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Conservation of steroidogenic acute regulatory (StAR) protein structure and expression in vertebrates

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Abstract

Complementary DNAs for the open reading frames of the chicken, *Xenopus* and zebrafish StAR homologs were cloned along with a partial cDNA of the zebrafish homolog to MLN64, a StAR-related protein. A comparison of the amino acid sequences of piscine, amphibian, avian and mammalian StARs, indicates strong conservation of the protein across divergent vertebrate groups. On Northern blots probed with species specific StAR cDNAs, expression of StAR transcripts was observed in the ovary and adrenal of chicken, and the ovary, testis, kidney and head of zebrafish. The expression of StAR mRNA in various compartments of the hen ovary was consistent with the results of past studies on steroidogenesis; expression was first observed in follicles selected into the preovulatory hierarchy and was greatest in the largest preovulatory follicle. The expression of StAR mRNA was also consistent with aromatase expression in zebrafish ovaries. The conserved deduced protein sequence and expression pattern of StAR transcripts in chicken and zebrafish tissues, strongly suggest that StAR is also involved in the regulation of steroidogenesis in nonmammalian vertebrates.

Keywords: StAR-related proteins; Expression; Amino acid sequences

1. Introduction

Steroids play critical roles in sexual development, homeostasis, stress-responses, carbohydrate metabolism, tumor growth, and reproduction. These hormones are primarily produced in specialized steroidogenic tissues and are synthesized from a common precursor, cholesterol. From comparative studies it appears that steroidogenesis and the key enzymes regulating steroid biosynthesis are conserved among members of different vertebrate classes (Bourne, 1991; Selcer and Leavitt, 1991). Alignments of GenBank sequences for pivotal steroidogenic enzymes, including 3β-hydroxysteroid dehydrogenase, P450 cholesterol side-chain cleavage (P450scc), P450 17α-hydroxylase/17,20 lyase (P450c17), and aromatase, reveal sequence identities that are greater than 45% between piscine, avian, and mammalian species. While these enzymes are required for the eventual conversion of cholesterol to active steroid hormones, they may not be the rate-limiting step in steroid production. It has been found that a mediator called the Steroid Acute Regulatory (StAR) Protein is required for cholesterol shuttling across the mitochondrial membrane and appears to regulate acute steroid production (Clark and Stocco, 1997). In contrast, chronically regulated steroid production appears to be largely mediated by increased transcription of steroidogenic enzymes (Hum and Miller, 1993).

StAR is rapidly synthesized in response to tropic hormone stimulation and cAMP, and is primarily localized to the gonads and adrenals (Clark et al., 1994; Stocco and Clark, 1996). Additionally, StAR activity can be suppressed by cycloheximide treatment (Stocco and Sodeman, 1991; Pescador et al., 1997), and StAR protein has been localized to the inner mitochondrial membrane in adrenal cells (Cherradi et al., 1997). In COS-1 cells co-transfected with plasmids encoding P450scc enzyme complexes and StAR, a greater than four fold increase in pregnenolone synthesis is observed (Sugawara et al., 1995). Possibly the most convincing

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data implicating StAR in acute regulation of steroidogenesis comes from studies of patients with lipoid congenital adrenal hyperplasia (lipoid CAH). This disease has been linked to mutations in the StAR gene (Lin et al., 1995) and is characterized by severe impairment of steroid production in the adrenals and the ovaries, and by accumulation of cholesterol in enlarged adrenals (Saenger, 1997). Moreover, mice in which there is a null mutation for StAR have a phenotype similar to lipoid CAH patients (Caron et al., 1997).

While steroidogenic enzymes appear to be conserved across vertebrate species, very little is known in non-mammalian vertebrates about proteins such as StAR that may be responsible for cholesterol transport across the mitochondrial membrane. In this paper, we describe for the first time the StAR homologs in piscine, avian, and amphibian vertebrates and provide evidence that the structure and expression of StAR is conserved within the Vertebrata. In addition, we also describe the sequence of a partial zebrafish cDNA that appears to be the homolog of mammalian MLN64, a protein that also enhances steroidogenesis and has sequence similarity to StAR (Watari et al., 1997).

2. Methods

Degenerative primers (forward-GCATGGA(A,G) GC(A,G,T,C)ATGGG(A,G,T,C)GAGTGGAA (amino acids 141-148 based on human StAR numbering: Fig. 1); reverse-T(C,T)TT(A,G,T,C)GGCAGCCA(A,G,T, C)CC(T,C)TT(C,G)AGGTC (amino acids 246-253 based on human StAR numbering: Fig. 1)) were designed by aligning mammalian and *C. elegans* StAR sequences as previously reported (Watari et al., 1997). These primers were used in polymerase chain reactions (PCR; 94°C, 1 min, 56–58°C, 2 min, 72°C, 2 min 34-38 cycles; *Amplitaq* DNA Polymerase; Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ) with cDNA obtained from chicken ovarian follicles, whole adult zebrafish bodies and mature *Xenopus* ovaries. In each case, a cDNA fragment of approximately 340 nucleotides was obtained that was cloned and sequenced. In the case of zebrafish and chicken, the species specific cDNA fragments were then used to screen an adult whole body (zebrafish) or embryo (chicken) cDNA library. Zebrafish and chicken cDNA

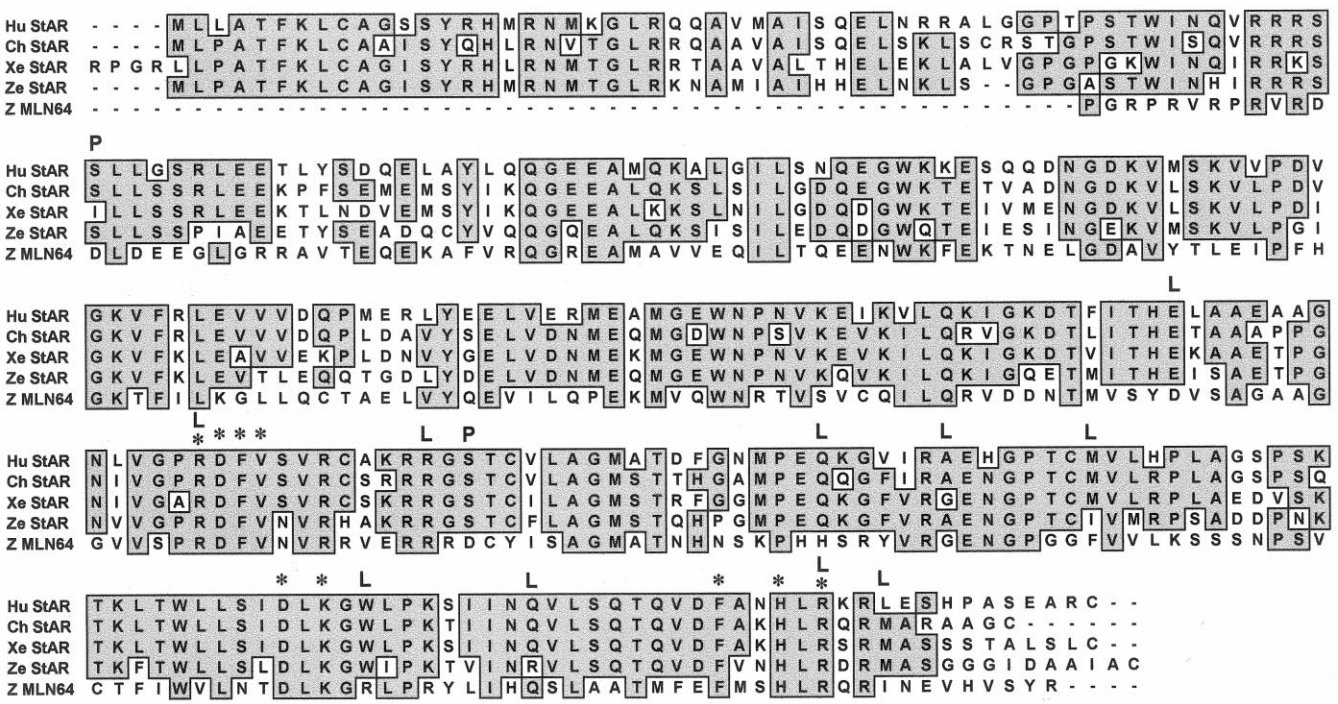

Fig. 1. Amino acid alignment of human (Hu: Accession # S79669), chicken (Ch: Accession # AF220436), *Xenopus* (Xe: Accession # AF220437), and zebrafish (Ze: Accession # AF220435) StARs and the zebrafish MLN64 homolog (Z: Accession # AF258786) using ClustalW (Thompson et al., 1994). *: Conserved residues (182–185, 246, 248, 267, 270 and 272—numbering based on the human StAR sequence) believed to be important for the steroidogenic function of StAR in mammals as determined by in vitro mutagenesis (Watari et al., 1997). L: StAR residues (169, 182, 193, 212, 218, 225, 250, 258, 272, 275) in which mutations have been observed in individuals with lipoid CAH (summary-Stocco, 1999b). P: Phosphorylation sites (57, 195) on human StAR (Strauss et al., 1999).

libraries were constructed in Unizap (Stratagene, La Jolla, CA). Positive plaques were rescreened once to homogeneity. Plasmids obtained by in vivo excision were grown for plasmid preparation, and the cDNAs were sequenced using Simultaneous Bi-directional Sequencing (SBS) reactions with the DYEnamic Cycle Sequencing Kit (US79535) from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec, Canada) and IRD700 and IRD800 labeled vector or internal primers (LiCor, Lincoln, NB). The sequencing reactions were separated on a LiCor 4200L sequencer (LiCor) and analyzed with Sequencher 4.0 (Gene Codes Corporation, Ann Arbor, MI). For *Xenopus*, nested gene specific primers were constructed from the sequence of the original 340 bp fragment and were used in 5′ and 3′ RACE (rapid amplification of cDNA ends: Marathon cDNA Amplification Kit; Clontech, Palo Alto, CA) reactions as previously described (Johnson et al., 1998; Lee and Goetz, 1998). All cDNAs obtained from RACE were fully sequenced on both strands as described above. In addition to StAR, a partial zebrafish homolog to MLN64 was also obtained by completely sequencing (as described above) a zebrafish Expressed Sequence Tag, # FC66D12.Y1 (Research Genetics, Huntsville, AL).

Various tissues were obtained from adult zebrafish and in certain cases were pooled within a tissue type to obtain sufficient RNA for Northern analysis. Ovaries were obtained from zebrafish females on the day of ovulation and 1–5 days postovulation. All tissues were processed individually for RNA using Tri-Reagent (Molecular Research Center, Cincinnati, OH) as previously described (Garczynski and Goetz, 1997), and mRNA was isolated from ovaries, testes and kidneys using the PolyAtract mRNA Isolation System (Promega, Madison, WI). Total cellular RNA was also extracted (as above) from steroidogenic and nonsteroidogenic hen tissues and used for Northern analysis. Specifically, granulosa and theca tissues from the largest (F1), second largest (F2) and third largest (F3) preovulatory follicles, follicles recently selected into the follicle hierarchy (9–12 mm diameter follicles), prehierarchal (6–8 mm and 3–5 mm) follicles, and ovarian stromal tissue were prepared as previously described (Tilly et al., 1991a).

Ribonucleic acid or polyA$^+$-rich RNA was separated on formaldehyde-agarose gels and transferred to nitrocellulose as previously described (Johnson et al., 1997). Blots were hybridized overnight with ^{32}P dCTP labeled probes produced by random prime labeling of the original 340 bp StAR cDNA fragment for chicken, or the full-length StAR cDNA in the case of zebrafish. The blots were washed under high stringency as previously described (Johnson et al., 1997) and were visualized on phosphor screens using a Storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA).

3. Results

3.1. Structure

Using the 340 bp StAR fragments as probes, cDNAs of 1304 bp (zebrafish: Accession # AF220435) and 1770 bp (chicken: Accession # AF220436) were obtained from screening the zebrafish and chicken cDNA libraries, respectively. For *Xenopus*, an 867 bp (Accession # AF220437) open reading frame was constructed by aligning three overlapping cDNA fragments; the original 340 bp fragment, together with one 5′ and one 3′ RACE product. The open reading frames of the chicken, *Xenopus* and zebrafish StAR cDNAs presumably encode proteins of 281, 289, and 285 amino acids respectively, and were 69, 67, and 63% identical to the most similar mammalian (human) StAR (Fig. 1). Both the chicken and zebrafish StAR proteins contained complete protein coding regions, however in all of the clones obtained from 5′ RACE reactions with *Xenopus* ovaries, the coding region was open on the amino end of the protein and thus did not contain a methionine. The amino acid sequence of the zebrafish form of MLN64 (Accession # AF258786), a protein similar in sequence to StAR, was 71% and 32% identical when compared with the corresponding sequences of human MLN64 and zebrafish StAR, respectively. Given the size of MLN64 in mammals, the complete coding region of the zebrafish MLN64 homolog was probably not contained within the zebrafish Expressed Sequence Tag.

3.2. Transcript expression

The 340 bp chicken StAR cDNA hybridized with a 2.8 kb transcript in steroidogenically active tissues from the hen, including those from the ovary (granulosa from the largest preovulatory follicle and stromal tissues) and adrenal (Fig. 2A). Among ovarian tissues, the highest level of StAR expression was observed in the granulosa layer of the F1 follicle, while expression decreased to undetectable levels in the postovulatory follicle (POF) collected within 18 h following ovulation (Fig. 2A). Transcript levels observed in the F2 and F3 follicles were lower than in the F1 follicle, and there was no detectable expression of the transcript found in prehierarchal (3–5 mm and 6–8 mm diameter) follicle granulosa. The first stage of follicle development when StAR mRNA could readily be detected occurred after follicle selection into the preovulatory hierarchy (F3-F1 follicles)(Fig. 2B). By comparison, within theca tissue StAR mRNA expression increased during follicle development through the preovulatory stage, and then dramatically decreased in the F1 follicle (Fig. 2C).

On Northern blots of zebrafish tissues probed with the full-length StAR cDNA, a primary transcript was

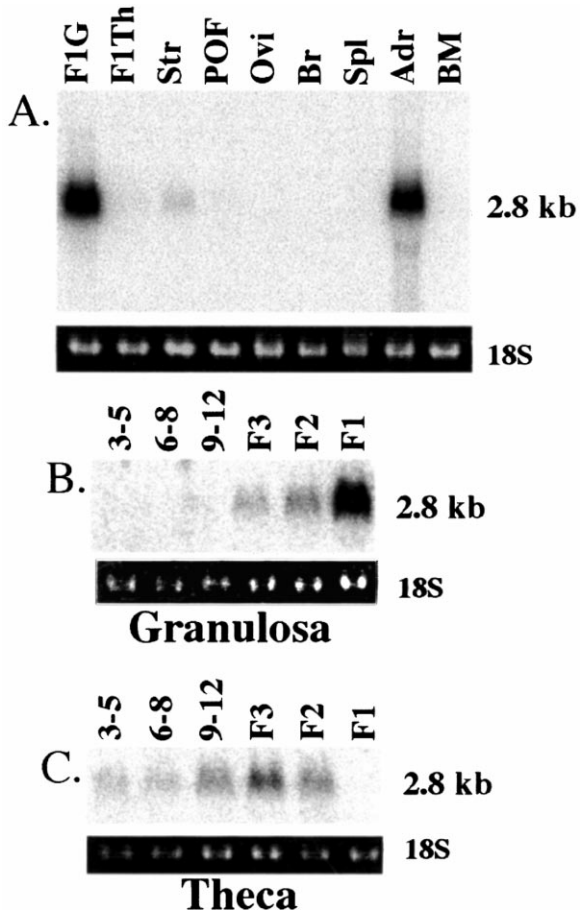

Fig. 2. Panel A: Comparison of chicken StAR transcript expression in hen tissues. F1G, granulosa layer from the largest preovulatory follicle; F1Th, theca tissue from the largest preovulatory follicle; Str, ovarian stromal tissue; POF, most recent postovulatory follicle; Ovi, oviduct; Br, brain; Spl, spleen; Adr, adrenal gland; BM, bone marrow. Expression of chicken StAR mRNA within granulosa (Panel B) and theca (Panel C) layers during follicle development. 3–5, 6–8, 9–12, size of follicle in mm; F3, F2, F1, third, second and largest preovulatory follicle, respectively. Ethidium bromide stained 18S ribosomal RNA serves as an indicator of equal loading. Each blot presented was replicated twice with similar results. All lanes contain 15 μg total RNA.

observed that was approximately 1.5 kb in length. This transcript was very highly expressed in the testis and at lower levels in the ovary, kidneys (head and trunk combined), and head (Fig. 3A). In the testis, two larger (~ 2.5 and 4.5 kb) transcripts were also observed but at much lower levels relative to the 1.5 kb transcript (results not shown). On Northern blots of ovaries taken at different stages and probed with both zebrafish aromatase (aromatase sequence—Bauer and Goetz, unpublished results, Accession # AF004521) and StAR cDNAs, there was generally a positive correlation between StAR and aromatase expression (Fig. 3B).

4. Discussion

The discovery of StAR, a factor required for shuttling cholesterol across the mitochondrial membrane, has had a dramatic impact in understanding the regulation of acute steroid production in mammals. Many of the features of steroidogenesis are conserved within different vertebrates, however, StAR has not been characterized from any nonmammalian vertebrate. Using degenerate primers constructed from the alignment of *C. elegans* and mammalian StARs as originally reported by Watari et al. (1997), it was possible for us to clone the StAR homologs from representatives of several divergent vertebrate groups. To our knowledge this is the first time this has been accomplished. [Note in proof: The cloning of the rainbow trout StAR cDNA was recently reported (Todo et al., 2000)]. In comparing the deduced protein sequences of these various StARs, there is high conservation of the protein among vertebrates, and the identity is particularly strong in the amino and carboxy regions. These regions have been suggested to be important for mitochondrial targeting and cholesterol shuttling activity, respectively (Stocco, 1999a). The most obvious difference between the cDNAs presented here is that the *Xenopus* StAR is longer on the amino terminus than all other reported StAR sequences. In fact, we were unable to obtain a 5′ RACE

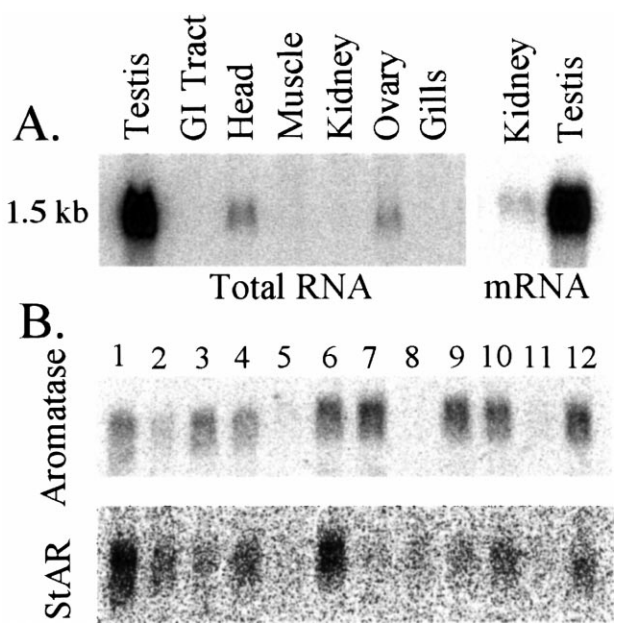

Fig. 3. Panel A: Northern blots of total RNA (20 μg/lane) and mRNA (1.0 μg/lane) from various zebrafish tissues. Panel B: Northern blot of ovarian mRNA (1.0 μg/lane) taken from individual females (1–12) at various times following ovulation (lanes 3, 7, 9, 10: 2 h, lanes 1 & 11: 26 h, lanes 2, 5, 8: 50 h, lanes 4 and 6: 74 h, lane 12: 98 h postovulation). Blots are representative of replicate Northerns and were probed with full-length zebrafish StAR cDNA and for panel B, nucleotides 1–1280 (ORF) of the zebrafish aromatase cDNA (Accession # AF004521)

product that contained an ATG start codon for the *Xenopus* StAR. However, the fact that the *Xenopus* did not have a methionine in the same position as the other StARs may simply be a cloning artifact that will be clarified in the future.

The results of this study demonstrate that the expression of StAR transcripts is also conserved in several divergent vertebrates. In both the zebrafish and chicken, StAR was expressed principally in steroidogenic tissues as it is in mammals. For example, relatively high expression of StAR was observed in the hen adrenal which most likely corresponds to the large quantities of glucocorticoids and lesser amounts of mineralcorticoids produced by this tissue (Carsia and Harvey, 2000). In addition, the expression of StAR in various compartments of the hen ovary was consistent with the results of past studies on steroidogenesis in that species. For example, granulosa cells from prehierarchal (3–8 mm diameter) hen follicles are reported to be incapable of synthesizing significant quantities of steroids due, in part, to the lack of sufficient cytochrome P450scc protein expression and enzyme activity (Tilly et al., 1991a,b). These follicles also failed to express detectable levels of StAR mRNA. In contrast, preovulatory follicles (e.g., F1–F3) secrete large quantities of progesterone, with the greatest steroidogenic capacity found in the granulosa of the F1 follicle (Etches, 1990). These follicles also had the highest expression of StAR. The absence of StAR mRNA expression in the postovulatory follicle is also consistent with previous reports showing that active steroidogenesis declines within the first 6–15 h after ovulation (reviewed by Johnson, 1990). The expression of StAR mRNA in the theca at all stages of follicle development except the F1 follicle, correlates with profiles of cytochrome P450c17 mRNA (Li and Johnson, 1993). Finally, the presence of StAR mRNA in the hen ovarian stroma was also not unexpected as this tissue expresses cytochrome P450c17 mRNA (Li and Johnson, 1993), and 3β-hydroxysteroid dehydrogenase and aromatase enzyme activities (Davidson et al., 1979).

Zebrafish have ovaries that are "asynchronous," containing oocytes in various stages of development. Zebrafish females were sampled at various times following ovulation to obtain ovaries that would presumably be undergoing different levels of steroidogenesis. On Northern blots we were able to show that the expression of StAR mRNA in the zebrafish ovary was correlated in most cases with the expression of aromatase, indicating that StAR is also linked to steroidogenesis in fish. In the zebrafish, the highest level of StAR expression was in the testis and the lowest was in the kidney. In this case, no attempt was made to separate the trunk kidney from the head kidney, which is the steroidogenic portion in fish. Finally, StAR transcripts were clearly detected in the head region of zebrafish, however further studies are needed to determine if this is localized to the brain since whole heads were assayed for RNA. Low levels of StAR expression have been observed in the brain of mammals (Stocco, 1999a). Multiple transcripts are common for StAR in mammals (e.g., Sugawara et al., 1995). In the present study, two larger transcripts were also observed in the zebrafish testis, most likely as a result of the high expression of StAR in that tissue. On blots of the adrenals and preovulatory follicles from the hen ovary, a transcript that was 200–300 bp smaller was occasionally observed but the expression was very low in comparison to the 2.8 kb form (e.g., adrenal, Fig. 2A).

Recently, MLN64 (a StAR related protein) has been shown to increase steroidogenesis in COS-1 cells when cotransfected with the cholesterol side-chain cleavage enzyme, suggesting that this gene product may regulate StAR-independent steroidogenesis (Watari et al., 1997). Here we report the isolation of a zebrafish cDNA fragment showing remarkable similarity to human MLN64 in the carboxy region which is presumed to contain the catalytic domain. Analyses of amino acid substitutions that inactivate StAR have identified several conserved residues that are required for full steroidogenic activity (Watari et al., 1997). Curiously, these residues appear to be conserved in both StAR and MLN64 proteins. We can now extend the comparison to show that these residues at position 182–185, 246, 248, 267, 270 and 272 (numbering based on the human StAR sequence) are identical between all human, chicken, *Xenopus* and zebrafish StARs and the zebrafish MLN64 homolog (Fig. 1). The conservation of MLN64 between mammals and fish in the carboxy region, and the conservation of amino acid residues required for StAR activity between these two proteins, strongly supports the hypothesis that MLN64 also regulates cholesterol transport.

Mutations in the StAR gene that presumably are responsible for lipoid CAH have been determined in a number of individuals (see Stocco, 1999b for summary). Of the amino acids in StAR for which changes have been documented in lipoid CAH, 6 residues (169, 182, 193, 212, 250, 272; numbering based on the human StAR) are identical in human, chicken, *Xenopus* and zebrafish StARs, while 3 residues (218, 225, and 258) are the same in 2 of the 3 nonmammalian vertebrate StARs (Fig. 1). One of the residues (275) is identical in chicken, *Xenopus* and zebrafish StARs, but is different from the amino acid residue in human StAR. However, the human leucine at position 275 is a conserved substitution for the methionine that is present in other vertebrates. Finally, a phosphorylation site at serine 195 in the human StAR (Strauss et al., 1999) is completely conserved in each of the vertebrate StARs whereas a phosphorylation site at serine 57 is conserved in chicken and zebrafish but not in *Xenopus* (Fig. 1).

In conclusion, using a combination of RT-PCR, library screening and RACE, we have cloned the chicken, *Xenopus* and zebrafish StAR homologs. A comparison of the amino acid sequences of the StAR protein of these species indicates strong conservation of the protein within divergent vertebrate groups. Further, the expression pattern of StAR transcripts in chicken and zebrafish tissues, strongly suggest that StAR is also involved in the regulation of steroidogenesis in non-mammalian vertebrates. The cloning of piscine, amphibian and avian StAR cDNAs will provide tools to study steroid regulation in other key vertebrate models. Isolation of StAR-like cDNAs in zebrafish, a model in which mutagenesis is possible, will likely lead to new insight into StAR regulation and possible identification of StAR interacting factors responsible for the acute regulation of steroid production. Additionally, understanding the functional relationships between StAR and MLN64 proteins in nonmammalian vertebrates may lead to new insights into conserved steroid-shuttling.

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