Genomic Structure and Chromosomal Mapping of the Nuclear Orphan Receptor RORγ (RORC) Gene

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The nuclear orphan receptor subfamily ROR/RZR is part of the steroid and thyroid hormone/retinoid receptor superfamily and consists of three different genes, α, β, and γ. In this study, we determined the genomic structure of mouse RORγ and the chromosomal localization of both mouse RORγ and human RORγ (HGMW-approved symbol RORC). The genomic structure of the mouse RORγ gene was derived from the analysis of P1 vector clones containing large genomic fragments encoding RORγ. These results revealed that the mRORγ gene has a complex structure consisting of 11 exons separated by 10 introns spanning more than 21 kb of genomic DNA. The DNA-binding domain is contained in two exons, 3 and 4, each encoding one zinc-finger. The splice site between exon 3 and exon 4 is identical to that found in RAR and TR3 receptors. RORγ is expressed as two mRNAs, 2.3 and 3.0 kb in size, that are derived by the use of alternative polyadenylation signals. We show by fluorescence *in situ* hybridization that the mouse RORγ gene is located on chromosome 3, in a region that corresponds to band 3F2.1–2.2. The human RORγ was mapped to chromosome region 1q21. The results demonstrate that the RORγ genes are located in chromosomal regions that are syntenic between mouse and human. © 1997 Academic Press

INTRODUCTION

The nuclear receptor superfamily constitutes a diverse group of ligand-activated transcriptional factors that share a common modular structure (Evans, 1988; Laudet *et al.,* 1992; Kastner *et al.,* 1995; Beato *et al.,* 1995). This superfamily includes receptors for steroid hormones, thyroid ho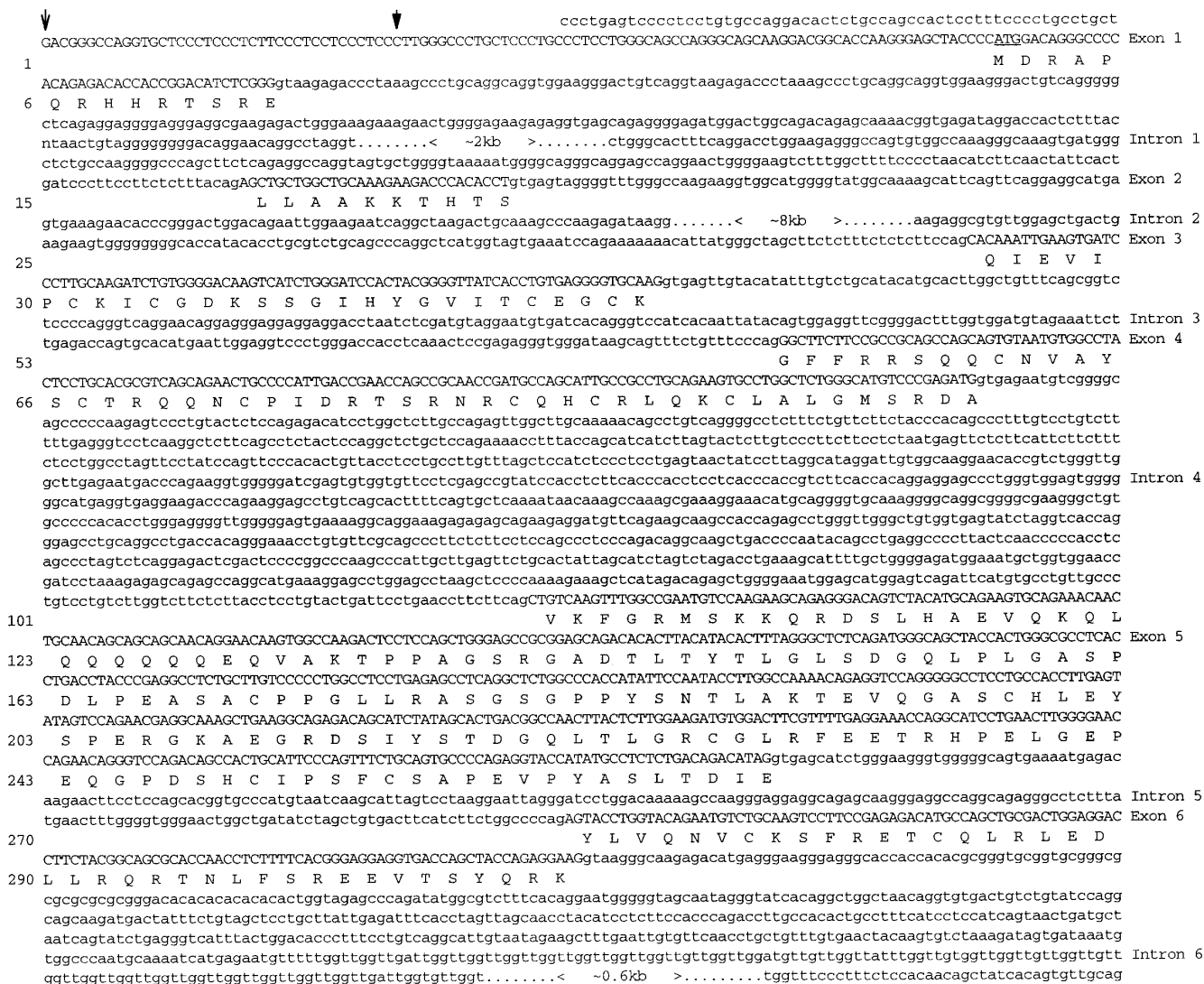rmone, retinoids, and vitamin D as well as a large number of receptors, referred to as orphan receptors, for which no ligand has been identified (Evans, 1988; Keller and Wahli, 1993; Wilson *et al.,* 1993; Qui *et al.,* 1994; Hirose *et al.,* 1994a, 1995). Based on their sequence aligments the nuclear receptors have been divided in several subfamilies (Laudet *et al.,* 1992; Escriva *et al.,* 1997). Most nuclear receptors contain five major domains: an A/B domain at the amino-terminus, a highly conserved DNA-binding (C) domain containing two "zinc-finger motifs," a hinge region (D domain), a ligand-binding (E) domain, and an F domain at the carboxyl-terminus. The A/B and E domains, which are poorly conserved, contain transactivation functions. Nuclear receptors bind as monomers or as homo- or heterodimers to response elements composed of a single core motif containing the consensus sequence PuGGTCA or to direct, palindromic, or inverted repeats of the core motif spaced by one or more nucleotides (Forman and Samuels, 1990; Truss and Beato, 1993; Glass, 1994). Important roles for nuclear receptors have been demonstrated in the control of embryonic development, cell proliferation, and differentiation (De Luca, 1991; Tontonoz *et al.,* 1994; Kastner *et al.,* 1995). In addition, genetic alterations in the expression and structure of these receptors have been implicated in various disease processes (Hughes *et al.,* 1988; De Luca, 1991; Chomienne *et al.,* 1990).

The superfamily of nuclear receptors can be divided into many subfamilies, each containing several closely related genes (Laudet *et al.,* 1992; Escriva *et al.,* 1997). Recently, our laboratory cloned mouse and human RORγ[2] (Hirose *et al.,* 1994b; Medvedev *et al.,* 1996), which constitutes the third member of the ROR (also named RZR) subfamily (Becker-André *et al.,* 1993; Carlberg *et al.,* 1994; Giguère *et al.,* 1994). The amino acid sequence of RORγ is highly conserved between species; the amino acid sequences between mouse and human RORγ exhibit 88% identity (Hirose *et al.,*

[2] The HGMW-approved symbol for the gene described in this paper is RORC.

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                                              ccctgagtcccctcctgtgccaggacactctgccagccactcctttcccctgcctgct
 GACGGGCCAGGTGCTCCCTCCCTCTTCCCTCCTCCCTCCCTTGGGCCCTGCTCCCTGCCCTCCTGGGCAGCCAGGGCAGCAAGGACGGCACCAAGGGAGCTACCCCATGGACAGGGCCCC  Exon 1
 1                                                                                                        M  D  R  A  P
 ACAGAGACACCACCGGACATCTCGGGgtaagagaccctaaagccctgcaggcaggtggaagggactgtcaggtaagagaccctaaagccctgcaggcaggtggaagggactgtcagggggg
 6  Q  R  H  H  R  T  S  R  E
 ctcagaggagggggagggaggcgaagagactgggaaagaaagaactggggagaagagaggtgagcagaggggagatggactggcagacagagcaaaacggtgagataggaccactctttac
 ntaactgtaggggggggacaggaacaggcctaggt........<   ~2kb   >........ctgggcactttcaggacctggaagagggccagtgtggccaaagggcaaagtgatggg  Intron 1
 ctctgccaagggggcccagcttctcagaggcaggtagtgctggggtaaaaatggggcagggcaggagccaggaactgggggaagtctttggcttttcccctaacatcttcaactattcact
 gatcccttccttctctttacagAGCTGCTGGCTGCAAAGAAGACCCACACCTgtgagtaggggtttgggccaagaaggtggcatggggtatggcaaaagcattcagttcaggaggcatga  Exon 2
 15                  L  L  A  A  K  K  T  H  T  S
 gtgaaagaacacccgggactggacagaattggaagaatcaggctaagactgcaaagcccaagagataagg.......<   ~8kb   >........aagaggcgtgttgggagctgactg  Intron 2
 aagaagtggggggggcaccatacacctgcgtctgcagcccaggctcatggtagtgaaatccagaaaaaaacattatgggctagcttctctttctctcttccagCACAAATTGAAGTGATC  Exon 3
 25                                                                                                        Q  I  E  V  I
 CCTTGCAAGATCGTGGGGGACAAGTCATCTGGGATCCACTACGGGGTTATCACCTGTGAGGGGTGCAAGgtgagttgtacatatttgtctgcatacatgcacttggctgtttcagcggtc
 30 P  C  K  I  C  G  D  K  S  S  G  I  H  Y  G  V  I  T  C  E  G  C  K
 tccccagggtcaggaacaggagggaggaggaggacctaatctcgatgtaggaatgtgatcacagggtccatcacaattatacagtggaggttcggggactttggtggatgtagaaattct
 tgagaccagtgcacatgaattggaggtccctgggaccacctcaaactccgagagggtgggataagcagtttctgtttcccagGGCTTCTTCCGCCGCAGCCAGCAGTGTAATGTGGCCTA  Exon 4
 53                                                                          G  F  F  R  R  S  Q  Q  C  N  V  A  Y
 CTCCTGCACGCGTCAGCAGAACTGCCCCATTGACCGAACCAGCCGCAACCGATGCCAGCATTGCCGCCTGCAGAAGTGCCTGGCTCTGGGCATGTCCCGAGATGgtgagaatgtcgggggc
 66 S  C  T  R  Q  Q  N  C  P  I  D  R  T  S  R  N  R  C  Q  H  C  R  L  Q  K  C  L  A  L  G  M  S  R  D  A
 agcccccaagagtccctgtactctccagagacatcctggctcttgccagagttggcttgcagaaagcctgtcaggggcctctttctgttcttctacccacagcccctttgtcctgtctt
 tttgagggtcctcaaggctcttcagcctctactccaggctctgctccagaaaaccttttaccagcatcatcttagtactcttgtccttctttcctctaatgagttctcttcattcttcttt
 ctcctggcctagttcctatccagttcccacactgttacctcctgccttgtttagctccatctccctcctgagtaactatccttaggcataggattgtggcaaggaaaccgtctgggttg
 gcttgagaatgacccagaaggtgggggatcgagtgtggtgttcctcgagccgtatccacctcttcacccacctcctcacccaccgtcttcaccacaggaggagcccgggtggagtgggg
 ggcatgaggtgaggaagacccagaaggagcctgtcagcacttttcagtgctcaaaataacaaagccaaagcgaaaggaaacatgcaggggtgcaaaggggcaggcggggcgaagggctgt  Intron 4
 gccccacacctgggagggttgggggagtgaaaaggcaggaaagaagacgagaggatgttcagaagcaagccaccagagctgggttgggctgtggtgagtatctaggtcaccag
 ggagcctgcaggcctgaccacagggaaacctgtgttcgcagcccttctcttcctccagccctcccagacaggcaagctgaccccaatacagcctgaggcccccttactcaacccccaacctc
 agccctagtctcaggagactcgactccccggcccaagcccattgcttgagttctgcactattagcatctcagtctctagacctgaaagcattttgctggggagatggaaatgctggtggaacc
 gatcctaaagagagcagagccaggcatgaaaggagcctggagcctaagctccccaaaagaaagctcatagacagagctgggaaatggagcatggagtcagattcatgtgcctgttgccc
 tgtcctgtcttggtcttctctcttacctcctgtactgattcctgaaccttcttcagCTGTCAAGTTTGGCCGAATGTCCAAGAAGCAGAGGGACAGTCTACATGCAGAAGTGCAGAAACAAC
 101                                                      V  K  F  G  R  M  S  K  K  Q  R  D  S  L  H  A  E  V  Q  K  Q  L
 TGCAACAGCAGCAGCAACAGGAACAAGTGGCCAAGACTCCTCCAGCTGGGAGCGCGGAGCAGACACACTTACATACACTTTAGGGCTCTCAGATGGGCAGCTACCACTGGGCGCCTCAC  Exon 5
 123 Q  Q  Q  Q  Q  Q  E  Q  V  A  K  T  P  P  A  G  S  R  G  A  D  T  L  T  Y  T  L  G  L  S  D  G  Q  L  P  L  G  A  S  P
 CTGACCTACCCGAGGCCTCTGCTTGTCCCCCTGGCCTCCTGAGAGCCTCAGGCTCTGGCCCACCATATTCCAATACCTTGGCCAAAACAGAGGTCCAGGGGGCCTCCTGCCACCTTGAGT
 163 D  L  P  E  A  S  A  C  P  P  G  L  L  R  A  S  G  S  G  P  P  Y  S  N  T  L  A  K  T  E  V  Q  G  A  S  C  H  L  E  Y
 ATAGTCCAGAACGAGGCAAAGCTGAAGGCAGAGACAGCATCTATAGCACTGACGGCCAACTTACTCTTGGAAGATGTGGACTTCGTTTTGAGGAAACCAGGCATCCTGAACTTGGGGAAC
 203 S  P  E  R  G  K  A  E  G  R  D  S  I  Y  S  T  D  G  Q  L  T  L  G  R  C  G  L  R  F  E  E  T  R  H  P  E  L  G  E  P
 CAGAAACAGGGTCCAGACAGCCACTGCATTCCCAGTTTCTGCAGTGCCCCAGAGGTACCATATGCCTCTCTGCAGACATAGgtgagcatctgggaagggtgggggcagtgaaaatgagac
 243 E  Q  G  P  D  S  H  C  I  P  S  F  C  S  A  P  E  V  P  Y  A  S  L  T  D  I  E
 aagaacttcctccagcacggtgcccatgtaatcaagcattagtcctaaggaatttagggatcctggacaaaaagccaagggaggaggcagagcaagggaggccaggcagagggcctcttta  Intron 5
 tgaactttggggtgggaactggctgatatctagctgtgacttcatcttctggccccagAGTACCTGGTACAGAATGTCTGCAAGTCCTTCCGAGAGACATGCCAGCTGCGACTGGAGGAC  Exon 6
 270                                                        Y  L  V  Q  N  V  C  K  S  F  R  E  T  C  Q  L  R  L  E  D
 CTTCTACGGCAGCGCACCAACCTCTTTTCACGGGAGGAGGTGACCAGCTACCAGAGGAAGgtaaggcaagagacatgagggaagggaggggcaccaccacacgcgggtgcggtgcgggcg
 290 L  L  R  Q  R  T  N  L  F  S  R  E  E  V  T  S  Y  Q  R  K
 cgcgcgcgcgggacacacacacacactggtagagcccagatatggcgtctttcacaggaatgggggtagcaataggggtatcacaggctggctaacaggtgtgactgtctgtatccagg
 cagcaagatgactatttctgtagctcctgcttattgagatttcacctagttagcaacctacatcctcttccacccagaccttgccacactgcctttcatcctccatcagtaactgatgct
 aatcagtatctgaggggtcatttactggacaccctttcctgtcaggcattgtaatagaagctttgaattgtgttcaacctgctgtttgtgaactacaagtgtctaaagatagtgataaatg
 tggcccaatgcaaaatcatgagaatgttttttggttggttgattggttggttggttggttggttggttggttgttggttggatgttgttggttatttggttgtggttggttgttggttgtt
 ggttggttggttggttggttggttggttggttggttgattggtgttggt........<  ~0.6kb   >.........tggtttccctttctccaacaacagctatcacagtgttgcag  Intron 6
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FIG. 1. Genomic structure of the mouse RORγ gene. Uppercase letters indicate exon sequences, and lowercase letters indicate intron sequences or upstream and downstream flanking sequences. Exons and introns are indicated on the right. The coding regions are marked by single-letter amino acid code. The numbers on the left indicate the amino acid in RORγ. The start codon (ATG), the stop codon (TGA), and the polyadenylation signals are underlined. The start of the sequence of the RORγ cDNA is marked by the solid arrow. The end of the primer extension is marked by the open arrow.

1994b; Medvedev *et al.,* 1996). The amino acid sequences of hRORγ and mRORγ are 50–51% identical to those of the RORα and β receptors. The A/B, C, D, and E domains between mRORγ and mRORα1 exhibit 50, 90, 34, and 57% homology, respectively (Giguère *et al.,* 1994; Medvedev *et al.,* 1996). These receptors do not have an F domain.

The RORs have been shown to bind as monomers to single core motifs preceded by an AT-rich sequence (Giguère *et al.,* 1994; Medvedev *et al.,* 1996; Greiner *et al.,* 1996). Recently, several putative target genes, including γF-crystallin, $p21^{WAF1/CIP1}$, laminin B1, and 5-lipoxygenase, have been shown to contain response elements in their promoter region that are able to bind RORα (Tini *et al.,* 1995; Schräder *et al.,* 1996; Matsui, 1996). Each member of the ROR subfamily exhibits a different tissue distribution, suggesting that these receptors have distinct biological roles. While RORβ appears to be uniquely expressed in brain and retina, RORα and γ were found to be highly expressed in several tissues (Becker-André *et al.,* 1993; Carlberg *et al.,* 1994; Hirose *et al.,* 1994b; Medvedev *et al.,* 1996). RORα and γ have been demonstrated to be induced during adipocyte differentiation in several preadipocyte cell lines (Adachi *et al.,* 1996; Austin *et al.,* 1997). A deletion in the RORα gene was found to be linked to defects in *staggerer* mice with severe cerebellar ataxia (Hamilton *et al.,* 1996; Matysiak-Scholtze and Nehls, 1997). These studies support important regulatory roles for RORs in different developmental and differentiation processes.

In this study, we describe the isolation of P1 vector

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gaaagccatcgcctcctatctggagccactccctgggaatccgggaagctctctggcaacccttttctctcccgtagcagaatcctaatacctagtattgaatggacttcacaagagacca
ggttatcagggagaaaggagtttgaagttaagaaattagaagttttcaacccttatataagtctctgggattccttgtactgagctggggatagcagtaactgtaactcactgacaggca
ctgaccctggctacatttgtgctaataggctctatggttgacaacaatatacaactcataccctaaagtttatggggtgcaggggcttgggggggatcacaggtctcctcctctatcccg
cagTCAATGTGGGAGATGTGGGAGCGCTGTGCCCACCACCTCACTGAGGCCATTCAGTATGTGGTGGAGTTTGCCAAGCGGCTTTCAGGCTTCATGGAGCTCTGCCAGAATGACCAGATC Exon 7
310     S  M  W  E  M  W  E  R  C  A  H  H  L  T  E  A  I  Q  Y  V  V  E  F  A  K  R  L  S  G  F  M  E  L  C  Q  N  D  Q  I
ATACTACTGACAGCAGgtgcacagggattggcaggccctggggtggggtggagcatggctgggggatggaaggacagacccagggccaagtggaggggaggtgacttnggnatcnagacacc Intron 7
349 I  L  L  T  A  G
gagggcttaacaagacactggtctgcttaacatggctacccatctttctcccctttatccagGAGCAATGGAAGTCGTCCTAGTCAGAATGTGCAGGGCCTACAATGCCAACAACCACAC Exon 8
355                                                              A  M  E  V  V  L  V  R  M  C  R  A  Y  N  A  N  N  H  T
AGTCTTTTTTGAAGGCAAATACGGTGGTGTGGAGCTGTTTCGAGCCTTGGGtgagggcagtgggaattgagaaagtcctgatgccaatcctatcacaagcctctctacatggttgtttgc Intron 8
374  V  F  F  E  G  K  Y  G  G  V  E  L  F  R  A  L  G
cccctgctctaaacacccaagagtggcatccttggggggagaggggaggcagctaccatgcttactctcttcatctccactccggcagGCTGCAGCGAGCTCATCAGCTCCATATTTGACT Exon 9
391                                                                             C  S  E  L  I  S  S  I  F  D  F
TTTCCCACTTCCTCAGCGCCCTGTGTTTTTCTGAGGATGAGATTGCCCTCTACACGGCCCTGGTTCTCATCAATGCCAGtgagtgtggctgggcatgggagcgggcactcgtggctgaga
402   S  H  F  L  S  A  L  C  F  S  E  D  E  I  A  L  Y  T  A  L  V  L  I  N  A  N
cacagcagctaatggcgaacctaggcctttggatgtgactaatgggagactgcttgaaataaggaaatgagctctatcccgtgcttctgaaaatataacagactaaagtatgcagaggaa
ccttaagacagggcgaggatagcatacagtggggcagtgggaacatggagaaggaggttggaaggaaatgagccacttccccaatagatgttaacctactagccagtgtagggcagtga
ctcctttacagtacacatgggaatcacctagggagctttaaggactaattgagtccctgggtcccacgtccagattttgacataactcatctaggcctgcacccaaaatttttaatcc... Intron 9
.....<  ~1kb  >........cctagagtcatacagtttgctgtagtcaaaccagtacacacatgtgggacacatgcatgggggtgggtggggagggacagagacacagagagaca
gaggcagaagagacggagacagagattctcttcaaaaagatacatgacaccatttttgttggtcgacgtgtctttgtgttttccgtttccgtttcactctggtcccaytcccaytcccttccttca
gACCGTCCTGGGCTCCAAGAAGAGGAGAGTGGAACATCTGCAATACAATTTGGAACTGGCTTTCCATCATCATCTCTGCAAGACTCATCGACAAGGCCTCCTAGCCAAGgtaggagca Exon 10
428    R  P  G  L  Q  E  K  R  R  V  E  H  L  Q  Y  N  L  E  L  A  F  H  H  H  L  C  K  T  H  R  Q  G  L  L  A  K
gtccacagggtagaagaaactaggcccactgccagtgtgtgacatcagggccagggtcttcgggtgcagaggaaggagtgctgcagtgctgggctatgtcagcgtttgcagcagctgtgg
gccctgctggagcatctgtttcc........<  ~3kb  >........ggtttccagatatgcatacatctaacaaatgggattggctgctcaagttgggtcaagggtcggggaggc Intron 10
464                                                                              L  P  P  K  G  K  L  R  S  L
TGCAGCCAACATGTGGAAAAGCTGCAGATCTTCCAGCACCTCCACCCCATCGTGGTCCAAGCCGCCTTCCCGCCACTCTATAAGGAACTCTTCAGCACTGATGTTGAATCCCCTGAGGGG Exon 11
474 C  S  Q  H  V  E  K  L  Q  I  F  Q  H  L  H  P  I  V  V  Q  A  A  F  P  P  L  Y  K  E  L  F  S  T  D  V  E  S  P  E  G
CTGTCAAGTGATCTGGAGGAAGGACAACTTTCTATTTCCTTCAGCCCTCTGACCCGTCTCCCTGGACTCCCTTCACCCAGCCTTTCCCTTTCTGCACTCTATGAAGGGTGGTATCCCTA
514 L  S  K
GGAGTAAGCAAATCCTAAGACTGATTTTCTGCCCCTAGGCTTGCCTTGTAGGACAACAGCAGCAAGTGATGGAGAAAAGGCTTGTTATGTTTGATTTCCCATAAGTTCCACCCTGGCTTC
TGGAAGCTGTGGGGTAGATGGGATAGAGATAGGATGACCAAGTCAAATAAAAAACAGACTGACAATCAGCAGGGATAAATCCAGGTACCTGGGATAAGGAGAACTCAAATCTAGGCTTGA
AAGCTAATAACAGTCCTTTCAATACCTCATTGTATTTCCCCATGGGTCCTCCTGGGGGGACATGGATCTAGCTCAGAGACTGGTGGCAAGCCCCCAGAAGGACCTGTATATAATAAGAAT
ATAGATTCCTGAGACTTTTCTGCCTTTCTTCTTCCTAGTTAAGAAATGTTGTTGACCCCCTCTGCCTGTTTTCTGGGACCTAAAATGCCTGGATGTGTAAAGAATGAGGGTGGGGTGGAG
ATAAGGTCCCAAGATAACTGTTTTATGGGGTTTGGGTATGAAGAAAAACATCACTGGAAAAATTAGAATGGAAACCTCTTTGCACACTTTAAAAGTGTCAGATTCGTTAGCAGTCTAATC
AGAGACACACATCCACACAGGTGGAGCACACAGAGGCTCTGCCCCCAGTGACACCATTCTGTAGACTTTCCCTCTGGCACACAATCTCTTCCTTGAGGTTGCAGCTCTGAGAAGCCTGAG
GTTCTAATTCATACAGGACACCAGAATTCATCCCAGCTCCAGCTGTCCTCTGTCCCTAAGAGAAGCAGAGGACCGGATACTAACCAGCCGGAAAACCCAAGGACCAGCACCCGGGAAGAT
GCCCTTGACTTCAGTCTCTACGCTATGAGGAAGGAAGGCAGCGCCATCCCCTGCTCTGTACCTGTGTGCTGCTGACTCCACATGATGGAGAGACTAGGAACAGGACAGGGACCTGTTTCT
CCTCCATAGTCTTGCTCAGAATTTCTCTCAGTTTTGTAAAGCTGCAGACTCTCCTAGGAGGTATAAGCAGCAGATGAGAGGGAGGGAGGTTTTTTTTTTTTTTTTTTTTTTTTTTCTCTC
AGAGGAAGGGTTTAGCCAAGTAAACATAAATCCCAACTTGTGCCATTCtttataaaacgatttcaaaggctggaagtgtatcgtgcctgcgtgcttgttacaaggggtgcagggcacacatg
tcgggtgttgggagacttgaatgtgactgcctaggatacatgcttgccctgcagttttgtttctgtgtcaagccagcaatttatctgttttataagaattttagcacacacacatcacaca
cacacaccgcccaagattctccctcagctaagcaatccaccagggagactggtgctcagatacctgacacaagagaaatggcaagctaaactgaaggaaggtattctagactaagaactt
ccaacaaatgataccacaggcccgtttagatttagaaattgcacagaattgccctgcatctaaaagactagaggctgtggaagcggtgtcccgggagctctctagctccccaggaggaa
ccaggatgtcaaaactctctcaagcttcccaggctgtctccgtagcagaccctgcccagccctgggtcccctcttcagttcctccccgattcgattcgtcatcccgtagtggcgcttg
ctgcagccctccctggttgctttatttatttatttgcaccaacagggtgctgcagactcattctggtttaaaaagagagaagaaga
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FIG. 1—*Continued*

clones containing genomic fragments of the mouse and human RORγ genes. With the help of these clones, we characterized the genomic structure of the mouse RORγ gene and determined the chromosomal localization of the mouse and human RORγ genes. The mouse RORγ gene was mapped to chromosome 3 and the human gene to chromosome 1. In addition, we demonstrate that RORγ is expressed as two transcripts that are generated by the use of two different polyadenylation signals.

MATERIALS AND METHODS

Isolation of P1 vector clones. The plasmids mRORγ-BSK and hRORγ-BSK containing the full coding region of the mouse and human RORγ, respectively, were described previously (Hirose et al., 1994b; Medvedev et al., 1996). From the cDNA sequence several primer pairs were selected and used in PCRs with mouse genomic DNA or mRORγ-BSK as template. The primer set (FP1, 5′-GTTATC-ACCTGTGAGGGGTG and RP2, 5′-GACATGCCCAGAGCCAGGCA) amplified a product of the expected size of 158 bp from mRORγ-BSK DNA and amplified a 400-bp fragment from mouse genomic DNA. The sequence of the 400-bp fragment demonstrated that it contains in addition to the 158-bp RORγ coding sequence, an intron of 255

bp. A new forward primer FP2 (5′-CACTTGGCTGTTTCAGCGGTC) designed from the intron sequence and a reverse primer RP2 (5′-ATCGGTTGCGGCTGGTTCGGT) designed from the exon sequence were synthesized and amplified a 313-bp product from genomic mouse DNA. This specific primer pair was used to screen by PCR a library of P1 vector clones containing 75- to 150-kb inserts of mouse genomic DNA (Pierce et al., 1992; Genome Systems, St. Louis, MO). A similar strategy was employed for the isolation of P1 vector clones containing the human genomic RORγ gene. The following amplification cycles were employed: 30 cycles of 94°C, 60°C, and 72°C for 1 min each, plus extension at 72°C for 7 min. Three positive clones were obtained. P1 vector DNA isolated by the standard NaOH-SDS lysis method was purified by phenol/chloroform extraction and precipitated with ethanol. DNA was digested with *Bam*HI and fragments were analyzed by Southern analysis using fragments from different regions of the mRORγ cDNA as a probe. One of two P1 vector clones (P1-mRORγ) that contained the full coding region of RORγ was used for further investigation. Fragments hybridizing to the RORγ probe were cut from the gel, purified with microcon 30 (Amicon), and subcloned into Bluescript SK II. The size and order of *Bam*HI fragments were subsequently confirmed by additional restriction analysis, PCR, and sequence analysis.

DNA sequencing. Plasmids were purified using Wizard miniprep or midiprep kits (Promega). Manual sequencing was performed using the dideoxynucleotide chain-termination method and the Sequenase Quick-denature plasmid sequencing kit (Amersham). Automatic se-

TABLE 1

Summary of the Size of the Exons and Introns and the DNA Sequence of the Exon/Intron Junctions in the Mouse RORγ Gene

Exon	Exon size	5′-Exon/intron junction	Intron size	3′-Exon/intron junction	aa interrupted[a]
1	146 bp	TCTCGGG$_{146}$gtaaga	~2.0 kb	ttacagA$_{147}$GCTGCT	Glu$_{14}$
2	31 bp	CACACCT$_{176}$gtgagt	~8.5 kb	ttccagC$_{177}$ACAAAT	Ser$_{24}$
3	82 bp	GTGCAAG$_{262}$gtgagt	255 bp	tcccagG$_{263}$GCTTCT	Lys$_{52}$/Gly$_{53}$
4	140 bp	CGAGATG$_{404}$gtgaga	1153 bp	cttcagC$_{405}$TGTCAA	Ala$_{100}$
5	507 bp	GACATAG$_{911}$gtgagc	217 bp	ccccagA$_{912}$GTACCT	Glu$_{269}$
6	121 bp	GAGGAAG$_{1033}$gtaagg	~1.4 kb	ccgcagT$_{1034}$CAATGT	Lys$_{309}$/Ser$_{310}$
7	132 bp	ACAGCAG$_{1166}$gtgcac	167 bp	atccagG$_{1167}$AGCAAT	Gly$_{354}$
8	107 bp	GCCTTGG$_{1275}$gtgagg	157 bp	cggcagG$_{1276}$CTGCAG	Gly$_{390}$
9	110 bp	AATGCCA$_{1385}$gtgagt	~1.3 kb	cttcagA$_{1386}$CCGTCC	Asn$_{427}$
10	109 bp	AGCCAAG$_{1495}$gtagga	~4 kb	tcctcagC$_{1496}$TGCCAC	Lys$_{463}$/Leu$_{464}$
11	1398 bp				

Note. Exon sequences are shown in uppercase letters, and intron sequences are shown in lowercase letters.

[a] The nucleotide numbers indicate the positive within the mouse RORγ mRNA.

quencing was carried out using a Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin–Elmer) and an ABI Prism 377 automatic sequencer. DNA and deduced protein sequences were analyzed by the UWGCG (Devereux *et al.,* 1984) and MacVector (Kodak) sequence analysis software packages.

Fluorescence in situ hybridization (FISH). The regional chromosomal localization was determined by FISH using fragments of genomic DNA containing either the human or the mouse RORγ gene cloned into P1 vectors as a probe (Stokke *et al.,* 1995). FISH was carried out by Genome Systems. Two different methods were employed. In the first method, DNA was labeled with digoxigenin–dUTP by nick-translation. Labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes derived from embryonic stem cells from male mice. Specific hybridization signals were detected by incubating the hybridized slides with fluoresceinated anti-digoxigenin antibodies followed by counterstaining with propidium iodide. These results indicated that the mouse RORγ gene was located on chromosome 3. This was confirmed by cohybridizing the labeled P1 vector DNA with a biotin-labeled probe specific for the centromeric region of chromosome 3 and counterstaining with 4′,6-diamidino-2-phenylindole (DAPI). The latter resulted in the specific labeling of the centromeric region of chromosome 3 after incubation with Texas red-labeled avidin. A total of 80 metaphase cells were analyzed, 71 of which exhibited specific labeling. Measurements were done on 10 specifically hybridized chromosomes 3.

To localize the human RORγ gene, digoxigenin-labeled DNA was hybridized to normal metaphase chromosomes derived from PHA-stimulated human peripheral blood lymphocytes from a male donor. After the initial indication that the human RORγ gene was localized on chromosome 1, the labeled P1 vector DNA was cohybridized with a biotin-labeled probe specific for the heterochromatic region of chromosome 1. A total of 80 metaphase cells were analyzed, with 73 exhibiting specific labeling. Measurements were performed on 10 specifically hybridized chromosomes 1.

Northern blot analysis. Total RNA from murine D1 adipocytes was isolated as described previously (Adachi *et al.,* 1996). Total RNA (30 μg) was electrophoresed through formaldehyde 1.2% agarose gel, blotted to Nytran membrane (Schleicher & Schuell), and UV-cross-linked. The blots were hybridized with either a ^{32}P-labeled, 2.1-kb 5′-fragment of RORγ cDNA (Medvedev *et al.,* 1996) or the 3′-end of the 3′-UTR of RORγ Hybridizations were performed for 1–2 h at 68°C using Quickhyb reagent (Stratagene), and blots were washed twice with 2× SSC, 0.05% SDS at room temperature for 15 min and subsequently with 0.1× SSC, 0.1% SDS at 60°C for 30 min. Autoradiography was carried out with Hyperfilm-MP (Amersham) at −70°C.

5′-RACE and nested primer PCR. Three reverse, mRORγ-specific primers NP4-6 (5′-GTGAGGCGCCCAGTGGTAGCT), NP5 (5′-GAC-ATGCCCAGAGCCAGGCA), and NP6 (5′-CAAGGGATCACTTCA-ATTTG) were used in 5′-RACE as well as in nested primer PCR. These primers were designed to be specific for regions in exon 5, 4, and 3, respectively. 5′-RACE was performed with a 5′/3′ RACE kit (Boehringer Mannheim) according to the manufacturer's protocol. The first strain cDNA was synthesized with AMV reverse transcriptase and the primer NP4. The cDNA was then purified and a poly(A) tail added to its 3′-end by terminal transferase reaction. After purification, this modified cDNA was used as a template in two subsequent rounds of PCR with oligo(dT) and the two remaining mRORγ-specific primers NP5 or NP6. The PCR fragments were agarose purified, subcloned, and sequenced. The inserts of all clones examined contained sequence identical to that of the mRORγ cDNA.

Nested primer PCR was performed in three rounds (25 cycles/round) with an Expand High Fidelity PCR System (Boehringer Mannheim) and three different sets of nested primers consisting of three forward vector primers (NP1-3) and three reverse, mRORγ-specific primers (NP4-6). Mouse embryonic (17th day) and liver cDNA libraries were used as a template in the first round of PCR (25 cycles) with primers NP1 and NP4. The PCR products obtained in each round were used in the subsequent rounds with NP2 and NP5 or with NP3 and NP6. The final PCR products were then agarose purified, subcloned, and sequenced. The inserts of all clones examined contained sequence identical to that of the mRORγ cDNA.

Primer extension. The oligonucleotide primer PE-1 (5′-GTGGGT-CTTCTTTGCAGCCA) was end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. After 10 min of labeling at 37°C, the enzyme was inactivated by incubation of the reaction mixture for 2 min at 90°C. The radiolabeled oligonucleotide was purified over NAP-5 columns (Pharmacia). For primer extension analysis 15 μg of total RNA from muscle or liver was annealed to the radiolabeled PE-1 and then reverse transcribed using an AMV Reverse Transcriptase Primer Extension System (Promega) according to the manufacturer's protocol. Primer extension products were analyzed on a denaturing, 6% polyacrylamide sequencing gel together with a sequence ladder obtained by dideoxy-sequencing of unrelated plasmid DNA, which served as a size marker.

RESULTS AND DISCUSSION

Genomic structure of the mouse RORγ gene. P1 vector clones containing large fragments of mouse genomic DNA encoding the RORγ gene were isolated by screen-
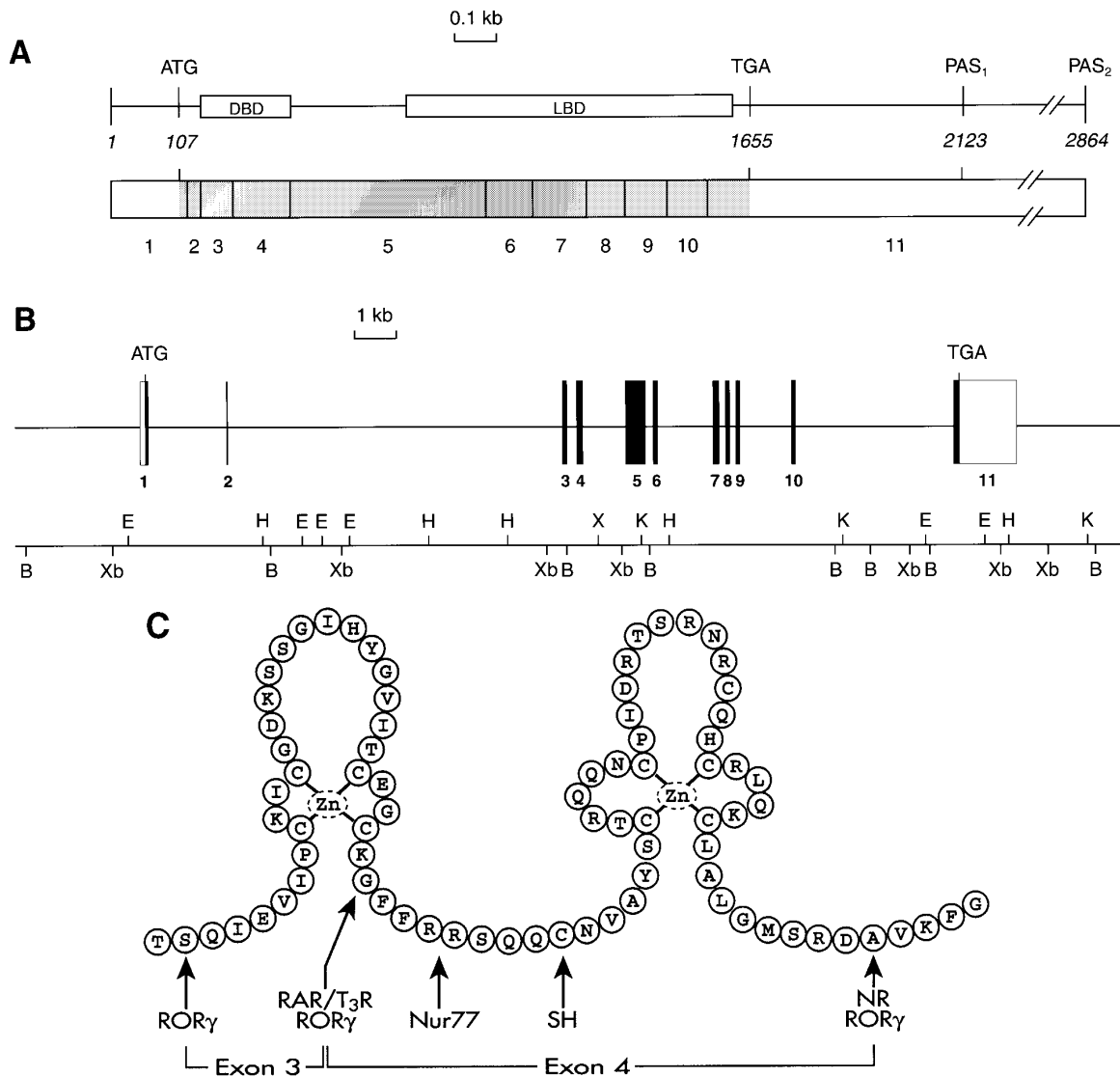
FIG. 2. Schematic presentation of the genomic structure of the mouse RORγ gene. (**A**) The positions of the 11 exons corresponding to the RORγ mRNA are shown. The sizes of the RORγ transcripts, 2.3 and 2.9 kb, are indicated. The translation start site, the stop codon, and the two major polyadenylation signals (PAS$_{1,2}$) are marked. Open boxes in top row indicate the DNA-binding domain (DBD) and putative ligand-binding domain (LDB). Shaded area represents open reading frame. (**B**) Genomic structure of the RORγ gene locus. Boxes indicate exons, black boxes represent open reading frames. *Bam*HI (B), *Kpn*I (K), *Eco*RI (E), *Hin*dIII (H), *Xho*I (X), and *Xba*I (Xb) restriction sites are marked. (**C**) Sequence of DNA-binding domain of RORγ in relation to genomic structure. Splice sites for the RORγ, thyroid hormone receptor (T3R), the retinoic acid receptor (RAR), the orphan receptor Nur77/NGFIB, and the steroid hormone receptors (SH) are indicated. NR indicates the splice site conserved among all nuclear receptors.

ing a P1 vector library by PCR using two specific primers, FP2 and RP2. These primers yielded under appropriate conditions a PCR product of about 300 bp with mouse genomic DNA as template. Three P1 vector clones containing the RORγ gene were obtained; one of two clones P1-mRORγ containing the whole coding region was selected for further analysis. The P1 vector was cut by *Bam*HI and subjected to Southern analysis using a ^{32}P-labeled cDNA fragment containing the entire cDNA of RORγ (Medvedev *et al.,* 1996) as a probe. Southern hybridization showed that *Bam*HI fragments of 8, 6.5, 4.7, 4.4, and 2 kb were recognized by the labeled cDNA probe; these fragments were subse-

quently subcloned into pBluescript and then sequenced using either internal primers derived from the cDNA sequence or vector primers. The order and orientation of the cloned *Bam*HI fragments were established by PCR using P1-mRORγ DNA as template and primer sets that anneal to the ends of two different *Bam*HI fragments. This PCR positioned all cloned *Bam*HI fragments and identified an additional fragment of 2.4 kb of the mRORγ gene that did not hybridize to the mRORγ cDNA probe in Southern analysis of *Bam*HI-digested P1-mRORγ DNA. This fragment was cloned and found to consist of two *Bam*HI fragments of 0.9 and 1.5 kb. The restriction map of the mRORγ gene was con-
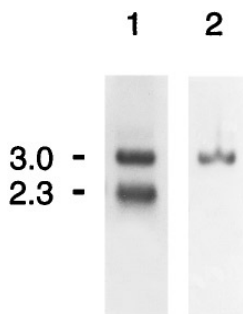
FIG. 3. The 2.3- and 3.0-kb RORγ transcripts are generated by the use of alternative polyadenylation signals. Total RNA (30 μg) isolated from D1 adipocytes was examined by Northern analysis using ^{32}P-labeled probes encoding a 2.1-kb 5'-fragment of the RORγ cDNA (lane 1) or a 0.8-kb genomic fragment encoding a sequence just downstream of the first polyadenylation signal (lane 2). The former fragment hybridizes to both transcripts while the latter hybridizes only to the 3.0-kb transcript.

structed based on the analysis of all cloned fragments with different restriction enzymes, including *Eco*RI, *Hin*dIII, *Xho*I, *Xba*I, and *Kpn*I, and analyzed by Southern blotting with radioactive probes specific for different regions of the cDNA. The exon–intron structure of the mRORγ gene was confirmed by PCR analysis using P1-mRORγ DNA as a template and various primer sets that amplify overlapping genomic DNA fragments. A summary of the PCR, sequencing, and restriction mapping analyses is presented in Fig. 1. These analyses revealed that the mouse RORγ gene spans more than 21 kb and is composed of 11 exons separated by 10 introns. A summary of the various sizes of the exons and introns and the sequence of the exon/intron junctions is shown in Table 1. The sequences of these junctions are consistent with the consensus ($A_{62}G_{77}$/$g_{100}t_{100}a_{60}a_{74}g_{88}$ for the 5' donor and $y_{87}ny_{97}a_{100}g_{100}$/$G_{55}$ for the 3' acceptor site) for known splice sites within eukaryotic genes (Mount, 1982). The DNA-binding domain (from Cys$_{31}$ to Cys$_{91}$) of RORγ is contained within two exons, 3 and 4 (Fig. 2). Exon 5 encodes the hinge domain while exons 6–10 encode the putative ligand-binding domain. A schematic view of the genomic structure and the relationship between the various exons and the DNA- and putative ligand-binding domains is presented in Figs. 2A–2B.

Some of the intron/exon junctions are conserved between members of the nuclear receptor superfamily. The location of the second intron (at Ser$_{24}$) in the RORγ gene is shared with a splice site in the RORα gene (Giguère *et al.,* 1994). The splice site (intron 3) between the two zinc-fingers of RORγ is located at the second amino acid from the last cysteine of the first zinc-finger (Fig. 2C); this position is shared with that of the thyroid hormone and retinoic acid nuclear receptors, including the retinoic acid receptor RARγ (Ritchie *et al.,* 1990; Lehmann *et al.,* 1991). This splice site is different from that of the steroid hormone receptor that is located 10 amino acids 3' from the last cysteine of the

first zinc-finger and different from that of the orphan receptors COUP-TF and Nur77/NGFI-B, which have either no splice site or a splice site at the fifth amino acid, respectively (Watson and Milbrandt, 1989; Ritchie *et al.,* 1990) (Fig. 2C). The position of the next splice site (intron 4) at the C-terminus of the DNA-binding domain of RORγ (Ala$_{100}$) is highly conserved among all nuclear receptors (Ponglikitmongkol *et al.,* 1988; Ritchie *et al.,* 1990; Lehmann *et al.,* 1991). Based on the positioning of these splice sites, the nuclear receptors have been divided into different groups that diverged from each other early during the evolutionary
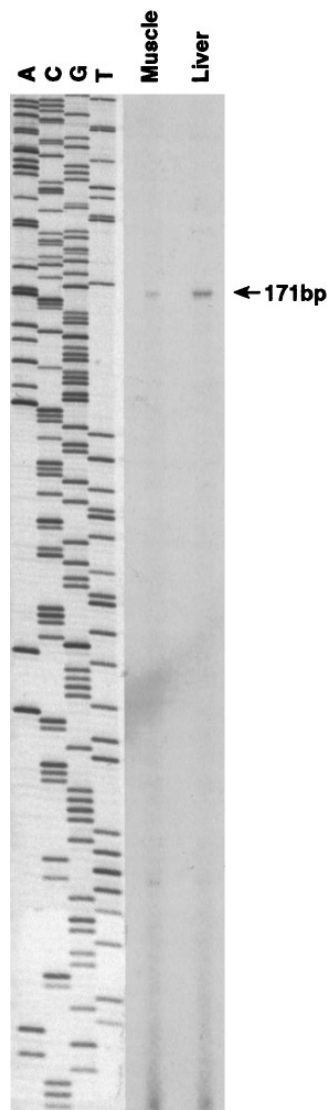

FIG. 4. Determination of the transcriptional initiation site of the mouse RORγ gene. For primer extension analysis, 15 μg of total RNA from muscle or liver was annealed to the end-labeled oligonucleotide PE-1 and then reverse-transcribed as described under Materials and Methods. Primer extension products were analyzed on a denaturing, polyacrylamide gel together with a sequence ladder (lanes **A, C, G,** and **T**) obtained by dideoxy sequencing of unrelated plasmid DNA that served as a size marker. The arrow indicates the major primer extension product of 171 bp.

process. In this respect, ROR fits in the T3R/RAR subgroup.

Alternative polyadenylation signals. Northern blot analysis with RNA from various tissues demonstrated that a probe consisting of a 2.1-kb 5′-fragment of the RORγ cDNA hybridizes to two transcripts about 2.3 and 3.0 kb (Hirose *et al.,* 1994b; Medvedev *et al.,* 1996) (Fig. 3). These transcripts could be generated by alternative splicing, use of alternative promoters and transcription start sites, and/or the selection of alternative polyadenylation signals. The RORα gene has been reported to generate through alternative splicing three isoforms that differ at the amino terminus (Giguère *et al.,* 1994). This alternative splicing involves exons upstream from the splice site that is conserved between RORα and RORγ (junction of exons 2 and 3 of mRORγ). To examine the possibility of alternative splicing of RORγ transcripts, we performed 5′-RACE using RNA from liver and muscle as a template and three nested reverse primers annealing to a region in exon 5, 4, or 3. The sequences of the inserts from seven different clones were found to be all identical to that reported for mRORγ and extended into the 5′-UTR. In a second approach, we performed nested PCR using a mouse embryonic and liver cDNA library. The inserts of the 19 clones analyzed varied in length; however, their sequence was identical to that of the 5′-end of mRORγ. These results of these two strategies did not provide any evidence for alternative splicing of mRORγ mRNA in the 5′ region.

We next examined the possibility of the use of alternative polyadenylation signals. The 3′-end sequence of the RORγ cDNAs described previously (Medvedev *et al.,* 1996) contained a poly(A)$^+$ tail eight nucleotides from the sequence AATAAGAATATA containing two consecutive polyadenylation signals (Wickens, 1990). To determine whether the larger transcript was derived by the use of an alternative polyadenylation signal, we amplified a 0.8-kb region just downstream from the first polyadenylation signal with genomic DNA as a template and used it as a probe in Northern blot analysis. As shown in Fig. 3, in contrast to the 2.1-kb 5′-probe, this probe hybridized only to the 3.0-kb transcript. Examination of the sequence of this fragment identified two consecutive polyadenylation signals, AGTAAA and CATAAA (Wickens, 1990), 0.7 kb downstream from the first site. A labeled probe derived from the genomic fragment just downstream from these two polyadenylation signals did not hybridize to these two RNAs (not shown). These results indicate that the generation of the 2.3- and 3.0-kb RORγ transcripts is related to the use of alternative polyadenylation signals. It is interesting to note that the ratio between the two transcripts can vary dramatically between different tissues (Medvedev *et al.,* 1996), suggesting that it is regulated in a tissue-specific manner. The different lengths of the 3′-UTR may be important
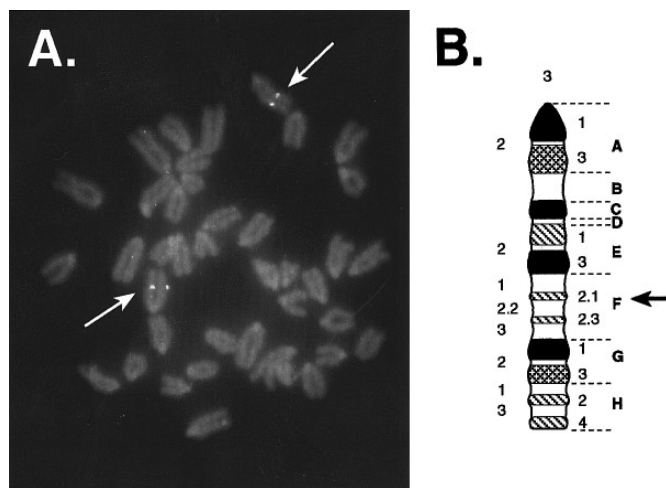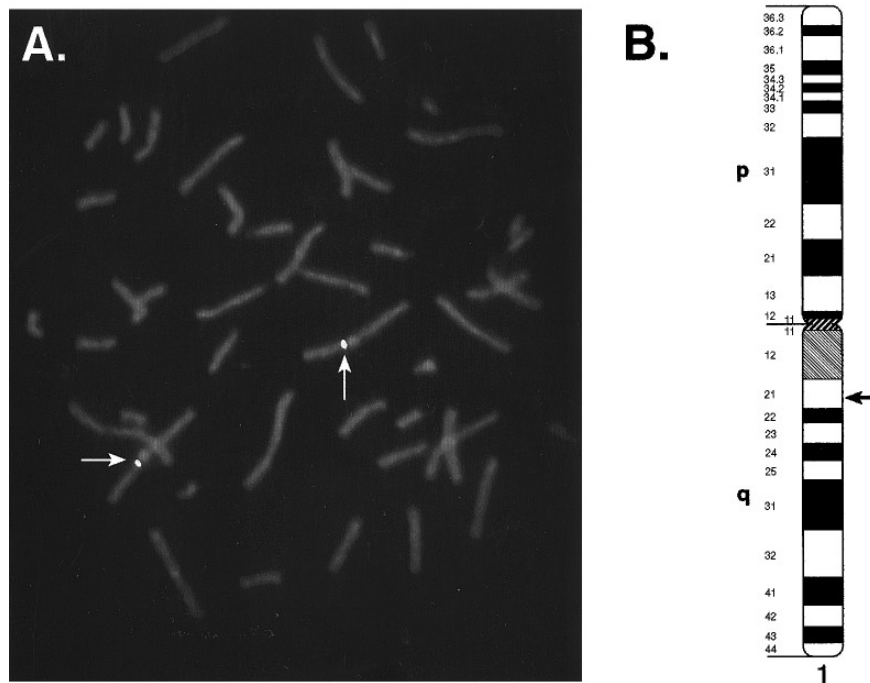

FIG. 5. Regional mapping of the RORγ gene by fluorescence *in situ* hybridization to mouse chromosome 3. (**A**) The location of the RORγ gene was identified on metaphase chromosomes from murine male embryonic stem cells using a digoxigenin dUTP-labeled, genomic fragment containing the RORγ gene. Chromosome 3 was identified by cohybridization with a biotinylated probe specific for the centromere of chromosome 3. Only the staining for RORγ is shown. (**B**) The idiogram indicates that mouse RORγ maps to band 3F2.1–2.2. The arrowhead indicates the interval within which the hybridization signal was detected on a sample of 71 chromosomes. No specific signal was detected on other chromosomes.

in the control of the stability or rate of translation of the RORγ mRNA.

Transcription initiation site. To determine the mRORγ transcription initiation site(s), primer extension was performed using RNA from mouse liver and muscle as a template and the reverse primer PE-1 designed from the sequence of the second exon (Fig. 4). Reactions with both templates identified a major extension product of 171 bp. A weak, 86-bp extension product was also observed. These results suggest that the transcription start site of RORγ mRNA is 106 bases from the start codon. Location of the transcription initiation site was confirmed by RT-PCR using mouse liver RNA as a template. Oligonucleotides just upstream and downstream from the putative transcription initiation site were used as forward primers and PE-1 was used as a reverse primer. Only the RT-PCR with the downstream primer yielded a PCR product of the predicted size while PCR with the upstream primer did not give a PCR product (data not shown). These results support the location of the transcriptional initiation site identified by primer extension.

Chromosomal localization of the mouse RORγ gene. The regional chromosomal localization was determined by FISH using fragments of genomic DNA of about 100 kb containing either the mouse or the human RORγ gene cloned into P1 vectors as a probe. These P1 vector clones were obtained by screening a P1 vector library by PCR using two specific primers that yielded a PCR product of about 300 bp with genomic DNA as template.

FIG. 6. Regional mapping of the RORγ gene by fluorescence *in situ* hybridization to human chromosome 1. (**A**) The RORγ gene was localized on normal metaphase chromosomes derived from PHA-stimulated human peripheral blood lymphocytes from a male donor using a digoxigenin dUTP-labeled, genomic fragment containing the RORγ gene. Chromosome 1 was identified by cohybridization with a biotinylated probe specific for the centromere of chromosome 1. Only the staining for RORγ is shown. (**B**) The idiogram indicates that RORγ maps to band 1q21. The arrowhead indicates the interval within which the hybridization signal was detected on a sample of 73 chromosomes. No specific signal was detected on other chromosomes.

DNA was labeled with digoxigenin–dUTP by nick-translation. Labeled probe was combined with sheared DNA and hybridized to normal metaphase chromosomes derived from male embryonic stem cells. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated anti-digoxigenin antibodies followed by counterstaining with propidium iodide. This resulted in the specific labeling of the fourth largest chromosome, suggesting that the RORγ gene was contained in chromosome 3 (not shown). This was confirmed by a second experiment in which the labeled P1 vector DNA was cohybridized with a biotin-labeled probe specific for chromosome 3. The latter resulted in the specific labeling of the centromeric region of chromosome 3 after incubation with Texas red avidin. The regional assignment of the RORγ probe was determined by the analysis of the fractional chromosome length; 10 specifically hybridized chromosomes were measured. These calculations indicated that the RORγ gene mapped to a position that is 54% of the distance from the heterochromatic–euchromatic boundary to the telomere of chromosome 3, an area that corresponds to band 3F2.1–2.2. This is schematically shown in Fig. 5.

Chromosomal localization of the human RORγ gene. Similar procedures were used to map the chromosomal localization of hRORγ. In this case, normal metaphase chromosomes were derived from PHA-stimulated peripheral blood lymphocytes from a male donor. The first

procedure mapped the RORγ gene to human chromosome 1. This assignment is in agreement with that obtained from Southern analysis using a BIOS Blot Somatic Cell Hybrid Panel (New Haven, CT) consisting of *Taq*I-digested genomic DNA isolated from a panel of human/hamster and human/mouse hybrid cell lines (Hirose *et al.,* 1994b). In the second experiment, a biotin-labeled probe specific for the heterochromatic region of chromosome 1 was cohybridized with the probe specific for hRORγ. The band assignment was determined by measuring the fractional chromosome length and by analyzing the banding pattern. Measurements of 10 specifically hybridized chromosomes 1 demonstrated that RORγ is located at a position that is 7% of the distance from the heterochromatic–euchromatic boundary to the telomere of chromosome arm 1q, an area that corresponds to band 1q21. This is shown schematically in Fig. 6.

The biological functions of RORγ have not been precisely established. RORγ has been shown to be expressed in a tissue-specific manner; it is highly expressed in liver, kidney, and thymus. Recent studies have indicated that members of the ROR/RZR family play important roles in the regulation of development, differentiation, and cell growth. A deletion in the RORα gene identified in *staggerer* mice (Hamilton *et al.,* 1996; Matysiak-Scholtze and Nehls, 1997) supports this conclusion. RORγ has been demonstrated to be induced

during fat cell differentiation in 3T3-L1 and D1 preadipocytes (Adachi *et al.,* 1996; Austin *et al.,* 1997), suggesting a role in the control of adipocyte function. In addition, RORγ is highly expressed in T-lymphocytes but is not expressed in B-lymphocytes (Hirose *et al.,* 1994b; Medvedev *et al.,* 1996; unpublished observations). The latter finding suggests a role for RORγ in regulating specific T-cell functions. Genetic alterations involving the human 1q21 region where the RORγ gene is located have been implicated in a variety of malignancies including lymphomas and renal carcinomas (Whang-Peng *et al.,* 1995; Weterman *et al.,* 1996). Future studies have to determine whether genetic alterations in the RORγ gene are involved in these disease processes.

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