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Energy deprivation and a deficiency in downstream metabolic target genes during the onset of embryonic heart failure in RXRα$^{-/-}$ embryos

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SUMMARY

RXRα null mutant mice display ocular and cardiac malformations, liver developmental delay, and die from cardiac failure around embryonic day (E) 14.5 pc. To dissect the molecular basis of the RXRα-associated cardiomyopathy, we performed subtractive hybridization and systematically characterized putative downstream target genes that were selectively lacking in the mutant embryos, both at early (E10.5) and late (E13.5) stages of mouse embryonic development. Approximately 50% of the subtracted clones (61/115) encoded proteins involved in intermediary metabolism and electron transport, suggesting an energy deficiency in the RXRα$^{-/-}$ embryos. In particular, clone G1, which encodes subunit 14.5b of the NADH-ubiquinone dehydrogenase complex, displayed a dose-dependent expression in the wild-type, heterozygous and RXRα mutant mice. This gene was also downregulated in a retinoid-deficient rat embryo model. ATP content and medium Acyl-CoA dehydrogenase mRNA were lower in RXRα mutant hearts compared to wild-type mice. Ultrastructural studies showed that the density of mitochondria per myocyte was higher in the RXRα mutant compared to wild-type littermates. We propose a model whereby defects in intermediary metabolism may be a causative factor of the RXRα$^{-/-}$ phenotype and resembles an embryonic form of dilated cardiomyopathy.

Key words: Retinoids, Heart, cDNA subtraction, In situ hybridization, Energy depletion, Mouse

INTRODUCTION

Vitamin A plays an essential role in the maintenance of normal growth and morphogenesis during mammalian embryonic development. Vitamin A deficiency can result in the onset of a specific subset of ocular and cardiovascular defects, including ventricular chamber hypoplasia, ventricular septal defects and aortic arch abnormalities (Wilson and Warkany, 1949; Dersch and Zile, 1993; Dickman et al., 1997). Presumably, these defects arise as a consequence of individual deficiencies in molecular and/or positional cues that guide sequential steps of cardiac development. Relatively little is known regarding the downstream target genes that are essential for these steps in cardiac morphogenesis and function, and which must ultimately be under the control of vitamin A signaling pathways.

The action of retinoids is mediated by nuclear receptors that are activated by signaling molecules and consequently function to control the activity of a panel of target genes (reviewed in Mangelsdorf et al., 1996). There are two distantly related families of nuclear retinoid receptors, RARs and RXRs, both of which are members of the superfamily of ligand-inducible transcriptional regulators (Evans, 1988; Green and Chambon, 1988). RARs are activated by both all-*trans* and 9-*cis* RA, whereas RXRs are only activated by 9-*cis* RA. The ligand-activated receptors differentially regulate gene expression through their ability to recognize specific hormone-responsive DNA elements in the promoter sequence of target genes, where the receptors can bind as heterodimers, homodimers or monomers (Glass, 1994).

The generation of knockouts for the nuclear receptors has led to advances into the knowledge of their function during embryonic development (Kastner et al., 1997; reviewed in Kastner et al., 1995). In particular, RXRα null mutant mice display malformations in the eye, defects in the ventricular compact zone and abnormalities in cushion tissue mesenchyme (Sucov et al., 1994; Kastner et al., 1994, Gruber et al., 1996), and die around E14.5 from heart failure (Dyson et al., 1995). Whether the onset of embryonic heart failure in RXRα$^{-/-}$ embryos reflects a primary defect in cardiac chamber

morphogenesis, contractile function or energy metabolism in cardiac cells is unclear. In this regard, a direct, mechanistic link between vitamin A signaling and in vivo energy metabolism during embryonic or postnatal development has not been clearly established. However, the nuclear receptor PPAR (peroxisome proliferator activated receptor), which requires heterodimerization with members of the RXR family of nuclear receptors for its function, has been shown to regulate the expression of enzymes of fatty acid oxidative enzymes in transient assays (Gulick et al., 1991; Leone et al., 1995). Previous studies have also documented that a subset of neonatal cardiomyopathies are caused by deficiency in these mitochondrial enzymes, which are involved in fatty acid β-oxidation (for a review, see Kelly and Strauss, 1994).

In the present study, we describe a collection of genes defectively expressed in the RXRα mutant embryos. Since a number of other gene-targeted mutations have been shown to produce a similar 'thin walled' appearance at this stage of cardiogenesis (Moens et al., 1993; Kreldberg et al., 1993; Jaber et al., 1996; Chen et al., 1994; Zhou et al., 1995), the possibility exists that this phenotype may represent a common final pathway for gestational lethality that occurs secondary to a form of dilated embryonic cardiomyopathy, where thinning of the myocardial wall and decreased compaction is a secondary effect of increases in wall tension. The current study employed a subtraction cloning strategy to identify the spectrum of downstream target genes in the RXRα$^{-/-}$ embryos. Utilizing this approach, we isolated cDNA clones that are putative RXRα downstream target genes. Genes in the metabolic category accounted for greater than 50% of the total isolated, suggesting that altered intermediary metabolism may contribute to the RXRα$^{-/-}$ phenotype.

MATERIALS AND METHODS

Generation of directional cDNA libraries and directional tag PCR subtraction

RXRα$^{+/-}$ mice were bred to generate 80 RXRα$^{-/-}$ and 80 wild-type day 13.5pc embryos. The embryonic hearts were individually microdissected to isolate the ventricular chambers and genotyping was performed by PCR of the individual yolk sacs. 2 μm of poly(A)$^+$ RNA were purified from the ventricular chambers of wild-type E13.5 (target) or RXRα null mutant embryos E13.5 (driver) and directional libraries were generated according to the method previously described (Usui et al., 1994). In addition, cDNA libraries were also generated from intact wild-type and RXRα$^{-/-}$ embryos. The target whole-embryo cDNA directional library (eWpT7) contained 1.3×10^6 recombinant clones, with an average insert size of 1.0 kb. The driver whole-embryo library (eKpG) contained 1.5×10^6 recombinants with an average insert size of 0.8 kb. The ventricular embryonic target (vWpT7) and driver (vKpG) libraries contained 1.3×10^6 and 1.4×10^6 recombinant clones respectively and displayed an average insert size of 0.9 kb. The cDNA sequence of selected clones was determined by standard methods using T7 DNA polymerase (GibcoBRL; Gaithersburg, MD).

Northern blot analysis

30 μg of total RNA from wild-type, heterozygous and homozygous RXRα mutant E13.5 whole embryos were separated on a 1.3% agarose/formamide gel, transferred to a nylon filter (N-Hybond, Amersham) and fixed by UV cross-linking. Filters were hybridized following manufacturer's directions, by the addition of 3×10^6 cpm/ml of random-primed ^{32}P-labeled probe, washed at 0.2× SSC, 65°C and exposed to autoradiography film.

In situ hybridization

Clones in pT7T3D were *Eco*RI digested and ^{35}S-UTP-labeled. Riboprobes were generated using T3 RNA polymerase. MCAD probe was generated by *Nar*I restriction of pGEM-MCAD plasmid (Kelly et al., 1989) and transcribed with Sp6 RNA polymerase. Each in situ hybridization was carried out using an MLC2a internal control for RNA integrity and efficiency of hybridization. MLC2a riboprobe was as previously described (Dyson et al., 1995). In situ hybridization was performed on 7 μm thick paraffin sections according to Lyons et al. (1995a).

Electron microscopy

Embryos were fixed for 1 hour in 2.5% glutaraldehyde 2%/paraformaldehyde, washed, stained in 2% aqueous uranyl acetate for at least 30 minutes, dehydrated and embedded in Durcopan. Sections through the ventricle were cut between 50 and 500 nm and poststained with 2% uranyl acetate and Sato lead (Sato, 1967). Images were recorded on film at 5,000 magnification on a JEOL4000EX electron microscope operated at voltages ranging from 120 to 400 kV, depending on the section thickness. Stereological observations were performed according to Poole and Mathieu-Costello (1996) and only 50 nm thick sections were used in order to approximate the section to a plane through the mitochondrion.

ATP determination

Rapidly frozen tissues were extracted in 10 volumes (v/v) 0.4 N perchloric acid and centrifuged at 10,000 ***g*** for 30 seconds. The supernatants were neutralized with one-quarter volume of 2.2 M $KHCO_3$ and the protein-containing pellets were dissolved in 50 μl of 0.1 N NaOH. ATP was measured in the neutralized supernatants by the luciferase method using a photon-counting luminometer (Lemasters and Hackenbrock, 1978). Protein was measured in the dissolved pellets (Bradford 1976). The data were expressed as nmol of ATP per milligram of protein, with duplicate samples generally being within 10% agreement.

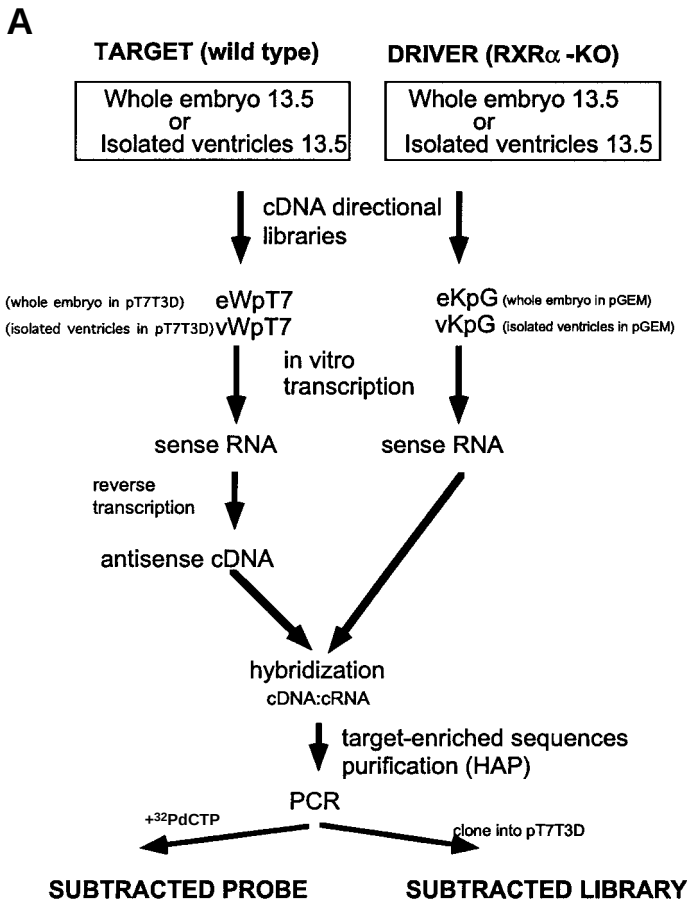
Production of retinoid-deficient rats

Retinoid-deficient rat embryos were produced according to the method described elsewhere (Dickman et al., 1997).

RESULTS

Isolation of RXRα downstream target genes via a directional tag subtraction cloning strategy with cDNA libraries derived from RXRα$^{-/-}$and wild-type embryos

To isolate putative RXRα downstream target genes, we used a subtractive hybridization method to generate a cDNA library of clones that were enriched in the wild-type versus the RXRα mutant embryos. Two independent strategies were employed based upon either the generation of whole-embryo cDNA (eWpT7 and eKpG) or embryonic cardiac ventricle (vWpT7 or vKpG) libraries derived from wild-type and RXRα$^{-/-}$ day 13.5 embryos. Subtraction was performed either through eWpT7 *minus* eKpG, or by vWpT7 *minus* vKpG. Purified single-stranded fractions corresponding to mRNAs enriched in the wild type were separated, PCR amplified and cloned to yield the wild-type subtracted libraries (eSpT7 and vSpT7), where 96% of the input target was removed (Fig. 1A). Subsequently,



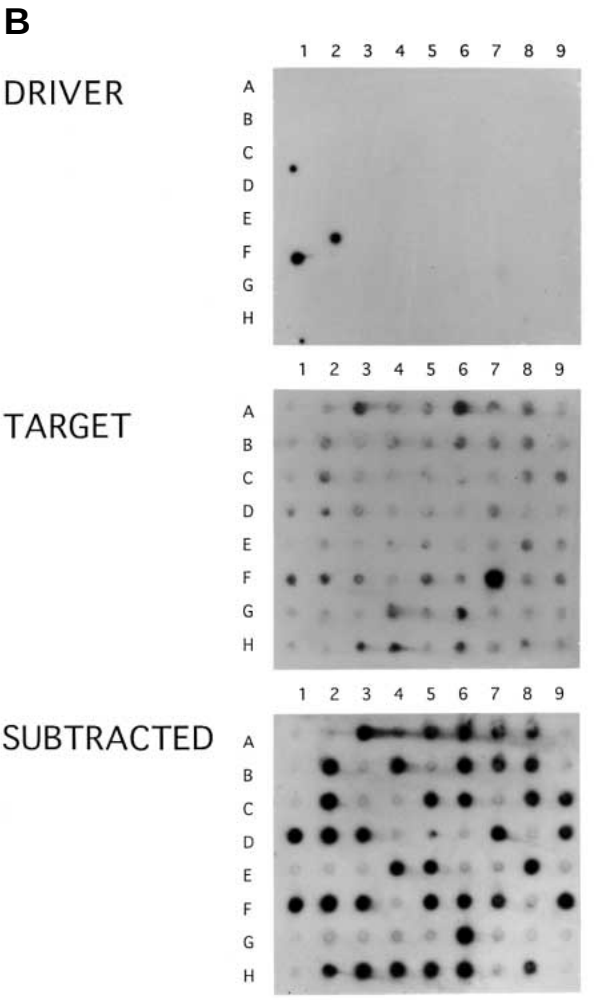


Fig. 1. Differential screening of the subtracted RXRα cDNA library. (A) Strategy of subtraction. Directional cDNA libraries from either whole-embryo or isolated cardiac ventricles were generated in two different phagemid vectors. Inserts were in vitro transcribed and antisense target cDNA was hybridized to sense driver cRNA. Target-enriched sequences were purified by HAP chromatography and used to generate either the subtracted probe or the wild-type subtracted library. (B) Clones from the RXRα$^{+/+}$ *minus* RXRα$^{-/-}$ subtracted libraries were picked and arrayed to make replica filters. Each filter was hybridized with unsubtracted RXRα$^{-/-}$ cDNA probe (DRIVER), unsubtracted RXRα$^{+/+}$ cDNA probe (TARGET), or RXRα$^{+/+}$-*minus*-RXRα$^{-/-}$ SUBTRACTED cDNA probe.

we picked and arrayed 432 clones randomly from the subtracted cDNA libraries and hybridized replica filters with the subtracted, the unsubtracted wild-type whole-embryo (target) and the unsubtracted mutant whole-embryo (driver) cDNA probes. These cDNA clones, enriched in the subtracted probe and non-detectable with the driver probe, were selected as candidates for RXRα downstream mRNAs (Fig. 1B). We determined the cDNA sequence of 115 selected clones, which resulted in the identification of three classes of cDNAs (Table 1). A portion of the subtracted clones represented novel sequences, which displayed both ubiquitous and tissue-restricted pathways of expression. A significant number of the subtracted cDNAs (50%; 61/115) encoded known enzymes involved in energy metabolism. For instance, clone G1 encodes the 14.5b subunit of the NADH-ubiquinone oxidoreductase complex (Arizmendi et al., 1992), and appeared 33 times in the subtracted library. Other clones involved in energy metabolism were also found in the subtracted library, including NADH-I (Noack et al., 1996), Fructose 1,6 bisphosphate aldolase (Buono et al., 1988) and the glucagon receptor (MacNeil et al., 1994; Lok et al., 1994).

Differential expression pattern of selected cDNAs during embryonic development of wild-type versus RXRα$^{-/-}$ embryos

We determined the differential expression of selected, subtracted clones in the wild-type mouse embryo day 13.5 and compared this to the expression pattern in RXRα null mutant littermates by either in situ hybridization (ISH) on paraffin-embedded embryos or northern blot analysis of total RNA samples. A summary of the validated clones is shown in Table 1 and several examples are provided in Fig. 2A,B. In particular, clone G1, encoding the 14.5b subunit of the NADH-ubiquinone oxidoreductase (mitochondrial Complex I), was ubiquitously expressed in the wild-type and showed a markedly reduced expression in the RXRα$^{-/-}$ embryo (Fig. 2A). Atrial myosin light chain-2 (MLC-2a) was persistently expressed in the RXRα mutant ventricles (Fig. 2A,B), as previously described (Dyson et al., 1995). The expression of MLC-2a demonstrates the integrity of RNA in these experiments and further supports the aberrant gene expression in the RXRα null mutant mouse. The novel cDNA sequences C8 and B2 were enriched in the fetal liver of wild-type mice

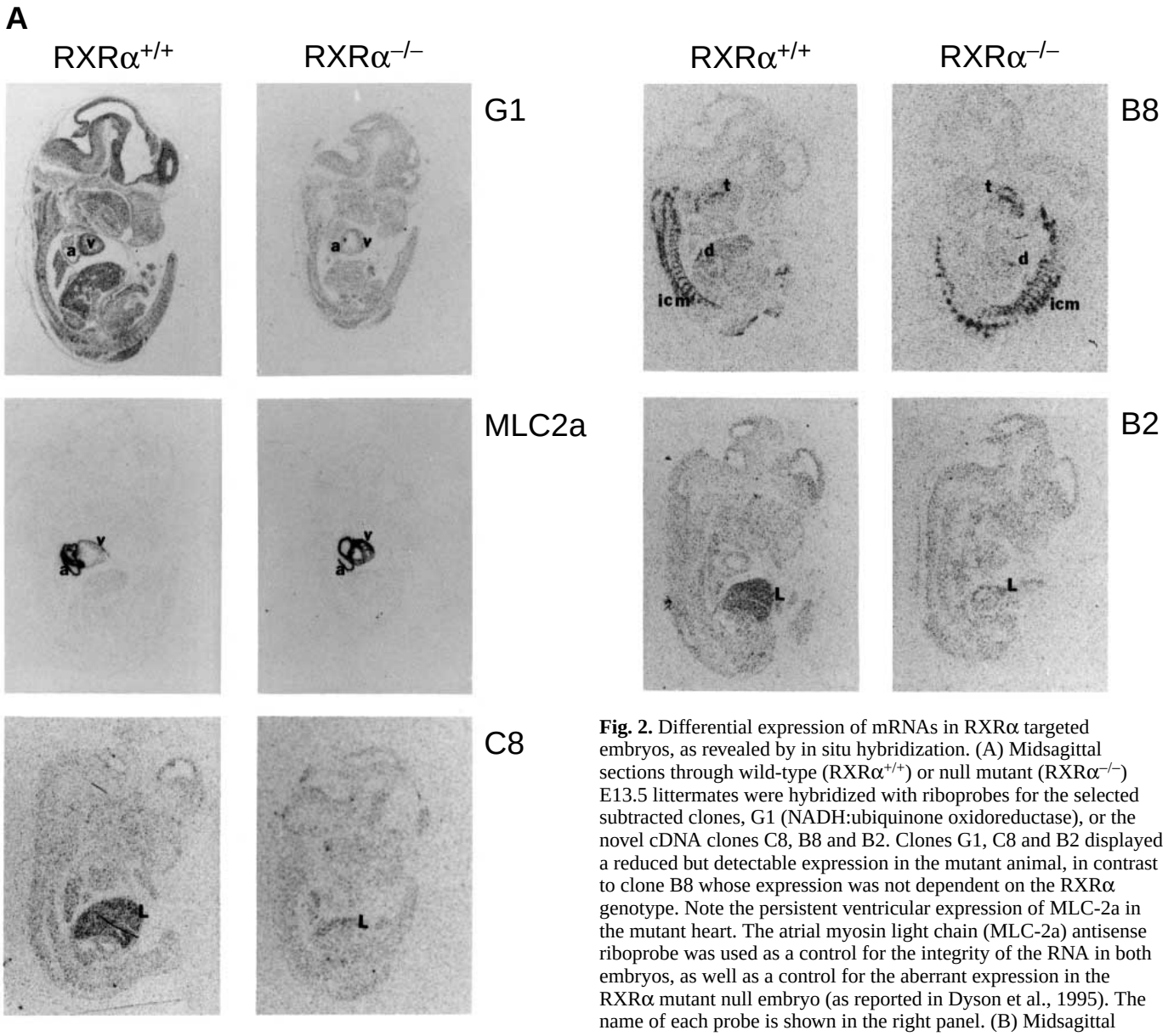
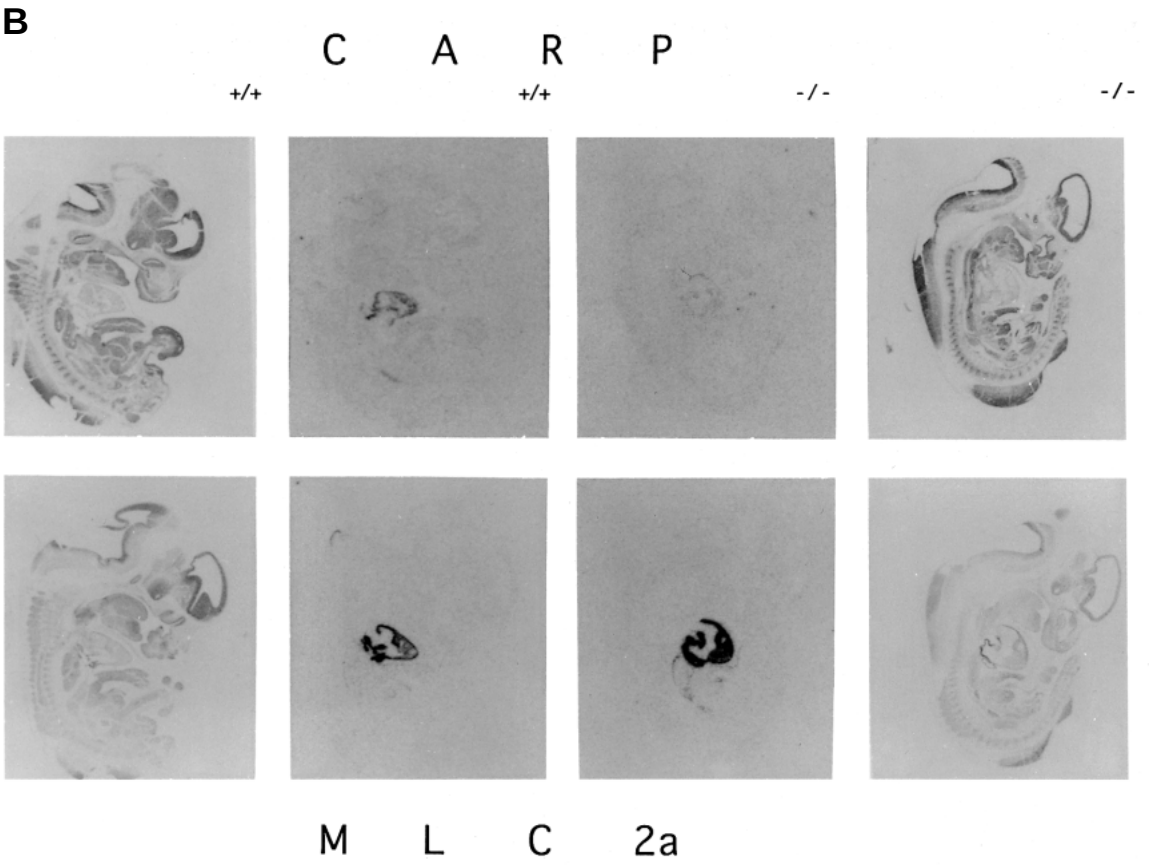


Fig. 2. Differential expression of mRNAs in RXRα targeted embryos, as revealed by in situ hybridization. (A) Midsagittal sections through wild-type (RXRα$^{+/+}$) or null mutant (RXRα$^{-/-}$) E13.5 littermates were hybridized with riboprobes for the selected subtracted clones, G1 (NADH:ubiquinone oxidoreductase), or the novel cDNA clones C8, B8 and B2. Clones G1, C8 and B2 displayed a reduced but detectable expression in the mutant animal, in contrast to clone B8 whose expression was not dependent on the RXRα genotype. Note the persistent ventricular expression of MLC-2a in the mutant heart. The atrial myosin light chain (MLC-2a) antisense riboprobe was used as a control for the integrity of the RNA in both embryos, as well as a control for the aberrant expression in the RXRα mutant null embryo (as reported in Dyson et al., 1995). The name of each probe is shown in the right panel. (B) Midsagittal sections through RXRα wild-type (+/+) or null mutant (–/–) E13.5 littermates were hybridized with either clone w70 (CARP) or MLC2a riboprobes (central panels). Right and left panels show hematoxylin staining of the embryos. Note the reduced expression of CARP mRNA in the RXRα mutant embryos, while MLC2a expression was higher in the mutant ventricle. v, ventricle; a, atria; L, liver; d, diaphragm; t, tongue.

and greatly attenuated in the RXRα$^{-/-}$ embryos (Fig. 2A). A few of the initially selected clones (e.g. clone B8, Fig. 2A) displayed no expression differences in RXRα mutant embryos compared to wild-type. A cardiac-restricted mRNA clone (w70) was also isolated in this subtractive screening. w70 encoded CARP mRNA (Zou et al., 1997). CARP expression in the mouse embryo was dependent on the RXRα genotype, being dramatically lower in the mutant E13.5 embryos (Fig. 2B).

The RXRα mutation results in embryonic lethality between E13.5 and E16.5 in homozygosity (Sucov et al., 1994; Kastner et al., 1994). Since our subtracted libraries and the initial validation of the clones were performed at E13.5, the possibility exists that this differential expression is not a primary consequence of the RXRα genotype, but rather represents a secondary effect due to embryonic lethality. To specifically address this issue, we performed a set of in situ hybridization experiments to analyze the expression of the subset of the subtracted clones at earlier time points in murine embryonic development. Homozygous embryos from E9.5-E11.5 are normal and identical to wild-type and heterozygous littermates (Sucov et al., 1994). Therefore, we performed expression analysis at E10.5, when no signs of embryonic lethality can be detected, a time point when any differential expression of candidate genes would be the consequence of RXRα deficiency. As shown in Fig. 3, clone G1 displayed differential expression in E10.5 RXRα$^{-/-}$ embryos compared to controls. The ubiquitous distribution of G1 was already detected at this developmental stage and its expression was significantly lower in the mutant embryos (Fig. 3A,B). A



tendency towards decreased expression was also detected for clone 3v11 (Fig. 3C,D). In addition, the hybridization signal of CARP (clone w70) was detected in E10.5 as strong cellular aggregates in the ventricular trabecular muscular layer (Fig. 3E-H). In RXRα mutant littermates, the CARP signal was barely detectable from background and displayed a more sparse signal (Fig. 3). Clone C8 did not show any significant difference in expression compared to controls at this developmental stage (not shown). Other subtracted clones had a later onset of expression and thus we were not able to detect

Table 1. Subclassification of subtracted cDNA clones isolated from wild-type versus RXRα$^{-/-}$ mouse embryos 13.5

Subclass	Sequence homology/motif	Embryonic expression pattern	Frequency	Downregulation, as per in situ hybridization
Metabolic genes				
G1	14.5bNADH-Q DH	Ubiquitous	33	Yes
w85	NADH-I	Tongue, pituitary	7	Yes
3v12	Glucagon receptor	Ubiquitous	7	Yes
3v11	F1,6 bP aldolase	Ubiquitous	10	Yes
3v16	Phosphorylase	Ubiquitous	4	Yes
Novel genes				
C8	Novel	Fetal liver	12	Yes
3v2	Novel	Ubiquitous	3	Yes
2B2	Novel	Fetal liver	8	Yes
10v2v5	Novel	Heart	1	No
B8	Novel	Skeletal muscle	2	No
Other				
w70	CARP*	Cardiac muscle	4	Yes
v17	PSD-95	Ubiquitous	2	Yes
3v9	Rhodopsin	Skeletal, brain, neural	7	Yes
4G6	Mitochrondrial DNA		15	ND
		Total	115	

*See Zou et al., 1997.

Name of the subtracted clones, sequence homology and frequency of a determined sequence among isolated clones are indicated. Individual clones were analyzed independently by in situ hybridization. Whether they were differentially expressed (downregulated) in the mutant RXRα compared to the wild-type embryos is indicated in the right column. (ND, not determined.)

hybridization signal in early wild-type embryos, making any comparison with mutant embryos difficult for certain subtracted clones. Taken together, these results suggest that differences in the expression of a subset of the subtracted clones are clearly a direct consequence of a lack of expression of RXRα in the mutant embryos.

Gene dosage effect of RXRα deficiency on expression of the NADH: ubiquinone oxidoreductase gene

Whereas the RXRα null mutation is lethal around E14.5, heterozygotes for this mutation survive and reproduce comparable to wild-type mice (Sucov et al., 1994). Nevertheless, a small proportion of heterozygote embryos have an intermediate phenotype and display some of the alterations observed when the RXRα gene is completely absent (Gruber et al., 1996). We determined whether heterozygosity implied an intermediate level of NADH:ubiquinone oxidoreductase 14.5b mRNA or alternatively, a two-copy RXRα gene threshold was required. We performed northern blot analysis on total RNA from E13.5 mouse embryos of the three RXRα genotypes. Clones C8 (novel) and G1 (NADH:ubiquinone oxidoreductase 14.5b) were abundantly expressed in the wild-type embryo, with dramatically attenuated but detectable levels in the null mutant. Whereas C8 mRNA content in the RXRα heterozygous embryos was similar to that in the wild type, G1 displayed intermediate concentrations of transcripts in the RXRα heterozygote (Fig. 4). These results document an intermediate molecular phenotype for a subset of the RXRα downstream target genes, such as this metabolic target gene, and provide a potential mechanism to explain the onset of defects in RXRα heterozygous deficient embryos (Gruber et al., 1996, unpublished data).

NADH:ubiquinone oxidoreductase is downregulated in the cardiac compartment of retinoid-deficient rat embryos

Retinoid-deficient (RDA) embryos share several phenotypic characteristics with the RXRα null mutation (Wilson and Warkany, 1949; Dickman et al., 1997). To test whether NADH:ubiquinone oxidoreductase 14.5b (clone G1) mRNA expression was potentially dependent upon ligand-activation of retinoid receptors in vivo, we studied G1 mRNA content in retinoid-deprived rat embryos. Retinoid-depleted embryonic hearts displayed significantly lower G1 mRNA expression than did their RA-sufficient controls, as shown by in situ hybridization (Fig. 5). These results suggest that both expression of retinoid receptors and their activation through specific ligands are required for the expression of this nuclear-encoded mitochondrial gene. However, a restricted number of tissues such as the neural tube and facial mesenchyme of the RDA embryos, seem to be less sensitive to retinoid depletion because G1 mRNA was clearly detected in these structures, with little downregulation.

Due to the altered expression of nuclear-encoded mitochondrial enzymes in RXRα-KO embryos, we tested whether mitochondrial structure or activity were modified in mutant embryos. Accordingly, we performed ultrastructural studies by transmission electron microscopy. In general,

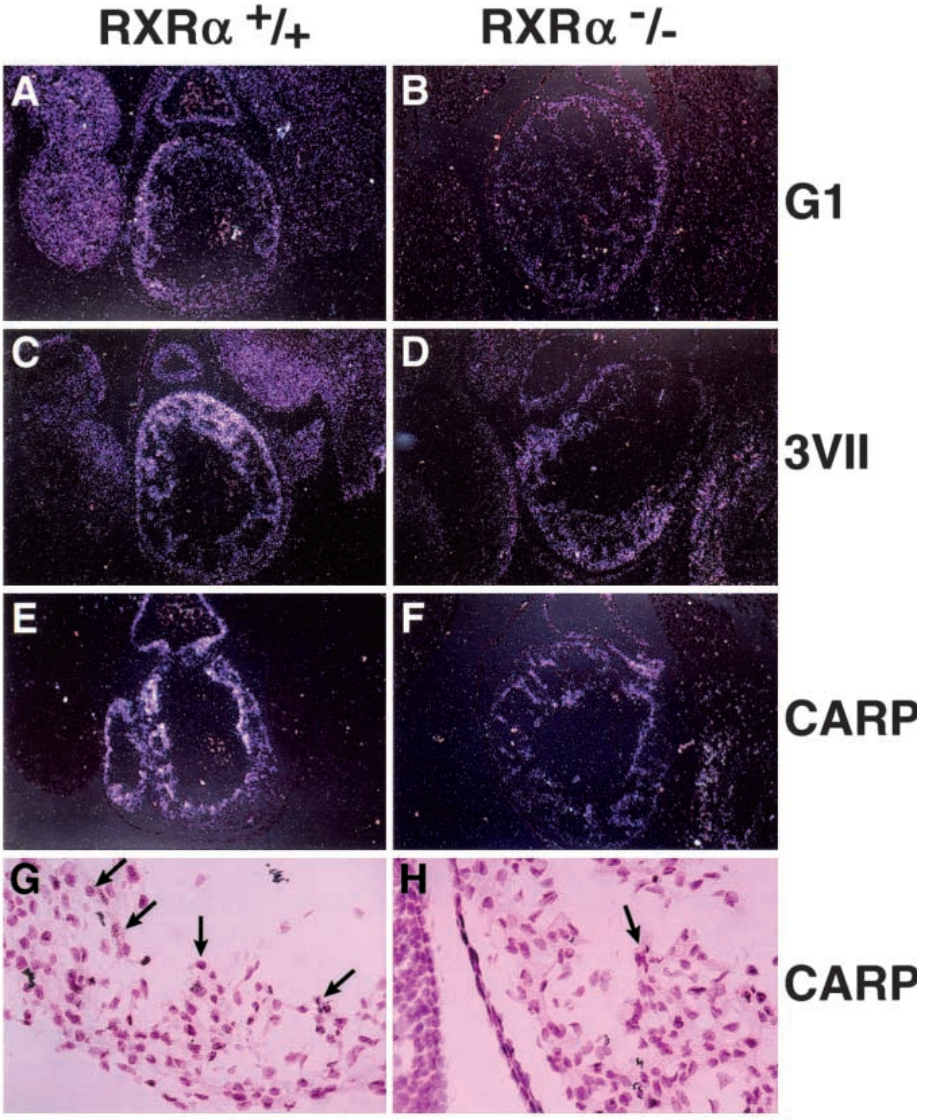


Fig. 3. Differential expression of several RXRα downstream genes occurs before overt signs of cardiac insufficiency. Midsagittal sections through wild-type ($RXR\alpha^{+/+}$; A,C,E,G) or null mutant ($RXR\alpha^{-/-}$; B,D,F,H) E10.5 littermates (age at which no signs of embryonic lethality are detected) were hybridized with riboprobes for the selected subtracted clones, G1 (14.5b NADH:ubiquinone DH), 3v11 (Fructose 1,6bP aldolase) and w70 (CARP). Note the clear reduction of G1 and w70 expression in the mutant embryos versus wild type, and the trend towards reduction in 3v11. **(**A-F) ×100 magnification; (G,H) higher magnification (×400) of E and F, respectively.

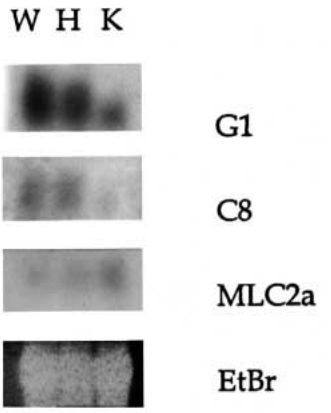


Fig. 4. Expression of some RXRα downstream genes is dependent on RXRα gene dosage. Northern blot of equal amounts of total RNA from the three different RXRα genotypes, W, wild type; H, heterozygote; K, mutant, was hybridized with probes for G1 and C8. As a control for mRNA integrity and aberrant gene expression, the blot was also probed using an atrial myosin light chain-2 (MLC-2a) cDNA. An ethidium bromide (EtBr) staining of the gel is also shown as a control for loading of the samples.

ultrastructure appeared normal in the mutant cells except for a higher degree of organization of contractile myofilaments (Fig. 6), which is in agreement with a previous report (Kastner et al., 1994). We subsequently measured the cellular volume that mitochondria occupied within the myocyte in the RXRα mutant embryo compared to the wild-type by stereological methods. Stereology demonstrated that wild-type mitochondria volume density was 7.56±0.82% of the total cell volume, in contrast to a 9.07±0.79% displayed by mutant littermates (Table 2). While the greater cellular volume that mitochondria occupies was not statistically significant (P=0.075) compared to the wild type, the trend towards a mitochondrial volume difference between wild-type and mutant embryos should be noted.

Table 2. Stereological measurements of mitochondrial volume in cardiac tissue from wild-type and RXRα mutant embryos

Volume density	Wild-type	Mutant
Mean	7.56 (16)	9.07 (28)
Standard	0.82	0.79

The percentage of total cellular volume occupied by mitochondria (Volume Density) as determined by electron microscopy on similar sections of day 13.5 wild-type and RXRα mutant embryos. The numbers in parentheses refer to the number of measurements performed on sections for a given genotype. Data shown are mean values. Volume density dispayed a trend towards a mitochondrial volume difference between wild-type and mutant embryos (P=0.075, Student's t-test).

in three independent experiments, using a total of 20 embryos (6 wild-type, 9 heterozygous, 5 homozygous). These results suggest that mutations affecting mitochondrial oxidative systems have pronounced effects in organs, such as heart, that are more reliant on oxidative energy production, and are in agreement with previous observations (Scarpulla, 1996).

Alterations of expression of MCAD/LCAD genes in RXRα$^{-/-}$ embryonic hearts

The misexpression of genes involved in electron transport in the RXRα mutant embryo suggests decreased oxidative phosphorylation. To test whether decreases in ATP content would be accompanied by modified expression of β-oxidation enzymes, we analyzed MCAD (medium-chain acyl-CoA dehydrogenase) and LCAD (long-chain acyl-CoA dehydrogenase) mRNA expression in embryonic tissues. MCAD and LCAD catalyze pivotal reactions in mitochondrial fatty acid oxidation and serve as indicators of mitochondrial β-oxidation. Since nuclear receptors regulate MCAD transcription, we studied MCAD expression as a candidate gene dependent on RXR signaling. MCAD mRNA expression was detected in the heart at E13.5 (Fig. 8A). Comparison of

Selective energy deprivation in the embryonic hearts of RXRα$^{-/-}$ embryos

To initially assess energy status in the RXRα mutant, we determined the ATP content in different embryonic tissues. ATP content in skeletal muscle and fetal liver was similar in the three RXRα genotypes. In contrast, a 60% reduction of the ATP content per milligram of total protein was observed in the isolated ventricles of RXRα null mutant embryos. Heterozygotes displayed an ATP concentration similar to wild-type samples in every tissue measured (Fig. 7). These data were reproduced

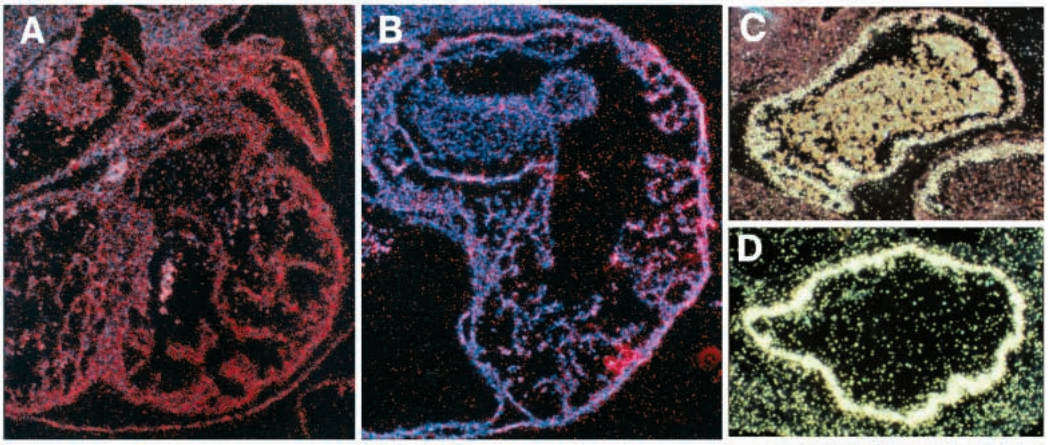


Fig. 5. Differential cardiac gene expression in retinoid-deficient rat embryos. In situ hybridization on embryonic hearts day 13.5 from retinoid-sufficient (A,C) and retinoid-deficient rat embryos (B,D). Cardiac expression of clone G1 (14.5b NADH:ubiquinone oxidoreductase) was determined using a G1 antisense riboprobe in retinoid-deficiency (B, red signal) and compared to retinoid-sufficiency (A, red signal). Hybridization signal is shown as red silver grains in dark field, with tissue counterstained with Hoechst dye (blue). The expression of the atrial myosin light chain (MLC-2a) was not reduced in retinoid deficiency (compare silver grain in sufficient (C) with RA-deficient (D)) and served as a control for the integrity of the RNA in both embryos, as well as a control for the specificity of G1 reduction.

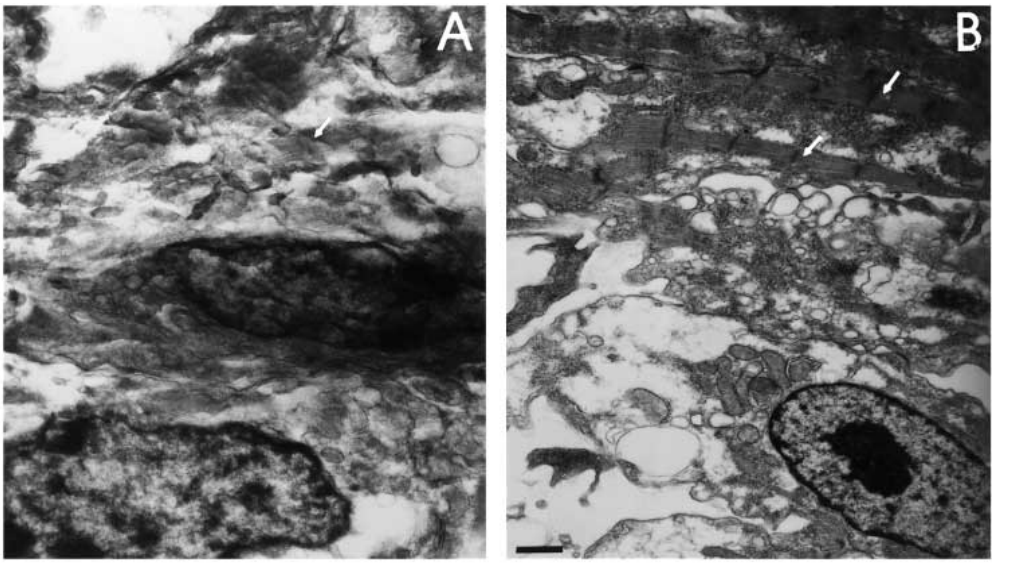


Fig. 6. Transmission electron microscopy of wild-type (A) and RXRα mutant (B) cardiac tissue at E13.5. The micrographs were taken from thin sections (50 nm). Morphological appearance was normal in both tissues. Note the more mature aspect of contractile myofibrils in the mutant sample (arrow). Scale bar: 1 cm = 1.3 nm.

wild-type embryos with RXRα mutant littermates at E13.5 revealed a dramatic reduction of MCAD mRNA concentration in RXRα null mutants (Fig. 8B,C). MCAD mRNA levels in heterozygotes were similar to wild-type embryos (not shown). LCAD mRNA was not detected in embryonic tissues by in situ hybridization. These results suggest that β-oxidation may be impaired in the setting of RXRα deficiency.

DISCUSSION

We have used subtracted hybridization to investigate the molecular basis of retinoid-associated cardiomyopathy (RAC) and have identified a collection of metabolic genes that suggest an intriguing relationship between cardiac morphogenesis and cardiac performance. This type of form and function linkage has not been previously described and may have broader applications to other developmental systems.

Utility of the PCR tag subtraction cloning strategy to identify a panel of RXRα downstream target genes

To dissect the complex molecular and positional cues that guide various states of cardiac morphogenesis, a number of laboratories have employed gene-targeting approaches to examine the physiological role of putative cardiogenic candidate genes. Various of these candidate genes were selected by virtue of their temporal and spatial patterns of expression during cardiogenesis (Lints et al., 1993), by their functional effects in vitro and in vivo (Srivastava et al., 1995, 1997), or by their ability to control the cardiac gene program (Jiang and Evans, 1996; Murphy et al, 1997; Lyons et al., 1995b; Kuo et al., 1997; Molkentin et al., 1997, Sucov et al., 1994; Moens et al., 1993; Chen et al., 1994). A wide variety of defects have been reported that include abnormalities in the aortic arch/aortic sac, conotruncal and outflow tract, individual defects in the right and left ventricle, defects in trabeculation, defects in the late stages of cardiac chamber morphogenesis and defects in the atrioventricular cushions (for review, see Fishman and Chien, 1997). In particular, the retinoid family of nuclear receptors appear to play an important role in cardiac

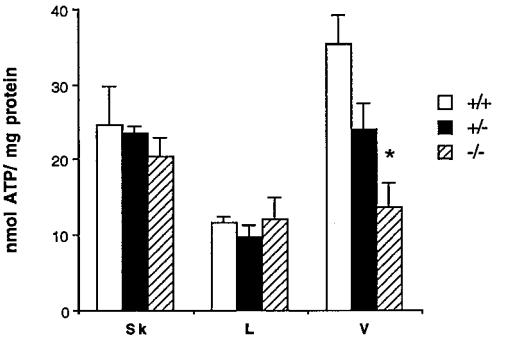


Fig. 7. ATP content. ATP content was measured in different embryonic tissues of various RXRα embryos (+/+, wild type; +/–, heterozygote and –/–, null mutant). Tissues were: Skeletal muscle (Sk), liver (L) and ventricle (V). ATP measures were normalized as nmol of ATP per milligram of total protein. Data are plotted as mean values and standard error. *Value statististically significant from control as per one factor ANOVA analysis (P<0.05).

function. A single mutation of RXRα is necessary and sufficient to yield profound defects in cardiac morphogenesis while RAR receptors have synergistic effects to mutations in RXRα, thus suggesting that RXR/RAR are the physiologically active partners (Kastner et al., 1997; Lee et al., 1997).

Several strategies have been described to search for components of the retinoid signaling pathway, including in vitro studies on retinoic acid-responsive cells (Jonk et al., 1994; Bouillet et al., 1995) and direct examination of candidate genes (Dyson et al., 1995). Via the isolation of in vivo targets of RXRα by subtractive hybridization, we have systematically analyzed the mRNA population that is downregulated in the mutant versus the wild-type embryos. To our knowledge, this study provides one of the first reported comprehensive analyses on gene expression based on a genetically modified mouse model and supports the utility of this approach toward the analysis of the growing list of mice that harbor a deficiency in a specific transcription factor. This could be of particular value in the event that early embryonic lethality is a feature of the

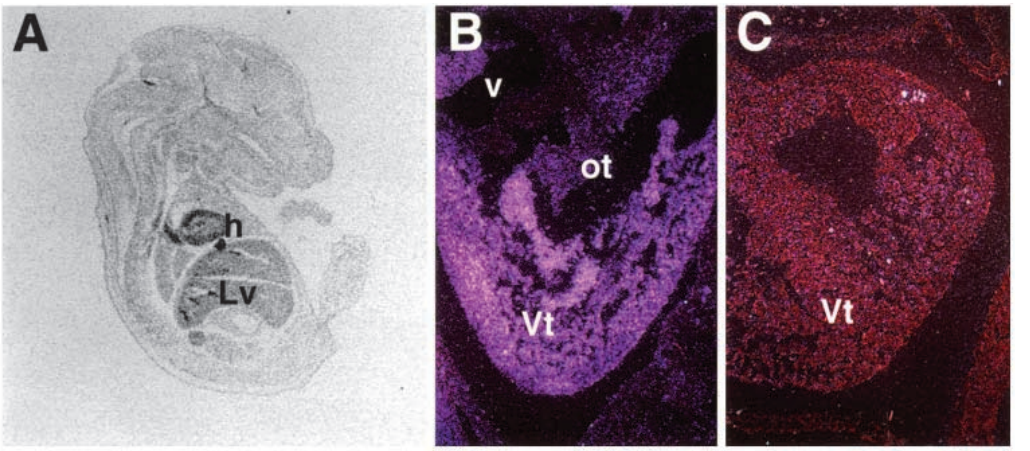


Fig. 8. Medium Acyl-CoA dehydrogenase (MCAD) mRNA expression in the mouse embryo. MCAD antisense riboprobe was used for in situ hybridization on: (A) mRNA is enriched in the day 13.5 embryonic heart; (B) MCAD mRNA expression in the wild-type ventricles and (C) in the heart of a RXRα mutant littermate. Lv, liver; h, heart; Vt, ventricle; ot, outflow tract.

phenotype. In this regard, the current study suggests that whole-embryo libraries may uncover downstream target genes in organ systems that do not display gross overt morphogenic defects. The strategy has the strength of allowing the subtraction to occur in either direction, such that one can isolate genes that are either activated or inhibited by RXRα. Evidence as to whether these represent direct or indirect target genes can be inferred by examining whether there is a gene dosage effect in the homozygous versus heterozygous deficient state, as noted for one of the target genes in the current study. Alternatively, transient assays and identification of known response elements can also be informative, as has been shown with the MLC-2a promoter, which appears to be under the control of a RXR/PPAR heterodimer pathway (Ruiz-Lozano, Merki and Chien, unpublished observations). In the current study, several genes have been found that encode novel proteins with unknown function and no homology to known motifs; others, like CARP, represent cardiac proteins whose sequences were previously reported and whose precise role in in vivo cardiac development have not yet been reported (Zou et al., 1997). A subset of mRNAs misexpressed in RXRα mutants is a collection of metabolic genes, suggesting the possibility of energy deficiency in the RXRα mutants.

RXRα pathways in the control of downstream metabolic target genes

In the systematic examination of putative downstream target genes by this subtraction cloning strategy, we have found that a high proportion of the subtractive clones encode proteins involved in energy metabolism. For example, bovine heart Complex I is composed of 41 different subunits and is the largest proton-translocating oxidoreductase in the mitochondria respiratory chain (Walker et al., 1995; Pilkington et al., 1993). This complex catalyzes the transfer of electrons from NADH to ubiquinone which is coupled with a vectorial transfer of protons across the mitochondrial membrane, thereby driving ATP formation (for a review, see Ohnishi, 1993). Interestingly, in the RXRα-deficient embryos, there was a virtual complete deficiency in the subunit 14.5b of Complex I. Since subunit 14.5b of Complex I (clone G1) was present at a 50% level in the RXRα heterozygous deficient embryos, this relationship supports the concept that this gene may indeed be a direct target of RXRα during the course of cardiac development in a manner dependent on RXRα gene dosage.

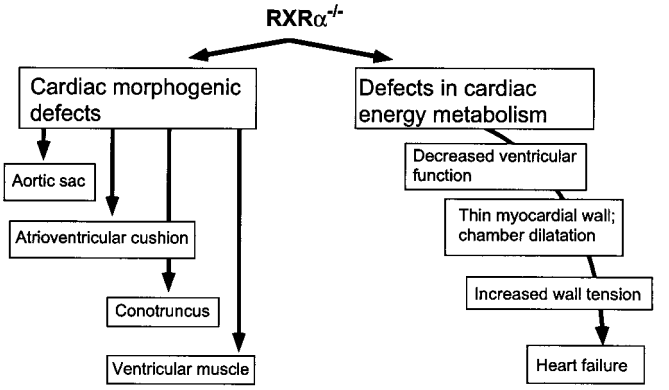


Fig. 9. Working model for the onset of ventricular chamber dysmorphogenesis in RXRα$^{-/-}$ embryos. RXRα deficiency affects the expression of genes responsible for morphogenesis and genes that regulate embryonic metabolism. A severe energy deprivation exists due to the downregulation of metabolic target genes associated with cardiomyopathy. Subsequent to a decrease in ventricular function, the chamber dilation, thinning of the ventricular wall and ultimately heart failure ensue as a secondary event.

G1 mRNA misexpression in retinoid-deficient rat embryos further supports a direct role of ligand-activated retinoid receptors on the regulation of metabolic genes.

A severe deficiency in this component would be expected to act in the mitochondrial respiratory chain in a manner analogous to the delivery of respiratory inhibitors that work at the ubiquinone step, such as synthetic capsaicin analogs (Satoh et al., 1996). Therefore, ATP depletion would be expected to be particularly prominent in organ systems that are highly dependent on high energy phosphates for the maintenance of normal function. Since the heart requires ATP for the maintenance of its normal contractility and displays a high degree of ATP utilization to maintain this function (Kelly and Straus, 1994), it would be expected that any impairment in this process might first appear in cardiac tissues. Consistent with this notion, the ATP levels in the ventricles of RXRα-deficient embryos were significantly lower than control and approached levels that are consistent with marked energy deprivation. Thus, associated with this deficiency in the mitochondria respiratory chain, there is clear evidence of energy deprivation. This effect appeared to be selective for the heart, as ATP

content in other tissues within the mutant embryo were maintained at control levels. The specific decrease in ATP content in the mutant heart may imply a metabolic deficiency for which the heart is a more sensitive tissue. The indication of an increased volume density of mitochondria in the RXRα mutant may suggest the activation of compensatory mechanisms in metabolically stressed cells that may be activating mitochondrial biogenesis. Similar mechanisms have been previously described in Huntington disease (Gobel et al., 1978; Brouillet et al., 1995) as well as in oxidatively stressed tissues (Steeghs et al., 1997).

In addition to this defect in mitochondrial electron transport, as reflected in reduced expression of the 14.5b subunit of mitochondrial Complex I, we also observed diminished expression of another critical enzyme in cardiac energy metabolism, the medium chain acyl-CoA dehydrogenase (MCAD) gene. MCAD catalyzes a pivotal step in mitochondrial fatty acid oxidation. Mutations in proteins involved in β oxidation are the most common known inherited metabolic diseases, with an estimated incidence of 1 in 10,000 to 1 in 15,000 live births (Coates, 1992). Several of these cardiomyopathies are caused by abnormalities in carnitine transport or by a deficiency in mitochondrial enzymes in fatty acid β-oxidation (for a review see Kelly and Strauss, 1994). Cardiac β-oxidation reaches its highest levels after birth, which represents the major source of energy in the adult mammalian heart (Neely et al., 1972). Restriction of MCAD expression to the mouse embryonic heart suggests that embryonic cardiac muscle is dependent to a certain extent on medium-chain fatty acids as an energy supply. Thus, the deficient expression of MCAD in the mutant embryos implies the impairment of this pathway in the absence of RXRα expression. These dual defects on both the mitochondrial respiratory chain and in fatty acid metabolism may contribute synergistically to the decrease in ATP levels and energy deprivation seen in the RXRα$^{-/-}$ embryos. Interestingly, previous studies have examined the pathways that are responsible for regulating MCAD expression in cardiac muscle. Recent studies have documented that a peroxisome proliferation activated receptor (RXR-PPAR) may be critical for the regulation of the MCAD gene transcription (Gulick et al., 1991; Leone et al., 1995). A promoter fragment that contains consensus retinoid response elements is required for the regulation of this gene in response to various hormonal stimuli (Sack et al., 1997).

The RXRα cardiac phenotype may represent a secondary effect of RXRα on metabolic pathways and consequent dilated cardiomyopathy

The phenotype seen in RXRα-deficient hearts is mimicked by a diversity of heart defects in mice that harbor deficiencies in a wide variety of transcriptional factors and other signaling molecules, including WT-1, βARK-1, myc and TEF-1 (Kreldberg et al., 1993; Jaber et al., 1996; Moens et al., 1993; Chen et al., 1994). This phenotype, known as the 'thin myocardial wall syndrome' (Rossant, 1996), has been thought to be due to a maturational arrest in ventricular lineages, reflecting an inherent defect in myocardial proliferation and/or to be an index of relative hypoplasia of the ventricular wall. However, given that the current study shows direct evidence for severe energy deprivation and downregulation of genes associated with cardiomyopathy in the human setting, a distinct alternative possibility exists that the RXRα phenotype, and perhaps the other phenotypes as well, actually represents the process of chamber dilation that is seen in the setting of adult heart failure. This view is supported by the recent finding that the requirement for RXRα in preventing 'thin wall myocardial' syndrome is not in ventricular muscle cells per se (Chen and Chien, 1998). In the mouse, and in other species, including man, heart failure is associated with cardiac chamber dilatation and a thinning of the ventricular wall. This phenotype is also seen in a genetically based mouse model in MLP-deficient mice, which display cardiac chamber dilation during the onset of dilated cardiomyopathy and heart failure (Arber et al., 1997). The finding of embryonic heart failure, as documented by a decrease in cardiac contractility (Dyson et al., 1995), in combination with the current study, which identify downstream metabolic target genes for RXRα, suggests a mechanistic model in which RXRα regulates at least two separate sets of genes: genes responsible for morphogenesis and genes that control energy status. Alterations in the expression of cardiac morphogenetic genes would result in defects in the aortic sac, atrioventricular cushion, conotruncus or ventricular muscle. A deficiency in maintaining energy status of cardiac muscle would result in a decrease in ventricular chamber function (Neubauer et al., 1997), concomitant dilatation and thinning of the myocardial wall, resultant increases in wall tension and finally heart failure (Fig. 9). Our studies underscore the potential regulatory importance of the maintenance of mouse cardiac function in the course of cardiac chamber morphogenesis, even though we can not rule out the possibility of a certain degree of cross-talk between the two pathways. Taken together, we propose a model whereby the relative thinning of the ventricular wall and dysmorphogenesis of the ventricular chamber in the RXRα-deficient mice may represent a secondary effect of the onset of a dilated cardiomyopathy due to energy deprivation as a result of dysregulation of a panel of metabolic target genes. Further studies on the mechanistic role of both the morphological and metabolic target genes in the onset of specific cardiac morphogenic defects is warranted to critically test this model.

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