Specificity of Zebrafish Retinol Saturase: Formation of All-*trans*-13,14-dihydroretinol and All-*trans*-7,8- dihydroretinol[†]

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ABSTRACT: Metabolism of vitamin A, all-*trans*-retinol, leads to the formation of 11-*cis*-retinaldehyde, the visual chromophore, and all-*trans*-retinoic acid, which is involved in the regulation of gene expression through the retinoic acid receptor. Enzymes and binding proteins involved in retinoid metabolism are highly conserved across species. We previously described a novel mammalian enzyme that saturates the 13−14 double bond of all-*trans*-retinol to produce all-*trans*-13,14-dihydroretinol, which then follows the same metabolic fate as that of all-*trans*-retinol. Specifically, all-*trans*-13,14-dihydroretinol is transiently oxidized to all-*trans*-13,14-dihydroretinoic acid before being oxidized further by Cyp26 enzymes. Here, we report the identification of two putative RetSat homologues in zebrafish, one of which, zebrafish RetSat A (zRetSat A), also had retinol saturase activity, whereas zebrafish RetSat B (zRetSat B) was inactive under similar conditions. Unlike mouse RetSat (mRetSat), zRetSat A had an altered bond specificity saturating either the 13−14 or 7−8 double bonds of all-*trans*-retinol to produce either all-*trans*-13,14-dihydroretinol or all-*trans*-7,8-dihydroretinol, respectively. zRetSat A also saturated the 13−14 or 7−8 double bonds of all-*trans*-3,4-didehydroretinol (vitamin A2), a second endogenous form of vitamin A in zebrafish. The dual enzymatic activity of zRetSat A displays a newly acquired specificity for the 13−14 double bond retained in higher vertebrates and also the evolutionarily preserved activity of bacterial phytoene desaturases and plant carotenoid isomerases. Expression of zRetSat A was restricted to the liver and intestine of hatchlings and adult zebrafish, whereas zRetSat B was expressed in the same tissues but at earlier developmental stages. Exogenous all-*trans*-retinol, all-*trans*-13,14-dihydroretinol, or all-*trans*-7,8-dihydroretinol led to the strong induction of the expression of the retinoic acid-metabolizing enzyme, Cyp26A1, arguing for an active signaling function of dihydroretinoid metabolites in zebrafish. These findings point to a conserved function but altered specificity of RetSat in vertebrates, leading to the generation of various dihydroretinoid compounds, some of which could have signaling functions.

Enzymes that process carotenoids or their cleavage products, the apocarotenoids that include retinoids, are found in every kingdom of life. Carotenoids synthesized by plants, bacteria, and fungi are needed for photosynthesis, whereas apocarotenoids play essential roles in photoreception (11-*cis*-retinal) and regulation of gene expression (all-*trans*-retinoic acid and abscisic acid). In vertebrates, vitamin A, that is, all-*trans*-retinol, is required for embryonic development and the maintenance of normal function of adult tissues. Biological transformations of carotenoids and apocarotenoids are carried out by enzymes that retain a high degree of protein sequence homology throughout nature (*1*). Homology searches using the sequence of plant carotenogenic enzymes led us to identify a novel vertebrate enzyme related to plant carotenoid isomerases (CRTISO[1]) (*2*, *3*), cyanobacterial carotenoid isomerases CrtH (*4*, *5*), and bacterial phytoene desaturases (CrtI) (*6*). The vertebrate relative carries out the saturation of the 13−14 double bond of all-*trans*-retinol to produce all-*trans*-13,14-dihydroretinol (*7*) and thus was designated as all-*trans*-retinol−all-*trans*-13,14-dihydroretinol saturase or RetSat (*7*). RetSat is an endoplasmic reticulum localized membrane protein found in most tissues and at highest levels in the liver, kidney, and intestine (*7*). Our results led to the general conclusion that enzyme members of the phytoene desaturase family catalyze the saturation−desaturation of double bonds in the polyene chains of

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[1] Abbreviations: ADH, medium-chain alcohol dehydrogenases; CRTISO, carotenoid isomerase; LRAT, lecithin-retinol acyltransferase; ER, endoplasmic reticulum; EST, expressed sequence tag; FAD, flavin-adenine dinucleotide; HPLC, high performance liquid chromatography; Pds, phytoene desaturase; RA, retinoic acid; RALDH, retinal dehydrogenase; RAR, retinoic acid receptor; (m, z)RetSat, (mouse, zebrafish) all-trans-(13,14)-retinol saturase; SCAD, short-chain alcohol dehydrogenase family of enzymes; T-REx-293, HEK-293 cells that stably express the tetracycline repressor protein.

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carotenoids or retinoids; moreover, such enzymes exist throughout nature in plants (CRTISO and PDS), bacteria and fungi (CrtI), cyanobacteria (CrtH and CrtP), and vertebrates (RetSat) (*1*, *8*).

All-*trans*-13,14-dihydroretinol, the product of RetSat, is present in tissues derived from animals maintained on a normal diet (*7*). The metabolism of all-*trans*-13,14-dihydroretinol was explored *in vivo* in lecithin−retinol acyltransferase (LRAT) knockout mice (*9*) gavaged with either retinyl palmitate or with all-*trans*-13,14-dihydroretinol (*10*). When enzymatic steps of the all-*trans*-13,14-dihydroretinol oxidation pathway were recapitulated *in vitro* with recombinantly expressed enzymes and synthetic substrates, we found that the same enzymes that are involved in the oxidation of all-*trans*-retinol to all-*trans*-retinoic acid and then to other more oxidized metabolites were also involved in the metabolism of all-*trans*-13,14-dihydroretinol (*10*). The oxidation of all-*trans*-13,14-dihydroretinol by medium (ADH) (*11*) and short-chain (SCAD) alcohol dehydrogenases (*12*, *13*) led to the formation of all-*trans*-13,14-dihydroretinal, which could be oxidized to all-*trans*-13,14-dihydroretinoic acid by retinaldehyde dehydrogenase (RALDH) enzymes 1−4 (*14*−*19*). All-*trans*-13,14-dihydroretinoic acid, being structurally similar to all-*trans*-retinoic acid, could activate transcription through the retinoic acid receptor (RAR) but not the retinoid X receptor (RXR) (*10*). All-*trans*-13,14-dihydroretinoic acid could be further oxidized by the same enzymes that oxidize retinoic acid (*20*−*23*), namely, Cyp26A1-C1, to more polar metabolites such as all-*trans*-4-oxo-13,-14-dihydroretinoic acid (*10*). The same enzymes involved in the synthesis and degradation of all-*trans*-retinoic acid also control the levels of all-*trans*-13,14-dihydroretinoic acid and, therefore, regulate its levels in a tissue specific and temporal manner.

RetSat is related to other members of the phytoene desaturase family, which includes phytoene desaturases and carotenoid isomerases from plants, bacteria, and fungi. Closely related RetSat homologues were identified in all chordate species examined, including nonvertebrate chordates, such as the ascidians *Ciona intestinalis* and *Ciona savignyi*, and even in nonchordate deuterostomes such as echinoderms. It is important to verify the function of these putative RetSat homologues to understand where and why during deuterostome evolution an ancestral phytoene desaturase evolved to become a retinol saturase enzyme. Here, we undertook the first evolution of function study of RetSat by characterizing homologues of RetSat in zebrafish.

EXPERIMENTAL PROCEDURES

Cloning and Expression Constructs. Full-length cDNA clones of zRetSat A (accession number BC090469, IMAGE: 6900936) and zRetSat B (accession number BG302971, IMAGE:3816518) obtained from Open Biosystems (Huntsville, AL) were subcloned into the *Xho I* site of pCDNA4/TO (Invitrogen, Carlsbad, CA) under the control of a tetracycline-inducible promoter. zRetSat A and zRetSat B were expressed in T-REx-293 cells (Invitrogen), which are HEK-293 cells (*24*) that stably express the tetracycline repressor protein. The transfection of T-REx-293 with zRetSat A or B expression constructs was performed with the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Stable expressing cells were selected with zeocin, and all resistant clones were pooled for activity assays. Cells stably expressing mRetSat were previously described (*7*). Cells were cultured in DMEM (Invitrogen), supplemented with 10% fetal calf serum, 0.5 mg/mL zeocin, and 2 μg/mL blasticidin and maintained at 37 °C, 5% CO_2, and 100% humidity.

Reagents and Chemical Synthesis. All reagents were purchased from Sigma-Aldrich and used without additional purification. Solvents were dried under standard procedures prior to use. For the synthesis of all-*trans*-7,8-dihydroretinol, 4-(2,6,6-trimethylcyclohex-1-enyl)butan-2-one was first condensed with triethylphosphonoacetate in anhydrous tetrahydrofuran (THF) using *n*-butyl-lithium as the base and *N*,*N*-dimethylpropyleneurea as the cosolvent to yield ethyl 3-methyl-5-(2,6,6-trimethylcyclohex-1-enyl)pent-2-enoate as a mixture of isomers. This mixture was then reduced with $LiAlH_4$ to the corresponding alcohols and subsequently oxidized to the aldehydes with MnO_2 under basic conditions. The aldehydes were separated by HPLC (95:5 hexane/ethyl acetate). The C15-all-*trans* aldehyde was used in a Horner−Wadsworth−Emmons condensation with triethyl 3-methyl-4-phosphonocrotonate in anhydrous THF to produce ethyl all-*trans*-7,8-dihydroretinoate that was reduced at −78 °C with Dibal-H to yield all-*trans*-7,8-dihydroretinol (*25*). All-*trans*-3,4-didehydroretinol was prepared as described previously (*26*).

Activity Assays and Retinoid Analysis. All procedures involving retinoids were performed under dim red light unless otherwise specified. Retinoids were stored in *N*,*N*-dimethylformamide (DMF) under argon at −80 °C. RetSat activity assays were performed as described previously (*7*). The expression of mRetSat or its putative fish homologues was induced by using tetracycline 48 h before analysis. Cells were incubated with 10 μM all-*trans*-retinol or all-*trans*-3,4-didehydroretinol substrates 24 h before analysis. Media and cells were collected by scraping and mixed with an equal volume of methanol. The methanol/water mixture was extracted with two volumes of hexane, after which the organic phase was dried, resuspended in hexane, and analyzed by normal phase HPLC (Beckman Ultrasphere-Si, 5 μm, 4.6 × 250 mm) with 10% ethyl acetate, 90% hexane at a flow rate of 1.4 mL/min, and an HP1100 high performance liquid chromatograph with a diode-array detector and HP Chemstation A 08.03 software. All-*trans*-7,8-dihydroretinol generated by zRetSat A catalyzed saturation of all-*trans*-retinol was purified by organic extraction, normal phase HPLC, and collection of the appropriate peak. Biosynthetic or chemically synthesized all-*trans*-7,8-dihydroretinol was oxidized in 600 μL of dichloromethane in the presence of manganese dioxide for 3 h at room temperature in the dark. The reaction was stopped by the addition of 3 mL of ethanol, and the resulting all-*trans*-7,8-dihydroretinal was derivatized with 10 mM hydroxylamine. Retinoids were extracted and analyzed by normal phase HPLC as described above.

Fish Maintenance and Embryonic Staging. Zebrafish were bred and maintained under standard conditions at 28.5 °C (*27*). Morphological features were used to determine the stage of the embryos in hours (hpf) or days (dpf) postfertilization according to Kimmel et al. (*28*). The embryos used for *in situ* hybridization experiments were raised in the presence

of 200 μM 1-phenyl-2-thiourea (Sigma-Aldrich, Germany) to inhibit pigmentation.

Whole Mount in Situ Hybridization. Whole mount *in situ* hybridization (WISH) was performed as described by Hauptmann and Gerster (*29*). zRetSat A and zRetSat B were cloned into the vector pCRII-TOPO (Invitrogen, Germany) by using the following oligonucleotide primers: for zRetSat A, 5′-AGGGTGGTGCTACTGAGATCGCCT and 5′-AGTTAGCAAAACTAACAACAGTCAGGGT and for zRetSat B, 5′-TGGTGCTAGTGAAATCCCGT and 5′-TGGATCACCATACGTGATTGCCCT. Antisense RNA probes were synthesized with the T7 RNA polymerase. The RNA probe used for the *in situ* hybridization of zebrafish Cyp26 A1 has been described elsewhere (*30*). RNA probes were generated with the Dig RNA labeling kit (Roche Molecular Biochemicals, Germany) according to the manufacturer's protocol.

Retinoid Treatments of Embryos. Fish embryos (6 hpf) were treated with 10^{-6} M all-*trans*-retinol (Sigma-Aldrich, Germany), 10^{-6} M all-*trans*-7,8-dihydroretinol, or 10^{-6} M all-*trans*-13,14-dihydroretinol in egg water. Sibling controls were treated with equivalent concentrations of DMSO alone. Embryos were kept under dim red light (620 nm) during treatment.

Photography. Stained, whole mount embryos were photographed in 100% glycerol on a dissecting microscope (Leica MZ FLIII) with an Axiocam (Zeiss).

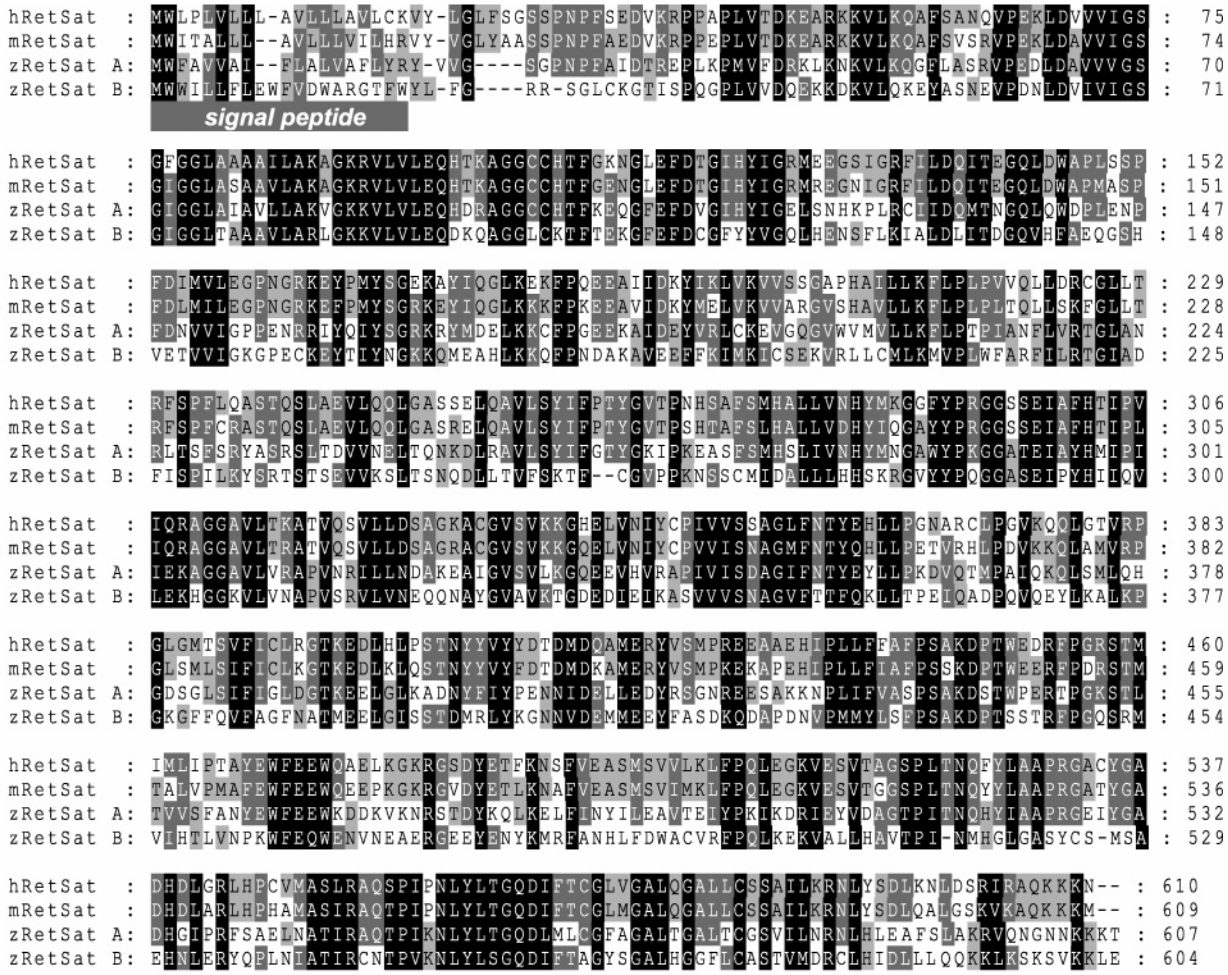
RESULTS

Identification of Putative RetSat Homologues in Zebrafish. We identified two zebrafish genes that could potentially code for the RetSat homologues of human and mRetSat proteins. One of the genes, zebrafish RetSat A (zRetSat A), was found on chromosome 3 (zgc:113107) and coded for a protein that shared 70% conserved and 49% identical residues compared with mRetSat protein (Figure 1A and B). The second gene, zRetSat B, was located on chromosome 9 (zgc:123334) and shared 59% conserved and 36% identical residues with mRetSat protein (Figure 1A and B). The exon−intron breaks were conserved within the aligned protein sequences of RetSat proteins and their putative homologues. The two RetSat homologues most likely evolved by gene duplication following the genome wide chromosome duplication proposed to have occurred in the teleost lineage after divergence from tetrapods (*31*−*33*). Interestingly, the two zebrafish RetSat homologues are less similar to one another than they are to either the human or the mRetSat sequences, indicating a high degree of divergence. Hydropathy analysis of the sequence of zRetSat A (Figure 1C) revealed the presence of a cleavable signal sequence that targets the nascent protein to the ER membrane (*34*) and a possible transmembrane domain located at the C-terminus of the protein. These observations suggest that RetSat proteins are targeted to the ER via their cleavable signal sequence and are anchored to the ER membrane via their C-terminal transmembrane domain.

Expression Specificity of zRetSat A and B. mRetSat is expressed predominantly in the liver, kidney, intestine (*7*), and adipose tissue (unpublished observations). A hybridization analysis *in situ* of zRetSat A showed that it was expressed in the liver and intestine of hatchlings 72 hpf and older (Figure 2A and B). Whole lysate derived from zebrafish at different developmental stages was analyzed by immunoblotting with an anti-mRetSat monoclonal antibody. Immunoblotting also confirmed that the zRetSat A protein was expressed mostly in hatchlings and older zebrafish (Figure 2C). Analysis of the expression of zRetSat B showed that it was expressed at earlier stages than zRetSat A and was distributed in cell layers adjacent to the anterior part of the yolk sack at 48 hpf, receding to the intestine at later stages (Figure 2D−I).

Analysis of zRetSat A Enzymatic Activity. We studied the enzymatic activity of mRetSat and zRetSat A and B in stably transfected cells. The cDNAs of zRetSat A and B were expressed in T-REx-293 cells under the control of a tetracycline inducible promoter. Assays were conducted by RetSat-expressing cells in media supplemented with all-*trans*-retinol. Cells expressing mRetSat incubated with all-*trans*-retinol gave rise to all-*trans*-13,14-dihydroretinol (Figure 3A, black, dashed line chromatogram, peak 1, and Figure 3B spectrum). The cells expressing zRetSat A also converted all-*trans*-retinol to all-*trans*-13,14-dihydroretinol (Figure 3, black, solid line chromatogram, peak 1, and Figure 3B) and gave rise to another product (Figure 3A, black, solid line chromatogram, peak 2, and Figure 3C) not seen in untransfected cells (Figure 3A, gray solid line chromatogram) or in cells expressing mRetSat. The unknown product, termed compound 2, was less polar than all-*trans*-retinol, has a λ_{max} = 280 nm, and was hypsochromic shifted compared to all-*trans*-retinol λ_{max} = 325 nm, indicating the saturation of one of the double bonds. The loss of conjugation in the polyene chain of all-*trans*-retinol by saturation of the terminal 13−14 double bond, as seen in all-*trans*-13,14-dihydroretinol, results in a tetraene with λ_{max} = 290 nm. The saturation of the 5−6 double bond would result in a tetraene, whereas the saturation of the 9−10 double bond would result in two dienes. The UV absorbance maximum of 280 nm exhibited by compound 2 indicates that it most likely is a triene resulting from the saturation of the 7−8 or 11−12 double bonds. We chemically synthesized all-*trans*-7,8-dihydroretinol and compared its chemical characteristics to those of compound 2. We also chemically synthesized all-*trans*-11,12-dihydroretinol, the other possible triene, and we found that neither its elution profile nor its λ_{max} = 264 nm matched those observed for compound 2 (results not shown). The products of the zRetSat A catalyzed reaction starting with all-*trans*-retinol were analyzed by normal phase HPLC after which the products were spiked with chemically synthesized all-*trans*-7,8-dihydroretinol and reanalyzed by normal phase HPLC. Compound 2 and chemically synthesized all-*trans*-7,8-dihydroretinol coeluted and had overlapping UV−visible absorption spectra (Figure 4, chromatograms and inset spectra). To distinguish between the different possible dihydroretinoids, we derivatized compound 2 and all-*trans*-7,8-dihydroretinol by oxidation to retinaldehyde and the formation of the corresponding oximes (structures shown in Figure 5A). The C−N double bond of the oxime is conjugated to the retinoid polyene chain, hence the absorbance of the derivatized compound should undergo a red shift. The UV−visible absorbance spectrum of the oxime and its red shift are characteristic, allowing us to distinguish the triene of all-*trans*-7,8-dihydroretinol from the shift of a diene in the case of all-*trans*-9,10-dihydroretinol or the

A



B

	mRetSat	zRetSat A	zRetSat B
hRetSat	90% (79%)	69% (48%)	60% (36%)
mRetSat		70% (49%)	59% (36%)
zRetSat A			63% (36%)

C

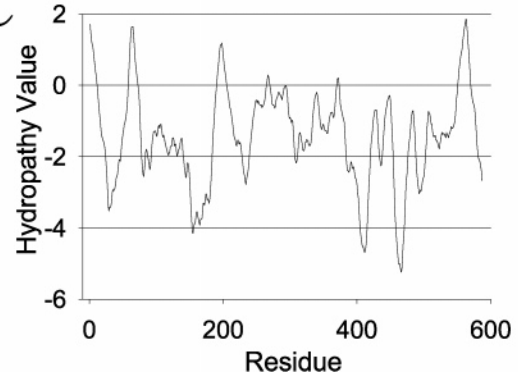


FIGURE 1: Analysis of zebrafish RetSat sequences. (A) Alignment of human RetSat (hRetSat) and mouse RetSat (mRetSat) and related hypothetical proteins from zebrafish. Protein sequences were derived from the conceptual translation of the cloned ESTs corresponding to zebrafish RetSat A (zRetSat A) and zebrafish RetSat B (zRetSat B) and compared to the sequence of mRetSat and hRetSat. White letters on a black background represent identical residues. White letters on a gray background represent conserved substitutions in all but one of the species examined, and black letters on a light gray background indicate substitutions conserved in two of the four species examined. Dashed lines represent gaps introduced to maximize the alignment built using the program T-Coffee and the matrix BLOSUM62 (*54*) with gap penalties existence-11 and extension-1. (B) Sequence homology between RetSat from different species. Percentages of residues conserved among the various RetSat proteins are shown, and the percentages of identical residues are indicated in parentheses. (C) Hydropathy plot for zRetSat A. The hydrophobic character of the sequence of zRetSat A was analyzed using the Goldman−Engelman−Steitz algorithm (*55*) processed by the TopPred II program (*56*) available from http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html.

corresponding shift of a single conjugated double bond in the case of all-*trans*-11,12-dihydroretinol. The oxime derivatives of compound 2 and chemically synthesized all-*trans*-7,8-dihydroretinol coeluted and have identical UV−visible absorbance spectra (Figure 5B, and inset spectra). Therefore, we conclude that compound 2 is all-*trans*-7,8-dihydroretinol and that zRetSat A catalyzes the saturation of all-*trans*-retinol to both all-*trans*-13,14-dihydroretinol and all-*trans*-7,8-dihydroretinol.

All-trans-3,4-didehydroretinol Is a Substrate for Both mRetSat and zRetSat A. All-*trans*-3,4-didehydroretinol, vitamin A2, is an endogenous retinoid that forms porphyropsin, a visual pigment found in freshwater fish, amphibians, and reptiles (*35*, *36*). Adult zebrafish also contain endogenous

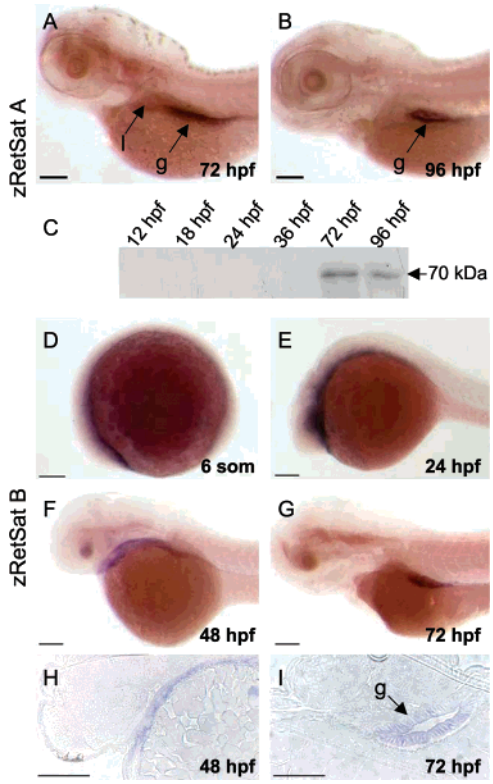


FIGURE 2: Tissue and stage-specific expression of zRetSat A and zRetSat B. (A and B) Hybridization *in situ* of zRetSat A reveals liver (l)- and gut (g)-specific expression at 72 h and gut-specific expression at 96 h. (C) Immunoblotting of protein lysates from different developmental stages with anti-RetSat monoclonal shows stage-specific staining in hatchlings and adults. (D−G) Hybridization *in situ* of zRetSat B indicates that at 48 h RetSat B is expressed in the cell layers adjacent to the anterior part of the yolk sack (heart region) and that by 72 h it recedes to the intestine. The scale bar in panels A, B, and D−H is 100 μm, and the scale bar in panel I is 50 μm.

all-*trans*-3,4-didehydroretinol (*37*), particularly in the liver (unpublished observations). The expression pattern of zRetSat A coincides with the time and location of the appearance of all-*trans*-3,4-didehydroretinol in adult fish. We incubated cells expressing zRetSat A or mRetSat with media supplemented with all-*trans*-3,4-didehydroretinol. Assays were set up in parallel with cells incubated with all-*trans*-retinol. mRetSat saturated both all-*trans*-retinol (Figure 6 B, peak 3) and all-*trans*-3,4-didehydroretinol (Figure 6E, peak 3′) to produce all-*trans*-13,14-dihydroretinol (Figure 6B, peak 1, and Figure 6G, dashed line spectrum) and all-*trans*-13,14-dihydro-3,4-didehydroretinol (Figure 6E, peak 1′, and Figure 6H, dashed line spectrum), respectively. In contrast, zRetSat A converted all-*trans*-retinol into a combination of all-*trans*-13,14-dihydroretinol (Figure 6C, peak 1) and all-*trans*-7,8-dihydroretinol (Figure 6C, peak 2). zRetSat A also converted all-*trans*-3,4-didehydroretinol into a combination of all-*trans*-13,14-dihydro-3,4-didehydroretinol (Figure 6F, peak 1′) and all-*trans*-7,8-dihydro-3,4-didehydroretinol (Figure 6F, peak 2′). We did not detect dihydroretinoid compounds in untransfected cells incubated with substrates (Figure 6A and D). The hypsochromic shift of all-*trans*-13,14-dihydro-3,4-didehydroretinol, λ_{max} = 331 nm, compared with that of all-*trans*-3,4-didehydroretinol, λ_{max} = 351 nm, is in good agreement with the saturation of one of the terminal double bonds of the six conjugated double bonds of all-*trans*-3,4-didehydroretinol resulting in the formation of a pentaene (Figure 6H, peak 1′). The loss of the 3−4 double bond would reconstitute all-*trans*-retinol, which has a different spectrum (Figure 6G, gray solid line spectrum) with a different λ_{max} = 325 nm than peak 1′. All-*trans*-7,8-dihydro-3,4-didehydroretinol is composed of a triene and an unconjugated diene (structure shown in Figure 7B), and its spectrum and λ_{max} = 282 nm are not very different from those of all-*trans*-7,8-dihydroretinol and λ_{max} = 280 nm (Figure 6I) that is composed of a triene and an unconjugated double bond (structure shown in Figure 7A). zRetSat A appeared to preferentially synthesize all-*trans*-7,8-dihydroretinol from all-*trans*-retinol, whereas all-*trans*-3,4-didehydroretinol is preferentially converted to all-*trans*-13,14-dihydro-3,4-didehydroretinol. This estimate is based on the absorbance of the corresponding peaks (Figure 6C, peak 1 vs 2; Figure 6F, peak 1′ vs 2′) at their λ_{max} (not shown). The bond preference of zRetSat A in relation to the substrate will have to be further verified by determining the molar ratio of the products of zRetSat A. We were not able to obtain synthetic all-*trans*-13,14-dihydro-3,4-didehydroretinol and all-*trans*-7,8-dihydro-3,4-didehydroretinol to determine their extinction coefficients and further substantiate this claim. In conclusion, both zRetSat A and mRetSat use both all-*trans*-retinol and all-*trans*-3,4-didehydroretinol as substrates. mRetSat saturates only the 13−14 double bond, whereas zRetSat A saturates either the 7−8 or the 13−14 double bond of all-*trans*-retinol or all-*trans*-3,4-didehydroretinol substrates (Figure 7).

Treatment with Exogenous Retinoids Results in the Induction of the Expression of Cyp26A1. Retinoic acid is oxidized by enzymes of the cytochrome P450 superfamily. The Cyp26 enzymes responsible for the oxidation of retinoic acid to more polar metabolites, such as 4-oxo-, 4-hydroxy-, and 18-hydroxyretinoic acid, were first identified in zebrafish on the basis of their induction in response to exogenous retinoic acid (*38*). All-*trans*-retinoic acid regulates its own catabolism by activating the RAR and upregulating the expression of Cyp26 enzymes. Exogenous all-*trans*-retinol can also induce the expression of Cyp26A1 (Figure 8B). This most likely occurs by oxidation of all-*trans*-retinol to all-*trans*-retinal and then to all-*trans*-retinoic acid, which induces the expression of Cyp26A1 via RAR. We show here that the products of zRetSat A, all-*trans*-13,14-dihydroretinol (Figure 8C) and all-*trans*-7,8-dihydroretinol (Figure 8D), can also upregulate the expression of zebrafish Cyp26A1. All-*trans*-13,14-dihydroretinol and all-*trans*-7,8-dihydroretinol are most likely first oxidized to the respective acids, which then can activate the RAR (*10*, *39*, *40*). Our results suggest that dihydroretinoic acid metabolites are capable of inducing the expression of Cyp26A1, thereby controlling their own oxidation.

DISCUSSION

Zebrafish Have Two Genes Related to Mammalian RetSat. The zebrafish genome, like that of other teleost fishes, has undergone large scale duplication of chromosomal segments that gave rise to several possible homologues for many genes found as single copies in mammals (*31*, *32*). The RetSat gene coded on human chromosome 2 has two related genes in zebrafish, zRetSat A coded on chromosome 3 and zRetSat B coded on chromosome 9. The zebrafish RetSat A and B

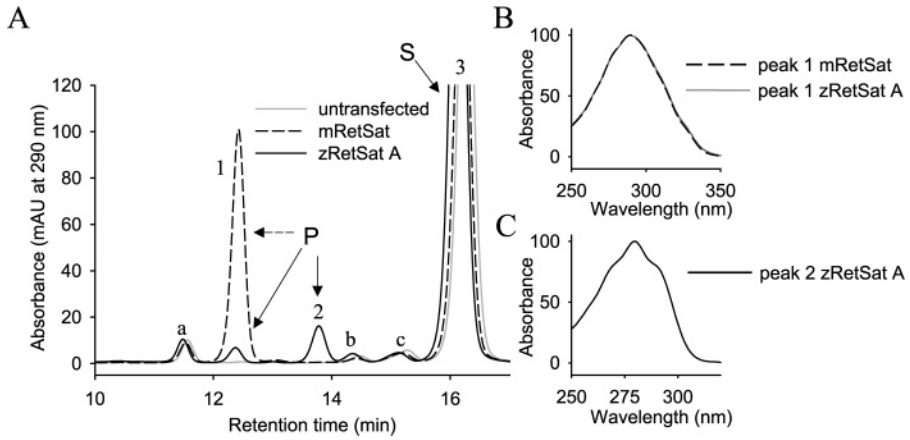


FIGURE 3: Enzymatic activity of zRetSat A compared to that of mRetSat. (A) Chromatogram of retinoids extracted from mRetSat- or zRetSat A-expressing cells treated with all-*trans*-retinol. HEKK cells stably transfected with either mRetSat or zRetSat A under the control of a Tet-inducible promoter were induced with tetracycline for 48 h and incubated with all-*trans*-retinol-containing media for 12 h. Nonpolar retinoids extracted from zRetSat A transfected cells were separated by normal phase HPLC and are represented by the black, solid line chromatogram. zRetSat A converted substrate (S) all-*trans*-retinol (peak 3) into two products (P) identified as all-*trans*-13,14-dihydroretinol (peak 1) and a novel compound (peak 2). Retinoids extracted from mRetSat transfected cells are represented by a black, dashed line chromatogram. mRetSat converted all-*trans*-retinol only to all-*trans*-13,14-dihydroretinol (peak 1). Other eluted compounds were identified as 13-*cis*-retinol (peak a) and 9-cis-retinol (peak c). The compound eluting as peak b was not identified but was also present in retinoids extracted from untransfected cells represented by the gray, solid line chromatogram. The assignment of compounds was based on their elution profiles and UV spectra as compared with chemically synthesized standards. (B) Spectra of all-*trans*-13,14-dihydroretinol products. The spectra of all-*trans*-13,14-dihydroretinol extracted from zRetSat A- or mRetSat-expressing cells were superimposable. (C) Spectrum of a novel product generated by zRetSat A. The spectrum of the novel product, peak 2, extracted from zRetSat A-expressing cells showed a $\lambda_{max} = 280$ nm.

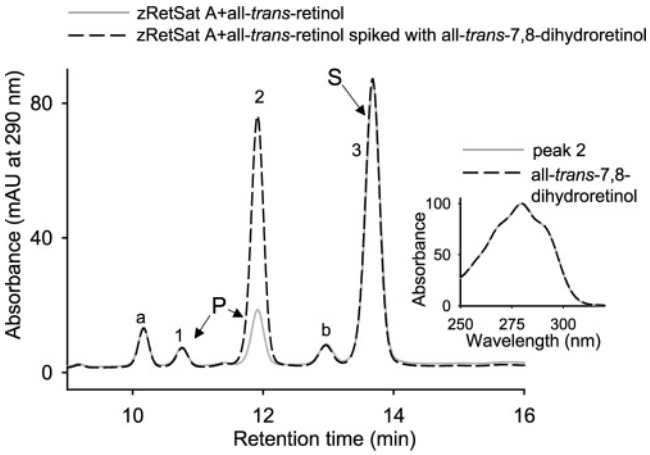


FIGURE 4: zRetSat A saturates all-*trans*-retinol to produce all-*trans*-13,14-dihydroretinol and all-*trans*-7,8-dihydroretinol. HEKK cells expressing zRetSat A were incubated with all-*trans*-retinol-containing media for 12 h. Nonpolar retinoids were extracted and separated by normal phase HPLC before (gray, solid line chromatogram) and after spiking with synthetic all-*trans*-7,8-dihydroretinol (black, dashed line chromatogram). The inset shows that the online absorbance spectra of synthetic and biologically produced all-*trans*-7,8-dihydroretinol examined separately by normal phase HPLC were superimposable. The peaks represent the reaction products: peak 1, all-*trans*-13,14-dihydroretinol; peak 2, all-*trans*-7,8-dihydroretinol and peak 3, the substrate, all-*trans*-retinol. Other compounds identified were 13-*cis*-retinol, peak a and 9-*cis*-retinol, peak b.

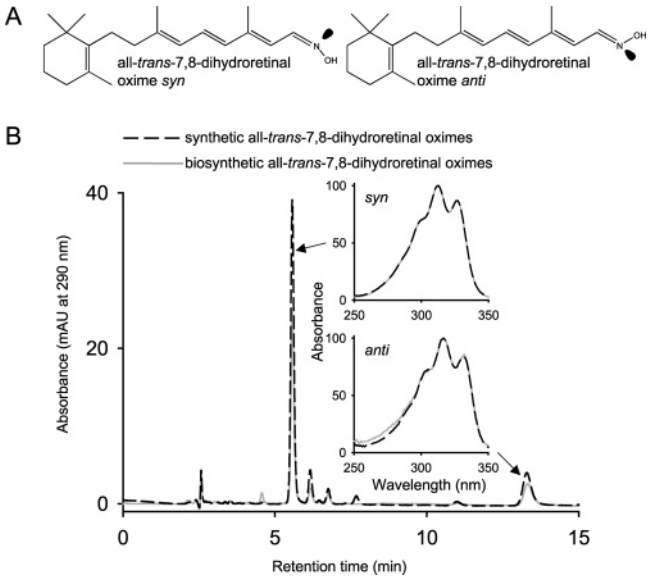


FIGURE 5: Separation of all-*trans*-7,8-dihydroretinal oximes. Derivatization of chemically or biologically synthesized all-*trans*-7,8-dihydroretinol yielded compounds with the same elution and absorbance spectra. All-*trans*-7,8-dihydroretinol purified from cells expressing zRetSat A incubated with all-*trans*-retinol or all-*trans*-7,8-dihydroretinol synthesized chemically were oxidized with manganese dioxide to produce all-*trans*-7,8-dihydroretinal and reacted with hydroxylamine to produce *syn* and *anti*-all-*trans*-7,8-dihydroretinal oximes. (A) The structures of the resulting oximes are shown. (B) The elution profiles and absorbance spectra of the derivatization products of biologically or chemically synthesized all-*trans*-7,8-dihydroretinal oximes overlap.

genes most likely evolved by gene duplication because the exon−intron junctions are retained within the sequence of the aligned RetSat and RetSat related proteins encoded by mammalian and zebrafish genomes. The zRetSat B homologue is found in linkage group 9, which is a region of conserved synteny with human chromosome 2 where human RetSat is located. We found that both zRetSat A and zRetSat B are expressed in digestive organs, such as the liver and intestine of adult animals, and that zRetSat B is expressed earlier in development than zRetSat A. This expression pattern is similar to that of mRetSat, which is expressed in the liver, kidney, and intestine. We were unable to detect RetSat activity in cells transfected with zRetSat B, suggesting that this enzyme may have either evolved a new function or become nonfunctional because of accumulated deleterious

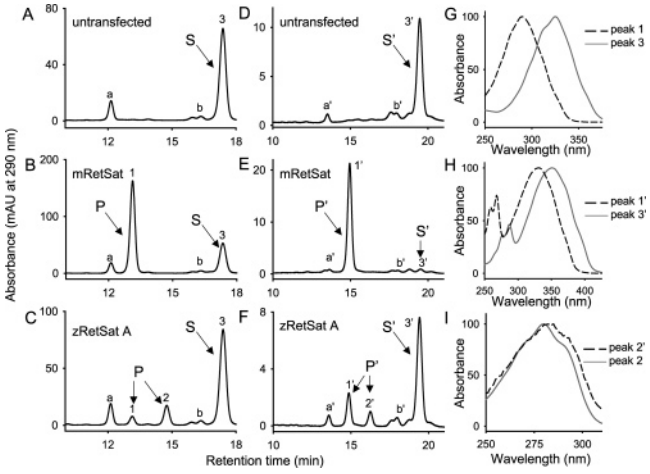


FIGURE 6: Specificity of mRetSat and zRetSat A. (A−F) Untransfected HEKK cells (A and D) or cells expressing mRetSat (B and E) or zRetSat A (C and F) were incubated with all-*trans*-retinol (A−C) or all-*trans*-3,4-didehydroretinol (D−F). Nonpolar retinoids were extracted and separated by normal phase HPLC. (B and C) The all-*trans*-retinol substrate (S) eluted as peak 3 was converted by both mRetSat and zRetSat A to a product (P), all-*trans*-13,14-dihydroretinol, that eluted as peak 1. (C) zRetSat A also converted all-*trans*-retinol to a second product, all-*trans*-7,8-dihydroretinol, that eluted as peak 2. (E and F) The all-*trans*-3,4-didehydroretinol substrate (S′), eluted as peak 3′, was converted by both mRetSat and zRetSat A to a product (P′), all-*trans*-13,14-dihydro-3,4-didehydroretinol, that eluted as peak 1′. (F) zRetSat A also converted all-*trans*-3,4-didehydroretinol to all-*trans*-7,8-dihydro-3,4-didehydroretinol, which eluted as peak 2′. (G) The spectra of peak 1 and 3 representing all-*trans*-13,14-dihydroretinol and all-*trans*-retinol, respectively, are shown. (H) The spectra of peak 1′ and 3′ representing all-*trans*-13,14-dihydro-3,4-didehydroretinol and all-*trans*-3,4-didehydroretinol, respectively, are shown. (I) The spectra of peak 2 and 2′ representing all-*trans*-7,8-dihydroretinol and all-*trans*-7,8-dihydro-3,4-didehydroretinol, respectively, are shown.

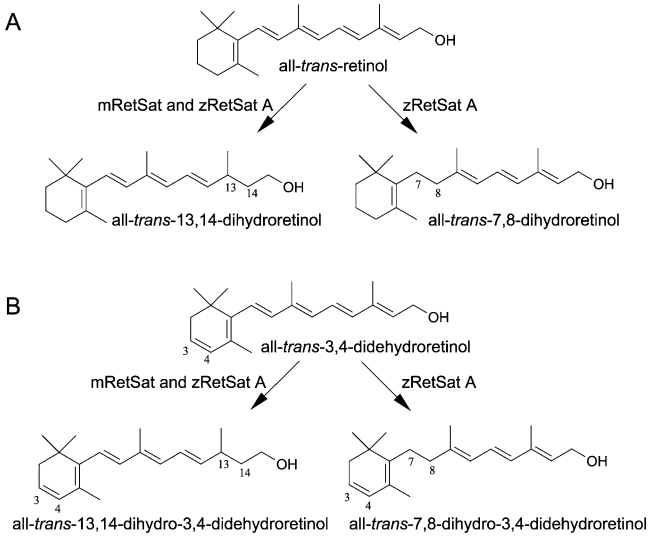


FIGURE 7: Saturation reactions catalyzed by mRetSat and zRetSat A have different bond specificities. mRetSat saturated the 13−14 double bond of all-*trans*-retinol (A) or all-*trans*-3,4-didehydroretinol (B). zRetSat A saturated either the 13−14 or the 7−8 double bonds of all-*trans*-retinol (A) or all-*trans*-3,4-didehydroretinol (B).

mutations. Possibly, zRetSat B has evolved an altered substrate specificity that does not include all-*trans*-retinol or its lack of activity was due to a cofactor or accessory protein missing from our assays.

The fate of duplicated genes is often explained by the "duplication-degeneration-complementation" model (*41*), whereby following duplication, one of the two genes becomes nonfunctional through the accumulation of deleterious mutations. Alternatively, the accumulation of gain-of-function mutations within one of the duplicated genes might lead to the acquisition of a new adaptive function. Another possibility is that both genes retain the activity of the original gene, but mutations accumulating within both of them lead to a more restricted and nonoverlapping expression pattern than that exhibited by the original gene. The function of the original gene is then shared by both members of the duplicate pair, resulting in complementation and subfunctionalization, and both duplicated genes are evolutionarily retained to replace the activity of the ancestral gene. Other genes encoding proteins involved in retinoid metabolism have been found as duplicate copies in zebrafish. In some cases, the paralogous copies of such genes are differentially expressed, as is the case for zebrafish CRBP I a and b and CRBP II a and b (*42*). This specific expression of the zebrafish CRBP I and II paralogues allow them to be retained in specific tissues and subfunctionalized. Thus, zRetSat A and zRetSat B might be similar to the zebrafish paralogues of CRBP I or II.

zRetSat A Catalyzes the Formation of Two Saturated Retinol Products. We present evidence here that zRetSat A is involved in the metabolism of all-*trans*-retinol and all-*trans*-3,4-dihydroretinol, both endogenous forms of vitamin A in zebrafish. zRetSat A can catalyze the saturation of either the 7−8 or the 13−14 double bond of its substrate in contrast to mRetSat, which saturates only the 13−14 double bond. Therefore, it appears that vertebrate RetSat enzymes saturate double bonds in the polyene chain of all-*trans*-retinol, but the particular bonds vary among species. Such altered specificity could lead to the generation of different sets of dihydroretinoid metabolites in different organisms. Our unexpected finding will have to be substantiated further by studying the specificity of RetSat enzymes from other vertebrate and nonvertebrate species. We probably failed to detect 13,14-dihydroretinoids or 7,8-dihydroretinoids in zebrafish fed a normal diet because they exist at levels undetectable by our analytical methodology. Also, knowledge of the enzymology of RetSat is just emerging, and the rate of product formation and/or expression of RetSat could be tightly regulated by levels of other retinoids or compounds. For instance, rat RetSat is upregulated by a high fat diet (*43*, *44*), and is upregulated in a rat model of gestational retinoid deficiency (*45*). These findings suggest that the formation of dihydroretinoid metabolites is affected by diet and by vitamin A status. Indeed, some dihydroretinoid metabolites can be detected both in rodents maintained on a normal diet and in patients. We have shown the existence of all-*trans*-13,14-dihydroretinol in mice maintained on a normal diet (*7*), and others have detected 9-*cis*-4-oxo-13,14-dihydroretinoic acid, a possible metabolite of all-*trans*-13,14-dihydroretinol, in the human liver (*46*) and in rodents (*47*, *48*).

The dual specificity of zRetSat A is of special interest from an enzymatic point of view. RetSat is evolutionarily related to plant CRTISO and cyanobacterial CrtH, enzymes that catalyze the isomerization of the 7−8 and 9−10 double bonds of 7,9,9′,7′-tetra-*cis*-lycopene. CRTISO catalyzes the

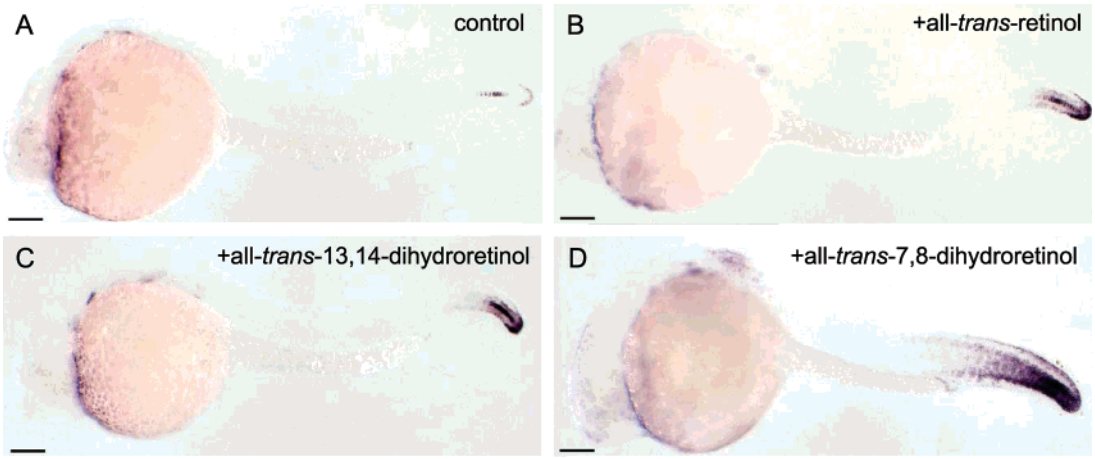


FIGURE 8: Upregulated expression of zebrafish Cyp26A1 in the caudal region of embryos treated with retinoids. Zebrafish embryos (6 hpf) were treated with the dimethylformamide control (A), 10^{-6} M all-*trans*-retinol (B), 10^{-6} M all-*trans*-13,14-dihydroretinol (C), or 10^{-6} M all-*trans*-7,8-dihydroretinol (D). The expression of zebrafish Cyp26A1 was evaluated by hybridization *in situ*. The scale bar represents 100 μm.

isomerization of prolycopene through a reversible redox reaction via saturation, rotation, and desaturation of the double bond (*49*). The 7−8 double bond of lycopene is equivalent to the 7−8 double bond in all-*trans*-retinol, which suggests that zRetSat A partially retains CRTISO saturase activity. However, zRetSat A also acquired a new activity toward the 13−14 double bond of all-*trans*-retinol, which becomes the sole activity of RetSat enzymes found in higher vertebrates, as observed earlier for the mammalian enzyme, mRetSat. A major difference between CRTISO and RetSat is that RetSat enzymes only catalyze the saturation of the double bonds. We did not detect isomerization of all-*trans*-retinol or the desaturation of all-*trans*-13,14-dihydroretinol in mRetSat-expressing cells.

The close relationship between retinoid- and carotenoid-processing enzymes can be explained by the fact that many carotenoid-processing enzymes actually recognize only half, that is, a retinoid unit, of their carotenoid substrate by catalyzing the asymmetric and sequential transformation of the substrate. CRTISO was shown first to isomerize the adjacent cis bonds of one end and then the adjacent bonds on the other end of the carotenoid (*7*, *49*). There is evidence that CrtI, the ancestral bacterial phytoene desaturase, also asymmetrically recognizes its substrate to produce 7,8,11,-12-tetrahydrolycopene (*50*). Therefore, it is easy to envision how an asymmetric-acting carotenoid enzyme could have evolved to recognize retinoid substrates.

Dihydroretinols Induce the Expression of Cyp26A1 in Vivo. We found that the treatment of embryos with all-*trans*-retinol or all-*trans*-7,8- or all-*trans*-13,14-dihydroretinols led to the induction of the oxidative enzyme Cyp26A1. The transcription of the Cyp26A1 gene is inducible by retinoic acid through the activation of RAR and the binding of response elements (RARE) within the Cyp26A1 promoter (*51*, *52*). The induction of Cyp26A1 in response to all-*trans*-13,14-dihydroretinol could be explained by the activation of RAR by all-*trans*-13,14-dihydroretinoic acid (*10*). The activity of several dihydroretinoic acids and their analogues have been studied by assays based on either the reversal of keratinized lesions in tracheal organ cultures or the reduction in size of chemically induced papillomas in mice. Although these biological assays do not directly address the activation of RAR, they do mimic the effects of all-*trans*-retinoic acid *in vivo*. Such assays showed that both all-*trans*-7,8-dihydroretinoic acid and other dihydroretinoic acids and their analogues exhibit all-*trans*-retinoic acid-like activity (*39*, *40*). Therefore, the induction of Cyp26A1 by dihydroretinols that we observed could be explained by the oxidation to their respective acids and the activation of the RAR. Alternatively, dihydroretinoid metabolites other than all-*trans*-13,14-dihydroretinoic and all-*trans*-7,8-dihydroretinoic acids might also be capable of activating RAR or RXR. Thus, the synthesis and oxidation of dihydroretinoids is tightly regulated, suggesting a signaling/hormone-like property of these retinoids.

Dihydroretinols as Potential Second Messengers. The identification of RAR, RALDH1, and Cyp26 homologues in nonchordate deuterostomes, such as echinoderms and hemichordates, suggests that the retinoic acid−RAR signaling system predates chordate evolution (*53*). On the basis of their homology to human RetSat, we identified RetSat related proteins in all vertebrate species examined thus far. Closely related homologues were also found in the echinoderm *Strongylocentrotus purpuratus* (purple sea urchin). The emergence of RetSat enzymes coincides with the acquisition of the retinoic acid receptor signaling system and the enzymes involved in the metabolism of retinoic and dihydroretinoic acids, such as RALDH and Cyp26 enzymes (*53*). Here, we provide evidence that zRetSat A can saturate retinol to produce novel dihydroretinoid metabolites and that the treatment of zebrafish embryos with dihydroretinol leads to the induction of Cyp26A1, as an autoregulatory loop. This suggests that the dihydroretinoid metabolic pathways and the regulatory mechanisms that exquisitely control the levels of dihydroretinoic acids in tissues have co-evolved.

All-*trans*-retinol is a unique chemical precursor of a potentially large number of compounds that could be used to control a set of cellular/physiological processes. The diversification of these compounds occurs not only by the oxidation of a functionalized −OH group but also by saturation of the polyene chain, as in the case of RetSat enzymes. Saturation of the 13−14 double bond introduces a bulkier structure at the end of a retinoid due to sp^3 instead of sp^2 hybridization, suggesting that dihydroretinoids acquire

a new structural feature that will not allow these compounds to bind to retinoid-binding sites. In contrast, traditional retinoids will likely bind, albeit with a lower affinity, to dihydroretinoid-binding sites. Future research should reveal the precise mechanism of action of these newly identified saturated retinols in physiological settings.

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