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Comparative genomic and phylogenetic analysis of short-chain dehydrogenases/reductases with dual retinol/sterol substrate specificity

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Abstract

Human short-chain dehydrogenases/reductases with dual retinol/sterol substrate specificity (RODH-like enzymes) are thought to contribute to the oxidation of retinol for retinoic acid biosynthesis and to the metabolism of androgenic and neuroactive 3α-hydroxysteroids. Here, we investigated the phylogeny and orthology of these proteins to understand better their origins and physiological roles. Phylogenetic and genomic analysis showed that two proteins (11-*cis*-RDH and RDHL) are highly conserved, and their orthologs can be identified in the lower taxa, such as amphibians and fish. Two other proteins (RODH-4 and 3α-HSD) are significantly less conserved. Orthologs for 3α-HSD are present in all mammals analyzed, whereas orthologs for RODH-4 can be identified in some mammalian species but not in others due to species-specific gene duplications. Understanding the evolution and divergence of RODH-like enzymes in various vertebrate species should facilitate further investigation of their in vivo functions using animal models.
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Short-chain dehydrogenases/reductases (SDR) comprise a large family of functionally heterogeneous proteins that participate in the metabolism of steroids, prostaglandins, retinoids, aliphatic alcohols, and xenobiotics (reviewed in Refs. [1–3]). Members of the SDR superfamily are found in the cytoplasm, mitochondria, nuclei, peroxisomes, and endoplasmic reticulum. Some of the SDR enzymes act on the same endogenous substrates but exhibit different subcellular localization, cofactor specificity, substrate affinity, and tissue distribution (reviewed in Ref. [4]). It is generally believed that due to the cellular ratios of $NAD^+/NADH$ and $NADP^+/NADPH$ [4], those SDR enzymes that prefer NAD^+ as a cofactor function in the oxidative direction in vivo, whereas those that prefer $NADP^+$ function in the reductive direction.

To date, about 3000 primary structures from various species have been annotated in sequence databases as members of the SDR superfamily based on SDR signature features such as the TGX$_3$GXG motif of the nucleotide binding region and the catalytic tetrad N-S-Y-K, which constitutes the active site [1]. Members of the SDR superfamily are found in all taxa— bacteria, plants, insects, and vertebrates. At least 63 SDR genes have been identified in the human genome database [1]. For many of these putative oxidoreductases, their cellular functions are yet to be determined.

Over the past decade, a number of SDRs have been implicated in the catalysis of the oxidation of retinol to retinaldehyde, which is the rate-limiting step in the biosynthesis of retinoic acid, a potent endogenous activator of the nuclear transcription factors retinoic acid receptors [5]. Efforts of this and other laboratories revealed that animal and human tissues contain numerous SDRs that are active toward retinol and retinaldehyde [6–11]. Within this rather large group of retinoid-active enzymes, there is a distinct subgroup of homologous SDRs that share over 40% protein sequence identity, exhibit similar subcellular localization in the membranes of endoplasmic reticulum, and prefer NAD^+ as a cofactor. One of the first members of this group, rat retinol dehydrogenase 1 (RODH-1),

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was purified from rat liver following its activity toward all-*trans*-retinol [7]. Subsequently, this enzyme was also shown to exhibit high activity toward androgenic 3α-hydroxysteroids and was implicated in the production of the most potent male hormone, dihydrotestosterone [12].

Proteins homologous to RODH-like SDRs were identified in all vertebrate species characterized thus far and also in a chordate, amphioxus (subphylum Cephalochordata), which has retinoid metabolism similar to that of vertebrates [13,14]. Amphioxus RODH-like proteins are believed to be the closest relatives to the single ancestral form of all RODH-like SDRs [13].

In humans, the RODH-like group of SDRs comprises four catalytically active proteins: retinol dehydrogenase type 4 (RODH-4) [8], RODH-like 3α-hydroxysteroid dehydrogenase (3α-HSD) [15], retinol dehydrogenase-like (RDHL) enzyme [16–18], and 11-*cis*-retinol dehydrogenase (11-*cis*-RDH) [6]. For the purpose of discussion, these enzymes will be referred to as RODH-like SDRs further in the text.

The four human RODH-like SDRs exhibit different specificity toward stereoisomers of retinol. RODH-4 recognizes all-*trans*-retinol, 13-*cis*-retinol, and 9-*cis*-retinol as substrates [8]. 3α-HSD is active toward all-*trans*-retinol but not toward *cis*-retinols [15]. RDHL (also known as nonhepatic 3α-HSD [16]) appears to have a rather low activity toward all-*trans*-retinol [16–18], whereas 11-*cis*-RDH prefers 11-*cis* and 9-*cis* retinols to all-*trans*-retinol [19]. In addition to retinoids, these human enzymes are highly active toward androgenic (C_{19}) and neuroactive (C_{21}) 3α-hydroxysteroids [15,16]. 11-*cis*-RDH was shown to contribute to the oxidation of 11-*cis*-retinol in the visual cycle in vivo [20] and was genetically linked with fundus albipunctatus, a rare form of stationary night blindness characterized by a delay in the regeneration of cone and rod photopigments [21,22]. Whether other RODH-like retinol/sterol dehydrogenases physiologically contribute to metabolism of retinoids and/or 3α-hydroxysteroids is not yet known.

In part, the difficulties in elucidation of the physiological functions of RODH-like dehydrogenases have been exacerbated by the apparent lack of clear orthologs for some of the human and animal enzymes, by the redundancy of RODH-like SDRs in mice compared to humans and rats, and by the differences in retinoid stereospecificity and tissue distribution of human versus rodent enzymes. Furthermore, it remains unclear whether enzymes homologous to RODHs are present in lower vertebrate species, all of which possess retinoid- and androgen-metabolizing pathways. Thus, to understand better the contribution of human RODH-like SDRs to retinoid and steroid metabolism, we investigated their origins and phylogenetic relationships with other members of the SDR superfamily in the present study.

Results and discussion

Phylogenetic analysis of RODH-like SDRs

The SDR superfamily contains many enzymes that were shown to be active toward steroid or retinoid substrates. In addition to RODH-like SDRs, these enzymes include 11β-hydroxysteroid dehydrogenases (11β-HSDs) [23], involved in metabolism of corticosteroids; numerous 17β-hydroxysteroid dehydrogenases (17β-HSDs) [24], involved in metabolism of androgens and estrogens; and several $NADP^+$-dependent oxidoreductases, such as retinal SDR1 [9], photoreceptor retinol dehydrogenase [10], and the RDH11–14 group of enzymes [25]. To determine how closely RODH-like SDRs are related to other SDR retinoid and steroid oxidoreductases, we aligned their protein sequences using ClustalW and generated a protein distance matrix based on the resulting alignment. The Fitch–Margoliash method was used to estimate phylogeny and to construct a tree. Phylogenetic analysis showed that RODH-like enzymes form a distinct clade within the group of retinoid and steroid-active SDRs (Fig. 1), being most closely related to the NAD^+-dependent type 2 17β- and 11β-HSDs (HSD17B2 and HSD11B2).

Proteins homologous to RODH-like SDRs are found in nonmammalian genomes of frog and fish and in invertebrate genomes of amphioxus (*Branchiostoma floridae*) [13,14] and worm (*Caenorhabditis elegans*) (Supplementary Table 1 and Fig. 1), indicating that RODH-like enzymes have an ancient ancestry. Human RODH-4 shares as high as 45 and 46% identity with two RODH-like *Branchiostoma* proteins (*Bf* Rdh1 and *Bf* Rdh2) and 29–36% identity with four RODH-like *C. elegans* proteins (*Ce* dhs16, *Ce* dhs20, *Ce* dhs2, and *Ce* Rdh member). Interestingly, the same *C. elegans* proteins are the closest nematode homologs of mammalian type 2 11β- and 17β-HSDs, in agreement with the proposed common origin for RODH-like enzymes and these hydroxysteroid dehydrogenases [26]. However, the nematode proteins are more similar to RODH-like proteins than to 11β- or 17β-HSDs (19–24% sequence identity).

Further evidence for the common origin of 11β- and 17β-HSDs and RODH-like SDRs comes from the comparison of their protein structures. Analysis of primary protein sequences of RODH-like proteins using ProDom 2005.1—a comprehensive database of protein domain families [27]—shows that these proteins contain a C-terminal motif, PD002736, which corresponds to amino acids 238–306 of RODH-4 (Fig. 2). This motif is characterized by conserved amino acids in positions corresponding to Y238, V264, A271, P277, Y281, G284, and P298 of RODH-4. In addition to the RODH-like subfamily, only 11β- and 17β-HSDs of type 2 contain this characteristic motif. Thus, PD002736 seems to be specific for these protein groups. Within the PD002736 sequence, RODH-like SDRs contain a block of 4 amino acids, C267-M268-E269-H270 (indicated by pound signs in Fig. 2) that appears to be highly conserved and characteristic of RODH-like SDRs. It was shown that hydrophobic fragment aa 289–311 of rat RODH-1, which constitutes a part of PD002736, improves protein association with microsomal membrane [28]. Our analysis suggests that the involvement of the C-terminal motif in membrane interaction might be potentially extended to all enzymes sharing the PD002736 sequence motif. Remarkably, the PD002736 C-terminal motif is present in all nematode and amphioxus homologs of RODHs. This, again, is consistent with
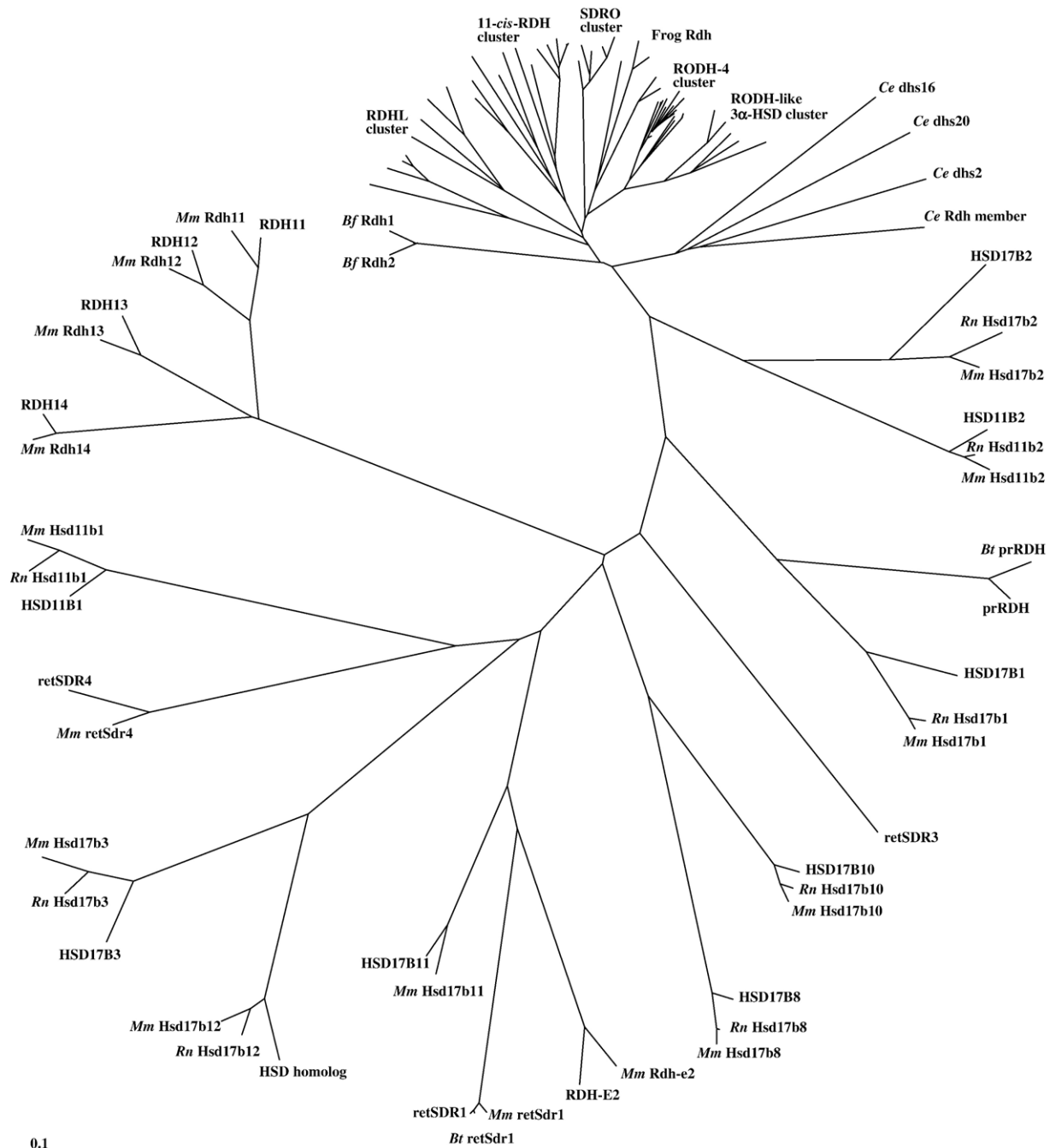
Fig. 1. Phylogenetic tree of retinoid- and steroid-active SDRs. Accession numbers and names of the proteins included in this analysis are listed in Supplementary Table 1. The protein sequence alignment file is provided in Supplementary Fig. 1. Prefix in italic designates a species. Abbreviations are as follows: *Mm, Mus musculus* (mouse); *Rn, Rattus norvegicus* (rat); *Bt, Bos taurus* (cow); *Bf, Branchiostoma floridae* (amphioxus); *Ce, Caenorhabditis elegans*. Human sequences do not contain prefixes. RODH-like proteins form a compact group; major clades within this group are labeled with names of representative proteins as 11-*cis*-RDH cluster and SDRO cluster. A detailed phylogenetic tree of the RODH-like group is shown in Fig. 3.

a common origin for RODH-like enzymes and type 2 11β- and 17β-HSDs.

To investigate the phylogenetic relationships within the group of RODH-like SDRs, a separate tree was constructed for a subset of 56 sequences from different species (Fig. 3). This tree revealed that RODH-like SDR proteins from clawed frog (*Xenopus tropicalis*), zebrafish (*Danio rerio*), and tiger pufferfish (*Fugu rubripes*) cluster with only two of the human

enzymes, RDHL and 11-*cis*-RDH, and not with RODH-4 or 3α-HSD. Because proteins from lower vertebrates are more related to RDHL and 11-*cis*-RDH, these two proteins appear to represent the most ancient forms of RODH-like SDRs. Furthermore, some of the lower vertebrates appear to have several RDHL and 11-*cis*-RDH homologs. For example, the clade containing 11-*cis*-RDH includes a single putative 11-*cis*-RDH protein from each of the mammalian species and chicken,
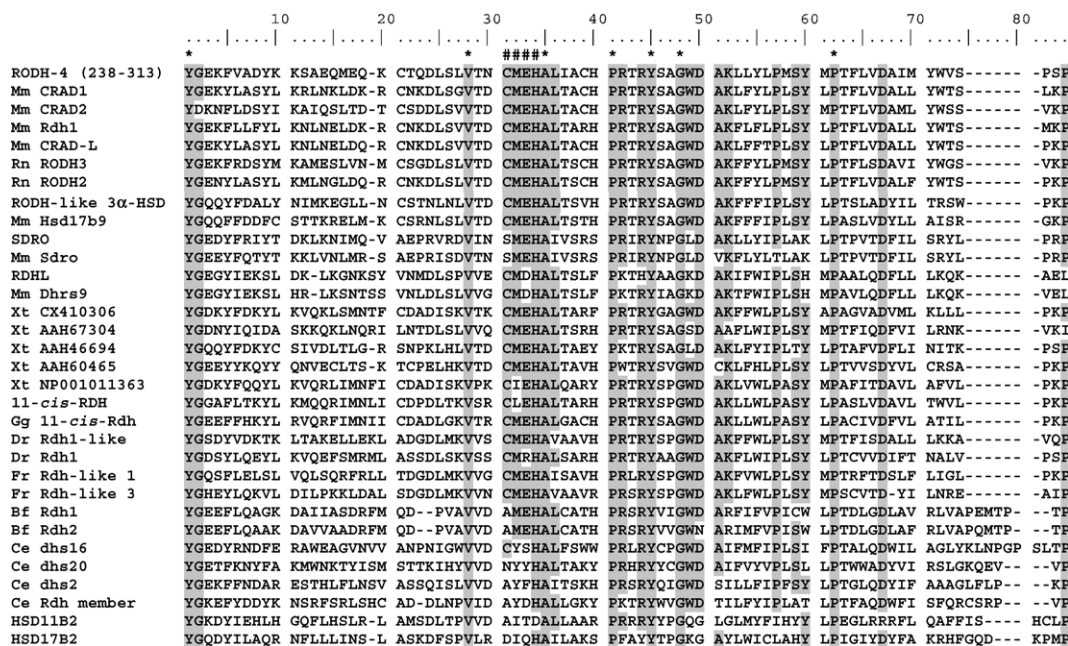
Fig. 2. Alignment of the ProDom PD002736 sequence of selected RODH-like SDRs, HSD11B2, and HSD17B2. The first position of the alignment corresponds to amino acid 238 of RODH-4. Amino acid residues conserved in more than 70% of the aligned proteins are shaded. Amino acid residues identical in all aligned proteins are marked with an asterisk. The CMEH consensus sequence conserved in most RODH-like SDRs is indicated by pound signs.

but two predicted proteins from frog (*Xt* AAH88529 and *Xt* CX410306) and from each of the two fish genomes (*Dr* AAH97151 and *Dr* Rdh1 from zebrafish and *Fr* Rdh-like 2 and *Fr* Rdh-like 5 from tiger pufferfish). The RDHL clade includes a single representative from each of the mammalian species, from frog (*Xt* AAH67304), and from zebrafish (*Dr* Rdh1-like), but three proteins from tiger pufferfish (*Fr* Rdh-like 1, *Fr* Rdh-like 3, and *Fr* Rdh-like 4).

RODH-like SDRs are also found in the genomes of African clawed frog (*Xenopus laevis*) and green pufferfish (*Tetraodon nigroviridis*). These proteins were not included in the trees presented in Figs. 1 and 2 to make the figures more compact; however, when they were included, they were assigned to the same clades as other fish and frog proteins and did not change the general topology of the tree.

Among mammals, both RDHL and 11-*cis*-RDH homologs appeared to be quite conserved. Human RDHL was 86% identical to rodent Rdhl proteins and 87% identical to its cow homolog (not included in the tree). Human 11-*cis*-RDH was 99% identical to that of chimpanzee, 91% identical to bovine and canine homologs, and 88% identical to mouse 11-*cis*-Rdh. High sequence conservation of 11-*cis*-RDH translates into functional correlation between enzymes from different species: human, mouse, and bovine enzymes highly prefer *cis*- over all-*trans*-retinoids (Table 1); also, 11-*cis*-RDH involvement in the visual cycle is supported by both in vivo studies in knockout mice [20] and association of mutated human 11-*cis*-RDH with congenital night blindness [21,22].

RODH-4 and 3α-HSD clades did not include any proteins from nonmammalian species (Fig. 3). This is consistent with interpretation that these clades were the latest to emerge in evolution and probably exist only in mammals. Although the proteins homologous to 3α-HSD formed a separate clade in

the tree constructed of RODH-like SDRs, the sequence conservation within this clade was much lower than that in the RDHL or 11-*cis*-RDH clades. For example, human 3α-HSD was only 76% identical to its canine and bovine homologs, 69% identical to mouse Hsd17b9, and 67% identical to rat Hsd17b9 (also known as 17βHSD type 6 [12]). This observation suggested that the function of 3α-HSD homologs in various species may be less conserved than that of RDHL or 11-*cis*-RDH. Consistent with this interpretation, the mouse enzyme was shown to be active toward a wide variety of substrates including all-*trans*-retinol and 17β- and 3α-hydroxysteroids (Table 1) [29]. In contrast, the rat protein was shown to be active primarily toward 17β-hydroxysteroids [12]. *Cis*-retinoids acted as effective inhibitors of steroid conversion by rat Hsd17b9 (Table 1) [12], but the activity of the enzyme toward retinoids has not yet been demonstrated directly.

The group containing RODH-4-similar proteins had the most complicated structure because of the numerous rodent homologs assigned to it (Fig. 3). Human RODH-4 protein clustered with proteins from chimp (*Pt* XP_522604), dog (*Cf* XP_531641), and cow (*Bt* Rdhs2), with which it shared 99, 77, and 79% sequence identity, respectively. At the same time, rat retinol dehydrogenases (RODH-1 to -3) formed a separate branch together with multiple mouse proteins. This suggested that RODH-4-like proteins underwent an extensive divergence in rodent species. Rodent proteins are only 69–74% identical to human RODH-4 and have 77–97% pair-wise identity among them. Enzymatic activity toward retinoid and/or steroid substrates was shown for rat RODH-1 [7], rat RODH-2 [30], mouse Rdh1 [31], and mouse *cis*-retinol/androgen dehydrogenases (CRAD) types 1–3 (Table 1) [32–34]. Only one of the mouse enzymes, Rdh1, was found to be active toward all-*trans*-
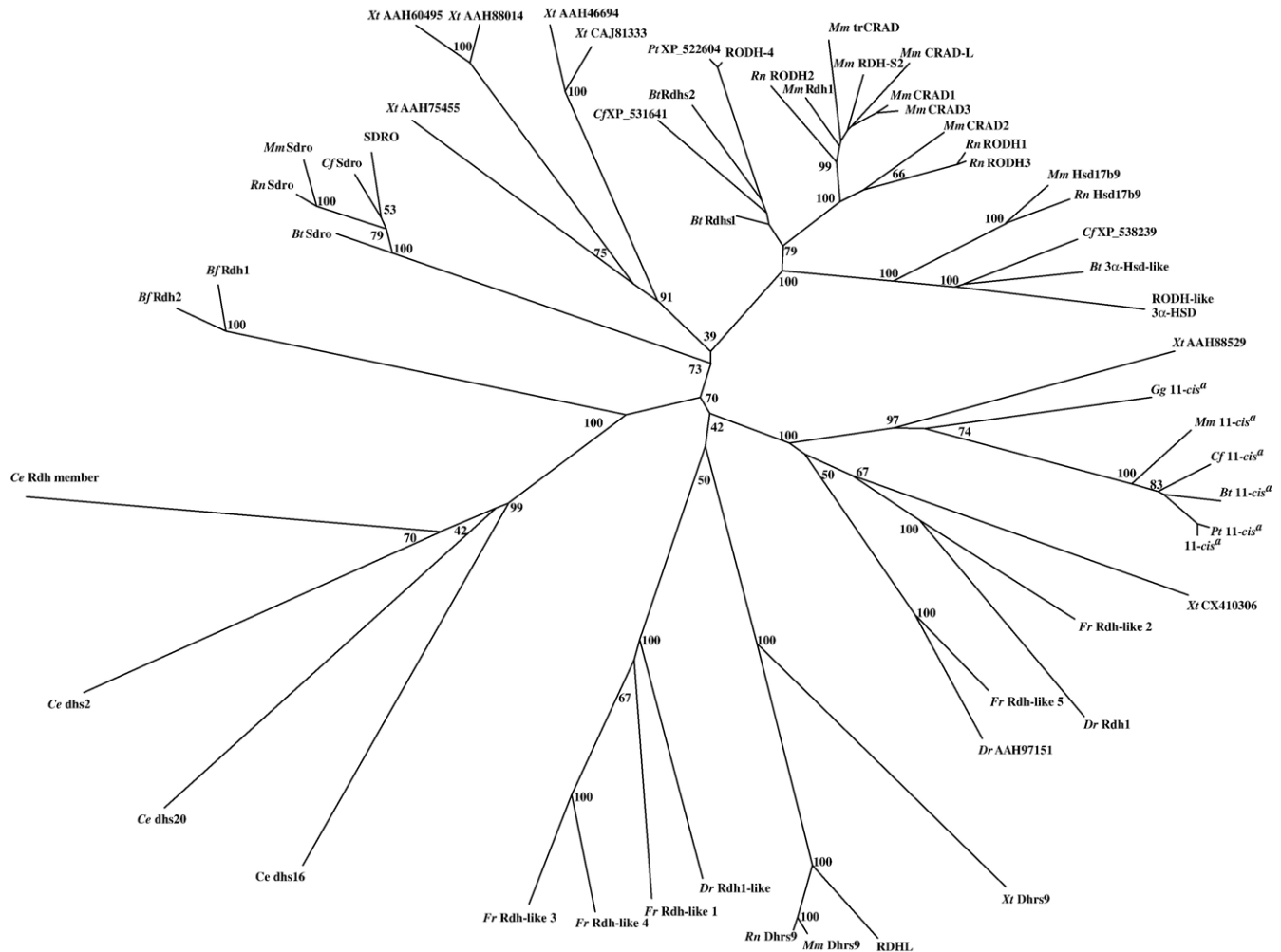
Fig. 3. Phylogenetic tree of RODH-like SDRs. Bootstrapping values (100 replicates) are shown on the tree. Prefix in italic designates a species as in Fig. 1; human sequences do not contain prefixes. 11-*cis*[a] refers to 11-*cis*-Rdh. Abbreviations are as follows: *Mm, M. musculus* (mouse); *Rn, R. norvegicus* (rat); *Cf, Canis familiaris* (dog); *Pt, Pan troglodytes* (chimpanzee); *Bt, Bos taurus* (cow); *Gg, Gallus gallus* (chicken); *Xt, Xenopus tropicalis* (clawed frog); *Dr, Danio rerio* (zebrafish); *Fr, Fugu rubripes* (tiger pufferfish); *Bf, B. floridae* (amphioxus); *Ce, C. elegans*.

retinol, while at least two all-*trans*-retinol-active enzymes were found in humans (RODH-4 and 3α-HSD) and rats (RODH-1 and RODH-2).

In addition to RODH-4, 3α-HSD, RDHL, and 11-*cis*-RDH, human tissues contain another protein that is closely related to this group of SDRs, SDR-orphan (SDRO). Proteins homologous to SDRO form a separate clade in the tree comprising RODH-like SDRs (Fig. 3). Human SDRO shares 89, 87, and 84% protein identity with dog (*Cf* Sdro), rat (*Rn* Sdro), and mouse (*Mm* Sdro) homologs, respectively. The function of SDRO protein remains unknown: in vitro assays showed that it is inactive toward either retinoid or hydroxysteroid substrates [35]. However, the high degree of sequence conservation suggests that SDRO performs an important function preserved in all mammals.

Several predicted RODH-like frog proteins included in this analysis (*Xt* AAH75455, *Xt* AAH60495, *Xt* AAH88014, *Xt* AAH46694, and *Xt* CAJ81333) formed a separate clade branching next to the SDRO clade node (Fig. 3). The topology of this clade suggested that AAH60495 and AAH46694 may

represent duplicated copies of AAH88014 and CAJ81333, respectively. The frog proteins were not significantly closer to any one type of human RODH-like SDR, suggesting that amphibians contain a diverged group of RODH-related proteins, none of which can be assigned to the RODH-4, 3α-HSD, RDHL, 11-*cis*-RDH, or SDRO subfamily. The properties of the frog proteins have not yet been characterized; therefore, it is not clear whether, together, they could be functionally equivalent to the sum of human RODH-like enzymes.

Thus, phylogenetic analysis showed that RODH-like proteins represent a separate subfamily of SDRs that are found in all vertebrates included in this study. This analysis also suggested that the proteins homologous to RDHL and 11-*cis*-RDH are more ancient and much more conserved than the proteins homologous to RODH-4 or 3α-HSD. However, phylogenetic analysis did not allow the identification of orthologous forms of RODH-like SDRs across species, which is important for designing animal models for retinoid and steroid metabolic studies. To address this question, we investigated the structure and organization of RODH-like SDR genes.

Table 1
Substrate specificity of RODH-like SDRs

Enzyme	Substrate		
	Retinoids	Hydroxysteroids	Ref.
RODH-like 3α-HSD	All-*trans*, no 13-*cis* or 9-*cis* activity	$3\alpha \gg 17\beta$	[12,15]
Mm Hsd17b9	All-*trans* > 11-*cis* > 13-*cis* > 9-*cis*	$3\alpha \cong 17\beta$	[29]
Rn Hsd17b9 (also known as Hsd17b6)	Retinoids? Inhibited by 9-*cis* and 13-*cis* retinoids	$17\beta \gg 3\alpha$	[12]
RDHL	Very low all-*trans*	$3\alpha \gg 17\beta$	[16,18]
Mm Dhrs9	No data	No data	
Rn Dhrs9	All-*trans*, others not tested	No data	[47]
SDRO	No activity detected	No activity detected	[35]
RODH-4	All-*trans* ≥ 13-*cis* ≅ 9-*cis*	$3\alpha \gg 17\beta$	[8,46]
Mm CRAD1	11-*cis* > 9-*cis* ≫ 13-*cis* ≫ all-*trans*	$3\alpha \gg 17\beta$	[34]
Mm CRAD2	11-*cis* ≫ all-*trans* ≫ 9-*cis*	$3\alpha \gg 17\beta$	[33]
Mm CRAD3	11-*cis* ≥ 9-*cis*, no all-*trans* activity	$3\alpha \gg 17\beta$	[34]
Mm Rdh1	All-*trans* ≥ 9-*cis*	$3\alpha \gg 17\beta$	[31]
Mm CRAD-L	No data	No data	
Mm RDH-S	No activity detected	No activity detected	[45]
Rn RODH-1	All-*trans* > 9-*cis*	$3\alpha \gg 17\beta$	[7,12]
Rn RODH-3	No data	No data	[48]
Rn RODH-2	All-*trans* > 9-*cis*	No data	[30]
11-*cis*-RDH	11-*cis* ≅ 9-*cis* > 13-*cis* ≫ all-*trans*	$3\alpha \gg 17\beta$	[19,43]
Mm 11-*cis*-Rdh	9-*cis* ≅ 11-*cis*, 13-*cis* ≫ all-*trans*	No data	[44]
Bt 11-*cis*-Rdh	11-*cis*, all-*trans* activity not detected	No data	[6,42]
Gg 11-*cis*-Rdh	No data	No data	
Dr Rdh1-like	Involved in all-*trans*-RA biosynthesis	No data	[37]
Dr Rdh1	Involved in all-*trans*-RA biosynthesis	No data	[37]
Bf Rdh1	No data	No data	
Bf Rdh2	No data	No data	

Genomic organization of RODH-like SDRs

Mammalian genomes

Analysis of genomic organization was carried out for those mammalian species for which whole-genome sequencing data were available in public domains at the time the search was performed. As shown in Fig. 4A, genes encoding homologs to human RDHL (gene name *DHRS9*) are localized on different chromosomes compared to the rest of the RODH-like SDR genes. Human *DHRS9* is localized on chromosome 2 at 2q31.1, while the genes encoding RDHL homologs, identified through phylogenetic analysis (Dhrs9 proteins), are found in syntenic regions of mouse chromosome 2, rat chromosome 3, and dog chromosome 36. Human *DHRS9* and its mammalian homologs are flanked by *ABCB11* and *LRP2* genes in human genome and by homologous loci in other mammalian species, indicating that *Dhrs9* genes are orthologous to the human gene for RDHL.

Genes for all other RODH-related proteins are localized on the same chromosome (Fig. 4A): at 12q13 in humans and in syntenic

regions in the mouse (chromosome 10), rat (chromosome 7), cow (chromosome 5), and dog (chromosome 10) genomes. Altogether, they span over 1 Mb of the genomic sequence. Much of this distance is taken up by the noncoding sequence between the genes for 3α-HSD and 11-*cis*-RDH (gene name *RDH5*) (1.04 Mb in human genome). The human gene for 11-*cis*-RDH is located between the *CD63* and the *BLOC1S1* loci, and genes for 11-*cis*-Rdh in other species occupy syntenic positions, thus representing single orthologs of the human enzyme.

Genes for SDRO-, 3α-HSD-, and RODH-4-like proteins in humans, mice, cows, and dogs form a tight cluster spanning 100–200 kb. This cluster is flanked by *ADMR* and *PRIM1* and is not interrupted by any known genes. The same position and the same orientation in different genomes easily define single orthologs for human *SDRO* and *HSD17B6* (gene name for human 3α-HSD). Orthologs of the human *SDRO* gene in mouse, rat, dog, and cow are *Sdro* genes. Genes orthologous to human 3α-HSD (*HSD17B6*) are as follows: in mouse and rat, *Hsd17b9*; in dog, the gene encoding XP_538239; and in cow, the gene encoding 3α-HSD-like protein.

In contrast, in place of a single *RODH4* in humans, other species tend to contain multiple homologs, all of which are grouped together with RODH-4 on the tree (Fig. 3). We were able to identify six RODH-4-like genes in mouse (encoding Rdh1, CRAD1, CRAD2, CRAD3, CRAD-like, and RDH-S), one in dog (encoding XP_531641), three in cow (encoding Rdh−1 to -3), and two in rat (encoding RODH-2 and RODH-3). Surprisingly, genomic sequence matching rat RODH-1, the first discovered microsomal all-*trans*-retinol dehydrogenase [7], was not found; this might be due to the current unfinished state of rat genome sequencing project.

All mammalian genes for RODH-like proteins are characterized by the same architecture with conserved exon–intron junctions, four protein-coding exons, and 5′-untranslated exon in many cases.

In addition to protein-coding genes, in human, mouse, rat, cow, and dog genomes, BLAST search revealed numerous loci with partial similarity to RODHs, sometimes designated as "retinol dehydrogenase similar" or unnamed, which are not supported by EST or mRNA data. We performed a detailed analysis of these loci to determine whether they are protein-coding genes or pseudogenes. We found that partial ORFs of these loci are often interrupted by stop codons; therefore, they most likely represent pseudogenes (marked as Ψ in Fig. 4). For example, rat locus *LOC500580* corresponding to the predicted "RODH-1-similar protein" is unlikely to encode a functional enzyme. Although two exons of the predicted mRNA encode polypeptides very similar to those encoded by the first two exons of *RODHs*, the downstream protein sequence is different and is interrupted by a stop codon at amino acid 291. Only the first two exons, very similar among rat *RODHs*, are supported by ESTs. Thus, rat *LOC500580* is a pseudogene. Another rat locus, *LOC503146*, encodes a predicted protein similar to N-terminally truncated RODH-2. Detailed analysis revealed that the genomic sequence homologous to the first exon of *RODHs* contains a frameshift, which results in a stop codon shortly afterward. Thus, *LOC503146* is another likely pseudogene.

The predicted protein product of the mouse AYL053573 locus is deposited with GenBank as a "truncated *cis*-retinol androgen dehydrogenase" (truncated CRAD); however, a stop codon disrupts its ORF so that the resulting polypeptide lacks the SDR consensus substrate binding sequence. This truncated product is not supported by any ESTs; therefore, this locus is also a pseudogene associated with the RODH-4-like cluster.
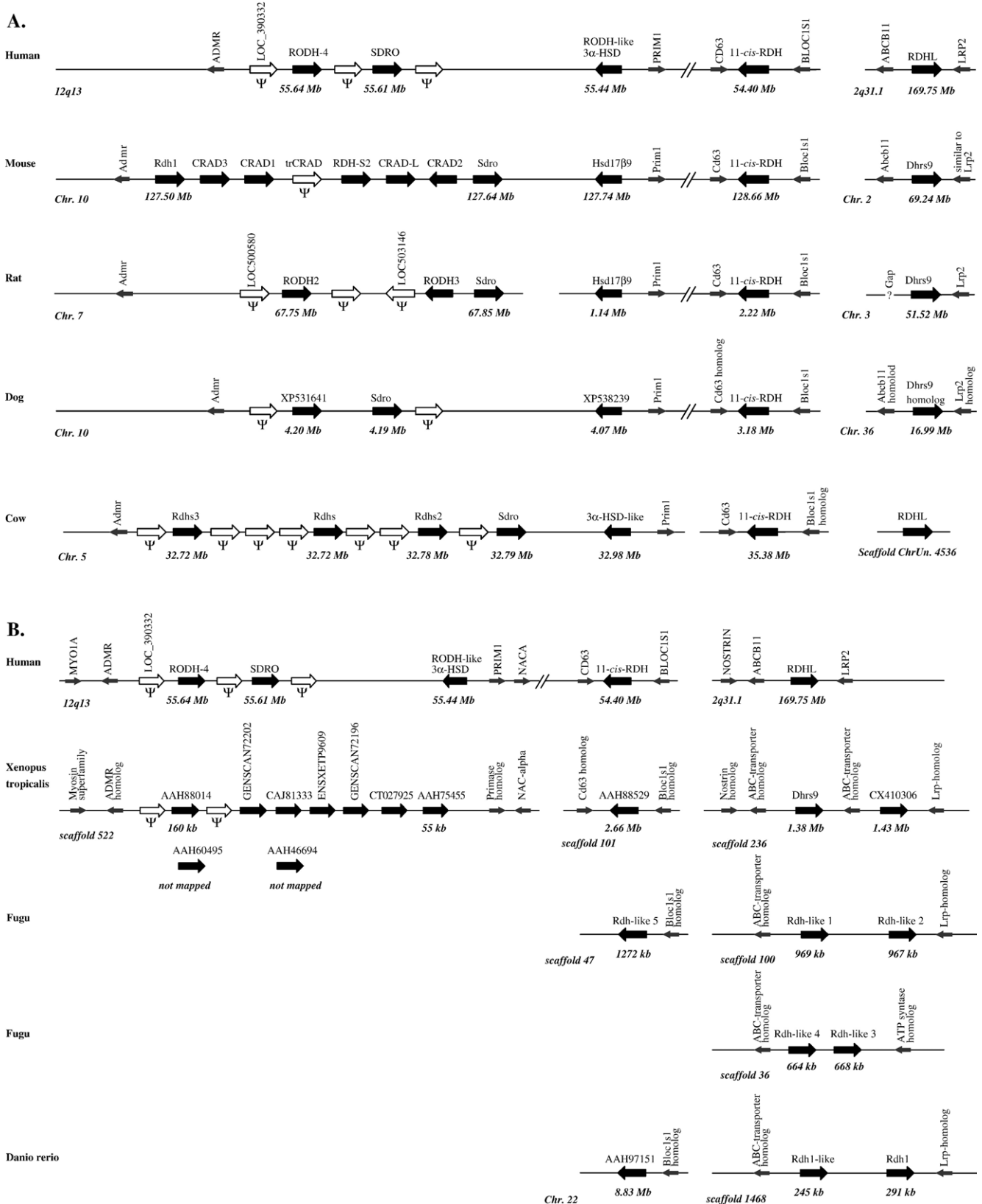
Nonmammalian genomes

In addition to mammals, we attempted to establish the orthologs of human enzymes in nonmammalian vertebrates, such as amphibians and fish, in hopes that this would provide some clues to the origin and functions of these proteins. Notably, RODH-like genes in amphibians and fish generally maintain the same exon–intron structure as their mammalian homologs, except in several of them the protein-coding regions are extended to encode a longer N- or C-terminus, as in zebrafish Rdh1. The similarity of exon–intron architecture between mammalian and amphioxus RODH-like genes was suggested to support the common ancestral RODH form for primitive chordates and vertebrate species [13].

Fig. 4B shows the genomic organization of nonmammalian RODH homologs in comparison to human. Genes for AAH67304 from *X. tropicalis,* Rdh-like 1 from *F. rubripes,* and Rdh1-like from *D. rerio,* which all belong to the RDHL clade in Fig. 3, occupy positions syntenic to that of human *RDHL*. Each of them is flanked by loci homologous to *ABCB11* and *LRP2,* neighbors of the human *RDHL* gene. The *Fugu* genome also encodes two additional RDHL homologs, Rdh-like 3 and Rdh-like 4, in a different scaffold. So far, it cannot be determined whether these two genes are specific for *Fugu* or are present in all fish. The phylogenetic tree suggests that one of them is the result of a relatively recent duplication of another.

Frog AAH88529, zebrafish AAH97151, and tiger pufferfish Rdh-like 5 seem to represent the orthologs of human 11-*cis*-RDH. Loci homologous to human *BLOC1S1* are present next to their genes in the same orientation as *BLOC1S1* in relation to the human gene for 11-*cis*-RDH (Fig. 4B). Surprisingly, the genes for CX410306 from frog, Rdh1 from zebrafish, and Rdh-like 2 from tiger pufferfish, which all belong to the 11-*cis*-RDH clade of the phylogenetic tree, are found next to *RDHL* orthologs in genomes of these species. Therefore, these extra 11-*cis*-RDH homologs in tiger pufferfish and zebrafish could not have appeared as a result of whole genome duplication proposed for ray-finned fish [36], but represent additional members of the retinol/sterol dehydrogenases subfamily missing in mammalian genomes.

Neither of the fish homologs could be identified as an ortholog of human RODH-4, SDRO, or 3α-HSD, which is in agreement with the absence of fish proteins in the corresponding clades of the phylogenetic tree. Complete genome sequences for fish species are not yet available; however, the fact that such orthologs were not found in any of the three analyzed fish species (including tiger pufferfish, for which more than 90% of the genome is sequenced) suggests that they do not exist in fish.

In frog, the genomic region in scaffold 522 between homologs of human ADMR and PRIM1 *(*corresponding to the human RODH-4–SDR-O–HSD17B6 cluster) contains seven predicted protein-coding genes and two pseudogenes (Fig. 4B). In addition to genes encoding AAH88014, CAJ81333, and AAH75455, which are shown on the phylogenetic tree, it also contains one more mRNA-supported gene (CT027925) and three automatically annotated genes for predicted RODH-similar proteins (ENSXET00000009609, GENSCAN00000072202, GENSCAN00000072196). When included in phylogenetic analysis, their predicted protein sequences were all assigned to the "frog Rdh" group on the tree (Fig. 1). All of these genes have the same orientation and span about 100 kb of the genomic sequence. From the analysis of their localization and orientation in relation to one another it appears impossible to establish orthology between any of these genes and the members of the human 12q13 cluster. Together with the phylogenetic tree topology, this suggests that frog retinol/sterol dehydrogenase-similar genes in scaffold 522 are the result of tandem duplications and divergence specific for amphibian lineage. Genes for two additional *X. tropicalis* proteins, AAH60495 and AAH46694, both supported by existing mRNAs, were not found in scaffold 522 and are currently unmapped. As mentioned above, the tree topology suggests that they may represent duplicated and translocated copies of AAH88014 and CAJ81333, respectively.

Origin and divergence

Based on the comparative analysis of SDR 11β- and 17β-HSDs and several RODH-like enzymes, Baker [26] suggested that HSD11B2, HSD17B2, and RODH-like enzymes diverged from the ancestral oxidoreductase, which duplicated early in the evolution of deuterostomes (Fig. 5, circled). In the lower taxa, such as nematodes, the ancestral oxidoreductase gave rise to several proteins similar to both RODH and type 2 11β- and 17β-HSDs, such as *C. elegans* homologs discussed above, which were not yet split into two separate subfamilies. Recently, retinoic acid biosynthesis was shown to exist in primitive chordates, such as amphioxus, consistent with the presence of RODH-like protein in this species. Based on these observations, Dalfo et al. [13,14] suggested that the single ancestral gene to RODH-like enzymes must have appeared before the divergence of cephalochordates (e.g., amphioxus) and vertebrates. Further reconstruction of the evolution of RODH-like enzymes is complicated by what appears to be several gene duplication events. Fish and amphibian genomes contain more than one homolog of 11-*cis*-RDH. As shown in Fig. 4B, the first 11-*cis*-RDH homolog, orthologous to human 11-*cis*-RDH, was found in all analyzed groups and must have been present in the common ancestor to teleost fish, amphibians, and mammals.

Fig. 4. Genomic organization of (A) mammalian and (B) nonmammalian RODH-like SDRs in comparison to humans. Genes are indicated by the names of encoded proteins. Physical distances on chromosomes and scaffolds (Mb or kb) are shown as available in the NCBI or Ensembl databases on April 7, 2006. The Ensembl database identifiers ENSXET00000009609, GENSCAN00000072202, and GENSCAN00000072196 are used for *Xenopus* predicted proteins not found in the NCBI database. Positions of RODH-like SDR genes are shown by large black arrows. Positions of genes unrelated to RODH-like SDRs are shown by small black arrows. Ψ indicates probable pseudogenes. In the currently available version of the rat genome, *Hsd17b9* and the gene for 11-*cis*-Rdh are found in a different contig of chromosome 7 compared to genes for RODH-2, RODH-3, and Sdro. This could be due to the unfinished state of the rat genome sequencing project or a chromosomal rearrangement. Notably, the distance between the genes for Hsd17β9 and 11-*cis*-RDH is the same as that in other mammalian genomes.
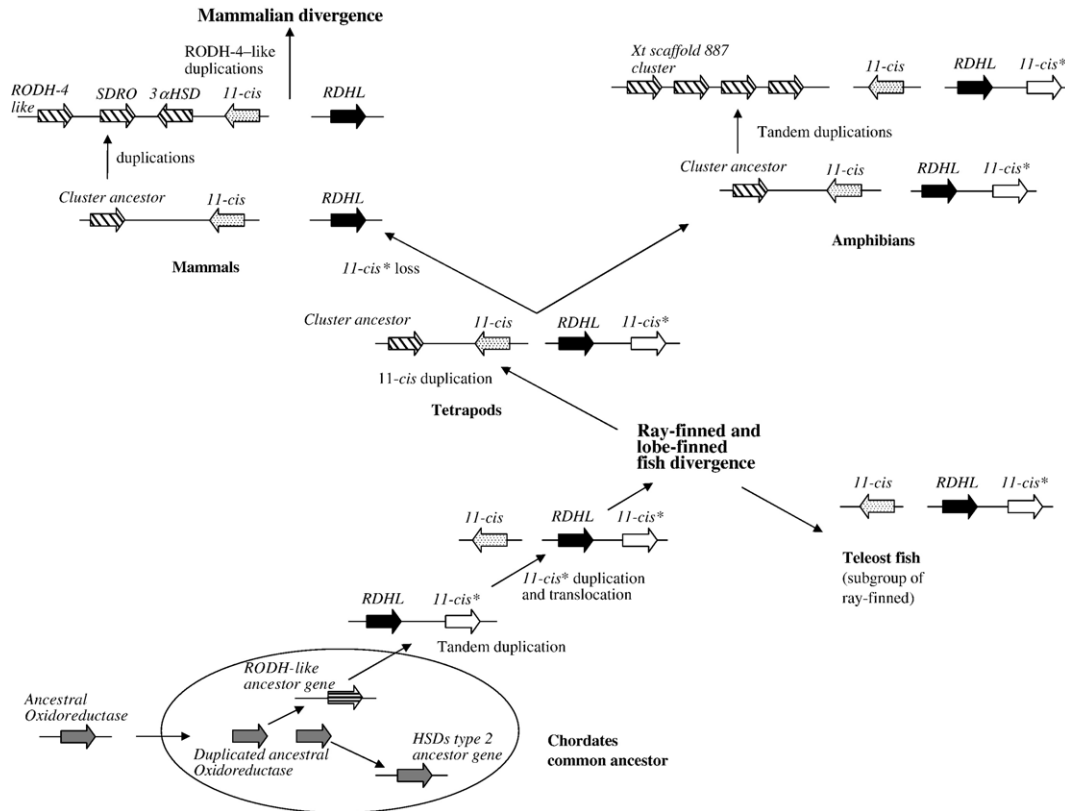
Fig. 5. Proposed model of origination and divergence of RODH-like subfamily of SDRs. 11-cis* denotes the predecessor for 11-cis-RDH (11-cis).

The same could be said about the highly conserved *RDHL* gene. The second *11-cis-RDH* homolog (*11-cis-RDH**) is located right next to the *RDHL* ortholog in both frogs and fish (Fig. 4B). This observation suggests that the tandem placement of *RDHL* and *11-cis-RDH** might have been present in, at least, the common ancestor of teleost fish (e.g., tiger pufferfish and zebrafish) and tetrapods (Fig. 5) and, therefore, before the divergence of ray-finned and lobe-finned fish. It is possible that the additional *11-cis-RDH* homolog originally linked to *RDHL* was later lost in mammals.

Based on these considerations, we propose a model for evolution of RODH-like SDRs (Fig. 5), which includes the appearance of tandem-organized predecessors of *RDHL* and *11-cis-RDH* (denoted as *11-cis** in Fig. 5) through duplication of the ancestral RODH-like gene with subsequent divergence. We propose that the *RDHL* and *11-cis-RDH** genes appeared from the RODH-like ancestor gene by tandem duplication (Fig. 5). *11-cis-RDH** was then duplicated by some mechanism that included translocation of the new copy to another region of the genome. These steps probably occurred before the vertebrate divergence, and as a result, the common ancestor to teleost fish and tetrapods possessed two copies of the *11-cis-RDH* gene (*11-cis* and *11-cis**) and a single predecessor of RDHL (Fig. 5). Teleost fish retained these three genes and acquired additional RDHL homologs (Rdh-like 3 and Rdh-like 4 in Fig. 3) as a result of fish-specific or species-specific gene duplications.

On the other hand, in the common ancestor of tetrapods this group of genes appeared to undergo a different development,

which resulted in acquisition of a new RODH-like cluster, e.g., *RODH-4–SDRO–HSD17B6* in humans and the RODH-like SDR cluster in scaffold 522 in *X. tropicalis*. In mammalian genomes, the *11-cis-RDH* gene is located on the same chromosome as genes encoding Sdro-, 3α-HSD-, and RODH-4-like proteins. Since *11-cis-RDH* seems to represent the most ancient form among them, the three other genes could have evolved from *11-cis-RDH* by several duplication events. It seems possible that the translocated copy of the *11-cis-RDH* predecessor (*11-cis* in Fig. 5) underwent duplication before the separation of amphibians and amniotes. The new copy evolved independently in descending lineages, with Sdro-, 3α-HSD-, and RODH-4-like clades eventually originating from this new copy in mammals and a separate clade (e.g., genes in *Xenopus* scaffold 522) appearing in amphibians. Although the orthologs between the members of the *Xenopus* clade and the mammalian genes were not identified, altogether these frog proteins may be functionally equivalent to Sdro-, 3α-HSD-, and RODH-4-like enzymes. Eventually, the copy of *11-cis-RDH** located next to *RDHL* was lost in mammals, but was maintained in the lower taxa, such as fish and amphibians. Interestingly, zebrafish Rdh1, corresponding to *11-cis-RDH**, was recently shown to contribute to retinoic acid biosynthesis [37]. Although the ability of Rdhl to oxidize retinol was not demonstrated directly, the observation that Rdhl contributes to retinoic acid biosynthesis in zebrafish implies that this protein (missing in mammals) carries out the same function as that attributed to mammalian RODH-4- and 3α-HSD-like proteins (missing in fish). Thus, although not directly orthologous,

zebrafish Rdhl and RODH-4/3α-HSD homologs may be functionally equivalent.

The ancestral cluster containing Sdro-, 3α-HSD-, and RODH-4-like genes underwent further divergence in mammalian species, leading to the generation of multiple functional enzymes in place of a single RODH-4 protein in humans. The existence of multiple RODH-4-similar proteins in mouse, rat, and cow and the presence of several adjacent RODH-like pseudogenes in human, cow, dog, rat, and mouse genomes suggest that the ancestral cluster was a hot spot of the duplication process that continued in different mammalian genera after their divergence. In mice, the largest number of functional copies seems to be preserved; each of them could adopt some functions of the ancestral gene, corresponding to RODH-4, or acquire new properties in addition to the function that RODH-4 plays in humans.

Thus, the data available to date show that mammalian species possess single orthologs for human RDHL, 11-*cis*-RDH, SDRO, and RODH-like 3α-HSD, which makes them obvious targets for animal knockouts. However, for RODH-4 the situation is less clear because of the lack of a clear ortholog. If the function of RODH-4 is "dispersed" among several enzymes in rodents, the targeting of a single gene most probably will not lead to conclusive results.

Materials and methods

The nonredundant NCBI nucleotide sequence database and NCBI and Ensembl project (v32, July 2005, http://www.ensembl.org) species-specific genomic sequence databases were searched using the tBLASTn algorithm with human RODH-4 protein sequence. When only partial sequence was retrieved through this search, the NCBI EST database was also screened for overlapping sequences to recover a full-length coding sequence. Mammalian sequences with more than 45% of protein identity were selected for analysis. Genomic sequence databases of other vertebrate species (*Gallus gallus, X. laevis, X. tropicalis, D. rerio, F. rubripes, T. nigroviridis*) and the nematode *C. elegans* were searched for homologs using human RODH-4, nonhepatic 3α-HSD, and 11-*cis*-RDH protein sequences as queries. Accession numbers for all sequences used in this work are provided in Supplementary Table 1. If the predicted full-length homolog is derived from several sequences, all accession numbers are provided. The complete protein alignment is provided in Supplementary Figure 1.

The Ensembl project database was used to compare the genomic organization of homologs in different species. At the time of analysis, the following versions of genomic sequences were available for search through the Ensembl project Web site: dog—CanFam 1.0 (July 2004), cow—Btau 1.0 (October 2004), chimpanzee—CHIMP 1 (November 2003), rat—RGSC 3.4 (December 2004), mouse—NCBI m34 (May 2005), chicken—WASHUC1 (March 2004), *X. tropicalis*—JGI 4 (June 2005), zebrafish—WTSI Zv5 (May 2005), *F. rubripes*—fourth genome assembly (October 2004), *T. nigroviridis*—TETRAODON 7 (Apr. 2003), *C. elegans*—WS 140 (March 2005). Protein sequences were aligned using ClustalW [38]; a phylogenetic tree was obtained using the Fitch–Margoliash algorithm in PHYLIP, version 3.65 [39,40]; TREEVIEW 1.6.6 [41] was used for graphic representation of the tree. Protein sequences of RODH-like proteins were analyzed using the ProDom database (release 2005.1, available at http://www.toulouse.inra.fr/prodom.html) [27].

Acknowledgment

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2006.06.004.

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