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# Two Novel RXR$\alpha$ Isoforms from Mouse Testis

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We report the isolation from mouse testis cDNA of two novel RXR$\alpha$ isoforms, mRXR$\alpha$2 and mRXR$\alpha$3, with distinct sequences upstream of exon 2. These two isoforms encode a similar protein (mRXR$\alpha$2/3) which lacks the 28 N-terminal amino acid residues of the major RXR$\alpha$ isoform, mRXR$\alpha$1. The N-terminal activation function (AF-1) of mRXR$\alpha$2/3 appears altered when compared to that of mRXR$\alpha$1. mRXR$\alpha$2 and mRXR$\alpha$3 are specifically expressed in the testis, and their expression is strongly upregulated in this tissue at puberty. These observations increase the molecular complexity of RXRs, and indicate that RXR$\alpha$ may play a specific function during spermatogenesis. 

There are three retinoid X receptor (RXR) isotypes in the mouse (RXR$\alpha$, $\beta$ and $\gamma$) (for reviews see 1-5). These receptors, which belong to the nuclear receptor superfamily, can function as homodimers activated by their specific ligand, 9-cis retinoic acid (9-cis RA) and are also thought to play a central role in several signalling pathways as heterodimeric partners involved in DNA binding of a number of other nuclear receptors [e. g. retinoic acid receptors (RARs), thyroid hormone receptors (TRs), vitamin D receptor (VDR); see 3].

The understanding of RXR function will ultimately require the precise knowledge of the molecular identity and properties of the various RXR proteins. In the case of RXR$\beta$ and RXR$\gamma$ isotypes, two distinct isoforms have been described, respectively RXR$\beta$1 and $\beta$2 (6-7) and RXR$\gamma$1 and $\gamma$2 (8), which in each case encode RXR proteins with distinct N-termini. As the N-terminal A/B region of RXRs contains a ligand independent transcription activation function (AF-1; see 9), the various isoforms of a given RXR isotype may possess some distinct transcriptional properties and differentially participate to the regulation of subsets of target genes. In the case of RXR$\alpha$, only one isoform has been described (7, 10-11). In a search for additional isoforms, we have now characterized two novel RXR$\alpha$ isoforms expressed in the mouse testis.

## MATERIALS AND METHODS

*Plasmids.* BSK-mRXR$\alpha$1(A/B-C) was generated by cloning the 600 bp EcoRI-ScaI fragment from the mRXR$\alpha$1 cDNA [corresponding to nucleotides 87 to 675 (Genbank sequence #M84817); see 7] between the EcoRI and EcoRV sites of pBluescriptIISK− (*Stratagene*). The mRXR$\alpha$2 expression vector was made as follows : a fragment was first amplified from a mRXR$\alpha$2 RACE-PCR clone (see below), with a mRXR$\alpha$2-specific primer (5′-ATTGAATTCTACCCTGTGGGGCTTGTGA-3′) and the mRXR$\alpha$1 region B-specific antisense primer P2 (5′-ATGAATTCGCGGACCCATGCCATTGATG-3′; nucleotides 300 to 281 in Genbank sequence #M84817), digested with EcoRI and SstI and then cloned between the EcoRI and SstI sites of pTL1 (7). The entire 3′ part of mRXR$\alpha$1 cDNA [A SStI-BglII fragment extending from the SstI site at nucleotide 277, to the BglII site 600 bp downstream of the stop codon (see 7)] was then cloned between the SstI and BglII sites of the latter plasmid. mRXR$\alpha$1(D1-28) and mRXR$\alpha$1(D1-65) were made as follows : PCR fragments amplified from mRXR$\alpha$1 with either the primer 5′-ATTGAATTCCACCATGGCTGTCCCCTCGCT-3′ or the primer 5′-ATTGAATTCCACCATGGGTCCGCCCTTCTC-3′, both specific for the A/B region, and the antisense primer 5′-GGAGAAGGAGGCGA-3′, specific for the D region, were digested with EcoRI and BamHI, and cloned between the EcoRI and BamHI sites of mRXR$\alpha$1 (7).

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*RNase protection.* The antisense RNA probe was produced from 1 $\mu$g of BamHI-digested BSK-mRXR$\alpha$1(A/B-C), in presence of 20 units of T7 RNA polymerase and 100 $\mu$Ci of [$^{32}$P]CTP. 30$\mu$g of total RNA were hybridized with $10^5$cpm of probe for 14-18h at 55°C, digested with RNase, and analyzed on a 6% polyacrylamide/urea denaturing gel (12).

*RACE PCR.* cDNA obtained from 2 $\mu$g of total RNA using reverse transcription with an antisense primer specific for the mRXR$\alpha$ C region (5′-GGAGCGGTCCCCACAGATAG-3′ ; nucleotides 536 to 517 in Genbank sequence #M84817), was poly-dG tailed and subjected to two rounds of PCR, according to Loh et al (13) and Zelent et al (14). The two successive mRXR$\alpha$ antisense primers were P1 (5′-GGGCTACCAGTCCCGAAGCC-3′ ; nucleotides 385 to 366 in Genbank sequence #M84817) and P2 (see above). The PCR products were digested with SacII and EcoRI, and subcloned in SacII-EcoRI-restricted pBSK−. Clones hybridizing with the exon 2-derived oligonucleotide P3 (5′-TTCTCTACCCAGGTGAACT-3′ ; nucleotides 146 to 165 in Genbank sequence #M84817), were further characterized.

*Reverse transcription-PCR (RT-PCR).* 1 $\mu$g of total RNA was used for RT-PCR reactions, with P2 as the antisense primer, and primers P4 (5′-ATGGACACCAAACATTTCCTGC-3′), P5 (5′-TGTGCAGGAGAGGTGTCCATT-3′) or P6 (5′-AAGCAGGTCTACAGGA-3′) (see Fig. 2), in order to amplify respectively mRXR$\alpha$1, mRXR$\alpha$2 and mRXR$\alpha$3 transcripts. After 3 min denaturation at 94°C, the samples were cooled down to 50°C in 10 min and a further 15 min step at 50°C allowed reverse transcription of the RNA according to Mallet et al (15). cDNAs were then subjected to 30 amplification cycles (30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C). 15 $\mu$l of the products were separated on a 2% agarose gel and analyzed by Southern-blotting with [$^{32}$P]-labelled oligonucleotide P3.

*Transient transfection of cells, Western-blot, and CAT-assay.* COS-1 cells (maintained in Dulbecco's modified Eagle's medium containing 5% delipidated fetal calf serum stripped of retinoids) were plated at 40-50% confluency and transfected using the calcium-phosphate procedure (12) ; the $\beta$-galactosidase expression vector pCH110 (*Pharmacia*) was used in all transfections as internal control (9). The total amount of DNA in each transfection was standardized to 20 $\mu$g, using carrier DNA (pBSK−). After a 20h period with the calcium-phosphate precipitate, cells were either directly harvested (for whole cell extract and Western-blot), or washed with medium, and further cultured for 24h in the presence or absence of $10^{-6}$ M 9-cis RA.

For Western-blotting, 10 $\mu$g of whole cell extracts from COS-1 cells transfected with 5 $\mu$g of expression vector, were separated by SDS-PAGE and analyzed with RPRX$\alpha$(D), a polyclonal antibody targeted against the RXR$\alpha$ D region, as described (16).

For CAT assays, COS-1 cells were transfected with 10 $\mu$g of either the reporter plasmid mCRABPII/CAT1 (17) or mCRBPII(17m-ERE)/CAT (9). With mCRABPII/CAT1, 1 $\mu$g of mRXR$\alpha$1, mRXR$\alpha$2 or mRXR$\alpha$1($\Delta$AB) (9) expression vectors were cotransfected. With mCRBPII(17m-ERE)/CAT, 250 ng of Gal4-RXR$\alpha$(DE) and 1 $\mu$g of mRXR$\alpha$1(AB)ER(C) or mRXR$\alpha$2(AB)ER(C) (9) expression vectors were cotransfected. Extracts corresponding to 25-30 units of $\beta$-galactosidase activity were used, and CAT activity was quantified by phospho-imaging.

## RESULTS

### *Novel RXR$\alpha$ Isoforms from Adult Mouse Testis*

Isoforms that diverge in their N-terminal sequence have been described for both the RXR$\beta$ and the RXR$\gamma$ genes (6-8). In the case of these RXRs, the 5′ sequence of the corresponding transcripts diverges either at the first (RXR$\beta$) or the second (RXR$\gamma$) exon boundary present in the A/B region-encoding sequences. To investigate whether similar isoforms exist for the RXR$\alpha$ gene, we analysed RNA derived from various mouse adult tissues, whole embryos or cell lines, using an RNase protection assay with an antisense riboprobe spanning a large portion of the 5′ region of the previously characterized mRXR$\alpha$ (hereafter designated mRXR$\alpha$1 ; see Fig 1A), which will detect any possible alternative transcripts diverging at either one of the boundary of the first two exons. mRXR$\alpha$1 form was the only type of RXR$\alpha$ transcript detectable in most of the RNA samples analysed (see Fig 1B). However, in testis and liver, a 36 nucleotides-shorter protected fragment was apparent, suggesting the presence of distinct mRXR$\alpha$ transcripts that might diverge from the known sequence upstream of the first exon boundary. We describe below the characterization of these testis isoforms.

RACE-PCR was performed on testis RNA, followed by subcloning and sequencing of the amplified products (see Material & Methods). In addition to clones corresponding to mRXR$\alpha$1, two additional mRXR$\alpha$ cDNA were identified, mRXR$\alpha$2 and mRXR$\alpha$3, whose sequences diverge from those of mRXR$\alpha$1 exactly upstream of exon 2 (see Fig 2). As neither mRXR$\alpha$2 nor mRXR$\alpha$3 sequences correspond to the intronic sequences located immediately 5′ of exon 2 (data not shown), these cDNA clones represent genuine mRXR$\alpha$ RNA isoforms, containing specific 5′ exonic sequences spliced to exon 2 transcript. Among the 31 independent clones

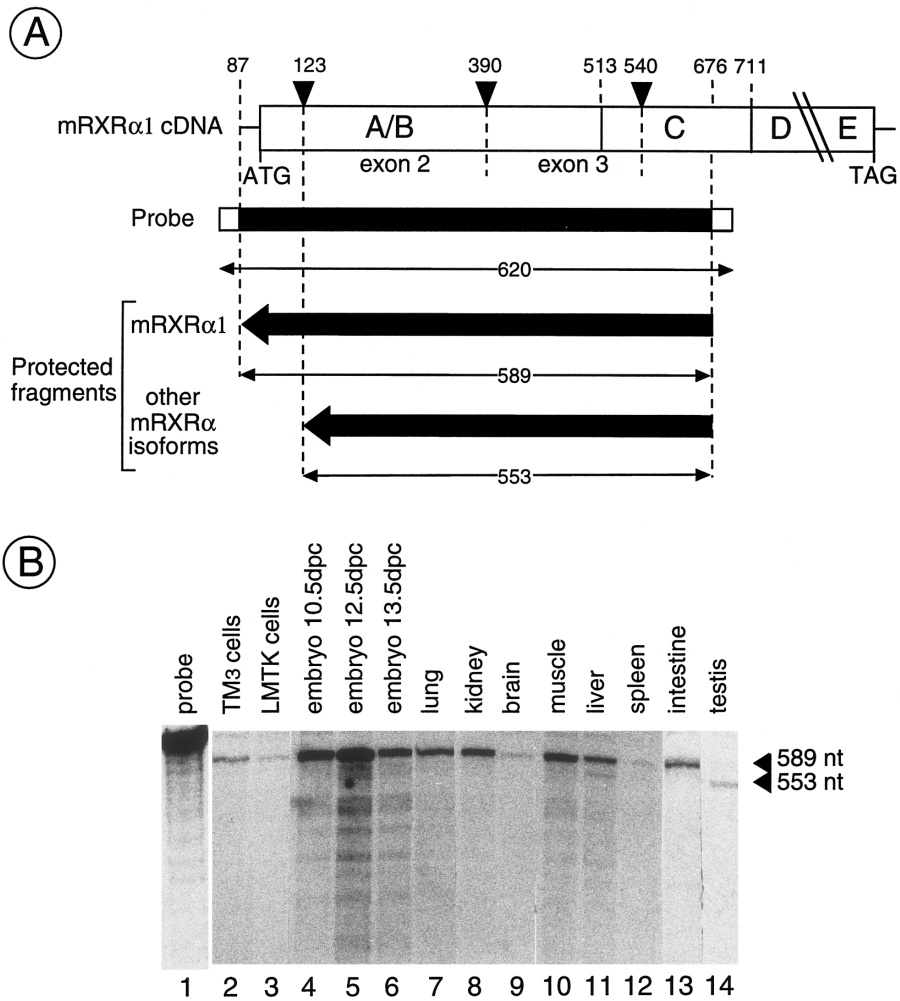


**FIG. 1.** Distinct RXR$\alpha$ transcripts in mouse testis and liver. (A) Experimental design (see text). An antisense RNA probe was synthesized from the T7 promoter (T7) of BSK-mRXR$\alpha$1(A/B-C). Following RNase digestion, mRXR$\alpha$1 and other transcripts isoforms, with diverging sequences upstream of the first exon boundary, should yield 589- and 553-nucleotide protected fragments, respectively. Filled triangles represent exon boundaries. (B) RNase protection analysis using 30 $\mu$g of total RNA, extracted from mouse organs or cell lines. A 589-nucleotide hybrid, corresponding to mRXR$\alpha$1, is detected in all samples. In contrast, a 553-nucleotide hybrid is detected only in liver (lane 11) and testis (lane 14).

sequenced, 15 corresponded to mRXR$\alpha$1, one to mRXR$\alpha$2 and 15 to mRXR$\alpha$3. Thus, among the novel testis isoforms, mRXR$\alpha$3 seems to be the predominant one.

Both mRXR$\alpha$2- and mRXR$\alpha$3-specific sequences contain upstream stop codons in frame with the common exon 2 coding sequence (Fig 2). Therefore, the translation initiation codon of these transcripts necessarily corresponds to one of the downstream mRXR$\alpha$ AUGs or possibly to non-AUG initiation codons. Expression of mRXR$\alpha$2 in COS-1 cells, and comparison of the size of the resulting protein with that of N-terminally truncated mRXR$\alpha$1, mRXR$\alpha$1($\Delta$1-28) and mRXR$\alpha$1($\Delta$1-65) initiated at Met1 and Met2 (Fig 3), respectively, showed that translation of the mRXR$\alpha$2-encoded protein is initiated at the first mRXR$\alpha$2 AUG, corresponding to Met1 of mRXR$\alpha$1 (Fig 2). Since the sequences surrounding this AUG

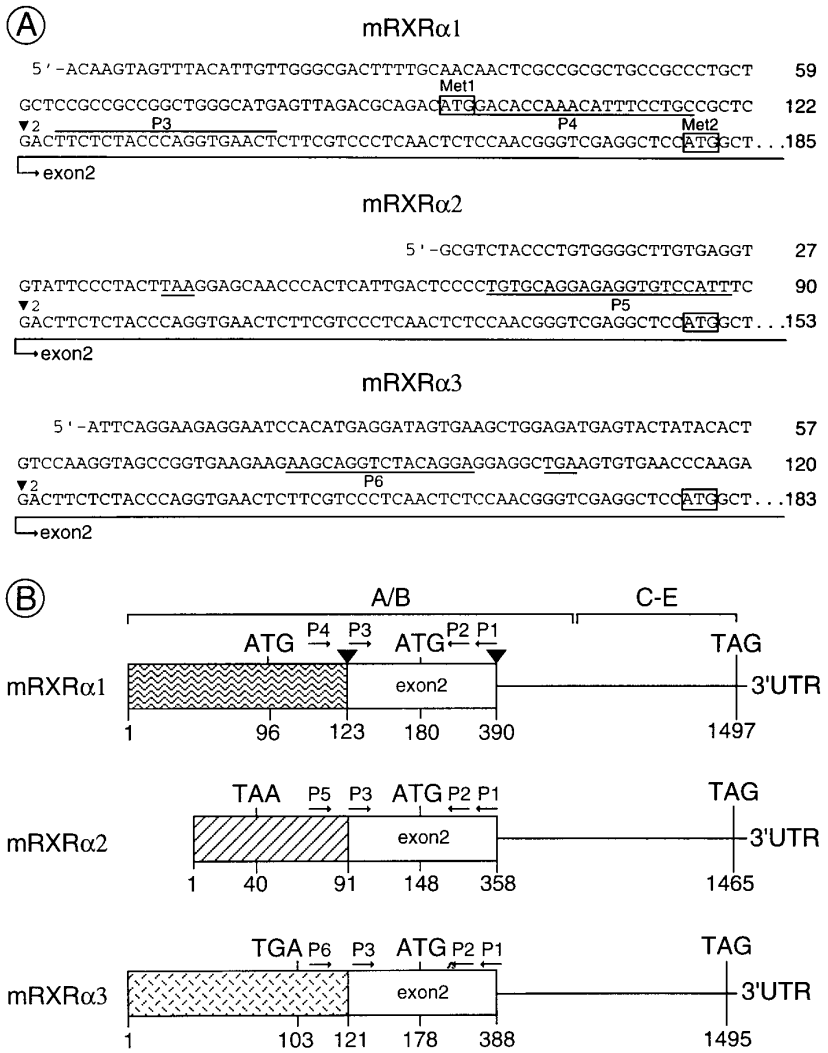


**FIG. 2.** Structure of mRXR$\alpha$ isoforms. (A) 5′ sequences of mRXR$\alpha$1, mRXR$\alpha$2 and mRXR$\alpha$3 cDNAs. The boundary between exons 1 and 2, determined by sequencing of RXR$\alpha$ genomic clones (our unpublished results), is represented with filled triangles. Initiator ATGs are boxed; upstream in-frame stop codons in mRXR$\alpha$2 and mRXR$\alpha$3 are underlined, as well as the sequence common to all isoforms and the P3 to P6 primers. (B) Schematic representation of mRXR$\alpha$ isoforms. Boxes represent sequences 5′ to the boundary between exons 2 and 3 and lines represent common sequences 3′ to it. Exon boundaries, when necessary, are indicated by filled triangles. The first exon is represented with different designs for mRXR$\alpha$1, mRXR$\alpha$2 and mRXR$\alpha$3. Initiator ATGs and upstream in-frame stop codons are shown as well as the position of primers P1-P6.

are identical in mRXR$\alpha$2 and mRXR$\alpha$3, it is highly likely that mRXR$\alpha$3 encodes the same N-terminally truncated mRXR$\alpha$ protein, hereafter designated mRXR$\alpha$2/3. Note that the sequence surrounding this AUG is in good agreement with a Kozak consensus sequence (18).

*AF-1 Activity of RXR$\alpha$2/3*

The N-terminal region of mRXR$\alpha$1 contains a transcription activation function (AF-1) that can, depending on the cell and promoter context, activate transcription on its own, or synergize with the ligand-dependent activation function AF-2 (9). Ligand-independent activation is

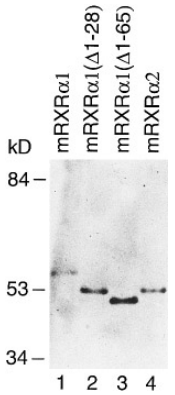


**FIG. 3.** Western-blot analysis of mRXR$\alpha$ isoforms. Proteins expressed in COS-1 cells from mRXR$\alpha$1, mRXR$\alpha$1($\Delta$1-28), mRXR$\alpha$1($\Delta$1-65), or mRXR$\alpha$2 expression vectors were detected with an antibody targeted against the D region of mRXR$\alpha$. Molecular weights are indicated on the left.

readily apparent from an ERE-containing CRBPII-promoter-based reporter in the presence of a chimeric activator consisting of the mRXR$\alpha$1 A/B region tethered to the DNA binding domain (DBD) of the estrogen receptor (ER) region C (9 ; Fig 4A, lane 2). In this assay, the mRXR$\alpha$2/3 A/B region could elicit a transcriptional activation, but its activity was consistently lower (about 50%) than that of the mRXR$\alpha$1 A/B region (Fig 4A, compare lanes 2 and 3). Thus, the ligand-independent activation function of the mRXR$\alpha$2/3 isoforms appears to be less efficient than that of mRXR$\alpha$1.

The ability of the mRXR$\alpha$2/3 AF-1 to synergize with the ligand-dependent activation function AF-2 was also tested. This synergy was previously shown to occur in the presence of 9-cis RA between co-expressed mRXR$\alpha$1(A/B)ER(C) and Gal4-RXR$\alpha$(DE) in the context of a CRBPII promoter containing binding sequences for both the ER and Gal4 DNA binding domains (9 ; Fig4B, compare lanes 4 and 5). A similar magnitude of activation was observed when either mRXR$\alpha$1(A/B)ER(C) or mRXR$\alpha$2(A/B)ER(C) were used in this assay (Fig 4B, lanes 5 and 6). A role of the mRXR$\alpha$ AF-1 in synergizing with the ligand-dependent activation can also be observed with the CRABPII promoter, for which full length mRXR$\alpha$1 mediates

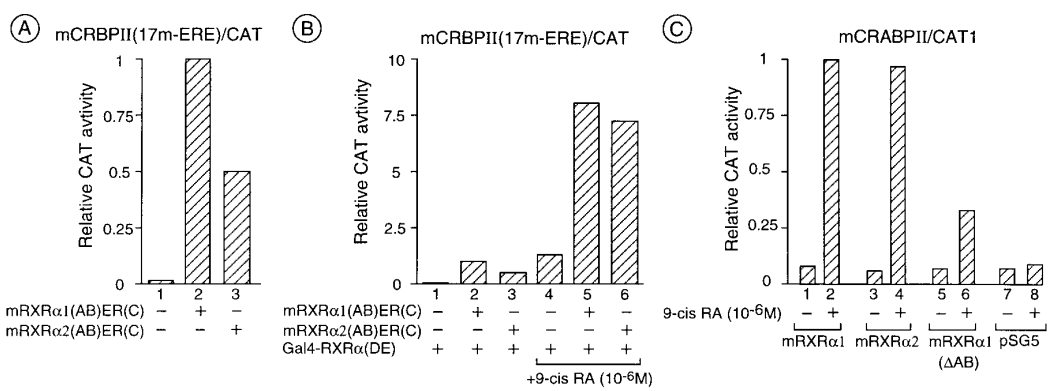


**FIG. 4.** AF-1 and AF-2 transcriptional activation functions of mRXR$\alpha$ isoforms. COS-1 cells were transfected with CAT reporter plasmids and expression vectors, as indicated. CAT activities were expressed relative to those obtained with mRXR$\alpha$1(AB)ER(C) [(A) and (B), lane 2] in the absence of 9-cis RA or mRXR$\alpha$1 [(C), lane 2], taken as 1. Each assay has been performed at least twice ($\pm$10%).

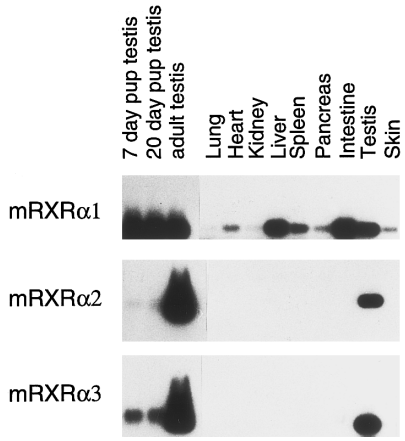


**FIG. 5.** Expression pattern of mRXR$\alpha$ isoforms. RT-PCR reactions performed on 1 $\mu$g of total RNA from various tissues were separated on an agarose gel, and hybridized to [$^{32}$P]-labelled oligonucleotide P3 (see Fig. 2B). Note that mRXR$\alpha$2 and mRXR$\alpha$3 are expressed at much higher levels in post-pubertal testis than in pre-pubertal testis, whereas mRXR$\alpha$1 levels remain unchanged. In each case, control reactions in which reverse-transcriptase was omitted did not yield any amplification product.

a much stronger 9-cis RA induction than an A/B region-truncated mRXR$\alpha$ (19 ; Fig 4C, compare lane 2 to lane 6). When tested in this system, mRXR$\alpha$2/3 had an activity comparable to that of mRXR$\alpha$1 (Fig 4C, compare lanes 2 and 4). Together with the above data, this result suggests that both mRXR$\alpha$1 and mRXR$\alpha$2/3 AF-1 activation functions similarly synergize with mRXR$\alpha$ AF-2.

*Expression Pattern of mRXR$\alpha$ Isoforms*

RT-PCR analysis of the expression of mRXR$\alpha$1 showed that this isoform is present in a number of adult tissues, as expected (Fig 5). In contrast, mRXR$\alpha$2 and mRXR$\alpha$3 transcripts could be detected only in the testis (Fig 5). Therefore, at least within the panel of tissues tested, mRXR$\alpha$2 and mRXR$\alpha$3 appear to be testis-specific. We next examined the expression of the different mRXR$\alpha$ isoforms during testis maturation. mRXR$\alpha$1 transcripts were present at comparable levels in prepubertal testes (7 day- and 20 day-old pups) and adult testes (2 month-old mice; Fig 5). In contrast, the expression of both mRXR$\alpha$2 and mRXR$\alpha$3 transcripts was strongly induced in post-pubertal testes (Fig 5). We have previously reported that in the adult testes, mRXR$\alpha$ is expressed in post-meiotic germ cells (round spermatids) of a subset of seminiferous tubules, as well as in Leydig cells (20). In this respect, the strong induction of the mRXR$\alpha$2 and mRXR$\alpha$3 transcript expression at puberty could possibly reflect their presence in these spermatids. Unfortunately, the small size of the specific sequences of the various isoforms did not allow their differential characterization by in situ hybridization (data not shown).

## DISCUSSION

*RXR$\alpha$ Isoforms*

We have reported here the characterization of two novel mRXR$\alpha$ transcript isoforms. RXR$\alpha$ joins therefore the many nuclear receptors for which isoforms with distinct N-terminal sequences have been described. These include notably the RARs (see 1 and 2 for review), TR$\beta$ (21), the progesterone receptor (22), PPAR$\gamma$ (23), the ecdysone receptor (24), HNF4 (25), RXR$\beta$ (6-7) and RXR$\gamma$ (8). In contrast to RARs for which clear homologies exist among the

N-terminal regions of some isoforms of different receptor isotypes, no such homologies are evident in the case of RXR isoforms where 5′ divergent exonic sequences are not even spliced to a common 3′ sequence, as it is the case for RAR isoforms (see 2 for review). Other mRXR$\alpha$ isoforms with distinct tissue specificities may possibly exist since a smaller hybridizing species was revealed in the liver using the present RNase protection assay.

The identity of the primary transcript(s), from which the two novel mRXR$\alpha$ isoforms originate, is presently unclear. However, it is not unlikely that these isoforms are transcribed from a promoter distinct from that generating mRXR$\alpha$1 transcripts, as mRXR$\alpha$1 and mRXR$\alpha$2/3 transcripts exhibit different expression profiles. The characterization of the mRXR$\alpha$ 5′ gene structure and the determination of the 5′ ends of mRXR$\alpha$2 and mRXR$\alpha$3 transcripts will be required to answer this question and also to establish whether both mRXR$\alpha$2 and mRXR$\alpha$3 originate from the same testis-specific promoter. However, the fact that the mRXR$\alpha$2 and mRXR$\alpha$3 specific sequences are entirely distinct, and that no RT-PCR product containing both types of sequences could be amplified (our unpublished data), argues in favor of a distinct origin.

The mRXR$\alpha$2/3 A/B region was less efficient than that of mRXR$\alpha$1 at inducing transcription from a CRBPII promoter-based reporter. Even though the AF-1 of mRXR$\alpha$2/3 did not appear to be compromised in its ability to synergize with the AF-2 function, this observation nevertheless suggests that the AF-1 function of mRXR$\alpha$1 and mRXR$\alpha$2/3 could be different. More drastic differences in the transcriptional potential of mRXR$\alpha$1 and mRXR$\alpha$2/3 may become more obvious in the context of other promoters and/or cell types. In this respect, we note that functional specificity for nuclear receptor isoforms has been clearly demonstrated in some cases. In the newt limb blastema, studies employing chimeric receptors comprising the ligand binding domain (LBD) of the TR and the RAR isoform-specific A/B region and DBD have shown that specific RAR isoforms mediate distinct physiological outcomes (see 26 for review). In vitro studies have also shown clear differences in the functional properties of the two progesterone receptor isoforms (22, 27-29), as well as between the AF-1 activation function of RXR$\alpha$1, RXR$\beta$2 and RXR$\gamma$1 (9,30).

### *Possible Function of RXR$\alpha$ Isoforms in the Testis*

RXR$\alpha$ null mutant mice cannot be used to investigate a possible function for mRXR$\alpha$ during spermatogenesis, as these mutants die in utero (31,32). However, since RAR$\alpha$ null mutant male mice exhibit a degeneration of their germinal epithelium which does not occur in RXR$\beta$ mutant males (20, 33), RXR$\alpha$ could be required during spermatogenesis as an RAR$\alpha$ heterodimeric partner. In addition to RAR$\alpha$, other nuclear receptors may also require RXR$\alpha$ as a partner in the testis, most notably TAK1 which is specifically localised in germ cells (34). These various observations suggest that RXR$\alpha$ may perform an important role during spermatogenesis. The present identification of testis-specific mRXR$\alpha$ isoforms should thus allow to genetically address this question, by the engineering of isoform-specific mutants which should not suffer from early lethality defects.

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