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Characterization and Expression of Steroidogenic Acute Regulatory Protein and MLN64 cDNAs in Trout

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Complementary DNA-encoding proteins with high homology to steroidogenic acute regulatory proteins (StAR) of mammals were cloned from rainbow trout head kidney and a mixture of several brook trout tissues. A cDNA encoding an MLN64 homolog was also cloned from brook trout. The C-terminal domains of rainbow trout StAR and brook trout StAR were very highly conserved compared with StAR of mammals. In rainbow trout, Northern and RT-PCR analyses showed abundant StAR transcripts in head kidney, ovary, and testis, and weaker signals were found in intestine, pyloric caeca, spleen, and kidney. Brief acute stress resulted in elevated plasma cortisol levels and a 2-fold increase in rainbow trout StAR transcripts in head kidneys sampled 3 h after exposure to the stressor. In brook trout, StAR transcripts were detected only in known steroidogenic tissues. Ovarian brook trout StAR mRNA was not seen until the onset of final maturation. Its abundance increased during germinal vesicle breakdown, peaked during and just following ovulation, and decreased by 2 wk post ovulation. Brook trout MLN64 transcripts were found in all tissues tested, and transcript abundance in ovarian samples did not vary during final oocyte maturation and ovulation. Both StAR structure and function appear to be highly conserved throughout the vertebrates. (*Endocrinology* 143: 2062–2070, 2002)

STEROID HORMONES ARE essential for maintaining diverse processes such as sexual differentiation, ion and carbohydrate homeostasis, immune system functioning, responsiveness to stress, and reproduction. The biosynthesis of steroids depends on the delivery of the common precursor, cholesterol, from the cytoplasm to the inner mitochondrial membrane in which it is converted to pregnenolone by the action of the cytochrome P450 side-chain cleavage enzyme (P450scc) (1). The aqueous layer between the outer and inner mitochondrial membranes represents a major barrier to diffusion of cholesterol (2). It is now known that the true rate-limiting step, and a key target for the acute regulation of steroidogenesis by tropic hormones and other mediators, is the rate of the delivery of cholesterol to P450scc by the sterol carrier protein, steroidogenic acute regulatory protein [StAR (2)]. A cDNA encoding a 30-kDa mouse StAR was first characterized by Clark *et al.* (3), who demonstrated that tropic hormones induced the rapid synthesis of this protein via cAMP/PKA. Since then, full-length cDNA-encoding StAR has been isolated from human (4), cow (5), hamster (6), pig (7), rat (8, 9), horse (10), and sheep (11). These StARs share high homology (85–88%). In mammals, studies have shown that StAR is expressed in adrenal cortex, testis, and ovary (4, 8, 12) and also in rat and marmoset brain (13, 14). StAR mRNA has also been detected in human kidney (4).

Another member of the sterol carrier protein family, MLN64, was identified from a breast cancer-derived metastatic lymph node cDNA library (15). MLN64 shares high homology with StAR at the C-terminal domain (16) and is associated with endosomes (17). Transient expression studies using COS-1 cells demonstrated that MLN64 was able to increase delivery of cholesterol to P450scc (18), although at rates lower than those seen in cells transfected with StAR cDNA. Mutant StAR (19) and MLN64 (18) from which the shared C-terminal domain was removed had no steroidogenic activity, suggesting that the C terminus of both StAR and MLN64 is the major functional region responsible for cholesterol transport. Recently the three-dimensional structure of the C-terminal domain, known as the StAR-related lipid transfer domain (START), was resolved (20), and models for StAR and MLN64 revealed that the START domain contributes to a hydrophobic tunnel structure that is considered to bind a single molecule of cholesterol.

Although the acute regulation of steroidogenesis by tropic hormones and other factors in mammals is now known to depend largely on the regulation of StAR gene expression, very little is known about the nature and regulation of homologous proteins in nonmammalian vertebrates. Recently, cDNAs for the chicken, *Xenopus,* and zebrafish StAR homologs along with a partial cDNA of the zebrafish MLN64 homolog (21) were cloned. The chicken, *Xenopus,* and zebrafish StAR homologs have reasonably high homology with the StARs from mammalian species, and preliminary analysis of StAR mRNA by Northern blotting indicated that StAR expression in nonmammalian vertebrates occurred in steroidogenic tissues. Recently regulation of chicken StAR expression by gonadotropins has been demonstrated (22). However, detailed analysis of the sites of expression of the StAR gene in lower vertebrates, particularly

Abbreviations: btMLN64, Brook trout MLN64; btStAR, brook trout StAR; DIG, digoxigenin; GVBD, germinal vesicle breakdown; P450scc, P450 side-chain cleavage enzyme; RACE, rapid amplification of cDNA ends; rtβ-actin, rainbow trout β-actin; rtStAR, rainbow trout StAR; StAR, steroidogenic acute regulatory protein; START, StAR-related lipid transfer domain.

the identification of the cells expressing this gene, and evidence for an association of StAR with acute changes in steroid hormone biosynthesis are currently lacking. In addition, expression of the mRNA for MLN64 in nonmammalian vertebrates has not been reported.

In this study, we report the cloning of cDNA-encoding StAR protein from rainbow trout head kidney (containing cortisol-producing interrenal cells; homologous to the adrenal cortex) and a mixed-tissue brook trout cDNA library and also the cloning of a brook trout MLN64 (btMLN64) cDNA. We demonstrate the sites of expression of StAR and MLN64 genes in trout, and we show that changes in steroid hormone production by interrenal cells and by ovarian follicles are associated with substantial changes in StAR mRNA.

Materials and Methods

Study animals

Investigations and animal care procedures for rainbow trout were approved by the University of Otago's (Dunedin, New Zealand) Animal Ethics Committee. Investigations and animal care procedures for brook trout were conducted according to the guidelines specified by the University of Notre Dame (Notre Dame, IN) Institutional Animal Care and Use Committee.

For rainbow trout StAR (rtStAR) cDNA isolation and characterization, 3-yr-old rainbow trout (steelhead strain, obtained from Inland Station, National Research Institute of Aquaculture, Tamaki, Mie, Japan) and 1-yr-old rainbow trout (Wanaka Trout Hatchery, Wanaka, New Zealand) were used. One- and 2.5-yr-old rainbow trout (Wanaka Trout Hatchery) were used for analysis of sites of and changes in StAR gene expression. Fish were killed with an overdose of anesthetic, 300 mg/liter MS222 (3-aminobenzoic acid ethyl ester, obtained from Sigma, St. Louis, MO) buffered in sodium bicarbonate. Tissues were frozen using liquid nitrogen and stored at −70 C until RNA extraction. For histological analysis, tissue fragments were fixed with Bouin's fixative to determine the developmental stage and with 4% paraformaldehyde in 0.1 M PBS for 24 h at room temperature for *in situ* hybridization analysis.

Mature brook trout (300–400 g) were purchased during the reproductive season from a commercial hatchery (Homestead Trout Farm and Hatchery, Grand Haven, MI) and held under natural photoperiod in 1100-liter tanks supplied with flow-through well water at 12 C. Before collecting tissue, the reproductive stage of individual trout was determined by sampling follicles *in vivo* as previously described (23). To collect tissues, trout were overanesthetized in 2-phenoxyethanol and decapitated. After removal, all tissues were frozen and stored in liquid nitrogen before RNA extraction.

Isolation of rtStAR cDNA

Total RNA was extracted from 1-yr-old rainbow trout head kidney using TRIzol (Life Technologies, Inc., Grand Island, NY) and was reverse-transcribed to first-strand cDNA using an oligo(dT) primer (Life Technologies, Inc.) and SuperScript II RNase H^- reverse transcriptase (Life Technologies, Inc.). Degenerate oligonucleotide primers [forward primer, ATG GAR GCN ATG GGN GAR TGG AAY CCN AA; reverse primer, ATN GTY TTN GGN ARC CAN CCY TTN ARR TC (R = A, G; N = A, T, G, C; Y = C, T)] were designed based on the alignment of human StAR cDNA (Ref. 4; GenBank accession no. U17280) and *Caenorhabditis elegans* cosmid F52F12.7 (GenBank accession no. Z83228). Thirty-five cycles of PCR amplification were performed under the following conditions: 30 sec at 94 C (denaturation), 30 sec at 60 C (annealing), and 1 min at 72 C (extension). The final extension was at 72 C for 5 min. A 350-bp product was isolated from the gel using QIAEX II (QIAGEN, Hilden, Germany) and ligated into the pGEM-T Easy vector (Promega Corp., Madison, WI). The cDNA was sequenced using an ABI PRISM 377 DNA Sequencer (PerkinElmer Applied Biosystems, Foster City, CA).

Subsequently, rapid amplification of cDNA ends (RACE) was performed to obtain a full-length cDNA-encoding StAR. Total RNA was extracted from 3-yr-old rainbow trout head kidney using TRIzol (Life Technologies, Inc.), and poly(A)$^+$ RNA was isolated from the total RNA using Oligotex-dT30 (Takara Shuzo, Shiga, Japan). This mRNA was used to synthesize rainbow trout head kidney cDNA for RACE using a Marathon cDNA amplification kit (CLONTECH Laboratories, Inc., Palo Alto, CA) as described by the manufacturer. Separate 5′ and 3′ RACE PCRs were performed using two gene-specific primers (5′ RACE primer, TGG TCC GTT CTC TGC CCT AAC AAC TC; 3′ RACE primer, ATC CTC CAG AAG ATA GGT CAG GAG AC) in conjunction with appropriate adaptor primers incorporated into the cDNAs. Takara LA *Taq* (Takara Shuzo) was used for all the RACE PCRs. The 5′ RACE and 3′ RACE PCR products were sequenced using an ABI PRISM 377 DNA sequencer (PerkinElmer Applied Biosystems). Nucleotide and amino acid analysis was performed using SeqEd v1.0.3 software (PerkinElmer Applied Biosystems).

Isolation of brook trout StAR (btStAR) cDNA

A 340-bp btStAR cDNA fragment was initially obtained by RT-PCR of a mass-excised, mixed-tissue (liver, brain, testes, skin, head kidney) brook trout cDNA library constructed in Zap Express (Stratagene, La Jolla, CA) using primers and conditions previously described (21). This fragment was used to screen the same library under high stringency, and positive plaques were rescreened once to homogeneity and *in vivo* excised. Plasmid preparations were made from clones containing the largest inserts, and two plasmids were sequenced on both strands as previously described (21).

Isolation of btMLN64 cDNA

A pair of degenerate primers (forward, GAR AAY TGG AAR TTY GAR AAR; reverse, RAA YTC RAA CAT IGT IGC IGC; I = inosine) were designed from an alignment of the human MLN64 (18) and a partial cDNA of zebrafish MLN64 (21). The primers were used to PCR-amplify a 500-bp btMLN64 cDNA fragment from a mass-excised, mixed-issue brook trout cDNA library (as described above). This fragment was used to screen the same library under high stringency, and positive plaques were rescreened once to homogeneity and *in vivo* excised. Plasmid preparations were made from clones containing the largest inserts and several plasmids were sequenced on both strands as previously described (21).

Transcript size and sites of expression of rtStAR

Total RNAs were extracted from 2.5-yr-old rainbow trout tissues (brain, pituitary, gill, heart, head kidney, posterior kidney, liver, spleen, pyloric caeca, intestine, testis, ovary, muscle, skin, and blood) and used for poly(A)$^+$ RNA isolation. Two separate fish were analyzed by either Northern blot or RT-PCR as follows.

Northern blotting. Poly(A)$^+$ RNA was electrophoresed on a 1% agarose gel containing formaldehyde and transferred to a BrightStar-Plus (Ambion, Inc., Austin, TX) nylon membrane. Probes for rtStAR mRNA and for rainbow trout β-actin (rtβ-actin; Kusakabe, M., and G. Young, unpublished data) mRNA were labeled with [α^{32}P]-dCTP (Amersham International, Little Chalfont, UK) by PCR amplification using specific primers. For rtStAR, the sense primer was ATG TTG CCT GCA ACT TTC AA and the antisense primer was TCA GCA TGC TGC TGC CAT CT. Hybridization was carried out using ULTRAhyb hybridization buffer (Ambion, Inc.) as described by the manufacturer. The membrane was exposed to BioMax autoradiographic film (Kodak, Rochester, NY) at −70 C.

RT-PCR. Tissue distribution of rtStAR mRNA was examined by RT-PCR. Twenty-five nanograms poly(A)$^+$ RNA from each tissue were reverse-transcribed to first-strand cDNA using oligo(dT) primer (Life Technologies, Inc.) and SuperScript II RNase H^- reverse transcriptase (Life Technologies, Inc.). Specific primers for rtStAR designed for the RACE PCR, and specific primers for rtP40scc (Ref. 24; GenBank accession no. S57305) and rtβ-actin were used for the RT-PCR amplification under the following conditions: denatured at 94 C for 10 sec, annealed at 60 C for 20 sec, and extended at 72 C for 30 sec. The final extension was at 72 C for 5 min. Thirty cycles were performed for rtStAR and rtP450scc and 20 cycles were used for the rtβ-actin PCR amplification.

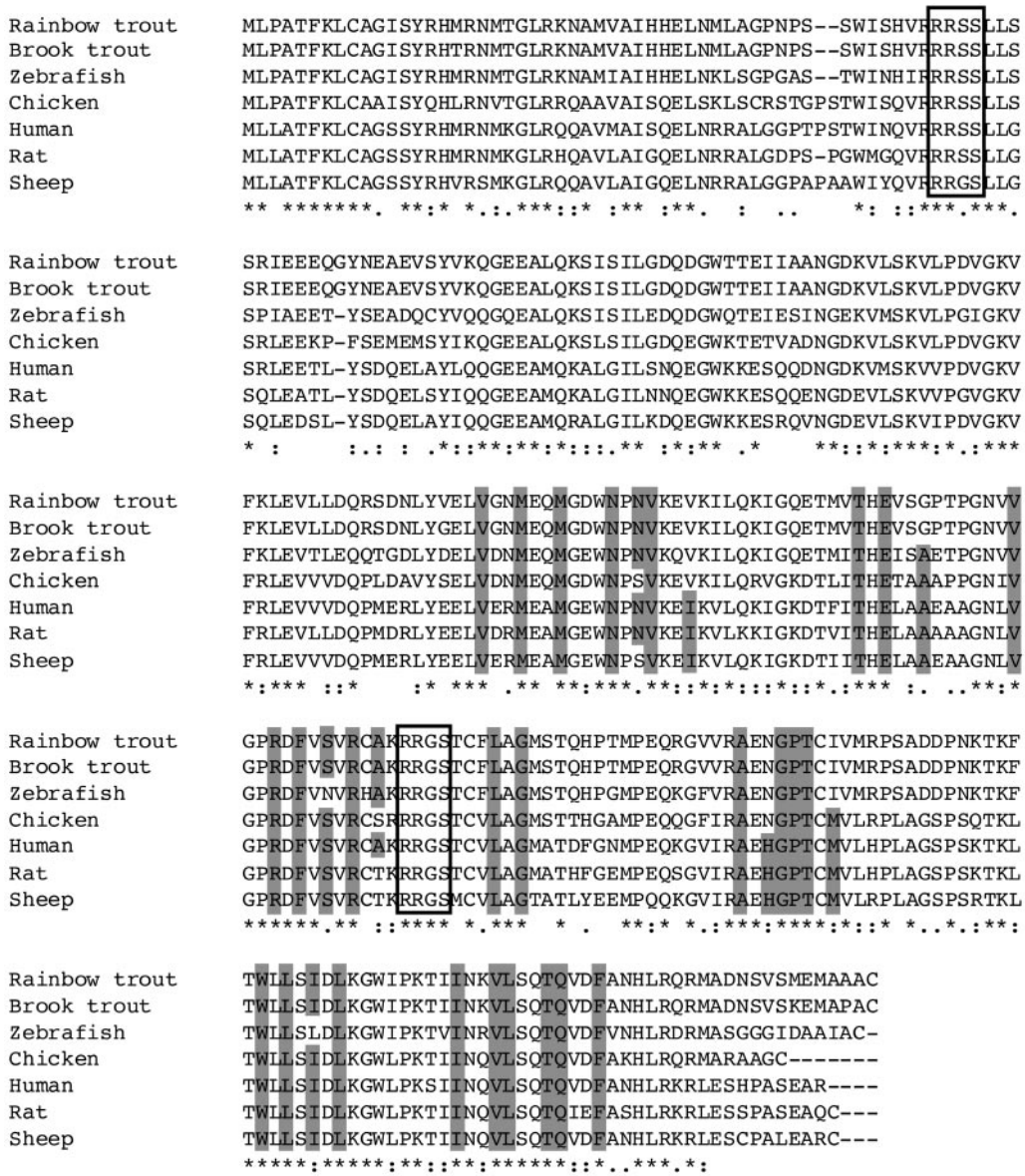

FIG. 1. Alignment of the predicted amino acid sequences of rtStAR and btStAR and those of other species analyzed by ClustalW multiple alignments. An *asterisk* (*) indicates identical residues in all sequences of the alignment; a *colon* (:) indicates conserved substitutions; and a *period* (.) indicates semiconserved substitutions. Putative amino acid residues directly contributing to the hydrophobic tunnel structure are highlighted by *gray boxes* (20). *Rectangles* indicate conserved PKA phosphorylation motifs (33).

In situ hybridization. The 350-bp rtStAR cDNA in pGEM-T Easy plasmids (Promega Corp.) was linearized using *Spe*I (Roche Molecular Biochemicals, Mannheim, Germany). Digoxigenin (DIG)-labeled sense and antisense cRNA probes were synthesized using a DIG RNA labeling kit (Roche Molecular Biochemicals). Paraformaldehyde-fixed head kidney, testis, and ovary fragments taken from 2.5-yr-old rainbow trout were embedded in paraffin, sectioned (8 μm), and mounted onto poly-*L*-lysine coated slides (BDH Laboratory Supplies, Poole, UK). *In situ* hybridization analysis was carried out with minor changes to the protocol of Braissant and Wahli (25). Hybridized probes were immunologically detected with anti-DIG antibody diluted 1:5000 for 2 h. Slides were stained with 4-nitro blue tetrazolium chloride (450 μg/ml) and X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate (175 μg/ml; Roche Molecular Biochemicals) for 16–18 h at room temperature. Staining was stopped with distilled water (2 $\times$ 10 min), and nonspecific staining was removed by washing in 95% ethanol for 1 h. Sections were mounted using Aquamount (BDH Laboratory Supplies).

Effect of acute stress on plasma cortisol and StAR mRNA in head kidney of rainbow trout

To investigate changes in StAR gene expression during acute stress, five juvenile rainbow trout were exposed to an acute stressor consisting of brief confinement in a bucket and light anesthesia in 0.5 ml/liter 2-phenoxyethanol for a total of 5 min, after which they were transferred to an experimental tank for recovery. Three hours after treatment, these fish were rapidly killed using an overdose of buffered MS222 (300 mg/liter). This treatment has been shown to prevent a handling-induced increase in circulating cortisol levels (26, 27). Blood samples from the caudal vasculature were taken with heparinized capillary tubes (Chase Scientific Glass, Inc., Rockwood, TN) and plasma obtained by centrifugation. Plasma samples were stored at −20 C until RIA. Whole head kidneys were removed and frozen in liquid nitrogen and then stored at −70 C until RNA extraction. Five undisturbed fish were also anesthetized with MS222, and plasma and head kidneys were sampled as

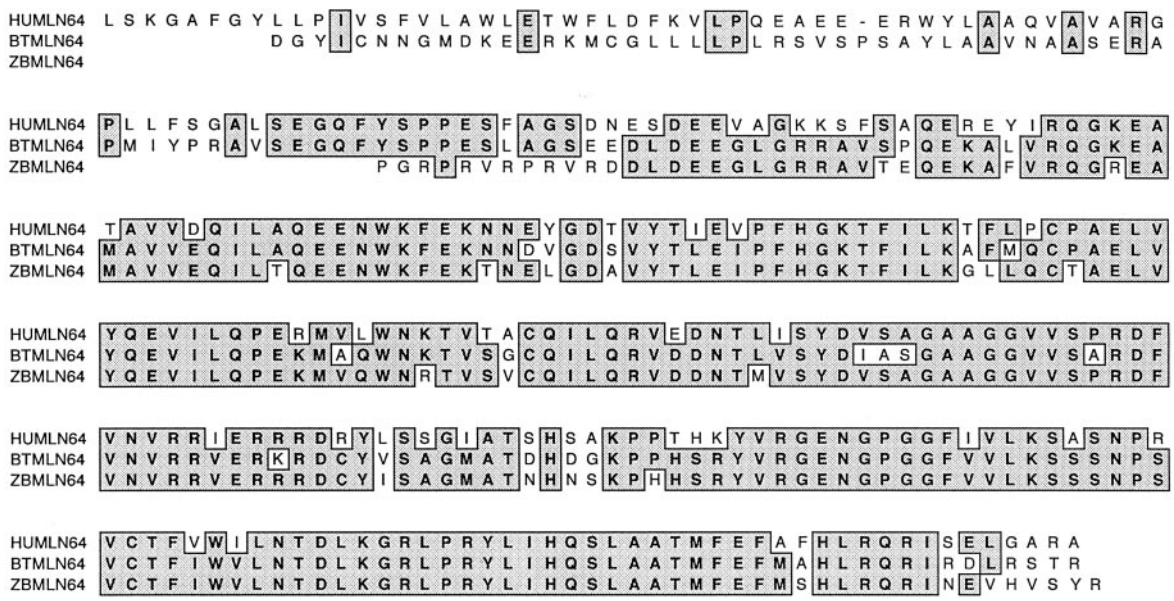

FIG. 2. Amino acid alignment of human HUMLN64 (amino acids 146–446), brook trout (BTMLN64), and zebrafish (ZBMLN64) MLN64 proteins. *Shading* indicates amino acid identity of at least two of three sequences.

described above to determine the basal levels of cortisol in plasma and rtStAR mRNA in head kidney.

Plasma cortisol levels were measured by RIA as described previously (27, 28). The antiserum used for the assay was purchased from Endocrine Sciences, Inc. (Calabasas, CA).

Head kidney rtStAR mRNA levels were assessed by Northern blot using 15 μg total RNA. After stripping hybridized rtStAR probe, the membrane was rehybridized with rtβ-actin probe to assess loading variation using the same procedures. The band intensities were measured using laser densitometry, and rtStAR mRNA levels were normalized to those of rtβ-actin.

Differences in plasma cortisol and rtStAR levels between control and 3-h post-acute-stress fish were analyzed by unpaired *t* test. Cortisol data were log transformed.

Tissue distribution and changes in btStAR and btMLN64 mRNAs during final oocyte maturation and ovulation

Ovarian tissues were collected from females before germinal vesicle breakdown (GVBD), within 24 h of GVBD, 24 h post GVBD, during ovulation (20–100% of the ovary ovulated at the time of sampling), at the completion of ovulation, and 48 h, 6–8 d, and 2 and 4–5 wk post ovulation. In addition, other tissues were removed from a female assayed at 48 h post ovulation, and testes were also obtained from spermiating males.

Northern blotting

Total RNA from tissues was extracted in Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) at a ratio of 50 mg tissue/1.0 ml reagent as previously described (29, 30). Poly(A)$^+$ RNA was isolated using the PolyAtract mRNA isolation system (Promega Corp.). Northern analysis of mRNA was performed as previously described (31, 32). In the case of ovarian RNA samples obtained at different reproductive stages, normalization of Northern blots was accomplished by loading equal amounts of mRNA/lane because expression of genes such as actin also change according to reproductive stage and cannot be used for normalization.

Results

Cloning and sequencing of rtStAR and btStAR cDNAs

A 1770-bp (excluding poly(A) tail) rtStAR cDNA (accession no. AB047032) was constructed from the products of 5′ and 3′ RACE. The open reading frame starting from the first ATG is 864 nucleotides, encoding a predicted protein composed of 287 amino acids. The cDNA contains a 5′ untranslated region of 158 bp and a 3′ untranslated region of 748 bp. A polyadenylation signal, AATAAA, is located 15 bp upstream from the poly(A) tail.

An 1802-bp btStAR cDNA (accession no. AF232215) was obtained from library screening that contained an 864-bp open reading frame encoding a protein of 287 amino acids. The two trout StARs were 97.7% identical at the amino acid level.

Alignment of the deduced amino acid sequence of rtStAR to those of other species is shown in Fig. 1. The rtStAR shares high homology with other species: 78.5% with zebrafish (21), 79.5% with chicken (21), 64.4% with human (4), 62.6% with rat (9), and 61.2% with sheep (11). However the C-terminal portion of trout StARs are more highly conserved. For example, 87.5% homology exists between rtStAR and human StAR in the hydrophobic residues identified as important in forming a hydrophobic tunnel structure capable of binding one molecule of cholesterol (20). Furthermore, predicted motifs for PKA phosphorylation sites (33) were 100% identical among all species except sheep (11).

Cloning and sequencing of btMLN64 cDNA

The largest btMLN64 cDNA (accession no. AF284379) obtained from library screening was 2707 bp and contained an open reading frame that was 885 bp. An inframe ATG site was present at nucleotides 25–27, but the reading frame was open on the 5′ end, so it is unclear whether this is an actual start site. The open reading frame is predicted to encode a protein of at least 294 amino acids, 151 amino acids less than the human MLN64. Over comparable regions, the btMLN64 protein is 65% and 81% identical with human and zebrafish MLN64s, respectively (Fig. 2).

Transcript size and sites of expression of rtStAR

The rtStAR transcript size was analyzed by Northern blot using either 2 μg or 10 μg poly(A)$^+$ RNA and exposing membranes for 1–5 d. The most abundant transcript was 2.3 kb in size, and less abundant transcripts were 4.4 kb and 9.9 kb in size (Fig. 3).

Northern blot analysis using 3 μg poly(A)$^+$ RNAs prepared from the rainbow trout tissues revealed, after a 1-wk exposure, abundant 2.3-kb rtStAR transcripts in head kidney, testis, and ovary samples and weaker signals in intestine, pyloric caeca, and spleen samples (Fig. 4A).

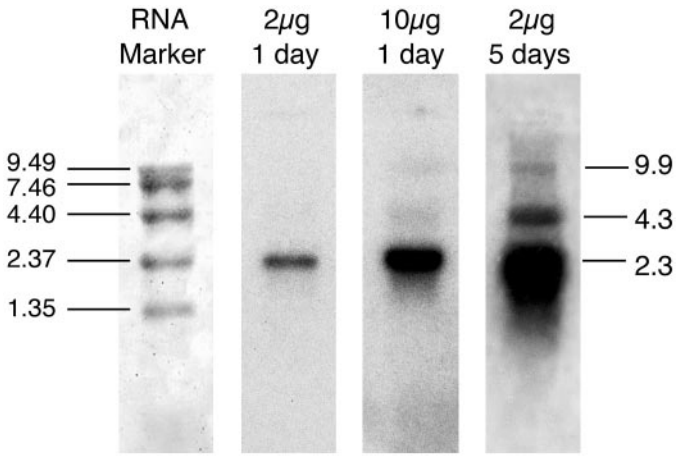

FIG. 3. Size of rtStAR transcripts. Poly(A)$^+$ RNA was prepared from head kidney, and either 2 μg or 10 μg poly(A)$^+$ RNA were subjected to Northern blotting. The membrane was exposed to autoradiographic film for 1–5 d.

Further analysis of rtStAR gene expression by RT-PCR showed the presence of rtStAR transcripts in head kidney, testis, ovary, intestine, pyloric caeca, spleen, and posterior kidney RNA (Fig. 4B). No signal was obtained from brain, pituitary, gill, heart, liver, muscle, skin, and blood RNA. Tissues were also examined by RT-PCR for P450scc: Intense 500-bp bands were obtained in head kidney, testis, and ovary, with a fainter band found in brain. Signals were not obtained in other tissues.

cRNA *in situ* hybridization (Fig. 5) revealed strong signals in interrenal cells of the head kidney of adult rainbow trout. The rtStAR mRNA signals were also found in Leydig cells of the spermiating testis and theca cells of the postovulatory ovary.

Effect of acute stress on plasma cortisol and StAR mRNA in head kidney of rainbow trout

Plasma cortisol (Fig. 6A) was significantly elevated in fish 3 h after exposure to an acute stressor, in comparison with levels in nonstressed controls ($P = 0.0048$). Northern blot analysis (Fig. 6B) showed that levels of rtStAR mRNA in head kidneys of acutely stressed fish were approxi-

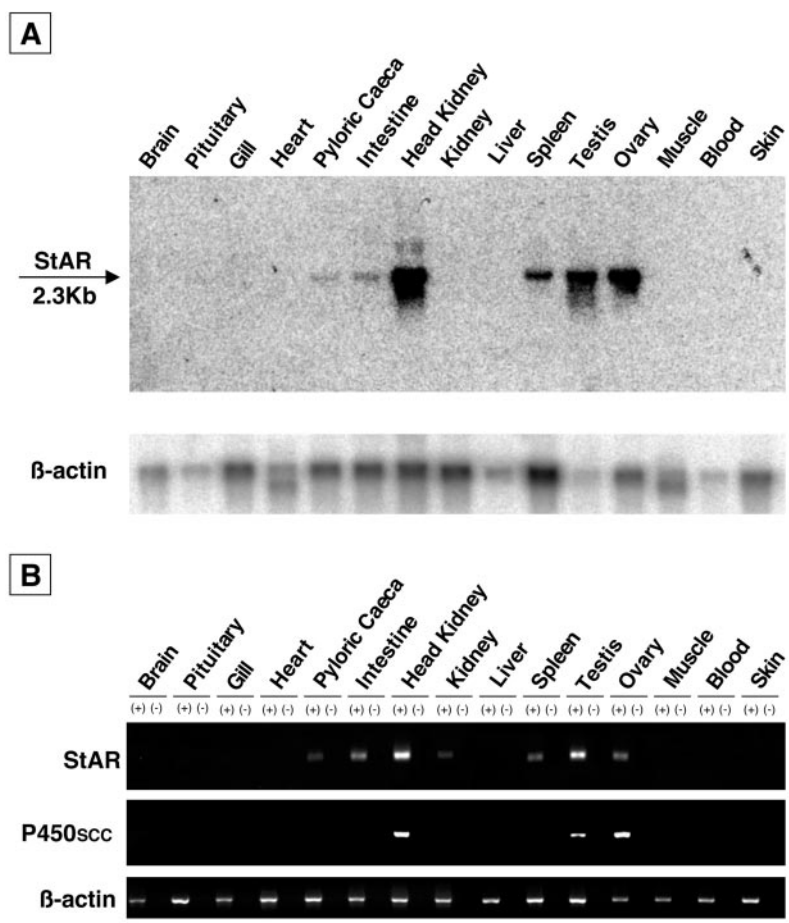

FIG. 4. Sites of expression of rtStAR gene. A, Northern blot analysis. Poly(A)$^+$ RNA was prepared from various tissues, and 3 μg was subject to Northern blotting. The membrane was exposed to autoradiographic film for 1 wk. B, RT-PCR analysis of tissue distribution of rtStAR and P450scc mRNAs. First-strand cDNAs were synthesized from 25 ng poly(A)$^+$ RNAs. Specific primers for rtStAR, P450scc, and β-actin were used for PCR amplification under the following conditions: denatured at 94 C for 20 sec, annealed at 60 C for 20 sec, and extended at 72 C for 30 sec. Thirty cycles were performed. +, Reverse transcriptase added; -, no reverse transcriptase added.

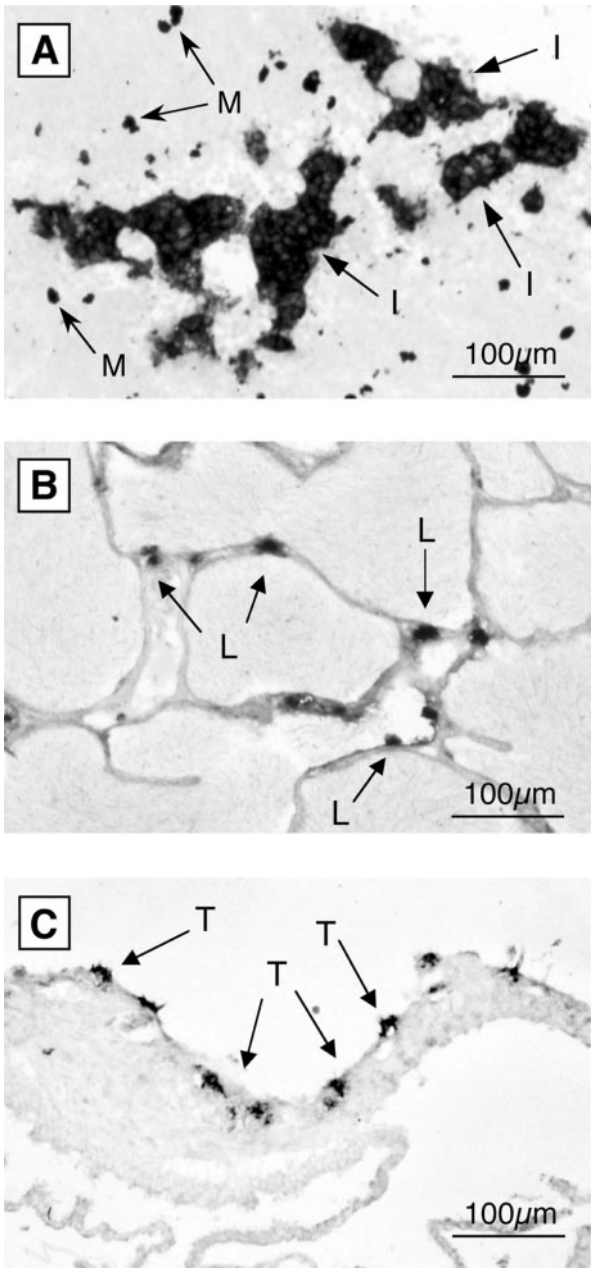

FIG. 5. *In situ* hybridization for rtStAR mRNA with DIG-labeled StAR cRNA probe. A, Head kidney. Signals were found in interrenal cells (I). M, Melanin granules (not StAR-positive cells). B, Testis-spermiating. Signals were found in Leydig cells (L). C, Ovary-postovulation. Signals were found in theca cells (T).

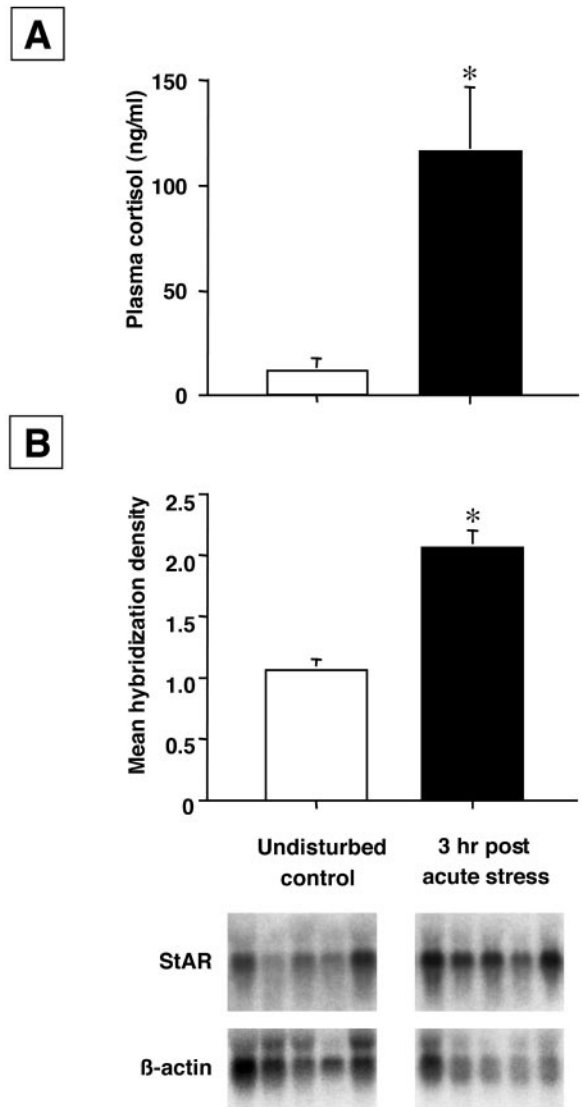

FIG. 6. Effect of acute stress on plasma cortisol and StAR mRNA levels in head kidney of rainbow trout. Fish were subjected to a capture and anesthesia stress and then allowed to recover for 3 h before sampling. Controls consisted of samples from undisturbed fish. A, Plasma cortisol levels 3 h after acute stress. B, Northern blot analysis for rtStAR mRNA levels in head kidney. rtStAR mRNA levels are presented as a ratio of the levels in control. Fifteen micrograms total RNA was hybridized with StAR and β-actin cDNA probes. StAR hybridization signals were normalized to those of β-actin. *, Values significantly different from undisturbed controls (cortisol, $P = 0.0048$; rtStAR mRNA, $P = 0.0003$).

mately 2-fold higher than those from nonstressed control fish ($P = 0.0003$).

Transcript size and sites of expression of btStAR

StAR transcripts were detected in head kidneys, testes, and ovaries by Northern blots of mRNA from brook trout but with a transcript size of 1.8 kb (Fig. 7A).

Transcript size and sites of expression of btMLN64

The btMLN64 cDNA hybridized with mRNA from all tissues sampled. However, two transcripts of 2.7 and 3.7 kb were observed in the testes and ovaries (Fig. 7B), but only the 3.7-kb transcript was observed in nongonadal tissues.

Changes in btStAR and btMLN64 mRNA levels in ovarian follicles of brook trout during final oocyte maturation and ovulation

The btStAR mRNA was barely detectable in fully grown ovarian follicles before the resumption of meiosis, and although there was some variation among individuals, transcript abundance increased following GVBD, peaked at ovulation, and was maintained at high levels for up to 6 d post

ovulation in some individuals (Fig. 8A). Transcript abundance declined by 2 wk post ovulation. In contrast, btMLN64 was expressed at low levels in all ovaries taken from females before the resumption of meiosis through 48 h post ovulation (Fig. 8B). btMLN64 expression appeared to be unrelated to reproductive stage.

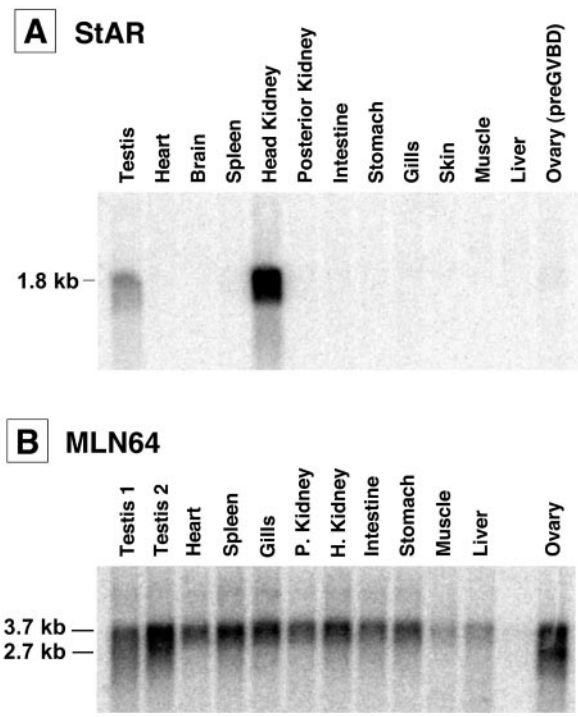

FIG. 7. Northern blot of brook trout mRNA (0.5 μg/lane) taken from various tissues and probed with (A) the full-length btStAR cDNA [ovary (preGVBD), ovarian follicles sampled before germinal vesicle breakdown] and (B) btMLN64 cDNA (samples taken from a female brook trout 48 h post ovulation and testes from two spermiating males). P. kidney, Posterior kidney; H. kidney, head kidney.

Discussion

In this study, we have cloned cDNAs from rainbow trout and brook trout that encode proteins with high homology to StARs of mammals. Notably, several regions were found to be very highly conserved, compared with other vertebrate StARs. These regions include the C-terminal START domain, which contains hydrophobic residues that contribute to forming a hydrophobic tunnel structure (20) and phosphorylation sites (33). C-terminal-domain amino acids are known to be critical to the function of StAR in cholesterol transfer: Truncation leads to loss of steroid production (19, 34). On the other hand, truncation of the N terminus resulted in a molecule capable of supporting steroidogenesis in mitochondria without being imported into mitochondria (19). These results indicate strong conservation of functional sites in StAR throughout the vertebrates.

Using Northern blot analysis, rtStAR and btStAR mRNAs were found in known steroidogenic tissues, including head kidney, testis, and ovary. In rainbow trout, one major transcript (2.3 kb) and two weak transcripts (4.4 kb and 9.9 kb) were detected. Similarly, three StAR transcripts (1.6 kb, 4.4 kb, and 7.5 kb) were found in the human ovary and testes (4). It appears that the size of the primary transcript may be different between rainbow trout (2.3 kb) and brook trout (1.8 kb), although it is possible that some of the variation seen between species may be because of the use of different molecular weight markers. Based on the sequences already obtained for these two species, a difference in transcript size would presumably reflect a difference in the size of the 5′ untranslated region. Cellular sites of expression of the rtStAR gene were identified as head kidney interrenal cells, Leydig cells of testis, and relatively scarce cells in the thecal layer of

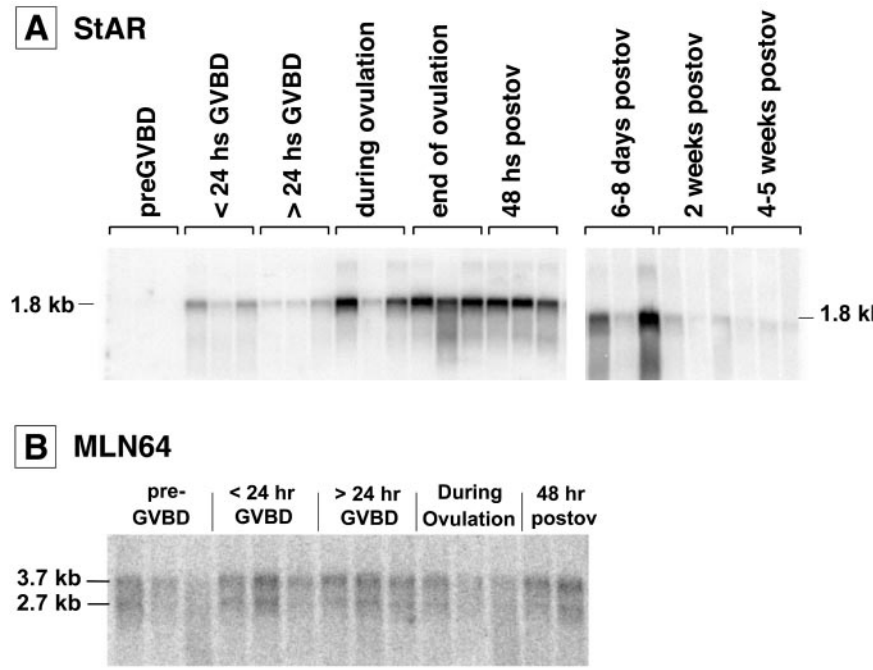

FIG. 8. Northern blots of brook trout ovarian follicle mRNA (0.5 μg/lane). A, Ovaries were sampled before germinal vesicle breakdown (pre-GVBD), within 24 h of GVBD (<24 h GVBD), greater than 24 h following GVBD but before ovulation (>24 h GVBD), during ovulation (20–80% ovulated), at the end of ovulation, and at various times following ovulation (48 h, 6–8 d, and 2 and 4–5 wk post ovulation). The blot was probed with the full-length btStAR cDNA. Samples from 6–8 d and 2 and 4–5 wk post ovulation were run on a separate Northern blot. B, MLN64 mRNA in ovarian follicles of brook trout. Follicles were obtained from ovaries sampled before germinal vesicle breakdown (pre-GVBD), within 24 h of GVBD (<24 h GVBD), greater than 24 h following GVBD but before ovulation (>24 h GVBD), at the end of ovulation, and at 48 h following ovulation.

the ovarian follicle. This latter observation further confirms results of studies showing that the thecal layer of salmonids functions to provide precursor steroids to the granulosa layer, the site of synthesis of 17β-estradiol and maturation-inducing steroid, 17α,20β-dihdroxy-4-pregnen-3-one (35).

StAR transcripts were undetectable in both rainbow and brook trout brains using Northern blots. We were also unable to detect StAR transcripts in rainbow trout brain using RT-PCR. However, low levels of P450scc transcripts were detected in rainbow trout brain using RT-PCR. *In situ* hybridization analysis and RNase protection assay of rat brain revealed the presence of StAR mRNA (13): Localization was restricted to the cerebral cortex, hippocampus, dentate gyrus, olfactory bulb, cerebellar granular layer, and Purkinje cells. A similar pattern of expression occurs in marmoset brain (14), and Northern hybridization with mRNA obtained from the head of zebrafish was recently reported (21). Therefore, it may be necessary to isolate RNA from specific brain regions to detect StAR transcripts in the trout brain.

Considering that expression of mammalian StAR genes is, with the exception of human kidney (4), restricted to steroidogenic tissue and brain, it is notable that we have found rtStAR transcripts in intestine, pyloric caeca, posterior kidney and spleen by both Northern blot and RT-PCR analysis, although P450scc transcripts were not detected in these tissues. Expression levels must be relatively low because detection of rtStAR transcripts in these tissues by Northern blot analysis required either prolonged exposure times or overloading gels. To our knowledge, the presence of StAR transcripts in nonsteroidogenic mammalian tissues has not been reported aside from human kidney (4). These findings are not restricted to trout. Similar evidence for StAR transcripts in nonsteroidogenic tissues has been found in zebrafish using RT-PCR (Bauer, M., and F. W. Goetz, unpublished data). Whether rtStAR may have a nonsteroidogenic role in these tissues is an intriguing question for future studies. In particular, it would be interesting to examine whether StAR may have a role as a sterol carrier in lipid-transporting tissues such as the intestine and pyloric caeca.

Although we have not yet completed transient expression studies to directly demonstrate the involvement of trout StAR in steroidogenesis, in this study, we showed that acute changes in steroid production are associated with substantial changes in StAR mRNA levels. The first example used the classical cortisol stress response to demonstrate that the increase in plasma cortisol levels resulting from exposure of trout to acute stress is accompanied by increased rtStAR transcripts in the interrenal cell-containing head kidney. Like other vertebrates, acute stress evokes a rapid increase in circulating ACTH levels followed by increased levels of glucocorticoids (36, 37). Previous studies on mammals have shown that ACTH stimulates *de novo* StAR synthesis via cAMP/PKA (4, 38). Results of this study are consistent with reports that bovine or rat adrenal cortex StAR mRNA levels increased within 0.5 h of ACTH treatment and peaked after 3–6 h after treatment *in vivo* or *in vitro* (9, 12, 38–40).

A second example of increased StAR transcripts in tissues exhibiting enhanced steroid production was based on previous reports of increased circulating levels of maturation-inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one, and increased potential for production of this steroid by brook trout ovarian follicles during final maturation and ovulation, presumably as a consequence of a preovulatory increase in LH (41, 42). We showed that btStAR transcripts increased markedly within brook trout follicles during this time and declined in older postovulatory follicles whose steroid-producing ability is known to be reduced in trout (43).

A MLN64 cDNA was also cloned from brook trout. Interestingly, although the btMLN64 homolog shares significant sequence identity with human MLN64, we have been unable to obtain a cDNA with an open reading frame even close to the size of that encoding the human protein. On Northern blots of brook trout RNA, there are clearly two transcripts in gonadal tissue, and the smaller transcript (2.7 kb) corresponds to the size of the largest cDNA (2707 bp) that we obtained from library screening. However, all other tissues have a much larger transcript (3.7 kb). Whether the additional bases in this larger transcript are an extension of the open reading frame or are primarily 5′ untranslated nucleotides is unknown. Clearly, the identity between btMLN64 and human MLN64s decreases greatly within the 50 amino acids of the N terminus, and there are potential ATG start sites on the extreme 5′ end of the brook trout cDNA (Fig. 2). It has also been shown that MLN64 in both zebrafish and humans is more homologous with StAR in the carboxy region and several key amino acids are conserved between MLN64 and StAR in that region (21). This is also the case for btMLN64.

MLN64 can increase steroidogenesis in COS-1 cells when cotransfected with P450scc (18), suggesting that it could regulate StAR-independent steroidogenesis. However, recently MLN64 was reported to be expressed at low levels in a variety of tissues and is associated with late endosomes (17). Given the pattern and location of expression, it is hypothesized that MLN64 may play a constitutive role in cholesterol trafficking. A constitutive role for MLN64 in trout is also supported by the low level of expression observed in all tissues examined. However, the one puzzling observation is the expression of two transcripts in gonadal tissues.

In summary, we have cloned and characterized cDNAs encoding trout StAR and MLN64 homologs. Sites of expression of brook and rtStAR genes are consistent with an involvement in steroidogenesis and the pattern of expression of btMLN64 is consistent with a constitutive role in cholesterol trafficking. Furthermore, we provide here the first evidence available from lower vertebrates for changes in StAR gene expression accompanying increased steroid production by interrenal cells and ovarian follicles. These data suggest that both StAR structure and function are highly conserved throughout the vertebrates.

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