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Steroidogenic Acute Regulatory Protein in Eels: cDNA Cloning and Effects of ACTH and Seawater Transfer on Its mRNA Expression

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ABSTRACT—Steroidogenic acute regulatory protein (StAR) is a key molecule for steroid production by translocating cholesterol from the outer to inner mitochondrial membrane. Two cDNAs of different length encoding StAR was cloned from the head kidney of the eel (*Anguilla japonica*). In the 3'-untranslated region (UTR) of the longer cDNA, two putative polyadenylation signals were found. The shorter one differed from the longer one solely by the lack of middle of 3'-UTR including the first polyadenylation signal. Reverse transcription-polymerase chain reaction (RT-PCR) that differentiates the two mRNAs showed that the ratio of the two was highly variable among individuals, and no preferential expression was detected between freshwater and seawater eels. The predicted protein consists of 285 amino acid residues with 64–83% identity to other StARs thus far obtained. RT-PCR analyses revealed that eel StAR mRNA was expressed abundantly in the head kidney and gonad, and faintly in the brain; but no expression was detected in the gill, heart, liver, intestine, kidney and skeletal muscle. Plasma cortisol concentration increased, but StAR mRNA content in the head kidney did not change, 3 and 24 h after transfer of freshwater eels to seawater, indicating that the transcriptional regulation of StAR may not be involved in cortisol production after seawater transfer. However, ACTH elevated both plasma cortisol and StAR mRNA levels in the head kidney 1.5 and 4.5 h after injection. Thus, the steroidogenic effect of ACTH is mediated by increased StAR production as observed in mammals.

Key words: steroidogenic acute regulatory protein (StAR), cDNA cloning, cortisol, head kidney, eel (*Anguilla japonica*)

INTRODUCTION

The biosynthesis of steroids begins with the conversion of cholesterol to pregnenolone, which is catalyzed by cytochrome P450 side chain cleavage enzyme (P450scc). It is known, in mammals, that the delivery of cholesterol from the outer to the inner mitochondrial membrane, where P450scc resides, is the rate-limiting step in steroidogenesis, and a mediator called steroidogenic acute regulatory protein (StAR) is required for this process (Stocco, 2001). It appears that this protein plays a critical role in the regulation of hormonally induced acute steroid production by stimulating cholesterol transfer through the hydrophobic tunnel structure formed within its molecule (Tsujishita and Hurley, 2000; Stocco, 2001).

The cDNA encoding StAR has been cloned in various mammalian species such as the mouse (Clark *et al*., 1994), human (Sugawara *et al*., 1995), rat (Ariyoshi *et al*., 1998), hamster (Fleury *et al*., 1998), and horse (Kerban *et al.,* 1999). In these animals, most steroidogenic actions of hormones and other factors are due to the increased StAR expression (Kerban *et al*., 1999; Stocco 2001). In non-mammalian vertebrates, StAR cDNAs have also been cloned in the chicken (*Gallus gallus*), *Xenopus laevis*, zebrafish (*Danio rerio*) and trout (*Salvelinus fontinalis* and *Oncorhynchus mykiss*) (Bauer *et al*., 2000; Kusakabe *et al*., 2002). The primary structure of StAR and tissue distribution of its transcripts are basically similar to those in mammals. However, little is known about the functional role of StAR in non-mammalian vertebrates except for the recent data that gonadotropins stimulated StAR expression in the follicle cells of the hen *in vitro* (Johnson and Bridgham, 2001), and that acute stress increased StAR transcripts in the head kidney of the trout (Kusakabe *et al*., 2002).

Cortisol is a principal corticosteroid secreted from the interrenal (the adrenocortical homologue) located in the head kidney of teleosts, and plays a pivotal role in adaptation to both freshwater (FW) and seawater (SW) environ-

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ments (for review, see McCormick 2001). Several hormones such as adrenocorticotropin (ACTH), natriuretic peptides (NPs), angiotensin II (ANG II) and urotensins have been shown to stimulate cortisol secretion in teleost fish (Henderson *et al*., 1976; Arnold-Reed and Balment, 1994; Li and Takei, 2003), and some of the hormones have been implicated in SW adaptation (Takei, 1993). However, little is known about the mechanisms by which these hormones stimulate cortisol production in fish. One possibility is that these hormones activate the steroidogenic enzymes involved in cortisol synthesis (Miller, 1988). Recent evidence in mammals raises the possible involvement of StAR in the hormonally induced cortisol production in fish. For examining it, the cDNA encoding StAR was cloned from the head kidney of a euryhaline teleost, the eel (*Anguilla japonica*) in the present study. The tissue distribution of StAR mRNA was examined by RT-PCR. In addition, changes in plasma cortisol concentrations were examined together with StAR mRNA expression in the head kidney after ACTH injection or transfer of eels from FW to SW, to assess whether StAR is involved in the cortisol production caused by hormonal and environmental stimuli.

MATERIALS AND METHODS

Animals and tissues collection

Cultured immature FW eels (*Anguilla japonica*) were purchased from a local dealer. The gonads were immature, and thus the sex was indistinguishable by naked eye. Eels were maintained under natural photoperiod in FW or SW tanks for more than one or two weeks, respectively, before experimental use. Water in the tank was continuously circulated, aerated, and regulated at 18°C. After anesthetization of eels with 0.1% (w/v) tricaine methanesulfonate (MS-222) (Sigma, St. Louis, USA), the tissues (see Tissue distribution) were surgically isolated and immediately frozen in liquid nitrogen, and were then stored at –80°C until RNA extraction.

RNA extraction

Total RNA was extracted from various tissues using ISOGEN (Nippon Gene, Toyama, Japan). For cDNA cloning, poly $(A)^+$-RNA was subsequently isolated from the total RNA extracted from the head kidney of a FW eel using Oligotex-dT30 (Japan Synthetic Rubber, Tokyo, Japan).

Cloning of eel StAR cDNA

The double-stranded cDNA pool was prepared from 0.5 μg poly $(A)^+$-RNA using SMART cDNA Library Construction Kit (Clontech Laboratories, Palo Alto, CA) as described previously (Inoue *et al*., 2003). Degenerate primer S1 [5'-GCAACHTTCAAAYTGT-GYGC-3'] and A1 [5'-TTCCASTCVCCCATYTGYTCCAT-3'] (Fig. 1), which were designed based on the StAR cDNA sequences of zebrafish (accession no. AF220435), trout (AF232215, AB047032), *Xenopus laevis* (AF220437) and chicken (AF220436), were used to amplify a partial cDNA fragment. Thirty cycles of amplification (denaturation at 94°C for 30 s, annealing at 61°C for 60 s and extension at 72°C for 90 s) were performed using Ex Taq DNA polymerase (Takara Shuzo, Kyoto, Japan). The amplified cDNA fragments were electrophoresed in 1.2% agarose gels containing ethidium bromide (EB), isolated from the gel using Geneclean Kit (Bio101, Vista, CA), subcloned into pT7Blue T-vector (Novagen, Madison, WI) and sequenced in a 310 DNA sequencer (PE Biosystems, Foster City, CA).

5'- and 3'-rapid amplification of cDNA end (RACE) were subsequently performed using the PCR protocol as above. The primers used were gene-specific primers (A2, 5'-CTCAGCACCTTGT CTC-CATTG-3'; S2, 5'-CAACCTGTATGGGGAGCTGGT-3') (Fig. 1) and adaptor primers (5' PCR primer and CDS III/3' primer from Clontech), respectively. The PCR products were isolated, subcloned and sequenced as above. The full-length of eel StAR cDNA was constructed by aligning the three overlapping cDNA fragments: i.e., the fragment amplified with primers S1 and A1, and the 5' and 3' RACE products. Finally, the full coding region was amplified using gene-specific primers S3 (5'-GTATTATCTCGACTGGCT-TCG-3') and A3 (5'-ATCAGCAAGCCTGTGCTGC-3'), which were designed, respectively, from the 5'-UTR end and termination codon region (Fig. 1), to confirm the sequence.

Tissue distribution of mRNA

The tissue distribution of StAR mRNA was evaluated by RT-PCR. Examined tissues included the head kidney, kidney, brain, heart, liver, skeletal muscle, immature gonad, intestine and gill from five FW eels (184.3±11.8 g BW) and two SW eels (173.5±10.4 g BW). Total RNA (2 μg) from each tissue was reverse transcribed (RT) with Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA). Negative control was also run, where the reverse transcriptase was removed from the reaction mixture when performed RT, to confirm the absence of genomic DNA contamination. PCR was carried out for 32 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 45 s. The expression of eel glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control as described previously (Takei *et al*., 2001). The PCR products were electrophoresed in 1–1.2% agarose gels containing EB, and observed under FAS-III Electronic U.V. Transilluminator (TOYOBO, Osaka, Japan).

Effects of ACTH and SW transfer on plasma cortisol and StAR mRNA levels

Effects of ACTH injection

After FW eels (186.5+13.4 g, n=20) were anesthetized in 0.1% MS-222, a polyethylene tube (SP 10, o.d.=0.61 mm, Natsume Seisakusho Co. Ltd, Tokyo) was surgically inserted into the ventral aorta for blood sampling and ACTH injection. After surgery, eels were held in individual plastic troughs, through which aerated and filtered FW was continuously circulated at 18°C. The troughs were covered with a black vinyl sheet to minimize the visual stress during experiments. On the day after surgery, each eel was given a single injection of ACTH (human, 1–24) (Peptide Institute Inc., Osaka) dissolved in 0.9% NaCl solution at 0.2 nmol/kg in a volume of 0.05 ml. Injection of the same volume of vehicle served as control. Eel ACTH (accession no. AF194969) is only one-amino acid different from human ACTH (P01189) in the N-terminal 18 amino acids, where the biological activity resides (Dewied, 1990). Since the results in mammals showed that the peak increase of StAR mRNA levels usually occurred at 3–6 hr after ACTH treatment, while plasma cortisol increased in a very short time (5–30 min) (Ariyoshi *et al*., 1998; LeHoux *et al*., 1998; Le Roy *et al*., 2000), we chose two time points (1.5 and 4.5 hr after injection), in this study, to collect blood (ca. 30 μl) and head kidney samples to assess whether StAR was involved in the ACTH-induced cortisol production in eels. Besides, blood was also collected at 0.5 hr after ACTH injection to examine whether cortisol was increased as quickly as observed in mammals. The removed blood volume was replaced immediately by a vehicle injection. After centrifugation, plasma was collected and stored at –20°C for subsequent cortisol determination. Head kidneys were collected for RNA extraction from half of the experimental eels at 1.5 and 4.5 hr after ACTH or saline injection, respectively.

Effects of SW transfer

Thirty FW eels (187.6±15.4 g BW) were equally divided into 5

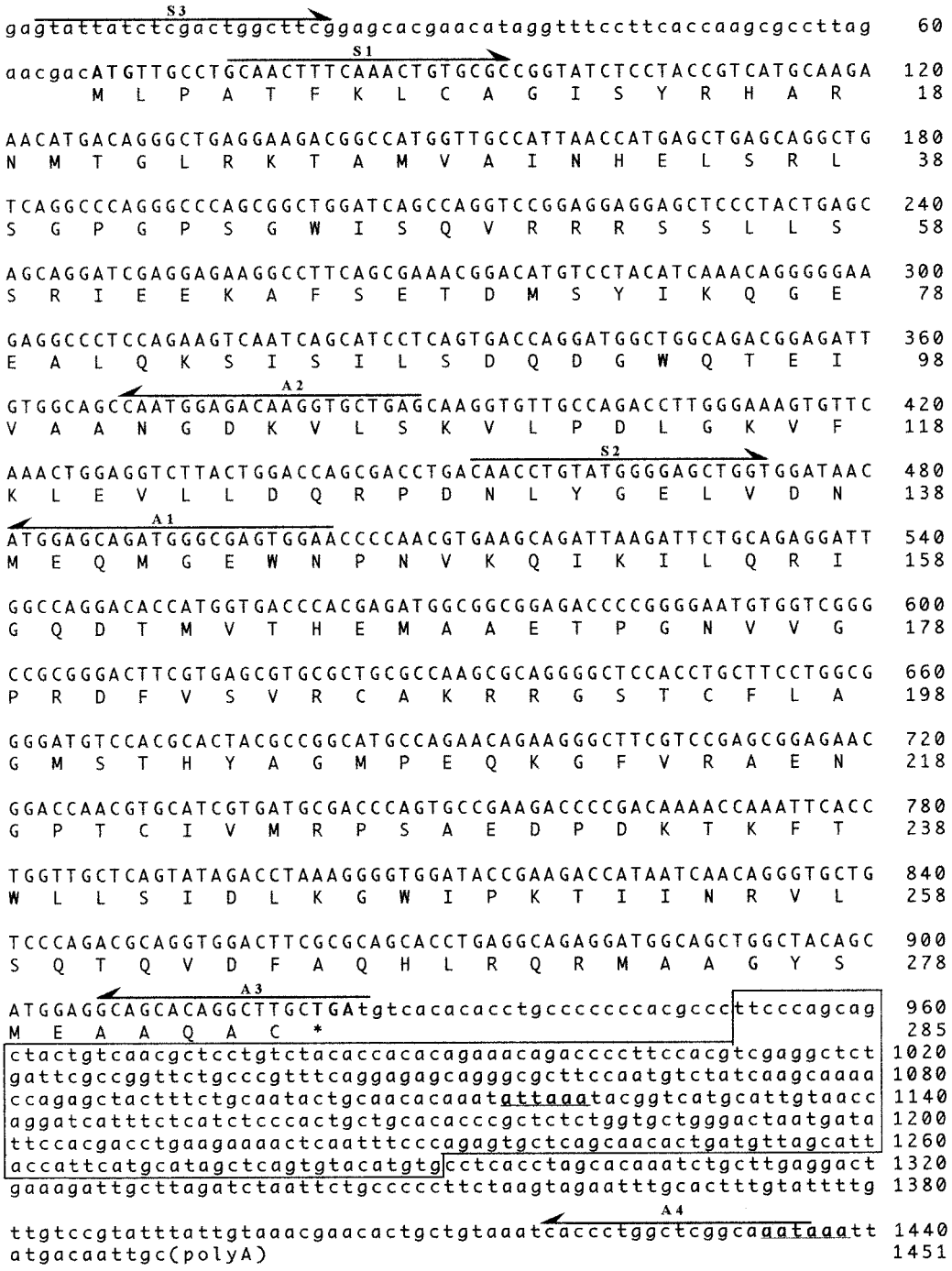

Fig. 1. Nucleotide and predicted peptide sequences of eel StAR cDNA. Two types of cDNA were isolated from the head kidney. Compared with the longer variant, the shorter one lacks a 340-bp sequence in the 3'-untranslated region (UTR) as indicated by the box. Both 5'- and 3'-UTRs are in lower case; the upper case letters indicate the nucleotides of the coding region. Nucleotide and amino acid numbers are shown on the right. The start (ATG) and termination (TGA) codons are boldfaced. Two putative polyadenylation signals, 'attaaa' and 'aataaa', are underlined. Primer sequences used for cloning and examining the expression of StAR mRNA are indicated by arrows (see details in Materials and Methods).

groups: (1) 0-hr controls, (2) 3-hr FW-FW group, (3) 24-hr FW-FW group, (4) 3-hr FW-SW group, and (5) 24-hr FW-SW group. Plasma cortisol increased within 24 hr after SW transfer in American eel (*Anguilla rostrata*) (Forrest *et al*., 1973), so we chose two time points (3 and 24 hr) in this study. The FW-FW groups served as matched time controls. Initially, eels were carefully transferred from a 1-ton FW culture tank to individual 55×40×30 cm experimental tanks with FW for acclimation to new environments. On the next day, water in the experimental tank was carefully decreased to 1/5. For 0-hr controls, eels were then anesthetized in 0.1% MS-222 neutralized with $NaHCO_3$; For FW-FW and FW-SW groups, FW and SW was carefully added, respectively, and fish were anesthetized at 3 or 24 hr after the add of water. Water in the tank was continuously aerated, and regulated at 18°C. The time schedule was

```
Eel       MLPATFKLCAGISYRHARNMTGLRKTAMVAINHELSRLSG..PGPSGWISQVRRRSSLLS  58
Trout     ----------------T--------N-----H---NM-A-..-N--S---H---------  58
Zebrafish ----------------M--------N--I--H---NK---..--A-T--NHI-------  58
Chicken   ----------A---Q-L--V----RQ-A---SQ---K--CRST---T-------------  60
Rat       --L--------S----M---K---HQ-VL--GQ--N-RALGD-S-.--MG----------G  59
Human     --L--------S----M---K---QQ-VM--SQ--N-RALGG-T--T--N----------G  60

Eel       SRIEEKA.FSETDMSYIKQGEEALQKSISILSDQDGWQTEIVAANGDKVLSKVLPDLGKV 117
Trout     -----EQGYN-AEV--V--------------G-----T---I--------------V--- 118
Zebrafish -P-A-ET.Y--A-QC-VQ--Q----------E---------ESI--E--M-----GI--- 117
Chicken   --L---P.---ME--------------L---G--E--K--T--D------------V--- 119
Rat       -QL-ATL.Y-DQEL---Q-----M--ALG--NN-E--KK-SQQE--DE-----V-GV--- 118
Human     --L--TL.Y-DQELA-LQ-----M--ALG---N-E--KK-SQQD--D--M---V--V--- 119

Eel       FKLEVLLDQRPDNLYGELVDNMEQMGEWNPNVKQIKILQRIGQDTMVTHEMAAETPGNVV 177
Trout     ----------S--------G------D------EV----K---E------VSGP------ 178
Zebrafish -----T-E-QTGD--D-----------------V----K---E--I---IS-------- 177
Chicken   -R---VV--PL-AV-S----------D---S--EV-----V-K--LI---T--AP---I- 179
Rat       -R-------PM-R--E----R--A---------E--V-KK--K--VI---L--AAA--L- 178
Human     -R---VV--PMER--E---ER--A---------E--V--K--K--FI---L---AA--L- 179

Eel       GPRDFVSVRCAKRRGSTCFLAGMSTHYAGMPEQKGFVRAENGPTCIVMRPSAEDPDKTKF 237
Trout     ---------------------QHPT----R-V----------------D--N---- 238
Zebrafish ------N--H-----------QHP----------------------D--N---- 237
Chicken   ----------SR------V------THGA----Q--I--------M-L--L-GS-SQ--L 239
Rat       ----------T-------V----A--FGE----S-VI---H----M-LH-L-GS-S---L 238
Human     ----------------V----D-DFGN------VI---H----M-LH-L-GS-S---L 239

Eel       TWLLSIDLKGWIPKTIINRVLSQTQVDFAQHLRQRMAAGYSMEAAQAC.           285
Trout     ------------------K---------N-------DNSVSKEMAPAC           287
Zebrafish -----L---------V----------VN---D---S-GGID--IAC.            285
Chicken   ----------L------Q---------K-------RAAGC.......            281
Rat       ----------L------Q------IE--S---K-LESSPAS--QC...            284
Human     ----------L--S--Q---------N---K-LESHPAS--RC...              285
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Fig. 2. Alignment of the deduced amino acid sequence of eel StAR with those of other species analyzed by ClustalW multiple alignment. The sequences were obtained from the GenBank database: trout (AAG39689), zebrafish (AAG28593), chicken (AAG28594), rat (P97826), Human (P49675). Amino acid numbers are shown on the right. Dashes (-) represent identical amino acid residues and dots (.) represent gaps. The N-terminal 25 residues are indicated by a solid horizontal line. Sites of protein kinase A-mediated phosphorylation of StAR are boxed. The conserved residues important for the steroidogenic function of StAR are indicated by asterisks (*); those putatively directly contributing to the hydrophobic tunnel structure are highlighted by gray boxes.

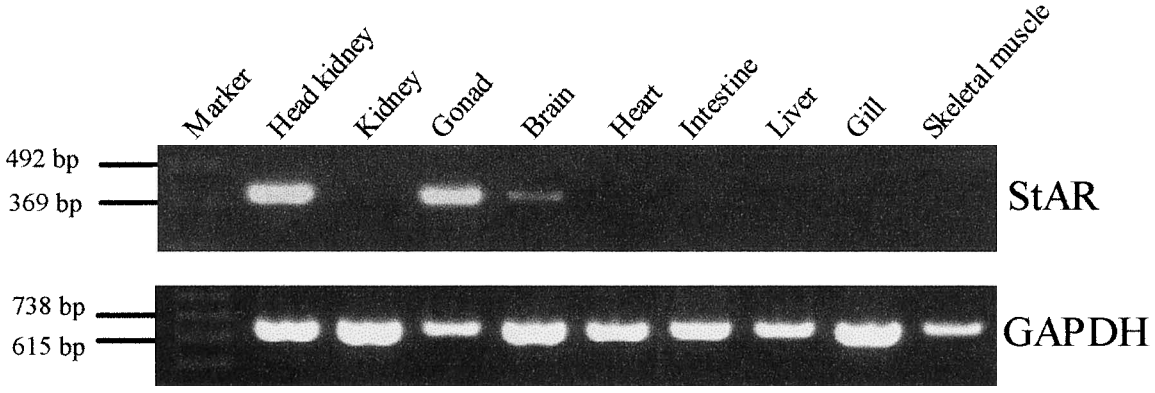

Fig. 3. Tissue-specific expression of StAR mRNA in eels examined by RT-PCR. The expression of eel glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. Five freshwater and two seawater eels were examined, and the expression patterns were the same. The results for a freshwater eel are presented here.

arranged to anesthetize the fish between 1300–1400 hr, because it has been shown that plasma cortisol concentration is relatively low and stable around the time of the day (Li and Takei, 2003). After anesthesia, plasma and head kidneys were collected for cortisol determination and RNA extraction, respectively.

Measurement of StAR mRNA content in the head kidney

The content of StAR mRNA in the head kidney was determined by RT-PCR using the protocol similar to that for examining tissue distribution. PCR cycles were changed to 24 for StAR (using primer pair S3-A2) and 26 for GAPDH. In pilot experiments using serially diluted templates, we confirmed that the signal intensity of PCR products and the amount of template exhibited linear relationship under these conditions, and the signal of all experimental samples were within the linear range (data not shown). Each PCR product (10 μl) was electrophoresed in 1.2% EB-stained agarose gels, and the signal was scanned and quantitated using a Fuji FLA-2000 imaging analyzer. The GAPDH signal was also quantified and used to normalize the results of StAR. This process was repeated three times.

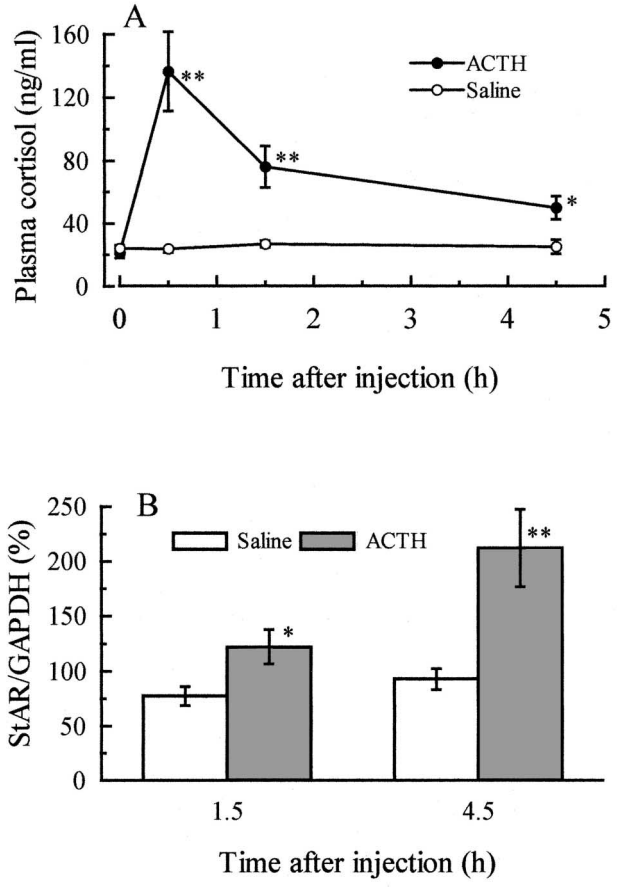

Fig. 4. Effect of ACTH injection on the levels of plasma cortisol and StAR mRNA in head kidney of freshwater eels. **A**, The plasma cortisol levels before and 0.5, 1.5, 4.5 h after ACTH injection were measured by enzyme immunoassay (EIA). Injection was made at 0hr. **B**, StAR mRNA expression in the head kidney at 1.5 and 4.5 hr post-injection was examined by RT-PCR. After electrophoresis, the StAR transcripts were quantitated and normalized to GAPDH levels. Values are means±SEM. Number of eels used for StAR mRNA quantitation was 5; that for cortisol measurement was 10 at 0, 0.5 and 1.5 hr post-injection, and 5 at 4.5 hr post-injection. Significant differences from control values at each time point are denoted by asterisks: $^{*}P<0.05$; $^{**}P<0.01$.

Comparison of expression of different StAR transcripts

As will be mentioned below, two transcripts with different length of 3'-UTR have been cloned (Fig. 1). The difference in their expression was examined in the head kidney by RT-PCR. PCR was first conducted using primer pair S3-A3, which amplifies the whole coding region of eel StAR, to confirm the normal expression of StAR in all eels. PCR was then performed using primers S2 and A4 (5'-TATTTGCCGAGCCAG GGTG-3'), of which A4 was located near the poly (A) tail, to distinguish the expression of two transcripts by the length of PCR products. Eels examined in this experiment were 0-hr controls, 3-hr FW-SW and 24-h FW-SW groups of the SW transfer experiment.

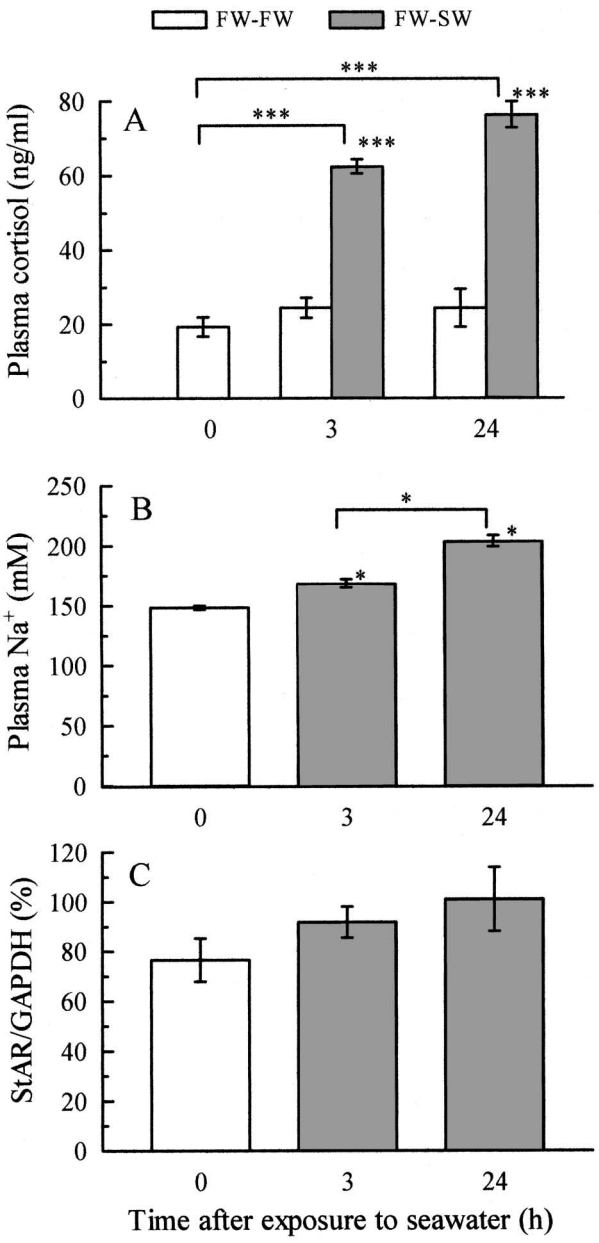

Fig. 5. Effect of seawater transfer on plasma cortisol (A) and Na^+ (B) concentration, and StAR mRNA content in head kidney (C) in eels. StAR transcripts were quantitated by RT-PCR and normalized to GAPDH levels. Values are means±SEM (n=6 for each group). Significant differences from the FW-FW controls are denoted by $^{*}P<0.05$, $^{***}P<0.001$.

Measurement of plasma cortisol and $Na^{\pm}$

Plasma cortisol was analysed by enzyme immunoassay (EIA) as described previously (Li and Takei, 2003). In brief, cortisol was extracted from plasma with diethyl ether. In plate wells, 50 μl of cortisol standard (0.04 to 10 ng/ml) or sample were incubated with 50 μl of anti-cortisol serum (1:50000 dilution) and 50 μl of enzyme conjugate (1:1000 dilution) for 1.5 hr at room temperature. After washing, 150 μl of substrate solution was added, incubated for 1 hr at 37°C, and the absorbance was measured at 492 nm in a microplate reader (MTP-300, Corona Electric Co., Ibaragi, Japan). The Na^+ concentration in plasma of SW-transferred eels was determined in an atomic absorption spectrophotometer (Z5300 Hitachi, Tokyo). Each measurement was performed in duplicate for cortisol and triplicate for Na^+.

Statistics

All data are presented as means ± SEM. Paired *t*-test was used to determine statistical differences of plasma cortisol concentrations before and after ACTH injection in the same fish. For comparison of plasma cortisol and StAR mRNA levels between ACTH- and saline-injected eels, or between FW- and SW-transferred eels, Student's *t*-test and ANOVA followed by Bonferroni's post-hoc test were applied. Significance was set at $p<0.05$.

RESULTS

Nucleotide and amino acid sequences of eel StAR

Using a combination of RT-PCR and RACE, two StAR cDNAs differing in the length of 3'-UTR were isolated from the eel head kidney (Fig. 1). The longer cDNA (accession no. AB095110) consisted of 1451 bp nucleotides (excluding poly (A) tail) containing an 858-bp open reading frame with 66 bp of 5'-UTR and 527 bp of 3'-UTR. In the 3'-UTR, two possible polyadenylation signals were found. The shorter cDNA consisted of 1111 bp, and had the same sequence as the longer one except for the lack of a 340-bp sequence at the central part of the 3'-UTR. The deleted sequence contained the first polyadenylation signal. Both cDNAs encoded an identical peptide with 285 amino acid residues. The percent homologies of eel StAR at the level of nucleotide and amino acid (in parentheses) were 78.2 (82.6) with trout, 73.7 (78.6) with zebrafish, 68.0 (75.2) with *Xenopus*, 75.0 (76.1) with chicken, and 66.4–70.0 (65.3–67.1) with mammals. The similarity is particularly high in the N-terminus and the functional domains of the C-terminus (Fig. 2).

Tissue-specific expression of eel StAR mRNA

The expression of StAR mRNA was consistently detected in steroidogenic tissues in both FW and SW eels examined: a high expression was observed in the head kidney and gonad, and a weak expression was encountered in the brain (Fig. 3). However, the expression in other tissues, such as the kidney, heart, intestine, liver, gill, and skeletal muscle, was too low to be detected under the current RT-PCR condition.

Effects of ACTH injection on plasma cortisol and StAR mRNA in head kidney

As shown in Fig. 4, plasma cortisol concentrations increased at 0.5, 1.5 and 4.5 hr after ACTH injection, respectively, compared with the pre-injection level (21.2±3.0 ng/ml, n=10). In contrast, plasma cortisol concentrations did not change during the 5-hr experimental period in controls injected with saline. Thus, the increases after ACTH injection were significant at all time points compared with the time controls. The StAR mRNA content in the head kidney increased gradually after ACTH injection, and the increase was significant at 1.5 and 4.5 hr post-injection compared with that of saline-injected controls.

Effects of SW transfer on plasma cortisol and Na^+, and StAR mRNA in head kidney

Plasma cortisol concentration increased at 3 and 24 hr after transfer of eels from FW to SW (Fig. 5A). However, the plasma cortisol levels in FW-FW groups had no obvious increase, indicating that the transfer itself did not cause stress-induced cortisol production. For examination of plasma Na^+ and StAR expression in head kidney, therefore, time controls were not prepared. As shown in Fig. 5B, plasma Na^+ concentration was elevated at 3 and 24 hr after SW transfer, and the value at 24 hr was also significantly higher than that at 3 hr. However, the StAR content in head kidney was not significantly different between FW and SW-transferred eels (Fig. 5C).

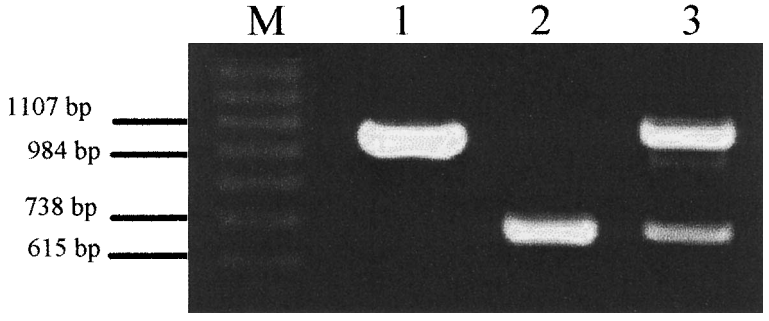

Fig. 6. Comparison of the two different StAR transcripts in freshwater- and seawater-transferred eels. The expression of the two different StAR transcripts in the head kidney was examined by RT-PCR using primers S2 and A4 (Fig. 1), which could amplify the two transcripts with different length of 3'-UTR. Among the 18 fresh water and seawater-transferred eels examined, a band corresponding to the 1451 bp transcript was detected in 1 eel (lane 1), that corresponding to the 1111 bp transcript was detected in 13 eels (lane 2), both bands were detected in 4 eels (lane 3). M: 123 bp DNA marker.

Comparison of expression of two different StAR transcripts

Prior to the comparison of expression of the two transcripts with different length of 3'-UTR, we confirmed that StAR mRNA was expressed in all eels (data not shown), using a primer pair (S3-A3) that amplified the coding region of StAR (Fig. 1). Then, the difference in expression of the two transcripts was examined by the size of amplified products using the primer pair S2-A4 that could amplify the whole 3'-UTR (Figs. 1 and 6). Among 18 eels examined, a single band corresponding to the longer transcript was observed only in one eel, while a single band corresponding to the shorter transcript was detected in thirteen eels. In 4 of 18 eels, both transcripts were expressed in the head kidney. No obvious differences in relative abundance of the two transcripts were obtained between FW and SW-transferred eels (data not shown).

DISCUSSION

In this study, two StAR cDNAs differing in the length of 3'-UTR have been cloned from the head kidney of Japanese eel. The eel StAR is composed of 285 amino acid residues, which is identical or similar in length to those of teleosts and mammals thus far identified. The eel peptide displays 82.6% similarity to that of trout, and 67.1% to human. Compared with the StARs of other vertebrate species, the putative phosphorylation sites by protein kinase A (RRSS) (Strauss *et al*., 1999) are conserved in eel (Fig. 2). In the N-terminal 25 residues, eel StAR displays 96% similarity to that of trout or zebrafish, and 84% to that of human, rat or chicken. This domain is composed of basic and hydrophobic amino acids that are characteristic of mitochondrial targeting sequences (Clark *et al*., 1994). In the C-terminal domain, the amino acid residues believed to be important for the steroidogenic function (Watari *et al*., 1997), and those contributing to the hydrophobic tunnel structure for lipid transport (Tsujishita and Hurley, 2000), are also conserved (Fig. 2). These data indicate that the primary structure of StAR, particularly in the functional domain, is highly conserved throughout vertebrates.

The tissue distribution of eel StAR mRNA in the head kidney and gonads is in agreement with the reports of other species where StAR is principally expressed in steroidogenic tissues (Sugawara *et al*., 1995; Bauer *et al*., 2000; Kusakabe *et al*., 2002). Weak but distinct expression of StAR was also detected in the brain of eel, as well as in the rat and marmoset (Furukawa *et al*., 1998; Bauer *et al*., 2000). Recently, it is reported that various neurosteroids are produced locally in the brain and play diverse functions (Tsutsui *et al*., 2000). The expression of StAR in steroidogenic tissues and brain is consistent with the role of StAR in the regulation of steroidogenesis (Stocco, 2001). Although StAR transcripts were also detected in the kidney of human (Sugawara *et al*., 1995) and in some non-steroidogenic tissues of trout (Kusakabe *et al*., 2002), the role of StAR in these tissues remains to be clarified.

Different lengths of StAR mRNAs have been reported in most species examined thus far. In some species, the difference in length can be attributable to the difference in the length of 3'-UTR (Fleury *et al*., 1998; Kerban *et al*., 1999). In bovine and rat StAR, multiple polyadenylation signals were found in the 3'-UTR, and it was suggested that different transcript sizes might represent the differential use of polyadenylation signals (Hartung *et al*., 1995; Pilon *et al*., 1997). In this study, two StAR transcripts differing in the length of 3'-UTR were identified in the eel. Since the longer mRNA contained two possible polyadenylation signals, while the shorter one lacks the first polyadenylation signal found in the longer one. This indicates that the shorter one was not generated by the use of the first polyadenylation signal. It is quite possible that the two mRNAs were generated by the alternative splicing from the same StAR gene, although we cannot exclude the possibility that they were encoded by different genes. The expression of the two StAR transcripts differed profoundly among eel individuals (Fig. 6). The reason and possible biological significance of two types of transcripts remain unknown.

In non-mammalian vertebrates, the induction of StAR expression by trophic hormones has not yet been demonstrated except for an *in vitro* study using the follicular cells of the hen (Johnson and Bridgham, 2001). In this study, we showed that both plasma cortisol and StAR mRNA levels in the head kidney were significantly enhanced at 1.5 and 4.5 hr after ACTH injection. The close relationship between the increase of StAR expression and cortisol production strongly suggests that StAR is involved in the ACTH-induced cortisol production in eels as has been demonstrated in mammals (Kim *et al*., 1997; Stocco, 2001). At present, although we do not know the StAR mRNA content in the head kidney at 0.5 hr after ACTH injection, the results showed that the StAR mRNA content increased from 1.5 to 4.5 hr after ACTH injection. These results are, to some extent, similar to those in mammals, where peak increase of StAR mRNA levels usually occurred at 3–6 hr after ACTH treatment (LeHoux *et al*., 1998; Le Roy *et al*., 2000). Since the plasma cortisol level increased immediately after ACTH injection (within 0.5 hr, Fig. 4), it seems that the transcriptional and translational regulation of StAR may not be directly involved in the initial acute cortisol production as observed in rats, where the expression of StAR mRNA and protein was delayed after the peak increase in plasma corticosterone concentration (Ariyoshi *et al*., 1998). Epstein and Orme-Johnson (1991) proposed that a labile pool of StAR could be responsible for this acute mediation. It is also possible that redistribution of StAR rather than its net synthesis is critical for the steroidogenesis (Ariyoshi *et al*., 1998).

SW adaptation is a complex physiological process, in which cortisol plays a critical role by remodeling chloride cells and up-regulating the expression/activity of Na^+, K^+-ATPase (McCormick, 1995). In the present study, after the transfer of eels from FW to SW, plasma Na^+ concentration

increased for 24 hr after transfer (Fig. 5). Thus plasma cortisol that increased in parallel appears to counteract the sudden increase in plasma Na^+ concentration and to promote SW adaptation. The increase in plasma cortisol remained until 24 hr after SW transfer, this suggested that the chronic regulation, but not the acute regulation, of cortisol production might be prevailing during SW adaptation. In contrast, StAR mRNA content in the head kidney had no obvious increase, suggesting that the transcriptional regulation of StAR might not be directly involved in the regulation of cortisol production after SW transfer. This is not surprising, because StAR is mainly involved in the regulation of acute steroid production (Stocco, 2001). Besides, in addition to cortisol, multiple hormones such as growth hormone/insulin-like growth factor I axis, thyroid hormones, ANP, ANG II and urotensins are also implicated in SW adaptation, and some of them have been shown to increase cortisol production in eels and other teleost fishes (Arnold-Reed and Balment, 1994; McCormick, 2001; Tsukada and Takei, 2001; Li and Takei, 2003; Takei and Hirose 2002). Actually, plasma ANG II and ANP concentrations increased transiently after SW transfer in eels (Tierney *et al*., 1995; Kaiya and Takei, 1996). In mammals, it has been shown that the chronic regulation of trophic hormones, such as ACTH and ANG II, on the production of corticosteroids is largely mediated by increasing the transcription of steroidogenic enzymes (Hanukoglu, 1992; LeHoux *et al*., 1998; Le Roy *et al*., 2000). Therefore, it is reasonable that the increased cortisol production after SW transfer in eels might be mainly due to the enhanced expression of steroidogenic enzymes, under the stimulation of hormones such as ACTH, ANP and ANG II. This can be clarified in the near future since the cDNAs encoding the cytochrome P450 11β-hydroxylase (Jiang *et al*., 1996), 17α-hydroxylase (Kazeto *et al*., 2000), and 21-hydroxylase (Li *et al*., 2003), all of which are involved in cortisol biosynthesis, have already been cloned in the eel.

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