

Characterization of three RXR genes that mediate the action of 9-*cis* retinoic acid

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An understanding of the differences and similarities of the retinoid X receptor (RXR) and retinoic acid receptor (RAR) systems requires knowledge of the diversity of their family members, their patterns of expression, and their pharmacological response to ligands. In this paper we report the isolation of a family of mouse RXR genes encoding three distinct receptors (RXR α , β , and γ). They are closely related to each other in their DNA- and ligand-binding domains but are quite divergent from the RAR subfamily in both structure and ligand specificity. Recently, we demonstrated that all-*trans* retinoic acid (RA) serves as a "pro-hormone" to the isomer 9-*cis* RA, which is a high-affinity ligand for the human RXR α . We extend those findings to show that 9-*cis* RA is also "retinoid X" for mouse RXR α , β , and γ . *Trans*-activation analyses show that although all three RXRs respond to a variety of endogenous retinoids, 9-*cis* RA is their most potent ligand and is up to 40-fold more active than all-*trans* RA. Northern blot and in situ hybridization analyses define a broad spectrum of expression for the RXRs, which display unique patterns and only partially overlap themselves and the RARs. This study suggests that the RXR family plays critical roles in diverse aspects of development, from embryo implantation to organogenesis and central nervous system differentiation, as well as in adult physiology.

[**Key Words:** RXR α , β , and γ ; vitamin A metabolism; RXR ligands; retinoid receptors in development; nuclear receptor superfamily; 9-*cis* retinoic acid]

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Derivatives of vitamin A, or retinoids [e.g., retinoic acid (RA)], are a group of signaling molecules that are essential for several life processes including growth, differentiation, and epithelial homeostasis (Goodman 1984; Sporn et al. 1984). In the embryo, RA has also been implicated in many roles, including the formation of the developing nervous system (Dencker et al. 1987; Durston et al. 1989; Wagner et al. 1990), craniofacial differentiation (Wedden et al. 1988), and limb morphogenesis (Maden 1982; Eichele 1989). In contrast to the physiologic requirement of vitamin A for these processes, retinoids may be toxic or severely teratogenic when applied at high concentrations or at inappropriate times (Lammer et al. 1985). The heterogeneity of these responses suggests the existence of complex signaling pathways to account for the diverse regulatory roles of retinoids. In the past several years, two distinct classes of nuclear hormone receptors were identified that mediate RA-dependent transcription. The first class is comprised of the RA receptors (RAR α , β , and γ), all of which bind all-*trans* RA with high affinity and share a high degree of structural conservation (Giguere et al. 1987; Petkovich et al.

1987; Brand et al. 1988; Krust et al. 1989; Zelent et al. 1989; Ishikawa et al. 1990). Recently, we described the existence of a second class of receptor that also responds to retinoids but is substantially different in primary structure and ligand specificity to the RARs (Mangelsdorf et al. 1990). Because this new receptor, termed the retinoid X receptor (RXR), is activated by but does not bind to all-*trans* RA, we speculated that its ligand would be an RA metabolite.

The discovery in RXR of a second retinoid transduction pathway has led us to investigate its functional properties and determine its relationship to the RAR system. The initial observation that RXR is abundantly expressed in metabolic organs, such as the liver, kidney, and intestine, suggested a role for RXR in the regulation of vitamin A metabolism itself (Mangelsdorf et al. 1990). More recently, we have shown that a potent target for RXR action is the gene for cellular retinol-binding protein type II (CRBP II), a protein involved in the processing of dietary vitamin A in the intestine (Mangelsdorf et al. 1991). The retinoid X response element (RXRE) found in the CRBP II gene promoter is strongly up-regulated by RXR but not by RAR. Furthermore, RXR activation through the CRBP II–RXRE can be completely inhibited

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by RAR. This interaction between RXR and RAR is made more intriguing by the recent finding that RXR can form stable solution heterodimers not only with RAR but also with vitamin D and thyroid hormone receptors (Kliwer et al. 1992). Thus, RXR may be a constituent in multiple hormonal signaling pathways. To better understand the complex functions of RXR, we have explored the diversity of the family, its patterns of expression, and its pharmacologic properties in response to ligand. In this paper we report the isolation of mouse cDNAs encoding three RXR proteins (mRXR α , β , and γ). Each of these RXRs is expressed throughout development and in the mature organism in patterns that are both unique and partially overlap those of the RARs and other receptors. Recently, we demonstrated that the RA isomer, 9-*cis* RA, is a high-affinity ligand for the human RXR α (Heyman et al. 1992). We extend those findings here to show that 9-*cis* RA is also "retinoid X" for the mouse RXR α , β , and γ . The discovery of three RXRs and their cognate ligand, along with their patterns of expression, helps to define a complex new regulatory system in which the RXRs may play a surprisingly widespread role in development and adult physiology.

Results

Molecular cloning of three RXR cDNAs

We observed previously by low-stringency-hybridization Southern analysis of human genomic DNA that the RXR belongs to a subfamily of genes comprised of at least two members (Mangelsdorf et al. 1990). To isolate and characterize the mouse counterparts to these potential RXR subtypes, mouse adult liver and mouse embryo cDNA libraries were screened at low stringency with a human (h)RXR α cDNA probe. Several positive clones were analyzed and divided into three different groups based on their sequence identity. The longest cDNA clone from each group was completely sequenced (Fig. 1). The three classes of cDNA clones encode three nonidentical RXR-like proteins designated mRXR α , mRXR β , and mRXR γ (Figs. 1 and 2). mRXR α is the murine homolog of the hRXR α (Mangelsdorf et al. 1990) with which it shares the greatest identity (Fig. 2). The predicted initiation codon for mRXR α is conserved in the hRXR α and occurs at the first in-frame ATG of the cDNA (Fig. 1a). The sequence surrounding this ATG conforms well with the Kozak consensus (Kozak 1987), and translation from this start site generates an mRXR α protein of 467 amino acids (M_r 51,000). The sequence designated mRXR β (Fig. 1b) is also homologous to a human counterpart that we have partially characterized (Mangelsdorf et al. 1990; data not shown). Although we have tentatively assigned a translation initiation start site at nucleotide 109, there is no in-frame terminator codon upstream of this putative start site, indicating that the region encoding the amino terminus may yet have to be defined. We note, however, that this mRXR β cDNA will produce a functional protein (see Figs. 3 and 5, below) of 410 amino acids (M_r 44,000). Interestingly, the mRXR β cDNA char-

acterized here is 100% identical in size and sequence to mH2RIBP, a cDNA isolated independently by Hamada et al. (1989), whose product binds a DNA site in the histocompatibility gene. The third receptor, mRXR γ (Fig. 1c), appears to be most closely related to the chicken (c)RXR reported by Rowe et al. (1991) (Fig. 2) and a recently described *Xenopus* homolog (Blumberg et al. 1992). The assigned initiation codon for mRXR γ is a canonical Kozak sequence with an in-frame termination site 12 bp upstream. The mRXR γ cDNA encodes a protein of 463 amino acids (M_r 51,000). The existence of RXRs in several species, including the related *Drosophila* protein *ultraspiracle* (Fig. 2) (Oro et al. 1990), indicates that this gene family has been well conserved throughout evolution. As seen in Figure 2, the RXRs comprise a closely related lineage with marked divergence from the RAR subfamily.

RXRs are activated by retinoids

The ability of all-*trans* RA to specifically activate the hRXR α demonstrated that this protein defined a pathway for retinoid action that was separate from the previously described RARs (Mangelsdorf et al. 1990). Because all-*trans* RA activates both RARs and RXRs, *trans*-activation experiments were performed in the *Drosophila* cell line Schneider S2, which unlike mammalian cells is devoid of endogenous retinoid receptors (Mangelsdorf et al. 1990). To examine the functional properties of each receptor subtype, expression plasmids containing the cDNAs for mRXR α , β , or γ were cotransfected into cells together with a reporter plasmid (ADH-CRBPII-CAT) containing the RXRE from the CRBPII promoter (Mangelsdorf et al. 1991). When these cells were incubated with increasing concentrations of all-*trans* RA, all three RXRs activated chloramphenicol acetyltransferase (CAT) expression (Fig. 3a), albeit with distinctive pharmacologic properties. The potent efficacy of RXR activation by RA on the CRBPII-RXRE is demonstrated by mRXR β , which even though it is the least sensitive of the RXRs, gives a robust induction (85-fold) to all-*trans* RA.

We have also tested the response of the mRXRs to the vitamin A metabolite 3,4-didehydroretinoic acid (ddRA). In addition to all-*trans* RA, ddRA is a potent morphogenetic signal in the developing chick wing bud, where its concentration is six times greater than that of all-*trans* RA (Thaller and Eichele 1990). As shown in Figure 3b, all three RXRs are responsive to ddRA, although at concentrations ~10-fold less than all-*trans* RA. We have observed similar results with the RARs (data not shown). These results indicate that although all-*trans* RA is a potent RXR activator, it is not the only biologically active retinoid that can stimulate the RXRs.

The RXR ligand-binding domain confers retinoid responsiveness

The activation of RXR by all-*trans* RA in insect cells (in comparison to mammalian cells, Fig. 3; Mangelsdorf et

for this activation. To localize more specifically the region of retinoid responsiveness in the RXR molecule, a series of experiments were performed utilizing RXR and

Figure 1. (See p. 333 for legend.)

ultraspiracle chimeric receptors (Fig. 4a). *ultraspiracle* is a *Drosophila* orphan receptor that is required both maternally and zygotically for pattern formation (Oro et al. 1990). Although *ultraspiracle* does not respond to all-*trans* RA, its high degree of structural similarity with RXR suggests that these two receptor-like proteins are ancestral homologs and may share other nonretinoid ligands (Oro et al. 1990). We therefore compared the abil-

ity of both RXR and *ultraspiracle* to respond to all-*trans* RA. When expression plasmids containing RXR or *ultraspiracle* are cotransfected independently with a reporter plasmid into Schneider S2 cells, only RXR is activated by all-*trans* RA (Fig. 4b). However, when a chimeric receptor containing the carboxy-terminal region of the RXR fused to the DNA-binding domain and amino terminus of *ultraspiracle* (UUX, Fig. 4a) is tested, this

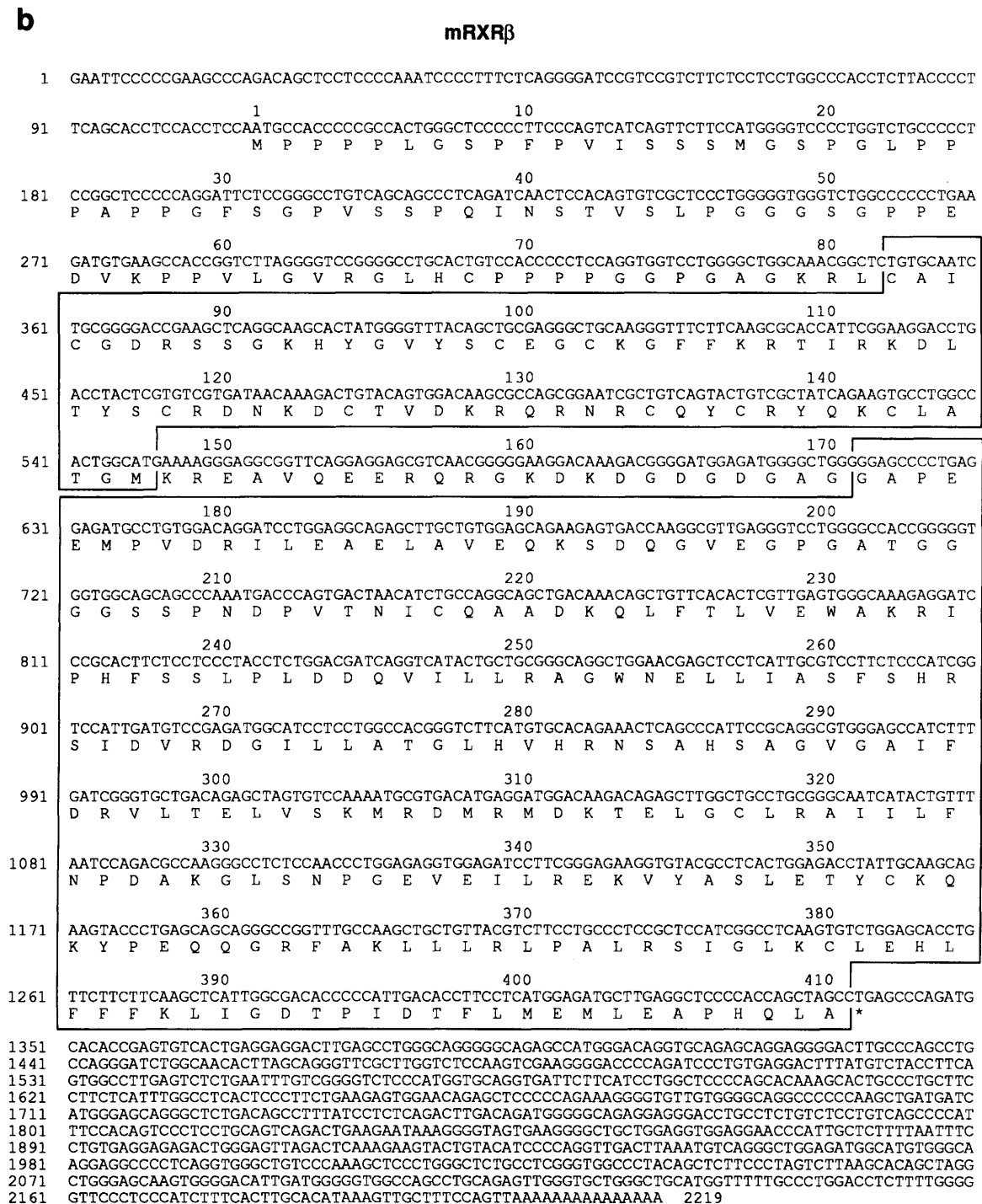


Figure 1. (See facing page for legend.)

sive to all-*trans* RA. These experiments demonstrate that the carboxy-terminal region of the RXR is required for activation by retinoids and that this region alone can

mRXR γ

Figure 1. Nucleotide and predicted amino acid sequence of mouse RXRs. (a) mRXR α ; (b) mRXR β ; (c) mRXR γ . The boxes indicate the regions of highest conservation in the DNA-binding and ligand-binding domains, which are compared in Fig. 2. The asterisk (*) marks the termination codon for the longest open reading frame in each sequence. The in-frame termination codon in the 5'-untranslated region of mRXR γ is underscored in c. The sequences for mRXR α and mRXR γ include the *EcoRI-NotI* cloning linkers. For mRXR β , the length of the poly(A)⁺ tail has not yet been determined accurately, and, therefore, the 3' *EcoRI* cloning linker has not been included in the sequence shown in b.

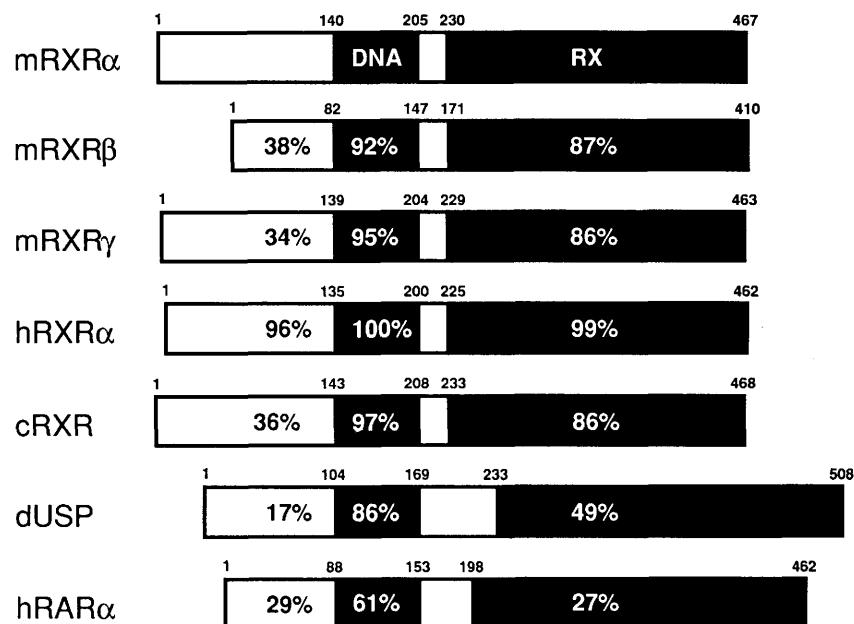


Figure 2. Amino acid comparison between mRXRα and the other members of the retinoid receptor family. Sequence alignments and percent identities were generated using the University of Wisconsin Genetics Computer Group programs (Devereux et al. 1984). Shown are the mouse (m)RXRs, human (h)RXRα, chicken (c)RXR, *Drosophila ul-traspiracle* (dUsp), and human (h)RARα.

transfer retinoid inducibility to another receptor. Previous studies have shown that this region of the steroid hormone receptors is the ligand-binding domain (Evans 1988; Green and Chambon 1988), supporting the proposal that RXR is a hormone-responsive receptor.

9-cis RA is a potent RXR ligand

Although all-*trans* RA induces RXR in transfection assays, high concentrations are required. Furthermore, direct binding of all-*trans* RA to RXR protein synthesized in eukaryotic or bacterial cells has not been demonstrated (Heyman et al. 1992; D. Mangelsdorf, unpubl.).

This led us to hypothesize that all-*trans* RA serves as a pro-hormone or metabolic precursor to a bona fide RXR ligand termed retinoid X. Recently, we have shown that for the hRXRα, one such retinoid X is 9-*cis* RA (Heyman et al. 1992). This RA isomer is metabolically converted from all-*trans* RA in cells, exists in RXR target organs in vivo, and unlike all-*trans* RA binds RXRα protein with high affinity (Heyman et al. 1992). The results shown in Figure 5 provide evidence that 9-*cis* RA is also a ligand for each mouse RXR subtype. In these experiments, *trans*-activation by RXR of a luciferase reporter ADH-CRBPII-LUC was tested in the presence of the biologically active RA isomers all-*trans* RA, 13-*cis* RA, and 9-*cis* RA. As with hRXRα, 9-*cis* RA was up to 40-fold

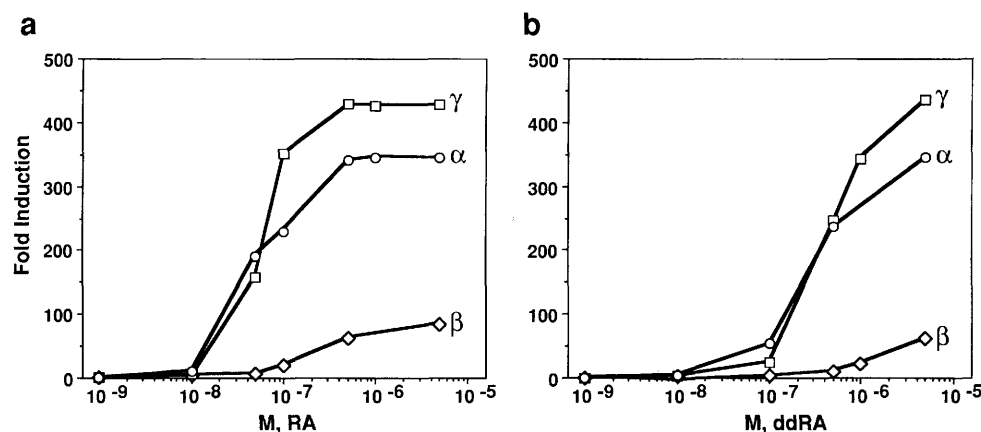


Figure 3. Concentration-dependent *trans*-activation of mRXR subtypes by retinoids. Schneider cells were cotransfected with A5C-mRXRα (○), A5C-mRXRβ (◇), or A5C-mRXRγ (□) and the reporter plasmid ADH-CRBPII-CAT and then incubated with increasing amounts of all-*trans* RA (a) or 3,4-didehydroretinoic acid (ddRA) (b). *Trans*-activation is expressed as induction of CAT activity (× control levels) in retinoid-treated cells compared with solvent-treated control cells and represents the average of duplicate assays.

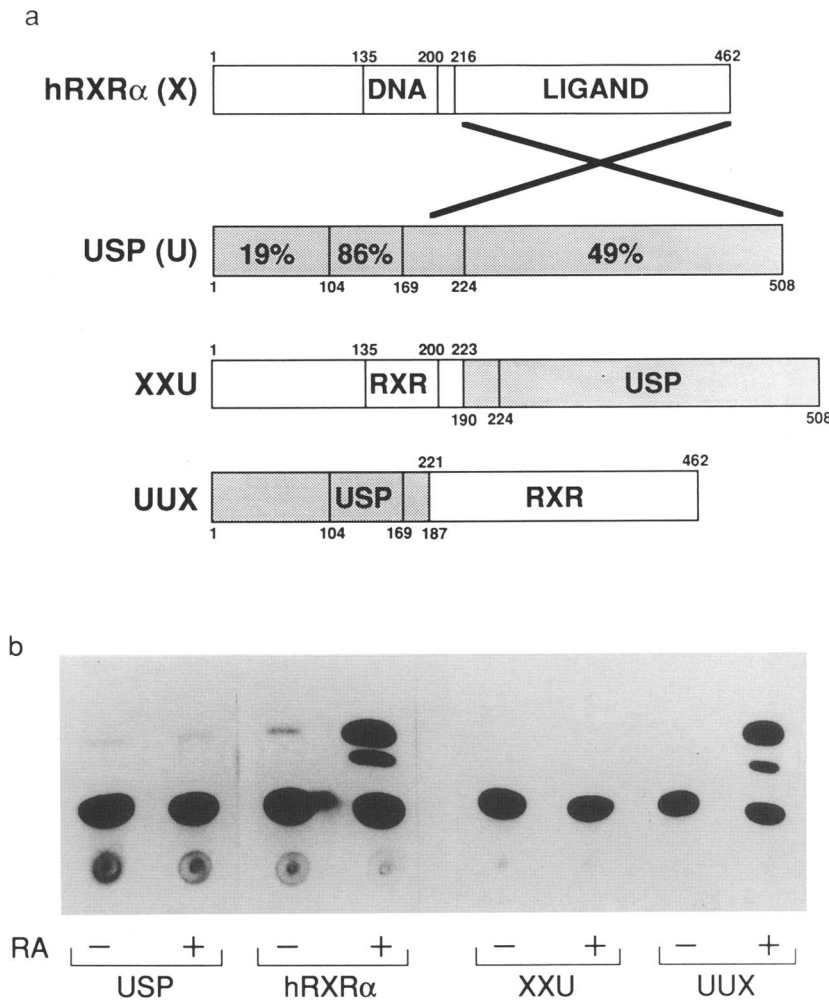


Figure 4. RXR retinoid responsiveness is localized to the ligand-binding domain. (a) Schematic showing the construction of RXR (X) and USP (U) chimeric receptors. The constructs XXU and UUX were created by reciprocally switching the putative ligand-binding domains of hRXR α and USP. (b) CAT assays of RA-dependent *trans*-activation of USP, hRXR α , XXU, and UUX. Cotransfections of Schneider cells in the absence (–) or presence (+) of all-*trans* RA were performed as in Fig. 3. The autoradiograph of TLC plates from individual CAT assays is representative of duplicate experiments. In these experiments, only hRXR α and UUX were activated by all-*trans* RA.

more potent than either 13-*cis* or all-*trans* RA in activating all three mRXRs. These responses were dose-dependent, saturable, and within the range of concentrations

by which all-*trans* RA activates the RARs. These results support the proposal that 9-*cis* RA is a signaling molecule that can mediate its action through the RXRs.

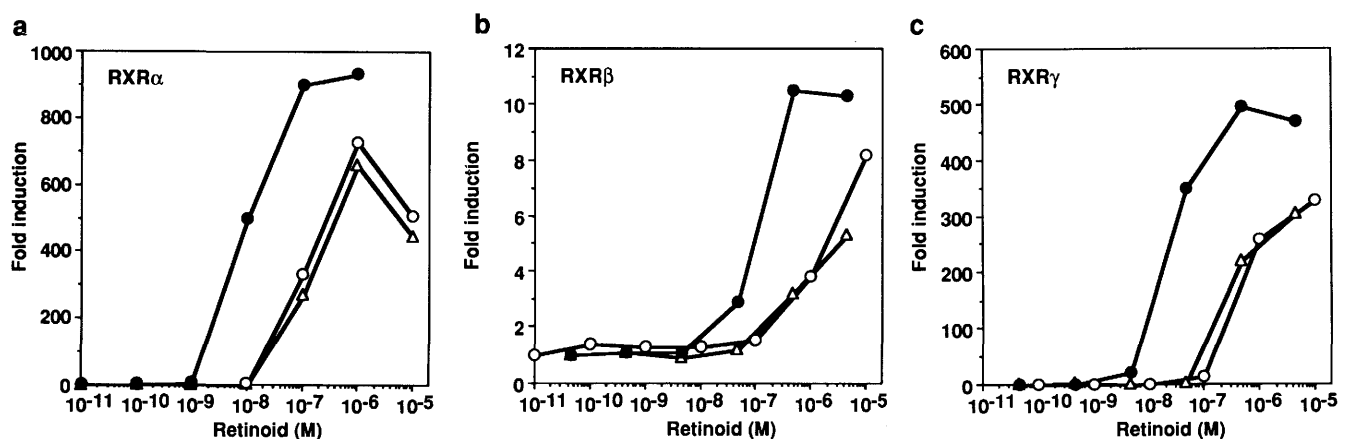


Figure 5. 9-*cis* RA is a high-affinity ligand for RXRs. Schneider cells were cotransfected with A5C-mRXR α (a), A5C-mRXR β (b), or A5C-mRXR γ (c) and the reporter plasmid ADH-CRBP-II-LUC and then incubated with increasing amounts of 9-*cis* RA (●), all-*trans* RA (○), or 13-*cis* RA (△). *Trans*-activation is expressed as induction of luciferase activity (\times control levels) in retinoid-treated cells compared with solvent-treated control cells and represents the average of triplicate assays.

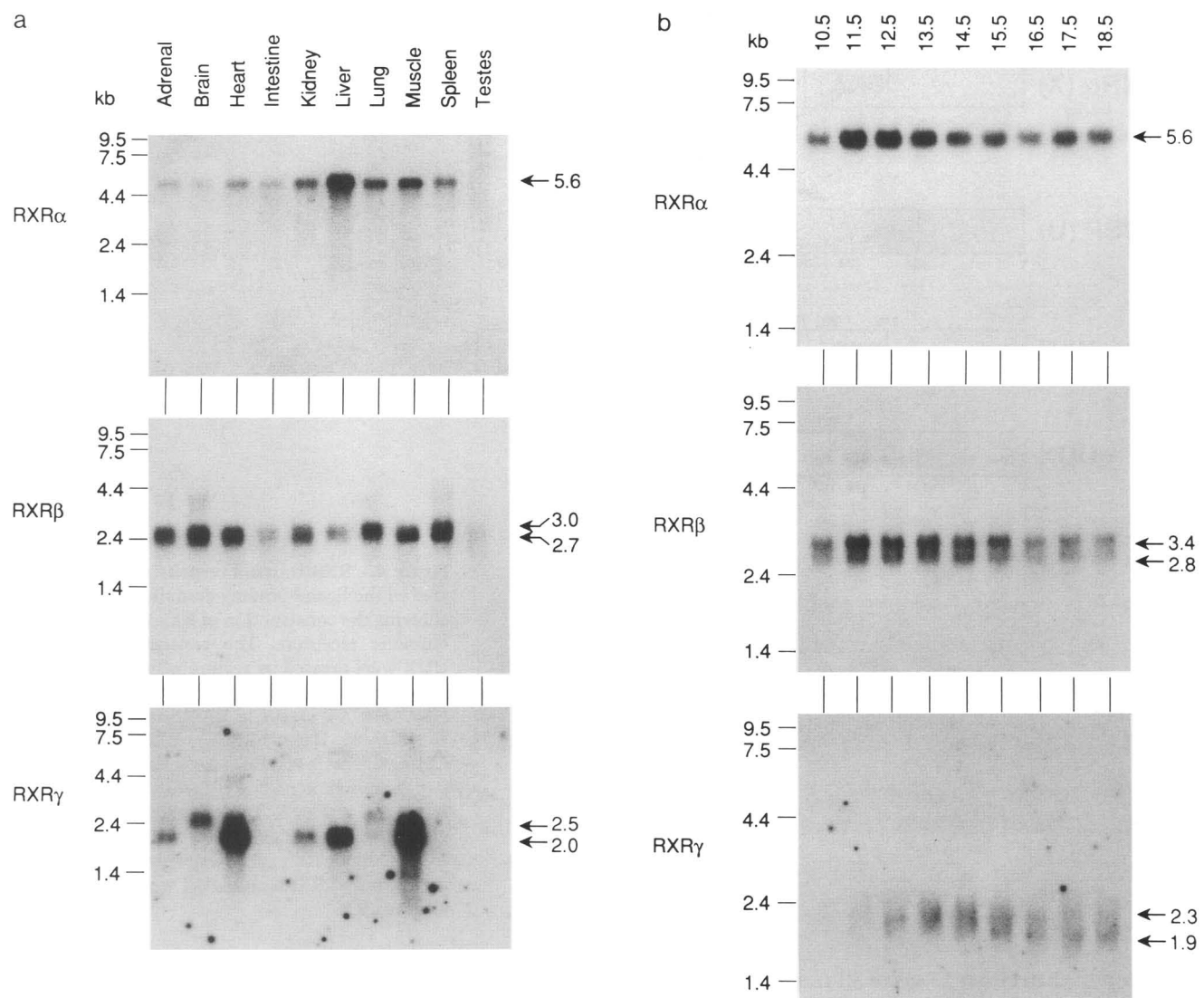


Figure 6. Northern blot analysis of RXR mRNA in adult and embryonic tissue. Adult male rat tissues (a) and mouse embryos (b) from gestation day 10.5 to 18.5. Ten micrograms of poly(A)⁺ RNA was analyzed in each lane as described in Materials and methods. Exposure times for each of the blots at -70°C with an intensifying screen were as follows: (a) RXRα, 50 hr; RXRβ, 25 hr; RXRγ, 7 days; (b) RXRα, 6.5 hr; RXRβ, 28 hr; RXRγ, 7 days. RNA size markers (in kb; BRL) are aligned at the left of each autoradiograph. The arrows on the right of each autoradiograph represent the sizes of different RXR-specific transcripts.

RXR expression in the adult

To understand the roles of the RXRs in hormonal signaling, we have examined their patterns of expression in the embryo and the adult. Northern analysis of poly(A)⁺ RNA from adult male rat tissues reveals the presence of both unique and overlapping patterns of expression for the three RXR genes (Fig. 6a). The rat RXRα mRNA migrates as a single species of 5.6 kb and displays the strongest level of expression for the RXRs in the adult. RXRα is noticeably abundant in visceral tissues such as the liver, kidney, lung, muscle, and spleen, but less so in adrenals, brain, heart, intestine, and testes (Fig. 6a, top). The RXRβ mRNA exists as two species of 2.7 and 3.0 kb,

and both are relatively abundant in all tissues except intestine, liver, and testes where they are expressed at lower levels (Fig. 6a, middle). In contrast to RXRα and RXRβ, RXRγ RNA is less abundant and more restricted in its distribution. The complexity of this expression pattern is increased by the detection of two RXRγ-specific RNAs (2.0 and 2.5 kb) that are also expressed differentially and may correspond to RXRγ isoforms, as has been observed with the RAR gene products (Giguere et al. 1990; Kastner et al. 1990). The 2.0-kb RXRγ mRNA is present in the adrenals, kidney, and liver, whereas the 2.5-kb RNA is present in the brain and lungs. In contrast, both mRNA isoforms are expressed strongly in the heart and muscle (Fig. 6a, bottom). In aggregate, the RXRs are

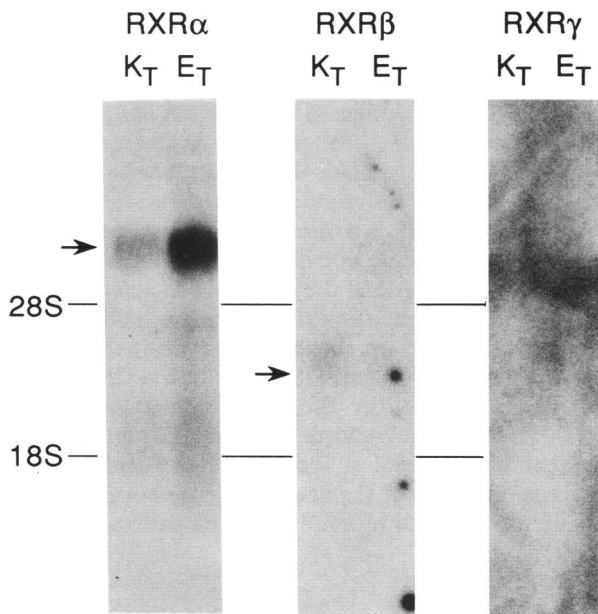


Figure 7. Northern blot analysis of RXR RNA derived from human skin. Each lane contains 40 μ g of total RNA isolated from human epidermis (E_T) or cultured human keratinocytes (K_T). Exposure times for each of the blots at -70°C with an intensifying screen were as follows: RXR α , 5 hr; RXR β , 25 hr; RXR γ , 39 hr. RNA size markers are given by 28S and 18S internal standards. The arrows depict RXR-specific transcripts.

expressed in all adult tissues examined, with individual tissues showing selective overlap with RAR subtypes (e.g., see below).

The skin is known to be a target organ for RA both therapeutically and during natural differentiation and a site of selective expression of RAR γ . An analysis of the expression of the RXRs in adult human epidermis and keratinocytes (Fig. 7) reveals high levels of RXR α mRNA. A very faint band can be detected for RXR β , but even after a relatively long exposure there is no detectable signal for RXR γ . These results suggest a combined and potentially interactive role for RXR α and RAR γ in human skin morphogenesis.

RXR expression during development

The critical requirement for RA during development has prompted a number of studies to explore the pattern of expression of the RARs and the cellular retinoid-binding proteins throughout embryogenesis (Dollé et al. 1989; Maden et al. 1989; Noji et al. 1989; Perez-Castro et al. 1989; Osumi-Yamashita et al. 1990; Ruberte et al. 1990; Smith and Eichele 1991). Because it is clear that such studies must now take into account the RXRs, we have focused next on the pattern of expression of the three RXR genes during mouse embryogenesis. Northern analyses of whole-embryo mRNA reveal that all three RXRs are expressed from at least day 10.5 postconception to parturition (Fig. 6b). As in the adult, RXR α and RXR β mRNAs are abundant, whereas RXR γ mRNA is present

at much lower levels. Again, there is a heterogeneity in the distribution of the two RXR γ transcripts, with the larger 2.3-kb band being predominant in the early embryo, followed by a transition to a 1.9-kb transcript at embryonic day 15.5 (Fig. 6b, bottom).

In situ hybridization has been used to extend the findings of Northern blot hybridization and localize more specifically the developmental expression of the RXRs (Figs. 8–11). A comparison on embryonic day 16.5 (E16.5) reveals a clear differential expression of the three RXR subtypes (Fig. 8). The RXR α transcript is found predominantly in the epithelia of the digestive system, the skin, and the liver (Fig. 8a). Expression is low in the central nervous system (CNS) and the skeleton. This pattern is found from the earliest stages of organogenesis through parturition (data not shown) and continues forward from newborn and adult (see Figs. 6a and 9a,b). High RXR α signals are easily detected in the liver, the villi of the small intestine and epithelia of the colon, and the skin (Fig. 9a,b). This pattern of expression supports the proposed role of RXR α in metabolism and retinoid physiology (Mangelsdorf et al. 1990, 1991). Before E10, we note intense hybridization signals present only in the extraembryonic tissue of the uterine wall (decidua), which can first be detected at E5 after implantation of the embryo (Fig. 9c). Other preliminary data suggest that in subsequent stages before organogenesis there is also expression in the placenta.

In situ hybridization did not reveal a high degree of specificity for RXR β transcription in the embryo, and in many cases the differences in signal intensity could not be distinguished from cell density (Fig. 8b). However, in contrast to RXR α , there were significant levels of RXR β transcripts detected in the CNS. The widespread embryonic expression of RXR β is similar to that found in the adult (Fig. 6a).

The RXR γ transcript displays the highest restriction of the three receptors (e.g., Fig. 8c). The intense hybridization signals seen at this stage are restricted to the corpus striatum (caudate putamen) and the pituitary, although weaker expression can also be detected in the neck and skeletal muscle, and tongue (Fig. 10 a–c). The high level of RXR γ expression in the striatum continues to be observed until at least 7 days after birth (data not shown). When the analysis of RXR γ is extended to earlier stages of development, the striking expression in the striatum could be identified as early as E13.5 (Fig. 11a) and, in the pituitary anlage, as early as E11 (Fig. 11b). At these earlier stages (E10–E13), prominent high signals were also detected in the somites, which are precursors to skeletal muscle (Fig. 11 b–f), and in the ventral horns of the spinal cord (Fig. 11f). In contrast to the RARs, prominent expression of any of the RXR subtypes was not observed in the developing limbs or skeletal system.

Discussion

The RXR Family

A striking observation to come from the original characterization of hRXR α was the apparent dissimilarity in

sequence to that of the RARs, providing evidence for an evolutionarily distinct retinoid-response pathway. An understanding of the differences and similarities of the RAR and RXR systems requires knowledge of the diversity of the family members, their patterns of expression, and their pharmacology in response to cognate ligands. In this paper we report the isolation of cDNAs encoding three mouse RXR proteins (mRXR α , β , and γ). These proteins are closely related to each other both in their DNA-binding and ligand-binding domains. Their homologies indicate that these receptors are likely to regulate common target sequences and respond to common ligands. However, they differ markedly in their amino-terminal domains, which could confer distinct *trans*-activation functions. Interestingly, although a *Drosophila* homolog of the RXR gene has been identified as the *ultraspiracle* locus, there is no apparent insect homolog of the RARs (Oro et al. 1990). This indicates the ancient evolutionary origin of the RXRs and raises the question as to whether RXRs might represent the original retinoid signaling system. In this respect, it will be of interest to discover the ligand for *ultraspiracle*.

A novel signaling pathway

hRXR α was identified originally by its ability to respond to all-*trans* RA, and similarly, murine RXR subtypes are also activated by all-*trans* RA. However, the inability of RA to bind to in vitro-expressed RXR and the high doses of RA required in transfections for transcriptional activation led us to propose that this retinoid served as a metabolic precursor to a higher-affinity ligand. Recently, we demonstrated that 9-*cis* RA is a naturally occurring, high-affinity ligand for the hRXR α as determined by a combination of binding and activation studies (Heyman et al. 1992). We also confirmed that all-*trans* RA shows

no detectable binding affinity for RXR α . We extend those findings here to demonstrate that 9-*cis* RA is also retinoid X for the mouse RXR α , β , and γ subtypes. Each of these receptors is activated by 9-*cis* RA with both increased potency and efficacy relative to all-*trans* RA. Although RXRs may interact with RARs (see below), they may also function independently. Expression in *Drosophila* Schneider cells is sufficient to reconstitute retinoid responsiveness. Thus, not only do the RXRs bind and respond to 9-*cis* RA but they are able to activate target genes in the absence of other RAR gene products. Furthermore, the RXRs are capable of activating through the recently described response element in the CRBP2 gene that is not induced by the RARs (Mangelsdorf et al. 1991). These results indicate that the RXRs are bona fide members of the nuclear receptor family and, together with the discovery of the new vertebrate hormone, 9-*cis* RA, define a second retinoid signaling pathway.

Tissue distribution

Although the RXRs can regulate distinct target genes from the RARs as described above, they may also regulate common target genes. The existence of three RARs and three RXRs that mediate the effects of the two retinoid hormones, all-*trans* RA and 9-*cis* RA, could help to account for the vast influence of retinoids on development and physiology. To understand the physiologic role of 9-*cis* RA, we have begun to analyze the target tissues for RXRs. In aggregate, in the adult organism, the RXRs are widely expressed. In general, for all the receptors, the adult pattern reflects a perdurance of that observed in situ in the embryo. Although there is no absolute correlation between the individual RAR and RXR subtypes, there are several interesting similarities. For example, both RAR α and RXR β are found in almost all tissues,

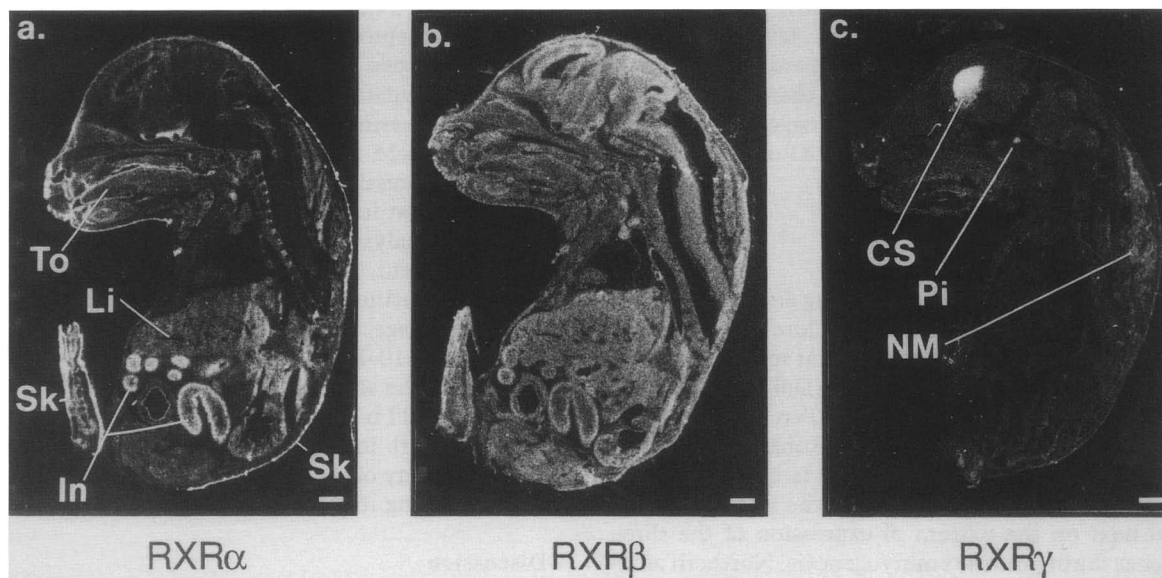


Figure 8. Localization of mRXR transcripts in E16.5 mouse embryos. Adjacent parasagittal sections of E16.5 embryos were hybridized in situ with probes for mRXR α (a), mRXR β (b), or mRXR γ (c). Photographs of sections were made by using the autoradiograph as a negative, giving white signals against a dark background. (CS) Corpus striatum; (In) intestine; (Li) liver; (NM) neck muscle; (Pi) pituitary; (Sk) skin; (To) tongue. Bar, 1 mm.

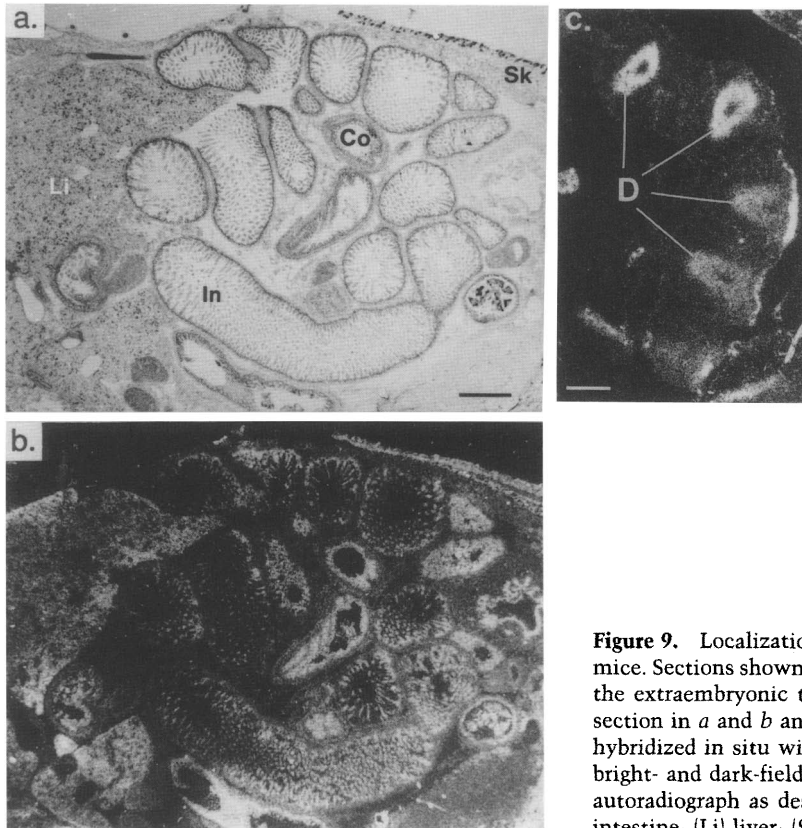


Figure 9. Localization of mRXR α transcripts in embryonic and neonatal mice. Sections shown are from the abdomen (*a,b*) of a 3-day-old mouse and the extraembryonic tissue (*c*) of an E5 mouse embryo. The parasagittal section in *a* and *b* and the cross section of implanted embryos in *c* were hybridized in situ with an mRXR α -specific probe. Photographs are from bright- and dark-field illumination in *a* and *b*, respectively, and from an autoradiograph as described in Fig. 8 in *c*. (Co) Colon; (D) decidua; (In) intestine; (Li) liver; (Sk) skin. Bar, 1 mm.

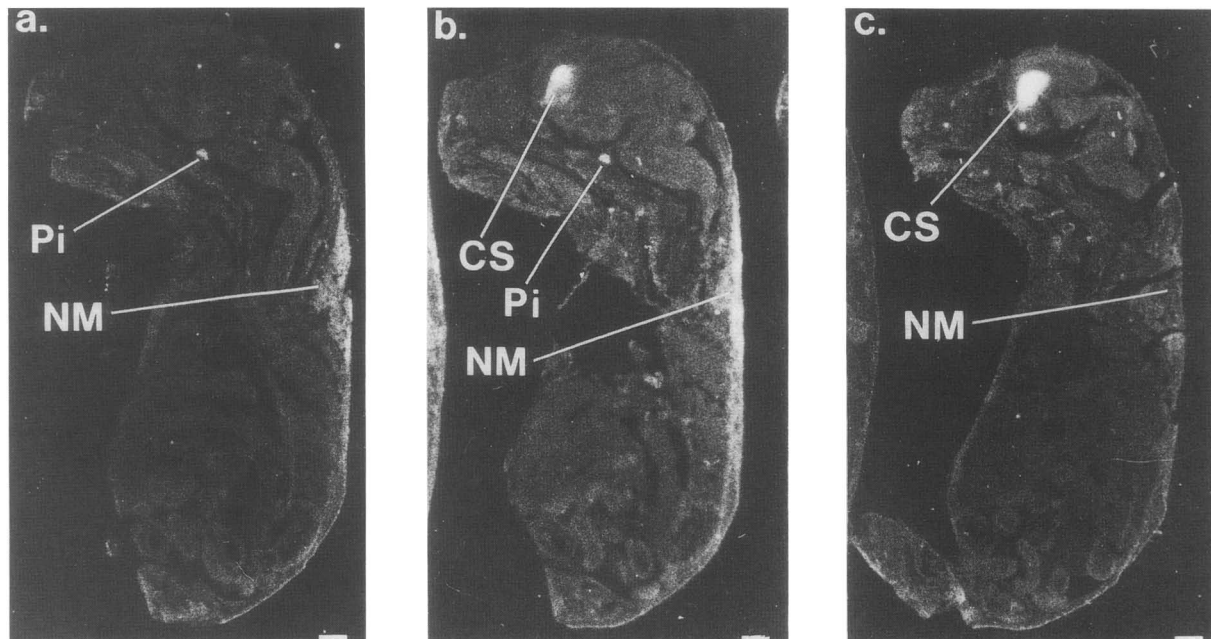


Figure 10. Localization of mRXR γ transcripts in an E16.5 mouse embryo. In situ hybridization using an mRXR γ -specific probe was performed on sections taken at the midline (mid-sagittal) in *a* and progressing laterally outward (parasagittal) in *b* and *c*. Thus, as expected, the signals in the pituitary are restricted to the mid-sagittal sections; the corpus striatum signals are localized in the more parasagittal sections. Photographs were made as described in Fig. 8. (CS) Corpus striatum; (NM) neck muscle; (Pi) pituitary. Bar, 1 mm.

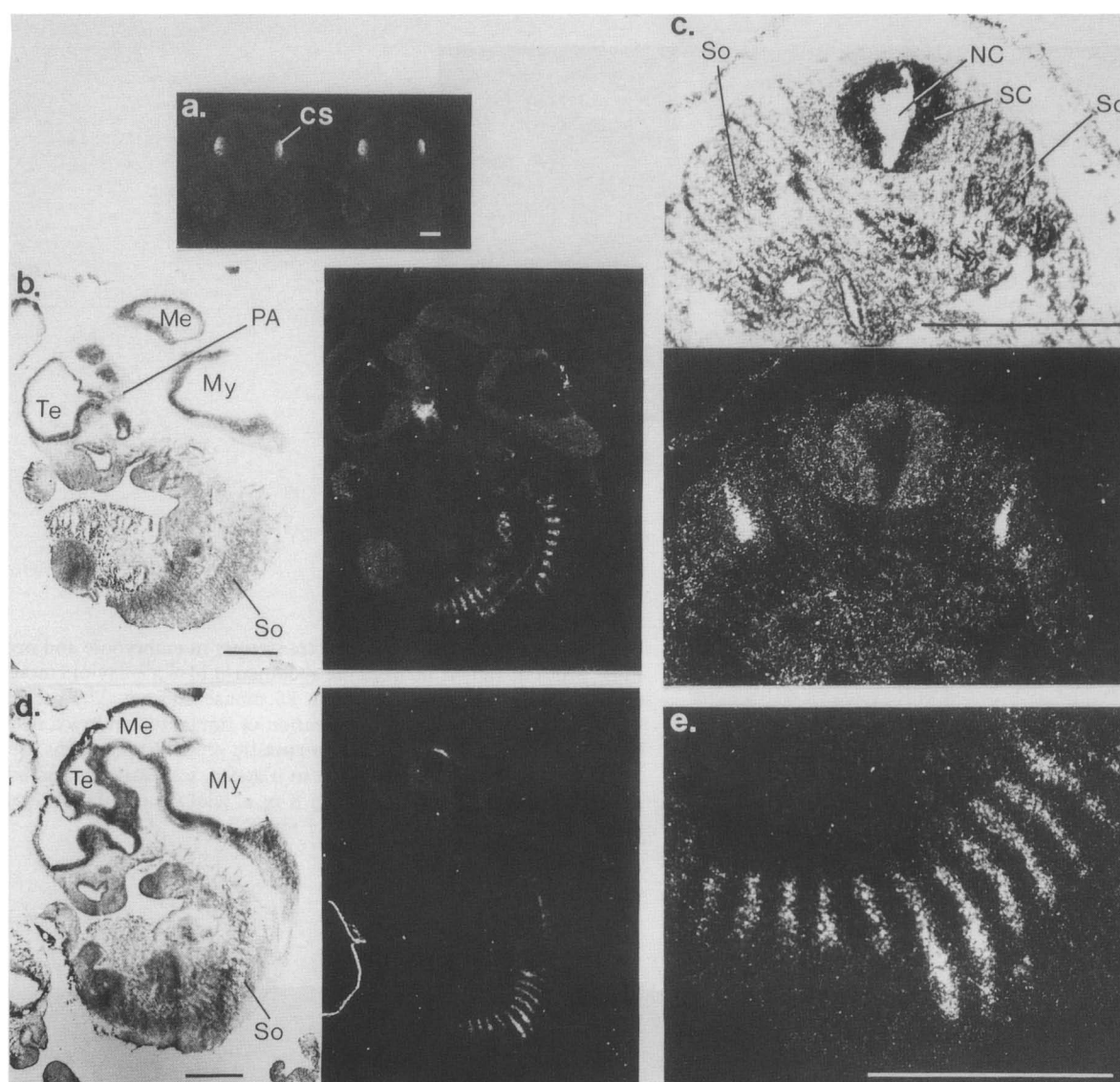


Figure 11. Localization of mRXR γ transcripts at various stages in the mouse embryo. In situ hybridizations were performed with the same mRXR γ -specific probe used in Figs. 8c and 10. (a) Two adjacent coronal sections through the head of an E13.5 embryo photographed as in Fig. 8. (b) Sagittal section of an E11 embryo. (c) Oblique section through the spinal cord and somites of an E10 embryo. (d) Sagittal section of an E11 embryo. (e) Higher magnification of somites shown in d. (f) Coronal section through the spinal cord of an E13 embryo. Bright- and dark-field illuminations are shown in b–f. (CS) corpus striatum; (DH) dorsal horn; (Me) mesencephalon (mesocoel); (My) myelencephalon (myelocoel); (NC) neural canal; (PA) pituitary anlage; (Pc) precartilag; (SC) spinal cord; (So) somite; (Te) telencephalon (teleocoel); (VH) ventral horn. Bar, 1 mm.

whereas RAR β and RAR γ and RXR α and RXR γ are much more restricted. Although RXR β shows no striking pattern, there is increased expression in the anterior extensions of the spinal cord and hind brain. RXR α shows abundant expression in liver, kidney, spleen, and a variety of visceral tissues that are in marked distinction to the RAR subtypes that are found in very low levels at these corresponding sites (Giguere et al. 1987; Brand et al. 1988; Zelent et al. 1989; Kastner et al. 1990). This pattern led us to propose that RXR α may be involved in retinoid and lipid metabolism, a hypothesis that was bolstered further by the demonstration that the CRBP II (Mangelsdorf et al. 1991) and apolipoprotein (apoA I) (Rottman et al. 1991) genes are RXR responsive. CRBP II helps to absorb dietary retinol and assist in its transfer to chylomicrons and ultimate transport to the liver. ApoA I is a major constituent of both chylomicrons and high-density lipoproteins, which are known to play an important role in cholesterol metabolism. Because high levels of retinoids are teratogenic, especially to the early embryo, the abundant early expression of RXR α in the decidua and placenta may indicate that RXR α has a role in feedback regulation of retinoid uptake and storage in the embryo. The expression of RXR α in vital organs suggests an additional role for retinoids in physiologic homeostasis. Interestingly, RXR α and RAR γ are both expressed abundantly in the epidermis (Fig. 7; Kastner et al. 1990), indicating that these two receptors are likely to be responsible for the dermatologic effects of retinoids.

The most restricted of the three receptors is RXR γ , which also shows a lower level of expression in the embryo and the adult. It is particularly common in the muscle and the brain. Thus, one might anticipate a role for RXR γ in aspects of muscle and CNS differentiation. In the chicken, a homolog of RXR γ has been shown to be expressed in the developing peripheral nervous system (Rowe et al. 1991). The CNS pattern of RXR γ expression in the mouse is particularly interesting, showing prominence in the embryonic anlage to the caudate putamen (basal ganglia). This is the major target of dopaminergic innervation by the substantia nigra. It is also the end site of impaired dopaminergic transmission found in Parkinsonian disorders (Yurek and Sladek 1990). Because the neurons of the corpus striatum tend to be generated along distinct spatiotemporal gradients, it will be of interest to discover whether these gradients can be influenced by retinoids. Interestingly, both RXR γ and RAR β are coexpressed in the motor neurons of the spinal cord, again suggesting a selective parity between these two receptor systems. We find prominent expression of RXR γ in the pituitary, suggesting a potential role for retinoids in the regulatory cascade associated with hypophyseal differentiation.

Family ties

It is generally believed that the nuclear receptors function as either homo- or heterodimers in binding to, and regulating expression of, target genes. Dimerization is apparently critical for high-affinity binding of receptor

with its cognate hormone-response elements (Kumar and Chambon 1988). Recent studies have shown that the binding sites for the vitamin D receptor (VDR), thyroid hormone receptor (TR), and RAR are closely related and comprise direct repeats spaced by 3, 4, or 5 nucleotides, respectively (Umesono et al. 1991). This underlying relationship suggested a commonality in the way in which these receptors recognize target DNA. It is believed that for some receptors, including VDR, TR, and RAR, interaction with other nuclear factors is required for DNA binding (Glass et al. 1990; Liao et al. 1990; Darling et al. 1991; O'Donnell et al. 1991; Yang et al. 1991). It has been proposed that such nuclear factors may be members of the steroid receptor family such that DNA binding would be mediated by a heterodimeric complex (Glass et al. 1990). RXR α unexpectedly serves as a common heterodimeric partner for the VDR, TR, and RAR (Kliwer et al. 1992). In vitro, each heterodimer displays cooperative binding to target DNA, suggesting that the interaction of the RXR with other receptors may yield a battery of factors with distinct functional properties. The range and diversity of effects that might be generated is augmented further by the existence of α , β , and γ subtypes. Thus, the interaction of multiple RXR isoforms with other nuclear receptors responsive to their own array of ligands is likely to play a critical role in creating the high degree of diversity and specificity necessary to regulate a large network of hormone-responsive genes. Why the VDR, TR, and RAR interact with a common RXR partner is not yet clear, particularly as the actions of vitamin D and thyroid hormones are apparently not RA dependent. RXR may function independently of vitamin A in this role. Adding further to the complexity is the fact that RXR clearly displays activities on the CRBP II response element in the absence of any of these other three receptor types.

Perspectives

The identification of three distinct genes constituting the RXR subfamily, their patterns of expression, and their responsiveness to the newly characterized metabolite, 9-*cis* RA, provides the foundation for a much broader view for the role of retinoids in vertebrate development and physiology. The discovery in 1987 by Giguere et al. and Petkovich et al. of a human RAR offered the hope of analyzing the mechanisms of morphogenesis in a vertebrate system. This discovery was made more meaningful by the recognition that the RAR was a member of the steroid/thyroid hormone receptor superfamily. It was believed initially that this molecule might provide a single molecular mechanism to account for the diverse range of effects of RA in vertebrate development. This problem has obviously become much more complicated by the identification of the RAR and the RXR families, the identification of distinct and common target genes, and the exploitation of retinoid isomers as ligands. This daunting complexity has been augmented further by the suggestion that the RXRs may form additional partners with the vitamin D and thyroid hormone

receptors, forcing a general re-evaluation of our current notions of signal transduction. Nonetheless, our evolving understanding of these molecular interactions provides us with a more comprehensive view of developmental and organ physiology and, most importantly, a fresh approach to the problem.

Materials and methods

Construction and screening of cDNA libraries

Subtype-specific mRXR cDNAs were isolated by screening an E16.5 mouse λ ZAPII cDNA library (for mRXR α), an adult mouse liver λ gt11 cDNA library (for mRXR β), and a E14.5 mouse λ ZAPII cDNA library (for mRXR γ). The mouse liver cDNA library was a gift of J. Darnell (Rockefeller University); the mouse embryonic cDNA libraries were constructed in λ ZAPII (Stratagene) as described by Kakizuka et al. (1991) except that for the E16.5 library, cDNA inserts were size-selected to >4 kb. To isolate mRXR α and γ cDNAs, the λ ZAPII libraries were screened at low stringency using the hRXR α cDNA as a probe (Mangelsdorf et al. 1990). To isolate mRXR β cDNA, the hRXR α probe was used to first isolate a human liver RXR β cDNA (Mangelsdorf et al. 1990), which was used subsequently to isolate mRXR β . All three mRXR cDNAs were excised or subcloned into the *Eco*RI site of pBluescript (SK) vectors (Stratagene) and sequenced using double-stranded DNA template. The orientation of mRXRs in pSK is as follows: For mRXR α , the *Kpn*I site is toward the 5' end; for mRXR β , the *Kpn*I site is toward the 3' end; and for mRXR γ , the *Kpn*I site is toward the 5' end.

Plasmids

The reporter plasmids ADH-CRBP-II-CAT and ADH-CRBP-II-LUC contain CRBP-II-RXRE (Mangelsdorf et al. 1991) inserted into position -33 of pD33-ADH-CAT (Mangelsdorf et al. 1990) or pD33-ADH-LUC (Heyman et al. 1992). These reporters contain the distal promoter of the *Drosophila* alcohol dehydrogenase gene linked to either the bacterial CAT gene (Krasnow et al. 1989) or the firefly luciferase gene (Heyman et al. 1992). The A5C-RXR and A5C-USP expression plasmids were constructed by inserting the full coding region of each respective receptor into an expression vector containing the *Drosophila* actin 5c promoter (Mangelsdorf et al. 1990). The A5C expression plasmid for the RXR-USP chimeric receptor XXU (Fig. 4) was created by replacing the region encoding amino acids 224-462 of hRXR α (Mangelsdorf et al. 1990) with the region encoding amino acids 190-508 of USP (Oro et al. 1990). Similarly, A5C-UUX was created by replacing the region encoding amino acids 188-508 of USP with the region encoding amino acids 221-462 of hRXR α . The points of fusion between RXR and USP were generated by PCR-directed site mutagenesis and confirmed by sequencing.

Cotransfection assays

Schneider line S2 cell culture, transfections, and CAT assays were performed as described previously (Mangelsdorf et al. 1990). Transfection of plasmid DNAs into S2 cells (Figs. 3 and 4) was accomplished in duplicate in 6-well (35-mm²/well) tissue culture plates. The amount of plasmids (in μ g) cotransfected per well was 1 : 0.5 : 0.5 : 8 [A5C-RXR/ADH-CRBP-II-CAT/A5C- β gal : pGEM4 carrier]. Cotransfection assays with the luciferase reporters (Fig. 5) were performed as described previously (Heyman et al. 1992) using Biomek (Beckman) automated work sta-

tions. Stock concentrations of all-*trans* RA (Sigma), ddRA (gift of Gregor Eichele), or the *cis* isomers of RA (Heyman et al. 1992) were made up in ethanol or dimethylsulfoxide solvents and delivered to cells at 10³-fold dilutions [0.1% (vol/vol) of solvent in media]. The characterization and synthesis of pure RA isomers has been described (Heyman et al. 1992). Transfections in which the control expression plasmid A5C-RXR_{rev} (Mangelsdorf et al. 1990) was substituted for A5C-RXR showed no activation at any ligand concentration (data not shown). Differences in the activation profiles shown in Figures 3 and 5 are the result of the inherent differences in the activation properties of the CAT and luciferase reporter plasmids and cannot be compared directly.

RNA preparation and Northern analyses

Murine poly(A)⁺ RNA used in these studies was derived from fresh tissue, as described previously (Mangelsdorf et al. 1990). Northern blot gels and hybridizations using murine RNA were performed as detailed elsewhere (Mangelsdorf et al. 1990). Equal amounts (10 μ g) of poly(A)⁺ RNA represented in each lane (Fig. 6) were verified by ethidium bromide staining and hybridizations performed with an actin cDNA probe as a control (data not shown). The human skin RNA blot (Fig. 7) was obtained from Gary Fisher (Department of Dermatology, University of Michigan, Ann Arbor). Northern hybridizations were performed on the same blots for each receptor by sequential hybridization and stripping of each RXR probe. In each case, the probes used were the entire *Eco*RI insert of RXR α , RXR β , or RXR γ .

In situ hybridization analyses

Antisense RNA probes labeled with [α -³⁵S]UTP (1000-1500 Ci/mmole, NEN) were generated with T3 or T7 polymerase from linearized pSK-mRXRs according to the manufacturer (Stratagene). For pSK-mRXR α , *Xho*I and T3 polymerase were used; for pSK-mRXR β , *Not*I and T7 polymerase; and for pSK-mRXR γ , *Asp*718 and T3 polymerase. Synthesized probes were purified by separating free nucleotides with a Sephadex spun column followed by ethanol precipitation. Probes of average size 300 bp were generated by partial alkaline hydrolysis. The in situ hybridizations were performed as described by Bettler et al. (1990). Specificity of the signals was verified by using unrelated probes and by using probes from nonoverlapping subclones. The sections were exposed to Hyperfilm Betamax (Amersham) for 10 days. Some sections were dipped for higher resolution in Kodak NTB-2 emulsion, developed after 4 weeks, and stained with Giemsa. Embryos and neonatal mice obtained from matings between ICR mice (HSD) were collected at the stages described. The occurrence of a vaginal plug was defined as E0 and the day of birth as P0. Morphological structures were identified by reference to Rugh (1990) and Theiler (1989).

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Characterization of three RXR genes that mediate the action of 9-cis retinoic acid.

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