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Identification of a Novel Isoform of the Retinoic Acid Receptor γ Expressed in the Mouse Embryo

VINCENT GIGUÈRE,[1,2]* MARY SHAGO,[1] RALPH ZIRNGIBL,[3] PERI TATE,[4] JANET ROSSANT,[2,3] AND SUE VARMUZA[4]

Division of Endocrinology, Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8[1]; Departments of Medical Genetics[2] and Zoology,[4] University of Toronto, Toronto, Ontario, Canada M5S 1A1; and Division of Molecular and Developmental Biology, Mount Sinai Hospital Research Institute, Toronto, Ontario, Canada M5G 1X5[3]

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Retinoic acid is known to have profound effects on developmental processes. It has been implicated as a putative morphogen in the developing chick limb bud and regenerating amphibian limb blastema and has been demonstrated to have powerful teratogenic effects in mammals, including humans. Recently, three specific retinoic acid receptors (RARs), RARα, -β, and -γ, were identified and shown to be members of the steroid receptor superfamily. We report the identification of a novel RARγ isoform, mRARγB, which differs from the previously described mouse RARγ at its amino terminus. In addition, we show that both RARγ isoforms are expressed maximally at midgestation in structures known to be affected adversely by retinoic acid administration to pregnant mice. Multiple RAR isoforms, each of which may play a unique or combinatorial role as a regulator of mammalian development, are thus expressed in the mouse embryo.

Retinoic acid, an active metabolite of vitamin A, has profound effects on vertebrate development. Teratogenic manipulations have demonstrated that local application of retinoic acid at the anterior margin of the developing chick limb bud induces a mirror-symmetrical digit pattern duplication (9, 26, 30, 31). Striking effects of retinoic acid have also been observed in the regenerating amphibian limb, where retinoic acid causes duplication along the proximodistal axis (19, 21). Retinoic acid is also known as a powerful teratogen whose effects are characterized by severe limb and cranial defects, including cleft secondary palate, midfacial malformations, abnormalities of the external and middle ear, and neural tube deformities both in mice (24) and humans (18).

The identification of a retinoic acid receptor (RAR) (13, 22) which belongs to the superfamily of steroid-thyroid hormone receptors provided a potential model for the molecular mechanism of action of retinoic acid. This family of nuclear receptors controls cell functions by directly regulating gene expression, and by analogy it has been proposed that the interaction between retinoic acid and its receptor induces a cascade of regulatory events that results from the activation of specific gene networks by the ligand-receptor complex (13). Since the initial discovery of the RAR, three different forms have been identified on the basis of their predicted amino acid sequences and designated RARα (13, 22), -β (2, 3), and -γ (16, 34). The three receptors are remarkably similar to one another except for their amino and carboxy termini. However, the different RARs can be distinguished by their affinities to retinoic acid (3; V. Giguère and R. M. Evans, Methods Enzymol., in press) and the distinct spatial pattern of expression of their mRNAs in adult mouse and human tissues (2, 6, 13, 34). These findings suggest that each receptor subtype may perform a specific biological function.

We now report the isolation of a cDNA encoding a novel isoform of mouse RARγ. The protein, designated mRARγB, differs from the previously characterized mRARγ (here referred to as RARγA) in its amino-terminal region. Both RARγA and -B transcripts are transiently expressed during mouse embryogenesis, with the highest level of expression detected in structures known to be adversely affected by retinoic acid administration to pregnant females. The abundant expression of RARγ in the developing mouse embryo suggests that the physiological and teratogenic activities of the retinoids might be mediated through these nuclear receptors.

MATERIALS AND METHODS

Isolation and sequencing of mouse RAR cDNA clones. The clone λmE7.1 (mRARγB) was isolated from a 7.5-day-old mouse embryo λgt10 cDNA library, and clone λmE13.20 (mRARγA) was obtained from a 12.5-day-old mouse embryo λgt10 cDNA library with hybridization probes derived from the cDNA encoding the human RARα (13). The hybridization mixture contained 35% formamide, 1× Denhardt solution, 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA [pH 7.7]), 0.1% sodium dodecyl sulfate, 100 μg of denaturated salmon sperm DNA ml^{-1}, and 10^6 cpm of ^{32}P-labeled DNA probes (>10^8 cpm μg^{-1}) ml^{-1}. Duplicate nitrocellulose filters were hybridized at 42°C for 16 h, washed three times for 20 min per wash in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 55°C and autoradiographed at −70°C with an intensifying screen. Clones λmE7.1 and λmE13.20 were subcloned into the *Eco*RI site of pGEM4 (Promega Biotec), resulting in the creation of pGmRARγA and pGmRARγB, and the inserts were digested with a number of restriction enzymes. The resulting fragments were subcloned in both orientations into the M13 sequencing vectors mp18 and mp19 and sequenced by the dideoxy procedure (25) with modified T7 polymerase (27) and dITP (20) to eliminate compression when required.

Recombinant plasmids. Plasmids pRSmRARγB and pRSmRARγA, which direct the synthesis of the two isoforms of mRARγ in COS-1 cells, were constructed as follows. To obtain pRSmRARγB, the human glucocorticoid

* Corresponding author.

receptor cDNA contained in the parent expression vector pRShGR (11) was excised by digestion with *Asp* 718 and *Bam*HI, and the plasmid DNA was blunted by end-filling with Klenow polymerase to yield the pRS vector. A fragment of the mRARγB cDNA containing the entire open reading frame was obtained by digestion with *Nru*I and *Xba*I followed by end-filling and ligation to the blunt pRS vector. To generate pRSmRARγA, the parent vector pRShGRN (12) was digested with *Bam*HI, end filled to generate a blunt end, and then digested by *Nco*I to remove the human glucocorticoid receptor cDNA insert. The sequence encoding the mRARγA was excised from the cDNA clone by digestion with *Xba*I, end-filling, and digestion by *Nco*I and was then inserted into *Nco*I-*Bam*HI (blunt)-digested pRShGRN to yield pRSmRARγA. To generate the expression vector pRShRARβ, an insert from the human RARβ cDNA (2) was excised by partial digestion with *Mae*I and ligated to the blunt pRS vector. Construction of reporter plasmid ΔMTV-pTRE-LUC, which contains the luciferase reporter gene linked to the mouse mammary tumor virus-long terminal repeat in which glucocorticoid-responsive elements have been deleted and replaced by a palindromic thyroid hormone responsive element (pTRE), was described elsewhere (33).

Transfection and luciferase and β-galactosidase assays. The recombinant RAR expression vectors and luciferase and β-galactosidase reporter genes were introduced into COS-1 cells by calcium phosphate coprecipitation as described previously (11, 15). Two micrograms of each RAR expression and luciferase reporter plasmid together with 500 ng of the β-galactosidase reporter vector were used per 6-cm-diameter dish. The following day, transfected cells were washed twice with phosphate-buffered saline and fed with Dulbecco modified Eagle medium supplemented with 5% (vol/vol) fetal calf serum containing the indicated concentration of retinoic acid or ethanol as control. As described elsewhere, luciferase (7) and β-galactosidase (14) assays were performed 24 h after induction of the cells with retinoic acid.

RNA analysis. Total RNA was isolated from whole mouse embryos or embryonic tissues by using guanidine thiocyanate (5), and the integrity of the RNA samples was confirmed by agarose gel electrophoresis and visualization of the rRNAs after ethidium bromide staining. For Northern (RNA) blot analysis, approximately 10 μg of total RNA obtained from mouse embryos sacrificed at different stages of development was fractionated in a formaldehyde-agarose gel, blotted to N-Hybond (Amersham Corp.), hybridized to ^{32}P-labeled DNA fragments, and washed as described previously (13). After exposure to the X-ray film, the filter was dehybridized by a 5-min treatment in a solution of 0.1% sodium dodecyl sulfate at 90°C, allowed to cool to room temperature, and rehybridized with the next probe. The three probes used in this study were obtained as follows. The probe common to both mRARγA and -B was excised from the RARγB cDNA clone with *Pst*I and encompassed a 1,282-base-pair (bp) region containing part of the carboxy-terminal coding region and 3′ untranslated sequence; the probes specific for mRARγA and -B were obtained by digesting their respective cDNA clones with *Eco*RI and *Asp* 718, giving fragments of 476 and 262 bp, respectively, that contain the 5′ untranslated sequence and coding region specific to each receptor subtype.

For the RNase protection assay, 20 μg of total RNA obtained from various mouse embryonic tissues was precipitated; suspended in hybridization buffer containing 80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.7], 0.4 M NaCl, and 1 mM EDTA; and hybridized overnight with antisense probes specific for RARγA or RARγB in 80% formamide at 50°C. The hybrids were digested with 40 μg of RNase A and 700 U of RNase T_1 per ml and then treated with proteinase K, phenol extracted, and precipitated. The hybrids were electrophoresed on a 6% sequencing gel with M13 sequence reactions as size markers. The antisense probes were derived from subclones of RARγA and RARγB consisting of the 5′ *Eco*RI-*Kpn*I fragments of each isoform subcloned into pGEM3 and pGEM7z, respectively. The RARγA subclone was digested to completion with *Nco*I and transcribed with SP6 RNA polymerase in the presence of [^{32}P]CTP to generate a 270-nucleotide probe. The RARγB subclone was digested to completion with *Eco*RI and transcribed with SP6 polymerase as well, which produced a 322-nucleotide probe.

RESULTS

Cloning of mouse RAR cDNAs. To explore the possible role of RAR(s) during mouse embryogenesis, we wished to isolate cDNAs encoding mouse homologs to the previously described human RARα and RARβ. Two λgt10 cDNA libraries made with poly(A)$^+$ RNA obtained from 7.5- and 13.5-day-old mouse embryos were screened under reduced stringency conditions with hybridization probes derived from the human RARα cDNAs (13). Several positive clones were isolated and characterized by restriction enzyme and sequence analyses. As expected, two groups of cDNA clones were shown to encode the mouse homologs to human RARα and RARβ (sequence data not shown). However, two other distinct clones, designated λmE7.1 and λm13.20, were determined to be identical to each other except for their respective 5′ ends (Fig. 1). Amino acid sequence analysis revealed discrete presumptive initiator methionine codons located at nucleotides 67 to 69 of clone λmE7.1 and 253 to 255 of clone λmE13.20 followed by open reading frames of 447 and 458 amino acid residues, respectively. After the terminator codon is a 1,088-nucleotide 3′ untranslated region with a consensus polyadenylation signal (AATAAA) found 20 nucleotides upstream of a polyadenylated tract. The protein encoded by clone λm13.20 was revealed to be identical to the recently described RARγ (34) and is here referred to as RARγA. Therefore, the gene product of λmE7.1, designated RARγB, constitutes a novel RARγ isoform with a different amino-terminal domain. DNA sequence analysis (Fig. 1) reveals that the point of divergence between RARγA and RARγB, nucleotides 1 to 435 and 1 to 216, respectively, corresponds to a splice junction between exons 1 and 2 of the human RARα gene (V. Giguère, unpublished data), suggesting that the two clones are generated by usage of two distinct promoters. Studies of the genomic organization of the mouse RARγ gene are in progress, and preliminary results indicate that the specific DNA sequences encoding the amino-terminal regions of RARγA and -B are closely linked, since a genomic clone which hybridizes with both 5′ probes (J. Rossant and R. Zirngibl, unpublished data) has been isolated.

Sequence comparison. As depicted schematically in Fig. 2, the mRARγB and mRARγA proteins show no amino acid sequence similarity at their amino termini, which is consistent with the observation of a hypervariable sequence in this region of different members of the steroid-thyroid hormone receptor superfamily (10). Interestingly, further amino acid comparisons of mRARγB and mRARγA with mRARα and mRARβ show 28% amino acid identity between the amino

RARγB

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  1                                                                                 MetTyrAspCysMetGluSerPhe
  1  AGGAGGGGGAATGGGCTGCGGGTCCTTGGTGTTCTAGCACCCAGTTTCTCTCCAAGCCGGGTCGCGATGTACGACTGCATGGAATCGTTT

  9  ValProGlyProArgArgLeuTyrGlyAlaAlaGlyProGlyAlaGlyLeuLeuArgArgAlaThrGlySerSerCysPheAlaGlyLeu
 91  GTCCCGGGTCCGCGACGGCTGTATGGGGCGGCCGGGCCCGGGGCCGGCTTACTACGCAGAGCCACTGGCAGCTCCTGCTTCGCCGGACTT

 39  GluSerPheAlaTrpAlaGlnProAlaSerLeuGln
181  GAGTCTTTTGCCTGGGCACAGCCTGCCAGTCTACAA
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RARγA

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     CGGGGCTCTGCGGCCTTCCTTCCCCCGCCCTCCCCTCCAGCAGTTTCCACCAGGTCCCTCACCTCAGCCTGGCCCAGTATGTAGGAGGGA     1

     ATCTCTGCAGAGGCCAGAGGGATCCTTGGAAACCCACTGGACAGACCAGGCAGGGTGGGCACGGAGCCTCCCAGGCCAGGGCAGTGGGCA    91

                                                                                   MetAlaThrAsnLysGlu     1
     TGGGCGGGGGCTGTAGCTGAAGACCACCCCCGCCTGCTGCAGAGTCCAAGGGATTCCCACGCCGCAGCTACCATGGCCACCAATAAGGAG   181

     ArgLeuPheAlaProGlyAlaLeuGlyProGlySerGlyTyrProGlyAlaGlyPheProPheAlaPheProGlyAlaLeuArgGlySer     7
     AGACTCTTTGCGCCCGGTGCCCTGGGGCCTGGATCTGGTTACCCAGGAGCAGGCTTCCCATTCGCCTTCCCAGGTGCACTCAGAGGGTCG   271

     ProProPheGluMetLeuSerProSerPheArgGlyLeuGlyGlnProAspLeuProLysGluMetAlaSerLeu                   37
     CCACCATTTGAGATGCTGAGCCCTAGCTTCCGGGGCCTGGGCCAGCCTGACCTCCCCAAGGAGATGGCTTCTCTC                  361
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COMMON

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  51  SerValGluThrGlnSerThrSerSerGluGluMetValProSerSerProSerProProProProProArgValTyrLysProCysPhe    62
 217  TCGGTGGAGACACAGAGCACCAGCTCGGAGGAGATGGTACCCAGCTCTCCCTCACCCCCACCACCTCCTCGGGTCTATAAGCCATGCTTT   436

  81  ValCysAsnAspLysSerSerGlyTyrHisTyrGlyValSerSerCysGluGlyCysLysGlyPhePheArgArgSerIleGlnLysAsn    92
 307  GTATGCAATGACAAGTCTTCTGGCTACCACTATGGGGTCAGCTCCTGTGAAGGCTGCAAGGGCTTCTTCAGACGCAGCATTCAGAAAAAC   526

 111  MetValTyrThrCysHisArgAspLysAsnCysIleIleAsnLysValThrArgAsnArgCysGlnTyrCysArgLeuGlnLysCysPhe   122
 397  ATGGTGTATACATGTCACCGTGACAAAAACTGTATCATCAACAAGGTCACCAGAAATCGATGCCAGTACTGCAGGCTACAAAAGTGTTTC   616

 141  GluValGlyMetSerLysGluAlaValArgAsnAspArgAsnLysLysLysLysGluValLysGluGluGlySerProAspSerTyrGlu   152
 487  GAAGTGGGCATGTCCAAGGAAGCTGTAAGGAACGATCGAAACAAGAAGAAAAAGGAGGTAAAAGAGGAGGGCTCGCCCGACAGCTATGAA   706

 171  LeuSerProGlnLeuGluGluLeuIleThrLysValSerLysAlaHisGlnGluThrPheProSerLeuCysGlnLeuGlyLysTyrThr   182
 577  CTGAGTCCACAGTTAGAGGAACTCATCACCAAGGTCAGCAAAGCCCACCAGGAGACTTTTCCCTCACTCTGCCAGCTGGGCAAGTACACC   796

 201  ThrAsnSerSerAlaAspHisArgValGlnLeuAspLeuGlyLeuTrpAspLysPheSerGluLeuAlaThrLysCysIleIleLysIle   212
 667  ACGAACTCCAGTGCAGATCACCGGGTGCAGCTGGACCTGGGGCTGTGGGACAAGTTCAGCGAGCTGGCCACCAAATGCATCATCAAGATT   886

 231  ValGluPheAlaLysArgLeuProGlyPheThrGlyLeuSerIleAlaAspGlnIleThrLeuLeuLysAlaAlaCysLeuAspIleLeu   242
 757  GTGGAGTTTGCGAAGCGGCTGCCTGGTTTTACAGGGCTCAGCATTGCCGACCAGATCACGCTGCTCAAGGCTGCTTGTCTGGACATCCTA   976

 261  MetLeuArgIleCysThrArgTyrThrProGluGlnAspThrMetThrPheSerAspGlyLeuThrLeuAsnArgThrGlnMetHisAsn   272
 847  ATGCTGCGGATCTGTACAAGGTATACCCCAGAGCAGGACACTATGACATTCTCGGATGGGCTGACCCTGAACCGAACCCAGATGCACAAT  1066

 291  AlaGlyPheGlyProLeuThrAspLeuValPheAlaPheAlaGlyGlnLeuLeuProLeuGluMetAspAspThrGluThrGlyLeuLeu   302
 937  GCTGGCTTTGGGCCCCTTACAGACCTCGTCTTTGCCTTTGCCGGGCAGCTGCTGCCCCTGGAGATGGATGACACCGAGACTGGGCTACTT  1156

 321  SerAlaIleCysLeuIleCysGlyAspArgMetAspLeuGluGluProGluLysValAspLysLeuGlnGluProLeuLeuGluAlaLeu   332
1027  AGTGCTATCTGCCTCATCTGTGGAGACCGAATGGACCTGGAAGAGCCCGAGAAGGTGGACAAGCTGCAGGAGCCCCTGCTGGAAGCCCTG  1246

 351  ArgLeuTyrAlaArgArgArgAspProAlaLysProTyrMetPheProArgMetLeuMetLysIleThrAspLeuArgGlyIleSerThr   362
1117  AGGCTCTATGCCCGGCGACGAGACCCAGCCAAACCCTACATGTTCCCAAGGATGCTGATGAAAATCACCGACCTCCGGGGCATCAGCACT  1336

 381  LysGlyAlaGluArgAlaIleThrLeuLysMetGluIleProGlyProMetProProLeuIleArgGluMetLeuGluAsnProGluMet   392
1207  AAGGGAGCAGAAAGGGCTATAACCCTGAAGATGGAGATTCCAGGCCCGATGCCACCCCTGATCCGAGAGATGCTGGAGAACCCGGAGATG  1426

 411  PheGluAspAspSerSerLysProGlyProHisProLysAlaSerSerGluAspGluAlaProGlyGlyGlnGlyLysArgGlyGlnSer   422
1297  TTTGAGGACGACTCCTCGAAGCCTGGCCCCCACCCCAAGGCTTCCAGTGAGGACGAAGCTCCAGGGGGCCAGGGCAAAAGGGGCCAAAGT  1516

 441  ProGlnProAspGlnGlyProSTOP                                                                   452
1387  CCCCAACCTGACCAGGGGCCCTGACCTACCCCGTTGTGGGGTTGGGCCCCAGGCAGCAGACTGACCATTTCCCAGATACCGCCAGTGACT  1606

1477  GGGGGAGGACCTGCCCCGCCCACTCTCCACCCTTTTAATGAGCTCGTTATTTTGCCAAAGTTTCTAGGGGTGCCTGTGTTCATCCCTGTC  1696

1567  CTGTTCTAACTGGCTCCCTCTACAGTCCCGCGGGGATCTGCTGCACACCTACCAGAAGAGCTGGAGAAGGGGCGAGCCTGGGTCTAGACT  1786

1657  CTAAAATCTCAGCACTGCCTTTCGGATTCAGGCCACAGGCTCCCGACGCAAGAGGAAGCCCCGCCTTCCCATAGCCTTTTCCTCTGCCAG  1876

1747  GTGCTTGGGCCTCTGGGAGCAAACAGGAACACTAGAGACCAAAAGGGGGGCCCCCCCCGAGGGGAGGGCTGAGCCACCCTTGTGCCCCAC  1966

1837  CCTGAGCGCCTGACTCTGTGTGATGAATCTGCCGCGCTCAGTGCCCCCGGTGCCCCATCCTAATCCTCCTGCCGGGTGTAGGGGCAGGCT  2056

1927  GGGGCCTGCGTTTTCTAGGGTGGGGCCTGAGGGGAAGGGAGAATGTGAGCCCGAGCCCGTCTGTACCTCTGCTGAAGTACAGCACTCATC  2146

2017  CTGGGCCCTCCATTATATAAGCCTCTCCCTTACTTCTGTCTCGTGCCTTGGCCTCCCGCCCTCATCTCAGCCATCAGGCAGGGCCCTCCT  2236

2107  ACACTACAGAGGGGCCAGGGGTCCCTCTCCTAGTGCCTTCTACCCTTGATCCCCCCAGAGCAGCTTGGCCCCGGGGAGGGGAGATGCTGC  2326

2197  TTAGCTGATCCCACCTTGACCCAGAGGAAGCCTCTATTTATTTATTAGCTTTTGTTTACACCCTGGAAATGACCCCTTCCTCCAGGGGTC  2416

2287  TTGGGAGGGGGAGCCCAGGGCCTCTGTGACCCCTTCCTTTCTTTCCCCAAGCCCCCAGTTTATATTTAGCTGCCAAATAAGATTCCTGCA  2506

2377  GGCTCCCCCATTTCTCTGGGGGGTTAGGGTGCTGTCCCCTCCCCTCTGTTTATATCTCCTACCCCGCTGTTCGCATATTGCTGAGTTTTC  2596

2467  TATTTTTGCAAAATAAAGTGATGGAAACTCAAAAAAAAAAAAAAAAAAAAAAAAA                                     2686
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FIG. 1. Nucleotide and deduced amino acid sequences of two mouse RAR isoforms. The sequence encoding the two proteins is divided into RARγB- and RARγA-specific and common regions. The RARγB and RARγA sequences were obtained from cDNA clones λmE7.1 and λmE13.20, respectively. The location of the polyadenylated tract is identical in the two cDNA clones. Nucleotides and amino acid residues are numbered on the right for RARγA and on the left for RARγB.

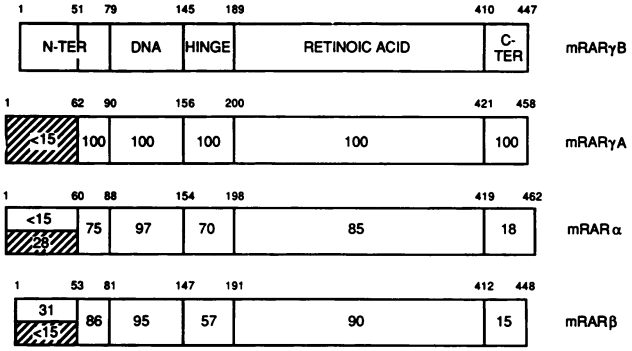

FIG. 2. Schematic amino acid comparisons among the four mouse RAR isoforms. Amino acid sequences have been aligned schematically according to the functional structure domain of the steroid-thyroid hormone receptor and RAR superfamilies. The percentage of amino acid identity between each receptor and mRARγB is indicated within the clear boxes, and identity with the specific N-terminal regions of RARγA is shown inside the cross-hatched boxes. The amino acid positions of domain boundaries are shown for each receptor.

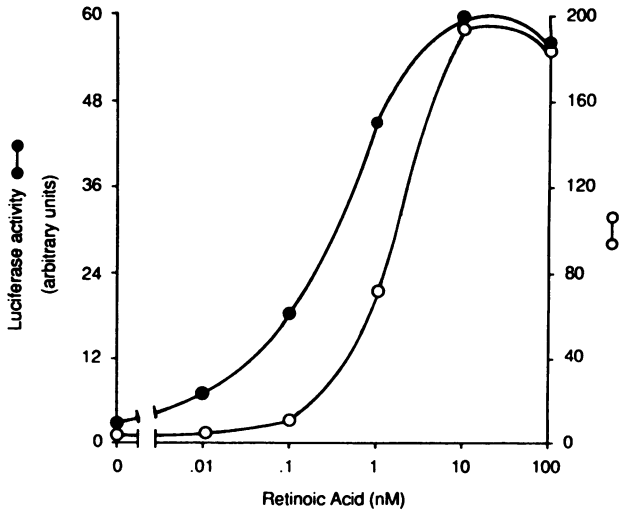

FIG. 3. Retinoic acid-dependent transcriptional activation by mouse RARγB and human RARβ. Luciferase activity was measured by using COS-1 cell extracts obtained after cotransfection of the reporter plasmid ΔMTV-pTRE-LUC, the expression vector carrying mouse RARγB (●) or human RARβ (○) and the plasmid RSV-βGAL as internal control. Because of significant differences in their potential to stimulate transcription in this assay, mouse RARγB and human RARβ responses to increasing doses of retinoic acid are shown on different scales (left and right, respectively) in arbitrary units of luciferase enzyme activity normalized for transfection efficiency, with β-galactosidase activity as an internal control.

termini of mRARγA and mRARα and 31% similarity between mRARγB and mRARβ in the same region. Additional amino acid sequence analysis reveals high degrees of conservation both in the DNA- and retinoic acid-binding domains between the mRARγs and mRARα (97 and 85%, respectively) and between the mRARγs and mRARβ (95 and 90%, respectively).

Expression of functional RARγB. To demonstrate the functional identity of the novel gene product as an RAR, expression vectors containing the cDNAs encoding either mRARγB or -A under the transcriptional control of the Rous sarcoma virus long terminal repeat were constructed and cotransfected in the mammalian cell line COS-1 together with the reporter plasmid ΔMTV-pTRE-LUC that has been shown to be responsive to both thyroid hormone receptors and RARs (29, 33). Transfection with the RARγB expression vector pRSmRARγB resulted in a two- to fourfold stimulation of luciferase activity in the absence of retinoic acid (data not shown). This constitutive activity was also observed when pRSmRARγA was introduced in COS-1 cells. Whether this constitutive activity is an intrinsic property of the RARγs or observed only in the context of this cotransfection assay remains to be investigated. However, increasing concentrations of retinoic acid led to a 20-fold stimulation of luciferase activity by mRARγB with a 50% effective dose of ~0.5 nM (Fig. 3). A similar dose-response curve was observed when mRARγA was substituted for mRARγB (data not shown). The response properties of mRARγB were also compared with those of human RARβ. In our assay, the 80-fold human RARβ activation of luciferase activity by retinoic acid was observed with a 50% effective dose of 2 nM. It has been previously demonstrated that human RARα mediates activation of transcription at concentrations of retinoic acid 10-fold higher than those for human RARβ (3; Giguère and Evans, in press). Thus, all three RARs respond to different concentrations of their ligand in this transcriptional assay. Whether these results are the reflection of different affinities of each RAR for retinoic acid remains to be investigated.

Expression of RARγ isoforms in the mouse embryo. To examine the temporal pattern of expression of the RARγs in the mouse embryo, we performed Northern blot analysis with RNA extracted from whole mouse embryos at different stages of development. When a 1,282-bp *Pst*I fragment common to both mRARγA and mRARγB cDNAs was used as a probe, two RNA species of 2.9 and 2.7 kilobases were detected as early as day 9.5. Levels of these mRNA species peaked around day 12.5 and declined substantially at day 16.5 (Fig. 4). Using probes specific to mRARγA and

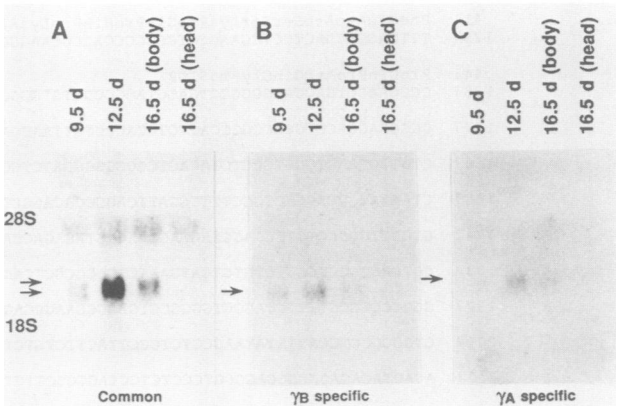

FIG. 4. Northern blot analysis of RARγ mRNAs in mouse embryos at different stages of development. The nylon filter was first hybridized with a DNA probe common to both mRARγ transcripts (A), washed, and exposed to an X-ray film for 4 days. The filter was then dehybridized and rehybridized with a DNA probe specific for mRARγB (B), washed, and exposed for 6 days. The procedure was repeated once more with an RARγA-specific DNA probe (C). Arrows indicate the position of each specific transcript. The size markers were the 28S (4,712 bp) and 18S (1,869 bp) rRNAs.

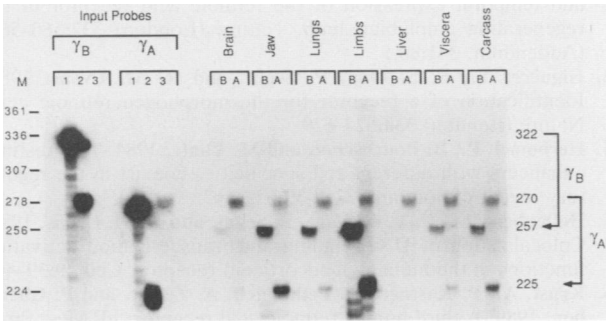

FIG. 5. RNase protection analysis of RARγB and -A transcripts in 13.5-day-old mouse embryo. Total RNA (20 μg) from various mouse embryo tissues was hybridized to either RARγB (lanes B)- or RARγA (lanes A)-specific antisense riboprobe overnight at 50°C. After digestion with RNases A and T_1 to remove nonhybridized probe, the resulting protected hybrids were electrophoresed on a 6% sequencing gel. The RARγA 270-base antisense probe was protected by embryo RNA to yield a 225-base protected fragment, while 257 bases of the 322-base RARγB antisense probe were protected. The leftmost two experiments show control hybridizations performed with sense RNA generated from *Cla*I and *Kpn*I digests of the RARγB and RARγA subclones, respectively, using T7 RNA polymerase in each case. The RARγB subclone contained an extra piece of polylinker which prohibited the use of *Kpn*I in this experiment, and consequently the sense RNA contained an extra 14 bases of polylinker which lengthened the protected fragment relative to that generated by embryo RNA. Lanes: 1, undigested probes; 2 and 3, probe hybridized, respectively, with or without sense transcripts and digested with RNases. Note that, as evidenced by its presence in the experimental lanes, RARγA probe was somewhat resistant to RNase.

mRARγB derived from the 5′ ends of the respective cDNAs, we could determine that the 2.9-kilobase mRNA encodes RARγA and the 2.7-kilobase mRNA encodes RARγB (Fig. 4). The sizes of the mRNAs indicate that we had isolated nearly full-length cDNAs for each RARγ isoform.

This result prompted us to investigate in more detail the spatial pattern of expression of each specific transcript in the 13.5-day-old embryo, when transcripts are most abundant (Fig. 4). When various tissues from 13.5-day-old embryos were analyzed by RNase protection assay, expression of both RARγs appeared to be concentrated in limbs, jaw, and carcass (which contains skeleton and skin) and to a lesser extent in lung and viscera, while it was not detected appreciably in brain or liver (Fig. 5). Northern blot analysis (Fig. 4) had suggested that RARγB was expressed to a lesser extent than RARγA. However, this may have been because of a lower specific labeling of the RARγB probe, a consistent observation. RNase protection analysis revealed roughly equal levels of both transcripts in those tissues in which the gene was strongly expressed.

DISCUSSION

The identification of two mouse RARγ isoforms, which differ solely in their amino termini, suggests that the two RARγ transcripts are derived from a single gene and therefore indicates a complex genomic organization for this particular member of the steroid hormone-thyroid hormone receptor superfamily. The most likely explanation at this time is differential promoter usage, as the 5′ untranslated sequences are distinct, although alternative splicing or other posttranscriptional events cannot be ruled out as explanations for the generation of these two transcripts. The dissimilarity of the amino termini of the putative RARγA and RARγB proteins is consistent with the observation that members of the steroid-thyroid hormone receptor superfamily contain a hypervariable region at their amino termini (10). However, amino acid sequence comparisons revealed significant identity between mRARγA and mRARα (28%) and between mRARγB and mRARβ (31%) in the hypervariable region. This is the first observation of amino acid identity between different members of the steroid-thyroid hormone and retinoic acid receptor superfamily in this region of the protein. Functional analyses of steroid hormone receptors have shown that the requirement for the N-terminal region of the receptors is target gene specific (17, 32). This suggests that mRARγA and mRARγB may possess different transcriptional properties and therefore regulate specific subsets of target genes. However, the demonstration of cell- and/or promoter-specific transactivation functions for each receptor subtype must await the identification and molecular characterization of a number of retinoic acid responsive genes.

The recent demonstration in the chick embryo of a gradient of endogenous retinoic acid across the limb anlage (28), coupled with the previous observation of concentration-dependent changes in digit identity following exposure of the limb bud to ectopic retinoic acid (26, 30), lead to the suggestion that retinoic acid is a natural morphogen that specifies pattern formation. Additional support for a morphogenetic role of retinoic acid in vertebrate development comes from studies in amphibians. *Xenopus* embryos treated with retinoic acid show dramatic anterior-to-posterior transition in the central nervous system (8), while retinoids can alter pattern formation in the regenerating limbs of urodele amphibians (for a review, see reference 4). Although the precise mechanisms through which retinoic acid may specify pattern formation are not known, the demonstration of transcriptional activation by RARs (13, 22, 33, 34; this work) indicates that retinoic acid may have an effect as a transcriptional activator through interaction with the RARs. One prediction of this model is that one or more RAR subtypes must be expressed in the developing tissue. The recent localization by in situ hybridization of high and uniform levels of RARβ mRNA in the mesenchymal regenerating blastema cells of the amputated limb of the adult newt (12) as well as the detection of RNA transcripts for the newt RARα and -δ in forelimb and tail blastemas (23) supports the idea that retinoic acid establishes positional information through differential receptor activation of gene expression (12).

Evidence that retinoic acid may also direct pattern formation in mammals was provided by the observation that retinoic acid is a powerful teratogen in both mice (24) and humans (18). Retinoic acid causes three general types of abnormalities, affecting limb, craniofacial, and neural-tube development, as well as various lesser deformities. These have been termed retinoic acid embryopathy, but the mechanism through which retinoic acid causes retinoic acid embryopathy remains to be elucidated. However, the specificity of the defects caused by systemic elevation of the putative morphogen suggests that the ability to respond may be limited to particular cell types and therefore argues against the possibility that the deleterious effects observed with large doses of retinoic acid are solely the result of generalized toxicity. The different patterns of expression of RARs, as evidenced from the distribution of transcripts in adult tissues (2, 6, 13, 34) and in embryonic tissues (this work) certainly support the idea of cell autonomous responsiveness. The high levels of expression of the RARγ isoforms in limb and facial structures but not in neural tissue

indicate that the effect of retinoic acid on the development of nonneural structures may be mediated by a different receptor combination than is the effect on the developing neural tube.

Finally, the recent demonstration that overexpression of a homeotic gene (Hox-1.1) in transgenic mice results in defects similar to retinoic acid embryopathy (1) is consistent with the idea that retinoic acid, through its interaction with an RAR, influences embryogenesis by regulating gene expression. Whether homeobox-containing or other regulatory genes are targets of the retinoic acid-RAR complex remains to be demonstrated. However, the presence of RARγA and -B in the developing mouse embryo in structures known to be affected by administration of retinoids provides strong evidence that the physiological and teratogenic effects of retinoic acid on mammalian development result from receptor activation of developmental control genes.

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