Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 1590–1594, February 1993
Developmental Biology

Normal development and growth of mice carrying a targeted disruption of the α1 retinoic acid receptor gene

(homologous recombination/gene targeting)

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Contributed by Ronald M. Evans, November 5, 1992

ABSTRACT **Three unlinked genes encode receptors for retinoic acid (RARα, -β, and -γ). Each gene expresses two major protein isoforms differing in the amino terminal A domain by alternative promoter use, fused to common exons encoding most of the receptor protein. The two RARα transcripts (RARα1 and -α2) are differentially expressed and evolutionarily conserved, as are the RARβ and -γ transcripts, suggesting that each isoform may have specific functions in the development of animals. To address the biological function of the α1 receptor, we have disrupted the portion of the RARα gene encoding this isoform by homologous recombination in mouse embryonic stem cells. Surprisingly, offspring homozygous for this mutation were viable and showed no apparently altered phenotype. RNA analysis confirmed that the RARα1 transcript was absent in homozygous tissues, and no evidence for a compensatory increase of RARα2 or of another RAR gene was obtained to account for the vitality of the mutant animals. These results clearly demonstrate that loss of RARα1 function does not disrupt embryonic development and argue for combinatorial or overlapping functions among the RAR isoforms.**

Retinoic acid (RA) is a vitamin A derivative that exerts profound influences in vertebrate development and physiology. Embryonic exposure to RA causes a wide spectrum of severe malformations in human (1), rodent (2, 3), chicken (4), and *Xenopus* (5, 6) offspring, including neural tube and central nervous system defects, skeletal defects, cleft palate, ear, and other craniofacial malformations, defects in the heart, thymus, and urogenital system, and limb and digit reduction or duplication. Because RA and related retinoids are found in at least some of these tissues at physiological concentrations (7–11), these experimental defects probably result from interference in processes that naturally use RA. In the neonate and adult, RA is likewise implicated in many diverse roles, including growth, epithelial homeostasis, and immunocompetence.

RA signaling is mediated by two distinct classes of receptors, RA receptors (RARs) and retinoid X receptors (RXRs). These receptors are members of the nuclear receptor family and function by directly activating transcription via binding to promoter elements of target genes. Each family is composed of three genes: RAR α, -β, and -γ, and RXRα, -β, and -γ. There is both convergence and divergence in the signal transduction pathways between the RAR and RXR families and between the different members of each family. For example, all trans-RA is a high-affinity ligand for the RARs but not for the RXRs, yet is isomerized *in vivo* to 9-*cis*-RA, which is a ligand for both receptor families (10, 12). The RARs must heterodimerize with RXR to bind DNA with high affinity, whereas RXR can form a functional homodimer (13–17); each complex transcriptionally activates through related promoter elements composed of direct repeats with different spacings (18, 19), and there is evidence for negative cross regulation via transcriptionally nonproductive binding (19). Each individual member of the RAR and RXR subfamily is differentially expressed and differs functionally from the other subtypes in both ligand binding and transcriptional activation profiles. For the RARs, additional variation is generated by alternative promoter use to generate two major isoforms (α1, α2, etc.) that differ in the amino terminal "A" domain (20, 21); this is likely to be true for the RXR genes as well (unpublished observations). The existence of distinct RAR and RXR subfamilies and of receptor isoforms indicates that these receptors might be involved in distinct physiological processes. Systematic application of homologous recombination techniques to generate mutations in individual genes provides an *in vivo* genetic approach to address the contribution of each receptor by determining the consequences of its absence.

To initiate this process, we have undertaken to determine the biological role of RARα1 by creating in the mouse germ line a targeted mutation in the α1-specific portion of the RARα gene. RARα1 is the most abundantly expressed of the RARs; it shows widespread, if not ubiquitous, expression in embryonic *in situ* hybridization studies (ref. 22; U. Borgmeyer and R.M.E., unpublished observations) and in RNA blots with embryonic and adult tissue RNAs (23–25). In comparison, α2 and the other RAR isoforms exhibit a more restricted pattern of expression. To our surprise, mice homozygous for this mutation are viable and show no obviously altered phenotype. This result suggests a redundancy in function between the retinoid receptors and illustrates the complexities of retinoid signal transduction.

MATERIALS AND METHODS

Targeting Construct. Genomic clones were isolated from a DBA/2 mouse liver genomic library (Clontech) screened with a RARα1 cDNA probe. A *Sal* I site was introduced into the coding region at the sixth codon by oligo-directed mutagenesis. The targeting vector was constructed by a deletion of the *Sal* I–*Xba* I segment replaced with a neo cassette. Both the *neo* and thymidine kinase genes are driven by the phosphoglycerate kinase 1 promoter (26). The construct was linearized by restriction at a unique vector *Not* I site before electroporation.

Cells. Embryonic stem cells of the J1 line (26) were grown on γ-irradiated embryonic fibroblast feeder cells, and at passage 8–10 were transfected by electroporation, as described (26). Selection with G418 (GIBCO) at 350 μg/ml (as dry powder) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosil)-

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Abbreviations: RT, reverse transcriptase; RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; FIAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosil)-5-iodouracil.

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5-iodouracil (FIAU) (Bristol-Myers Squibb, Wallingford, CT) at 0.2 μM was begun 36 hr after plating. Doubly resistant colonies were pooled, and DNA isolated from each pool was screened by Southern blot hybridization. Individual clones from positive pools were thawed and genotyped, and clones containing the targeted disruption were identified and used for generation of chimeric mice, as described (26). Chimeric males were outcrossed or were bred to 129/terSv females to produce heterozygotes on an outbred or inbred genetic background.

Hematopoietic Cell Analysis. Splenocytes, thymocytes, and bone-marrow cells isolated from adult heterozygotes and homozygotes were analyzed by analytical flow cytometry (FACS), using the following cell-surface markers: CD3-ε, CD44, TH B, B220, IG, GR-1, MAC-1, CD4, CD5, CD8, and HSA.

Skeleton and Organ Analysis. Adult animals were sacrificed, and internal organs were removed. Carcasses were cleared by extended soaking in 0.25 M NaOH, and skeletal elements were stained by adding a few drops of a saturated solution of alizarin sodium sulfonate/ethanol.

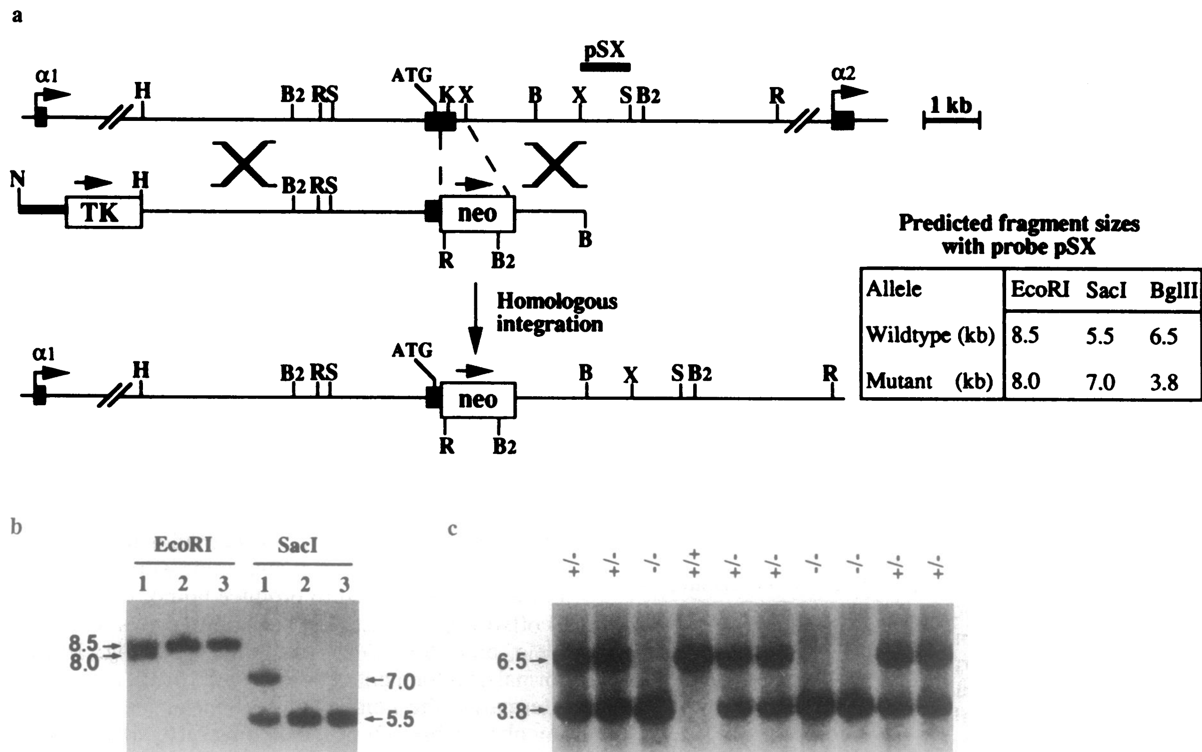
RNA Analysis. Total RNA was isolated from tissues of wild-type, heterozygous, and homozygous *RARα1⁻* adult mice by Polytron disruption in guanidinium thiocyanate, followed by acid phenol extraction and isopropanol precipitation (27). For Northern blots, 12 μg of total RNA was electrophoresed over a formaldehyde denaturing agarose gel and then transferred to nitrocellulose. The probes used were all derived from mouse cDNA clones (U. Borgmeyer and R.M.E., unpublished work). The RARα probe was a 1.3-kb *Pst* I-(*Eco*RI) fragment containing a small part of the C domain through the 3′ untranslated region; the RARβ probe was a 600-bp (*Eco*RI)-*Eco*RI fragment from the E domain; and the RARγ probe was a 1-kb (*Eco*RI)-(*Eco*RI) fragment containing domains B–E. Hybridization was in 50% (vol/vol) formamide at 42°C, followed by three washes at 60°C with the final wash in 0.7 × standard saline phosphate/EDTA (SSPE; 1× SSPE is 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/0.1% SDS. Reverse transcription reactions used 1.5 μg of total RNA and 100 ng of gene-specific primer in a 10-μl reaction volume at 37°C for 2 hr with Superscript reverse transcriptase (RT) (BRL); the reactions were then heat killed and diluted with Tris/EDTA buffer. Amounts of the RT reaction to use in PCR amplification were determined empirically for each set of samples; the amount of cDNA amplified between wild-type, heterozygous, and homozygous samples always represented the same amount of starting RNA. Standard PCR conditions used 200 ng of each primer in a 30-μl vol with 2.5 units of *Taq* polymerase (Boehringer Mannheim) in the recommended reaction buffer and were run at 92°C for 2 min, followed by 35 cycles of 92°C for 30 sec, 60°C for 30 sec, 72°C for 90 sec, with a 5-min final extension at 72°C. Twenty microliters of each reaction was electrophoresed over a 1.4% agarose gel. The specificity of the products was determined by blotting and hybridization (data not shown); note also that the PCR products span an intron/exon boundary and so cannot be accounted for by genomic DNA contamination. RT primers were from the E domains; PCR reactions used a common B domain antisense primer and isoform-specific A domain sense primers.

RESULTS

Targeting of the RARα1-Encoding Gene. A replacement-type targeting vector (28) was constructed, as diagrammed in Fig. 1*a*. The second exon of the RARα gene, which contains the protein-coding region of the α1 isoform, was disrupted by a neomycin-resistance gene under the control of the phosphoglycerate kinase 1 promoter. The 5′ and 3′ homologous genomic sequences were 5.5 kb and 1.5 kb long, respectively. A herpes simplex virus thymidine kinase gene was incorporated into the targeting construct to allow selection against cells that had undergone nonhomologous integration (29, 30). The linearized targeting vector was introduced into embryonic stem cells of the J1 line (26) by electroporation, and cells were selected in medium containing G418 and FIAU. Pools of doubly resistant colonies (three colonies per pool) were screened by Southern blot analysis for the presence of a 7.0-kb *Sac* I fragment derived from the mutated allele (Fig. 1*a*). Of 102 pools, 6 were positive for the disrupted allele. As FIAU selection resulted in a 5.8-fold enrichment, the frequency of homologous recombination was calculated to be ≈1 in 300 integration events. Individual clones containing the targeted disruption were identified from positive pools by a second round of Southern blot hybridization of DNA digested with *Eco*RI and *Sac* I (Fig. 1 *a* and *b*).

Production of RARα1⁻ homozygous mice. Four of the correctly targeted clones were used to make chimeric mice, all of which have transmitted the mutant allele to their offspring, as confirmed by Southern blot analysis of tail DNA (data not shown). Heterozygous mice were outwardly normal and were intercrossed to produce homozygous offspring. Of 65 offspring, 14 (21.5%) were homozygous for the disrupted allele, and 16 (24.6%) were wild type (Fig. 1*c*), indicating normal development of homozygous mice. As compared with littermates, the homozygous offspring were not unusual in any observable manner, including birth size, growth rate, or behavior. Furthermore, upon autopsy no obvious anatomical differences in any major organs were seen. Homozygous skeletons showed the normal C7/T13/L6 vertebral pattern. FACS analysis of spleen, thymus, and bone-marrow cells indicated that all major hematopoietic subtypes were present in normal frequencies (data not shown). Homozygotes have been raised to sexual maturity and when bred, produced normal offspring. Homozygous embryos isolated at 10.5 and 12.5 days of gestation appeared to have developed normally and synchronously with heterozygous littermates. Finally, when the mutant allele was crossed back into 129/terSv mice from which the J1 cells were derived, no obvious mutant phenotype was observed in *RARα1⁻* homozygotes in any of the above categories on the inbred genetic background.

RNA Analysis. Total RNA was prepared from skin, muscle, and liver of wild-type, heterozygous, and homozygous adult mice and was identified on Northern blots with a probe common to both RARα1 and α2 isoforms. Previous studies (23) have measured the relative amounts of α1 and α2 transcripts in a variety of tissues: skin, muscle, and liver show an α1/α2 ratio of 12, 48, and 0.8, respectively. As seen in Fig. 2*a*, the total amount of RARα transcript decreased in these three tissues as a consequence of the α1 mutation, to an extent proportional to the relative contribution of α1 to the total of all RARα messages. The most dramatic effect was seen in muscle, in which the level of RARα decreased by approximately half in heterozygous tissue and to below detectable levels in homozygotes. Because the α1 and α2 transcripts comigrate (the two bands seen on Northern blots represent differential 3′ processing), an RT/PCR assay was used to specifically visualize the α1 and α2 isoforms independently. As demonstrated for muscle RNA in Fig. 2*c*, the level of α1 transcript decreased ≈2-fold in heterozygous tissue relative to wild-type animals and was not detectable in *RARα1⁻* homozygous tissue, whereas the level of the α2 transcript was unaffected. The faint background bands seen in some lanes are not genuine RARα transcripts, as they were not labeled when the gels were blotted and appropriately probed (data not shown). These results are as predicted from the structure of the mutant allele and confirm at the RNA level that the α1 gene was disrupted with no effect on the α2 message.



Allele	EcoRI	SacI	BglII
Wildtype (kb)	8.5	5.5	6.5
Mutant (kb)	8.0	7.0	3.8

FIG. 1. Targeted disruption of the RARα1 gene in the germ line of mice. (*a*) Homologous integration of the targeting vector into the RARα locus. The targeting vector, shown in the middle line, contains a *neo* gene inserted into the α1-specific second exon (filled box) immediately downstream of the initiation ATG codon and a thymidine kinase (TK) gene to allow counter selection. Integration into the target locus (*Upper*) disrupts the RARα gene (*Lower*). Arrows indicate transcriptional start sites and orientation of α1- and α2-specific transcripts and transcriptional orientation of the *neo* and thymidine kinase genes. Homologous recombination was detected by probing Southern blots with probe pSX, and the expected sizes of diagnostic restriction fragments are at right. Restriction sites are as follows: B, *Bam*HI; B2, *Bgl* II; R, *Eco*RI; H, *Hin*dIII; K, *Kpn* I; S, *Sac* I; X, *Xba* I. (*b*) Southern blot hybridization of embryonic stem cell clones from a positive pool. DNA of individual clones was digested with the indicated restriction enzymes and hybridized with probe pSX. Clone 1 contained a targeted disruption of the RARα1 gene, as shown by the 8.0-kb *Eco*RI and the 7.0-kb *Sac* I fragments derived from the disrupted allele. (*c*) Genotypic analysis of offspring derived from an intercross of RARα1 heterozygous parents. Tail DNA digested with *Bgl* II was blotted and hybridized with probe pSX. Of 10 progeny, 3 were homozygous (−/−), 6 were heterozygous (+/−), and 1 was wild type (+/+).

Of the tissues analyzed and in all three genetic backgrounds, only the RARγ gene is expressed at a level sufficiently high to permit detection by Northern blotting and only in skin. Fig. 2*b* shows that the level of RARγ transcripts in skin did not change significantly as a consequence of the RARα1 mutation. These results indicate that no quantitatively major change in transcription of the RARβ and -γ genes occurs in the mutant background. By using RT-PCR, low expression levels of additional RAR isoforms could be detected, as shown for muscle in Fig. 2 *d* and *e*. In muscle, no apparent alteration in the expression of the RARβ2, -γ1, or -γ2 messages was noticed in *RARα1*$^-$ heterozygous or homozygous mice; β1 transcripts were not detected (data not shown), in agreement with previous studies (20). These data argue against a compensation mechanism in which the expression of another RAR gene, or of the α2 isoform, would be up-regulated so as to substitute for inactivation of the RARα1 gene. We have also searched for but failed to find either an aberrant neo/RARα fusion transcript (note that the *neo* gene is in the same transcriptional orientation as the mutated RARα gene (Fig. 1*a*) or a shortened transcript that might have been derived from RARα exon I being spliced to an exon downstream of exon II.

DISCUSSION

The studies described here, in which the α1 isoform of the RAR gene has been specifically disrupted by homologous recombination, show that no obvious morphogenic or essential function depends upon the amino-terminal portion of the RARα gene. The A domains of corresponding isoforms are extremely well conserved between species (94–98%; ref. 21); however, there is only marginal homology in the amino terminal (A) domain between isoforms of the same gene or between different genes of the same species. This observation has led to a widespread belief that the amino-terminal portion of the receptor protein may possess a set of unique functional properties. For example, there is corroborating evidence that the amino terminus of the steroid receptors may be involved in target gene specificity (31, 32). Although the RAR isoforms are comparable in their ability to activate most of the known repertoire of RA-responsive genes, a small number of exceptions have been reported (33–35), but none described for RARα1. Our results indicate that any specific function encoded in the amino terminus of the RARα gene is phenotypically very subtle or nonexistent and in the absence of other genetic lesions is not essential.

It seems unlikely that the highly conserved and abundantly expressed RARα1 isoform has no function. Rather, we presume that, for the most part, the developmental and physiological role of RARα1 is supplanted by the other RARs in *RARα1*$^-$ homozygous mice. We note that in muscle, four of the five other major RAR isoforms are also expressed, although at levels considerably less than RARα1, and that reported Northern blots (23) indicate RARα2 to be expressed in all tissues studied, although at various and usually markedly lower levels than RARα1. Furthermore, the transcriptional activation of RARβ2 (36) and RARα2 (37) in response to RA may represent a natural process by which the α1 deficiency is overcome. The viability of mice lacking a

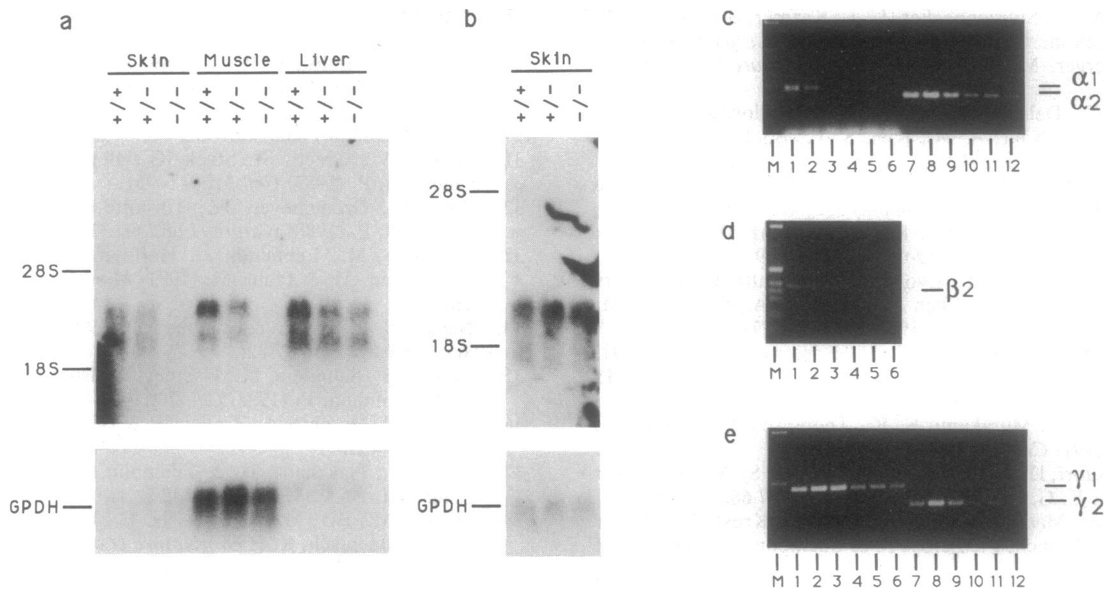


FIG. 2. Analysis of RNA in *RARα1⁻* tissues. (*a*) Northern blot of RARα transcripts in skin, muscle, and liver. Total RNA isolated from tissues of adult mice of the indicated genotypes was blotted and labeled with an RARα probe common to both α1 and α2 transcripts. The relative migration of 28S and 18S ribosomal RNA is indicated. After exposure, the blot was stripped and reprobed to identify the ubiquitously expressed glyceraldehyde phosphate dehydrogenase (GPDH) transcript. (*b*) Northern blot of RARγ transcripts in skin. Total skin RNA of the indicated genotypes was blotted and labeled with an RARγ probe common to both γ1 and γ2 transcripts and then stripped and reprobed to detect the GPDH transcript. (*c*) RT-PCR analysis of RARα1 and α2 transcripts in muscle. Muscle RNA was reverse transcribed; then aliquots were taken and amplified with a common-region antisense primer and α1- or α2-specific sense primers. Lanes: 1–6, α1 amplification; 7–12, α2 amplification. To ensure that the amount of product was proportional to the input amount of cDNA, parallel PCR reactions were run with a 4-fold difference in amount of starting material from the reverse transcription reaction. Lanes 1–3 represent PCR products from the same amount of cDNA from wild-type, heterozygous, and homozygous *RARα1⁻* muscle, respectively; lanes 4–6 represent PCR products from one-fourth the amount of cDNA used in lanes 1–3. Likewise, lanes 10–12 represent one-fourth the input amount of lanes 7–9. (*d*) RT-PCR analysis of RARβ2 transcripts in muscle. Lanes 1–3 and 4–6 are loaded as in *c*. (*e*) RT-PCR analysis of RARγ1 and γ2 transcripts in muscle. Lanes 1–3, 4–6, 7–9, and 10–12 are loaded as in *c*.

functional RARα1 gene is reminiscent of observations made on mice bearing loss-of-function mutations in other genes, including those encoding β_2-microglobulin (38, 39); c-src (40); En-2 (41), c-abl (42, 43), or the nerve growth factor receptor (44). These animals are viable and have no obvious phenotype or display a phenotype much less severe than might have been predicted considering the expression patterns of the respective wild-type genes. Functional redundancy between *Wnt* genes in the development of the caudal central nervous system, for example, has recently been suggested (45). Our results with the *RARα1⁻* mice suggest this may represent a general principle in regulatory networks controlling complex developmental processes. Nonetheless, complete redundancy for the function of RARα1 is surprising for at least two reasons—(*i*) the evolutionary conservation of the amino-terminal sequence of RARα1 indicates continued selection for a common function, although this function is apparently not essential, and (*ii*) RARα1 is clearly the most abundant of the RAR isoforms in most tissues. Although viability and gross anatomical inspection suggest a normal phenotype, a more detailed examination of the homozygous mice may reveal defects not readily apparent from the preliminary examination described here. Because RA has been most strongly implicated in embryonic development, as opposed to postnatal physiology, our initial expectation was to observe defects in embryogenesis. Although it is clear that embryonic development can proceed in homozygotes, certain developmental processes may be delayed or accelerated, or such embryos may have different sensitivity to the teratogenic effects of RA, or to retinoid deprivation. It is also possible that the consequences of the RARα1 mutation might only be seen in conjunction with a second mutation, in an RXR or RAR gene or other genetic loci. Furthermore, the mild environment and enriched diet of the laboratory setting does not provide the most rigorous test of gene function. Clearly, mutant animals will have to be analyzed under a variety of conditions for unambigously assessing the *in vivo* function of RARα1.

We are grateful to Bob Hyman (Salk Institute) for the FACS analysis and to Ron Wisdom (Salk Institute) for the GPDH probe. This work was supported by a grant from the National Institutes of Health/National Cancer Institute to R.J. (5-R35-CA44339-05) and by grants from the National Institutes of Health and the Mathers Foundation to R.M.E. R.M.E. is an Investigator of the Howard Hughes Medical Institute at the Salk Institute for Biological Studies. H.M.S. was supported by a Merck Postdoctoral Fellowship and by the National Institutes of Health. K.-F.L. was supported by an Anna Fuller Cancer Foundation Fellowship.

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