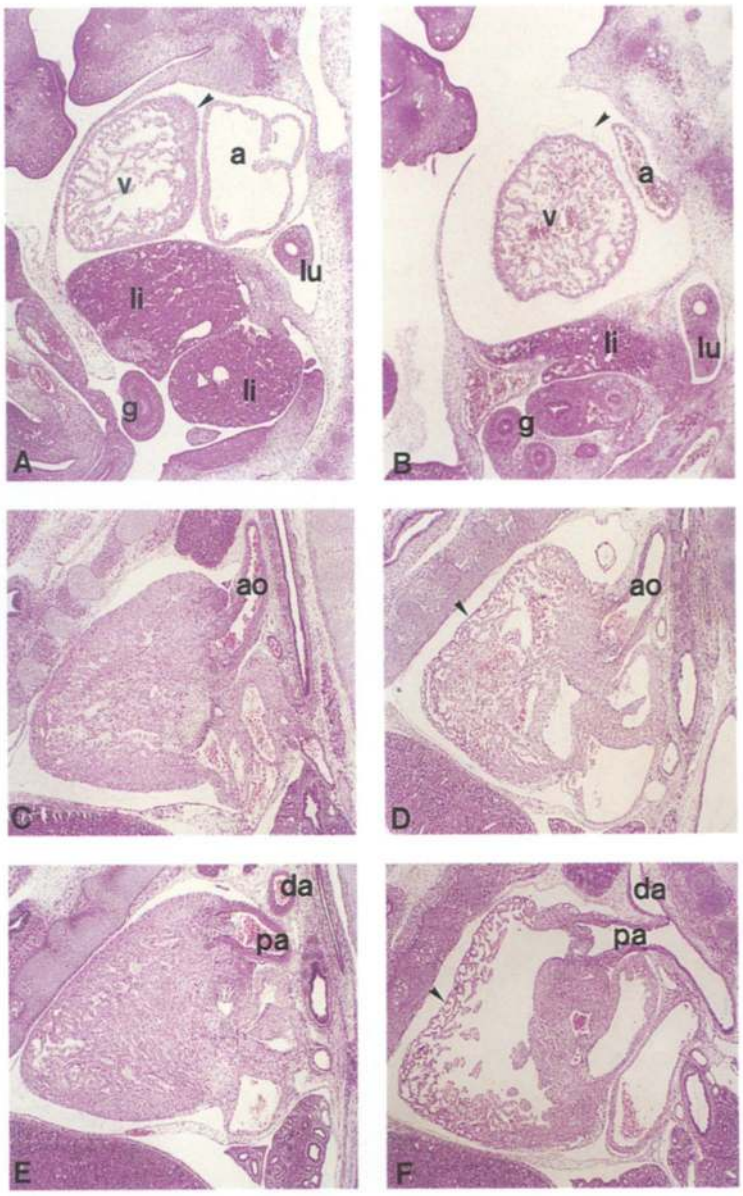

Figure 6. Morphology of the liver and cardiac outflow tracts. *A*, *C*, and *E* are from wild-type embryos; *B*, *D*, and *F* are from homozygous littermates. (*A*,*B*) Saggital sections through E12.5 embryos, showing both the diminished amount of liver present in homozygotes, as well as the altered morphology of the heart, both in the uneven contour of the ventricle and in the loose attachment of the pericardium. Arrowheads point to the pericardial layer. (*C*,*D*) Saggital sections through the aorta of E14.5 embryos. (*E*,*F*) Saggital sections through the pulmonary arteries of E14.5 embryos. The arrowheads in *D* and *F* point to the thinned ventricular wall of the homozygous embryo. Abbreviations are (a) atria, (ao) aorta, (da) ductus arteriosis, (g) gut, (li) liver, (lu) lung, (pa) pulmonary artery, and (v) ventricle.

Genotyping

Two probes were used for screening ES colonies and for initial screening of animals. Probe A is contained in the targeting construct and represents the 1.0-kb 3′ homology fragment. Probe B lies outside the targeting construct. Genomic DNA from ES cells or from mouse tails was restriction digested, electrophoresed, blotted, and probed by standard procedures. PCR primers derived from the *neo* gene and from sequence outside the targeting construct in the fourth intron were used to confirm results from Southern blots; a 1.7-kb amplification product was seen only from the targeted allele. Routine genotyping of animals and embryos was done subsequently by PCR using a combination of three primers: the *neo* primer above, a primer from the B domain of the RXRα gene that is deleted in the targeted allele, and a common primer from within the third intron; this results in the simultaneous amplification of both the wild-type and the targeted alleles as fragments of 600 and 900 bp, respectively, which can be easily resolved by electrophoresis. Primer sequences and PCR conditions are available on request. Embryos were individually isolated and genotyped by extraction and analysis of yolk sac DNA.

RT–PCR

Embryos were isolated at E13.5 and immediately homogenized individually in 4 ml of guanidinium thiocyanate buffer; total RNA was isolated as described (Chomczynski and Sacchi 1987) Yolk sac DNA was extracted separately for genotyping. One microgram of total RNA from wild-type, heterozygous, and homozygous embryos was reverse transcribed using an RXR-specific gene primer derived from the ligand-binding domain (LBD) of the protein coding region, which is conserved between the RXRα and RXRβ genes. PCR amplification utilized a common antisense primer from the LBD, and sense primers from exons 2

and 5 (domains A2 and D) of the RXRα gene, domain D of the RXRβ gene as a control, and a *neo*-specific coding region primer. Typical PCR reactions contained cDNA equivalent to 30 ng of total RNA and 200 ng of each primer, and proceeded for 45 cycles. For analysis of RXRα expression in the heart, RNA was isolated as above from heart tubes of wild-type E8.5 embryos and from ventricles of wild-type E12.5 and E13.5 embryos, and reverse transcribed and amplified as described above using the exon 5 sense primer. Parallel RNA samples were amplified without prior reverse transcription as controls. Primer sequences and exact PCR conditions are available upon request.

Histology

Embryos were isolated, immediately fixed in 10% formalin in PBS, and stored at room temperature. Embryos were dehydrated, paraffin-embedded, sectioned at 5 or 10 μm thicknesses, and stained with hematoxylin–eosin.

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