Two Distinct c-*ski* cDNAs of Fish, Tilapia (*Oreochromis aurea*)

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ABSTRACT Two classes of tilapia c-*ski* cDNA (accession nos. AJ012011, AJ012012), designated as t*ski*1 and t*ski*2, respectively encoded a 687 and a 714 AA protein and shared a 57% AA identity. Comparison with the Ski proteins of chickens, humans and *Xenopus*, tilapia TSki polypeptides shared a 60, 57, and 57% (TSki1) and 67, 63, and 61% (TSki2) AA identity, respectively. The most and the least abundant c-*ski* mRNAs are located in the brain and the skeletal muscle, respectively. Both t*ski*1 and t*ski*2 were widely expressed in the adult tissues examined, but t*ski*2 transcripts were at higher levels except in the ovary and oocytes: t*ski*1 transcripts were predominant in the ovary, whereas t*ski*2 transcripts were predominant in the testes. In the oocytes, the t*ski*1 mRNA was a maternally-inherited stockpile that subsequently was degraded, so that the expression ratio of t*ski*1 to t*ski*2 transcripts declined gradually as the fish developed from oocyte to 4-cm fry. *Mol. Reprod. Dev. 54:223–231.* © 1999 Wiley-Liss, Inc.

Key Words: cDNA; c-*ski*; fish; oncogene; Ski protein

INTRODUCTION

c-*ski* is proto-oncogene which encodes an 84 kDa nuclear protein (Nagase et al., 1990). The c-Ski protein stimulates proliferation, induces morphologic transformation, and promotes myogenic differentiation on cultured quail embryo fibroblasts (Stavnezer et al., 1981; Li et al., 1986; Stavnezer et al., 1986; Colmenares and Stavnezer, 1989; Colmenares et al., 1991). Transgenic mice show a distinctive muscular phenotype due to a hypertrophic effect on the formation of the fast muscle IIb fibers and concomitant decrease in body fat (Sutrave et al., 1990). c-Ski activates a muscle-specific enhancer resulting in the transcriptional regulation of muscle differentiation (Engert et al., 1995). c-*ski* is widely expressed in many tissues, although some studies that investigated skeletal muscle in *Xenopus* and chicken found no evidence of c-*ski* expression (Sutrave et al., 1990; Sleeman and Laskey, 1993). Namciu et al. (1994) reported c-*ski* expression at a high level in the skeletal muscle of mouse embryos at 12.5 days of gestation. Knockout mice die at birth and show a defective closure of the rostral neural tube and a decrease in skeletal muscle (Berk et al., 1997). Recently, Nicol and Stavnezer (1998) demonstrated that c-Ski acts as a repressor rather than an activator, and depends on the cellular environment that allows c-Ski to bind to the GTCTAGAC site. Ichikawa et al. (1997) also reported that c-Ski functions as suppressor of the myogenic gene in proliferating myoblasts and as a potentiator in post mitotic myotubes. The fact that c-Ski apparently plays many important roles suggests that its functionality as a transcriptional factor may be modified by interactions with various other transcriptional factors (Tarapore et al., 1997). It would therefore appear to be impossible to understand clearly and completely the biological properties of c-Ski without studying c-Ski in more diverse species. However, only the cDNA-derived c-*ski* gene of chicken (Sutrave and Hughes, 1989), human (Nomura et al., 1989), mouse (Namciu et al., 1995), *Xenopus* (Sleeman and Laskey, 1993) and axolotl (Ludolph et al., 1995) have been defined. To date, no such definition has been available for the cellular c-*ski* gene of the lowest vertebrates, fish.

As a step toward providing this information, we herein describe two classes of c-*ski* cDNAs of tilapia (*Oreochromis aurea*), one of the most popularly farmed fish species around the world. These date provide a wider base of knowledge on the primary structure and the gene expression of fish c-*ski*.

MATERIALS AND METHODS

RNA Isolation and RT-PCR (Reverse Transcription PCR)

RNAs were extracted from the whole bodies of tilapia fry (1.6 cm) using TRI reagent (Molecular Research Center). Polyadenylated RNA (0.2 μg) isolated with a poly(A) mRNA Isolation System (Promega) was reverse-transcribed using an oligo$(dT)_{12–18}$ primer and Super-Script reverse transcriptase (BRL). The single strand cDNA products were subjected to PCR with primers 1′F (5′ATCTSTGCTTYGTGGTGGGMGGMGA3′) and 1′R (5′ATCTSTGCTTYGTGGTGGGMGGMGA3′), which were designed from the predicted well-conserved sequences of exon 1 in chicken (Sutrave and Hughes,

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1989), human (Nomura et al., 1989) *Xenopus* (Sleeman and Laskey, 1993) c-*ski*. PCR was carried out for 34 cycles in a 50 µl solution containing 500 ng of the oligo(dT)$_{12–18}$, 5 µl of 10× PCR reaction buffer, 1 µl 2.5 mM dNTP, 3 µl 25 mM $MgCl_2$, 10 µl of each amplifier and 2.5 U Taq polymerase (Promega). Each cycle consisted of 94°C, 1 min; 65°C, 1 min; and 72°C, 2 min. After the RT-PCR product was cloned and sequenced (Sanger et al., 1977), another RT-PCR was performed using primers E-1–5′ (5′GAAACGGCTGTGTCTGCCGCAGATTCT3′) and P2R (5′AGGYTTCTCYTCYTGYTTCAC3′). Primer E-1–5′ was derived from the sequence of the first RT-PCR product using primers 1′F and 1′R. Primer P2R was the most well-conserved sequence of exon 6 of known c-*ski*.

Rapid Amplification of cDNA End (RACE)

For the 5′ end, the first strand of cDNA was synthesized with 3 µg of total RNA using SuperScript II (BRL) in the presence of 0.1 µM of primer 5′RA1 (5′GGTCCGCTGCACCTGG3′). The cDNA was homotailed with poly(dA) at the 5′ end using 18 U of terminal transferase Tdt (BM). The resulting tailed cDNA was then used to generate double stranded cDNA by amplification in the presence of 200 nM of each primer RAAP (5′GGCCACGCGTCGACTAGTAC(T)$_{18}$3′) and 5′RA2 (5′GTCGCACACCGAGTTGATCTG3′), 200 µM dNTP, and 1 U Taq polymerase (Promega). The PCR reaction was run over 30 cycles of: 1 min at 94°C, 1 min at 64°C and 2 min at 72°C. The first PCR product were specifically amplified using a nested PCR performed with the RAUAP primer (5′GGCCACGCGTCGACTAGTACT3′) and an internal 3′ oligonucleotide 1–5′RA3 (5′GGGAGAAATCTCTGAGAACGC3′; nucleotide 859–880) for t*ski*1 or 2–5′RA3 (5′GAAGTCCCGCAGCACCGTGTT3′; nucleotide 709–687) for t*ski*2 under above PCR conditions. The procedure for 3′ RACE was basically as same as that for 5′ RACE except that (1) primer RAAP was used to synthesize the first strand of cDNA; (2) the RAUAP primer with either 5′CATGGCTCGGGCTCAGGAGAGG3′ for t*ski*1 or 5′CGGTGTCAGCACCAGCCCCT3′ for t*ski*2 were used to generate double strand cDNA; (3) the annealing temperature was 70°C; and (4) the RAUAP primer with either 5′CTTGACACCAAGGAGGCGCG3′ for t*ski*1 or 5′GAGGCAGGCGCTGGACAGC3′ for t*ski*2 were used in a nested PCR amplification.

Deduced AA Sequence Analysis and Comparison

The deduced AA sequences of c-Ski from previously investigated species were retrieved from the GenBank (NCBI blast database search) for comparison. Alignment of the sequences was carried out by using the CLUSTAL 4 program (Higgins and Sharp, 1988) of DNASIS (Hitachi software). The weight matrix method (Bishop, 1994) based on the eukaryotic translation initiation context (Kozak, 1978) was used to analyze the initiation codon of TSki1 polypeptide. Chou-Fasman (Chou and Fasman, 1978), Garnier-Osguthorpe-Robson (Garnier et al., 1978) and hydrophobic moment methods (Eisenberg et al., 1984) were used to analyze the secondary structure of T*ski* polypeptides.

Genomic DNA Extraction and Southern Blot Analysis

Genomic DNA was extracted from red blood cells of tilapia (Sambrook et al., 1989). For the Southern blot analysis (Sambrook et al., 1989), 10 µg of genomic DNA were digested respectively with *Bam*HI, *Eco*RI, *Pst*I, and *Sac*I, and hybridized to a [α-^{32}P]dCTP-labeled class-specific probe, which was obtained by digesting plasmid containing RT-PCR product of the t*ski*1 (1290 bp) or t*ski*2 (1452 bp) fragment with *Nco*I and *Sep*I, respectively. Hybridization was carried out before autoradiography on X-ray film (Kodak BioMax MS-1) for 16 hr at −70°C.

Specific Primers for Amplifying tski1 and tski2 From Genomic DNA

Class-specific primers were used to detect the t*ski*1 and t*ski*2 genes, respectively. For t*ski*1 detection, forward primer 5′GAGTGAGTGGGGAGACAG3′ and reverse primer 1–5′RA3 were used, whereas t*ski*2 detection used forward primer 5′TCCGGGACTGCAACAAACT3′ and reverse primer 2–5′RA3. PCR was carried out 2.5 U VioTaq DNA polymerase (Viogene), and consisted of 25 cycles at 94°C for 1 min, annealing at 58°C for t*ski*1 or 64°C for t*ski*2 for 1 min, and extension at 72°C for 1 min. The expected M_r of the generated PCR products were 688 and 305 bp for t*ski*1 and t*ski*2, respectively.

RNase Protection Assay

The 1.3 and 1.4 kb PCR products containing cDNA sequences corresponding to nucleotide 831–2129 of t*ski*1 and nucleotide 659–2120 of t*ski*2 were inserted into the pGEM-T (Promega) and linearized at the *Xho*I and *Esp*I sites to generate two riboprobes of 517 and 340 bp, which were respectively designed to protect a 453 bp t*ski*1 mRNA fragment and a 244 bp t*ski*2 mRNA fragment. In vitro transcription for t*ski*1 and t*ski*2 were performed with 20 U of T7 and SP6 RNA polymerase (Promega), respectively, in the presence of 50 µCi [α-^{32}P]UTP. Total RNA samples (20 µg) were extracted from oocytes, stage 15 embryos (freshly hatched embryos), stage 23 larvae (complete yolk-absorption), fry (1-, 2-, and 4-cm body length) and various tissues (brain, eye, gill, heart, intestine, skeletal muscle, stomach, ovary, and testis) using TriZol reagent, and then hybridized overnight at 42°C with 10 µg of [α-^{32}P]UTP-labeled probe with a 5×10^8 cpm/µg specific activity. RNA-RNA hybrids were digested with RNase A (0.05 U) and T1 (20 U) (Ambion) and analyzed in a 5% (w/v) polyacrylamide gel. The dried gel was exposed to X-ray film for 2 hr at –70°C. For normalization purposes, tilapia β-actin mRNA hybridized with a β-actin specific riboprobe served as an internal control. RT-PCR was used to amplify a DNA fragment from total RNA of tilapia larva by using oligo(dA)$_{12–18}$ primer, forward primer 5′TGCGGTATCCATGAGACCAC3′ (nucleotide

2883–2903) and reverse primer 5′GAAGCATTTGCGGTGGACGA3′ (nucleotide 3295–3275). The last two primers were corresponding to exons 5 and 6 of the common carp β-actin genomic DNA (Liu et al., 1990). The 312 bp RT-PCR product was subcloned into the pGEM-T vector and linearized at the *Dde*I site to generate the riboprobe using T7 RNA polymerase. For quantifying the varying levels of *ski* expression in the tissues, the image of RNase protection assay data was taken from Kodak DC120 Zoom Digital Camera. Kodak Digital Science 1D Image Analysis Software was use to analyze the mean intensity. The total sum of intensity of two protection fragments resulting from β-actin probe were counted to normalize.

RESULTS

Two Classes of Products Produced by RT-PCR

A 350-bp DNA fragment was amplified after RT-PCR using primers 1′F and 1′R. After subcloning and sequencing, a forward primer (E-1–5′) specific for exon 1 of tilapia c-*ski* was designed to perform another RT-PCR with a reverse primer P2R. Two DNA fragments around 1.3 and 1.5 kb were shown on the gel. The identities of the nucleotide sequence and deduced AA sequence between these two clones were 76.2 and 53.5%, respectively, and we therefore named the 1.3 kb clone t*ski*1 and the 1.5 kb clone t*ski*2.

Primary Polynucleotide Sequences and Deduced AA of Tilapia c-Ski DNAs

Figure 1 depicts the full length polynucleotide sequences and deduced AA sequences of tilapia t*ski*1 and t*ski*2 cDNA. t*ski*1 cDNA contains a single open reading frame (ORF) of 2061 base (nucleotide 541–2601) encoding 687 AA residues. The stop codon TAA is followed by an untranslated region of 572 nucleotides at the 3′ end, in which two potential instability sequences (ATTTA) (Sleeman and Laskey, 1993) and a potential polyadenylation signal are included. t*ski*2 cDNA contains an single ORF of 2057 (nucleotide 370–2511) bases which encode 714 AA residues. At the 3′ end, one potential instability sequence and two potential polyadenylation signals are included.

Comparison of the Deduced AA Sequence of Tilapia TSki1 and TSki2 With c-Ski and SnoN of Some Other Known Species

In Figure 2, the deduced AA sequences of tilapia TSki1 and TSki2 are compared with those of the Ski proteins of other species. The shared identity between tilapia TSki1 and TSki2 is 57.2%. Tilapia TSki1 shares 59.7% identity with chicken c-Ski, 56.9% with human, and 57.1% with *Xenopus* Ski proteins. The corresponding shared identities for tilapia TSki2 are respectively 66.5, 62.7, and 61.3%. *ski*-related cDNA, sno, has been reported in humans (Nomura et al., 1989) and chickens (Boyer et al., 1993). However, the AA identity between human SnoN and tilapia TSki1 and TSki2 was as low as 38 and 39%, respectively. The comparison data also reveal that highly conserved domains are located at the termini, exon 1 (AA 1 to 298 of TSki1) and exons 6 and 7 (AA 489 to 655 of TSki1). In contrast, the middle segment of tilapia TSki protein is a highly variable region. Several characteristics of Ski protein found in humans, chickens, and *Xenopus* are also conserved in tilapia TSki polypeptides. Both TSki1 and TSki2 contained heptad repeats (Nagase et al., 1993), a 25-mer repeat element and an alternating hydrophobic-basic motif at the C-terminus (Sleeman and Laskey, 1993).

Two tski Genes Detected by Southern Blot Analysis and PCR

Genomic DNA was isolated from tilapia blood, digested with four restriction enzymes, and analyzed using Southern blot hybridization with probes for t*ski*1 and t*ski*2, respectively. The resulting restriction fragment analysis profiles were different (Fig. 3A), and only two common bands were found: an 8 kb *Bam*HI-band and a 27 kb *Eco*RI-band. Genomic DNA was extracted from individual fish and served as a template for PCR amplification using class-specific primers. PCR product around 700 bp was generated using the t*ski*1-specific primers, while the t*ski*2-specific primers generated a product of around 300 bp (Fig. 3B).

Expression of tski1 and tski2 Genes in Various Tissues and During Development

Tilapia t*ski* genes were expressed in all tissues examined, with the brain and the skeletal muscle containing the most and the least abundant c-*ski* mRNAs of both classes, respectively (Fig. 4A). In general, tissues such as the heart, intestine, and brain had more t*ski*2 transcript than t*ski*1, and in the testes, where t*ski*2 gene was also expressed dominantly, expression of the t*ski*1 gene was extremely rare. In contrast, in the ovary, t*ski*1 transcript was much more abundant in expression than the t*ski*2 transcript. Figure 4B shows that during development the expression of t*ski*1 appeared to undergo a stepwise decline as embryos developed, with a rapid decline over the hatching stage and relatively little change otherwise. However, the expression of t*ski*2 showed exclusively a high peak at the hatching stage. Consequently, the quantitative ratio of t*ski*1 to t*ski*2 decreased as the tilapia grew to fry of 4-cm body length.

DISCUSSION

Northern blot analysis of c-*ski* expression showed that there were two major mRNA species in chickens (Li et al., 1986) and humans (Nomura et al., 1989). This evidence in turn suggests that there might be more than one species of the c-*ski* gene, resulting either from an alternative splice and/or from two independent genes. Three classes of chicken c-*ski* cDNA have been found that the multiple cDNAs were derived from alternative splicing (Sutrave and Hughes, 1989). Sleeman and Laskey (1993) also detected two distinct fragments on Southern blot analysis of *Xenopus* genomic DNA at high stringency, but when the two respective genes were cloned out and sequenced, the

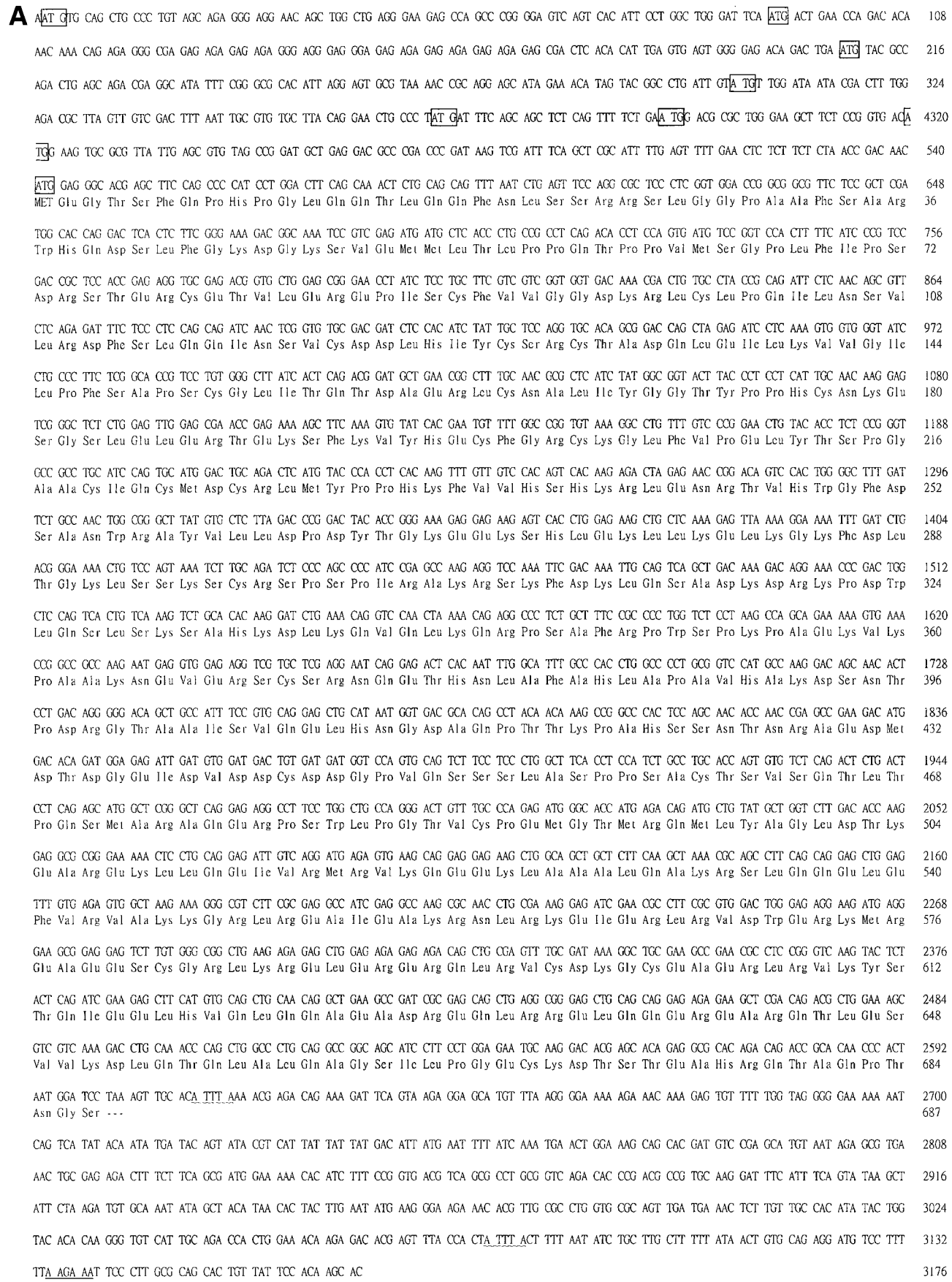


Fig. 1. Polynucleotide sequences of tilapia t*ski*1 (**A**) and t*ski*2 (**B**) cDNA and their deduced AA. The nucleotides are numbered beginning with the first nucleotide at the 5′-end, and where it appears, the second smaller number indicates the order of the AA position. The potential initiation codons in the 5′ region are boxed. The potential instability sequences (rapid decay sequences, Sleeman and Laskey, 1993) underscored with wavy lines and the possible polyadenylation signals are underlined.

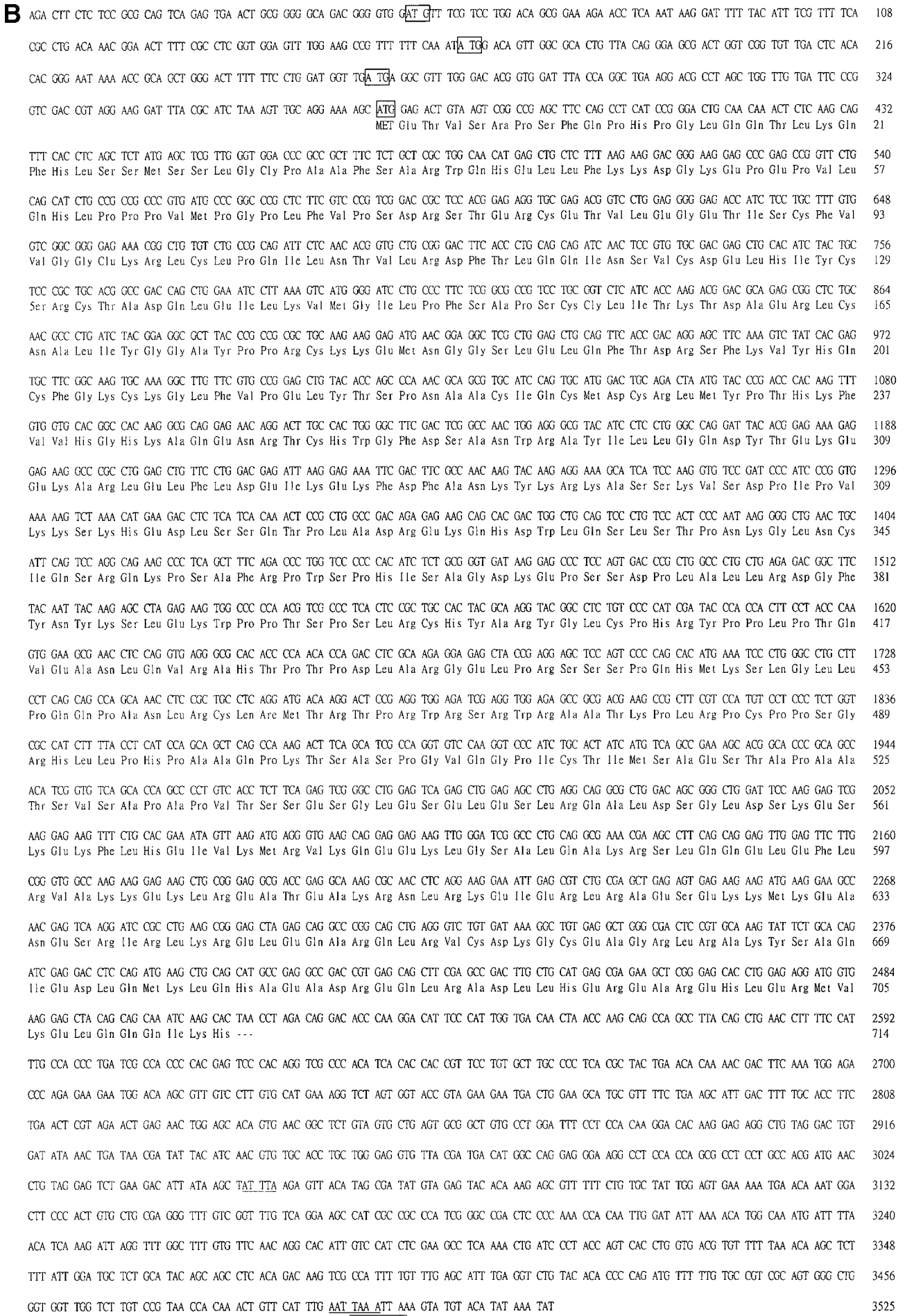


Figure 1. (Continued.)

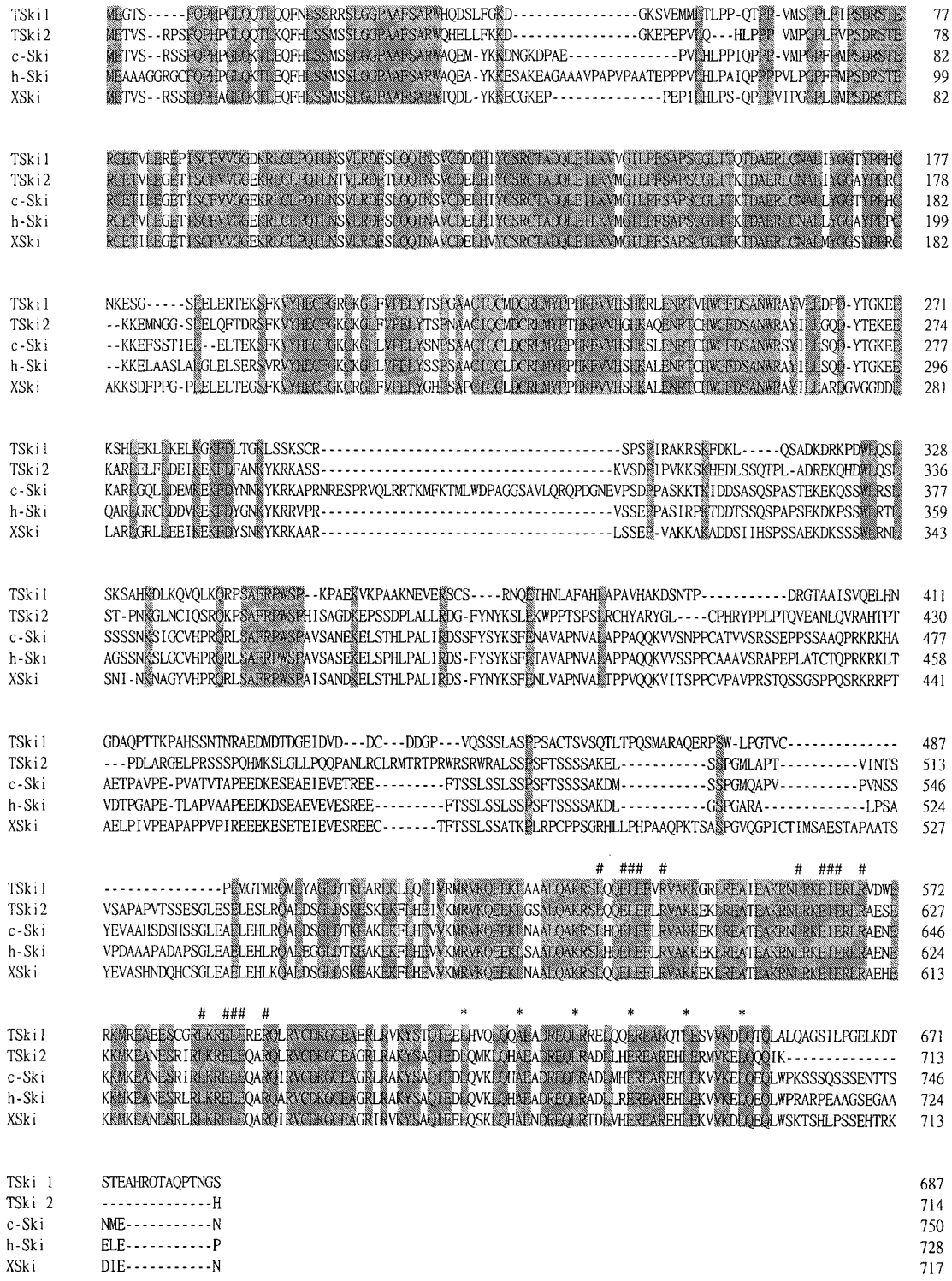


Fig. 2. Comparison of the deduced AA sequences of tilapia TSki proteins (TSki1 and TSki2) with those of chickens (c-Ski), humans (h-Ski) and *Xenopus* (XSki) Ski proteins. The numbers start at the first AA residue. Identical AA residues are boxed in shadow. Dashed lines represent gaps inserted to maximize the identity among all of the sequences compared. The well-conserved AA residues at the tandem repeat domain and at the leucine zipper domain are indicated by hashes (pound signs) and asterisks, respectively.

difference between them was only an extra C residue at position 848. Such a slight difference, however, cannot explain why two RNA bands of about 3.4 and 3.9 kb were detected when X*ski*1 was used as a probe in a Northern blot analysis (Sleeman and Laskey, 1993); on the contrary, this latter evidence suggests that another class of *Xenopus* c-*ski* remains to be discovered. The present study is the first that clearly demonstrates that

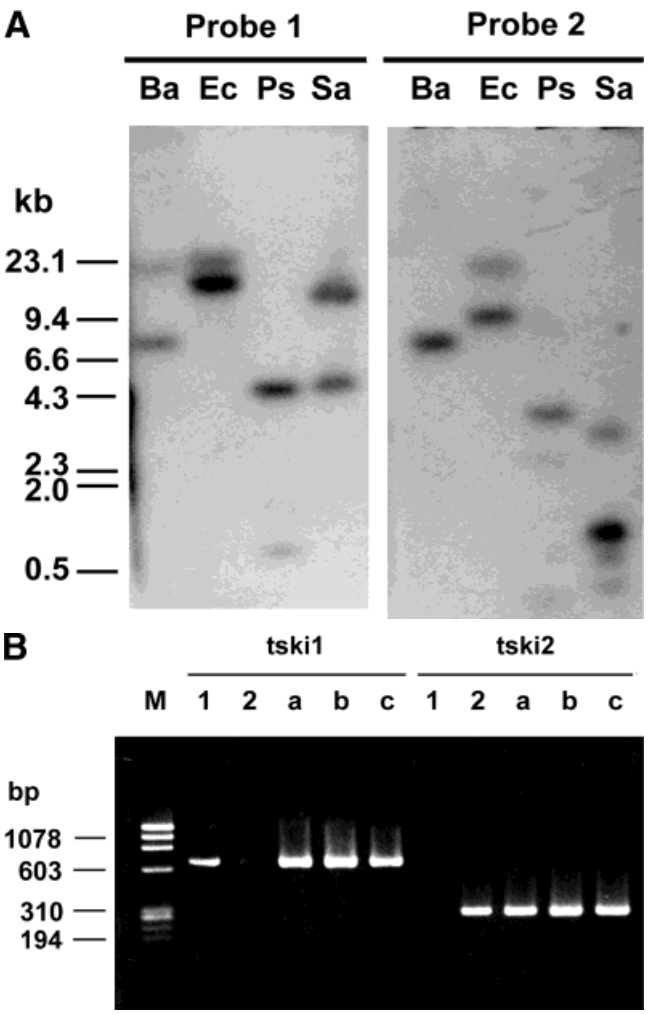
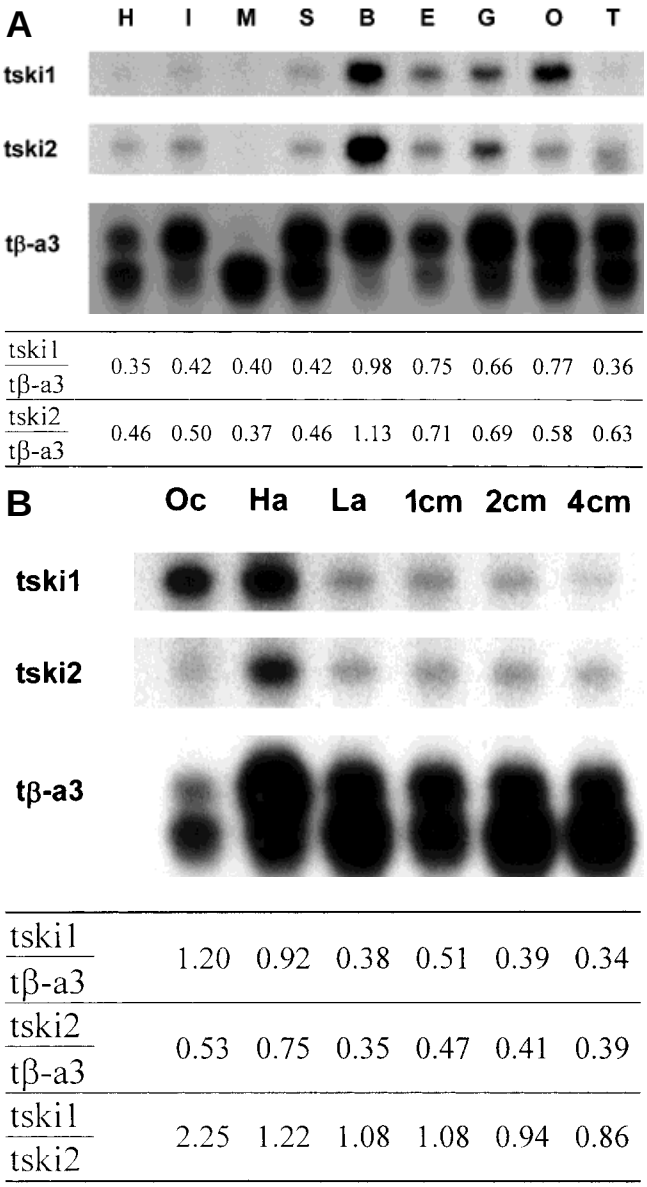


Fig. 3. (**A**) Southern blot analysis of enzyme-digested genomic DNA isolated from red blood cells of tilapia fry. *Bam*HI (Ba)-, *Eco*RI (Ec)-, *Pst*I (Ps)- and *Sal*I (Sa)-digested genomic DNA were electrophoresed on a gel, transferred to the nylon membrane and hybridized with [α-^{32}P]dCTP-labeled probe 1 for t*ski*1 and probe 2 for t*ski*2. Hybridization was carried out in a solution (Sambrook et al., 1989) of 50% formamide, 5× Denhardt's solution, 0.5% SDS, 6× SSPE and 100 mg/ml fragmented calf thymus DNA at 42°C for 16 hr with a specific activity of 10^8 cpm/μg. Membranes were washed in 2× SSC and 0.5% (w/v) SDS for 25 min at room temperature, 2× SSC and 0.1% SDS for 20 min at room temperature, followed by another washing in 2× SSC and 0.5% SDS for 20 min at 37°C. Molecular sizes in kilobase pairs (kb) were given by *Hin*dIII-digested lambda. (**B**) PCR analysis for the presence of t*ski*1 and t*ski*2 in tilapia genome. Genomic DNA was isolated from three individual tilapia fry (lanes a–c) and amplified using specific primers for t*ski*1 and t*ski*2, respectively. Lane M, the molecular marker, *Hin*dIII-digested lambda genome; lane 1, plasmid containing t*ski*1 cDNA insert; and lane 2, plasmid containing t*ski*2 cDNA insert.



tski1 / tβ-a3	0.35	0.42	0.40	0.42	0.98	0.75	0.66	0.77	0.36
tski2 / tβ-a3	0.46	0.50	0.37	0.46	1.13	0.71	0.69	0.58	0.63

tski1 / tβ-a3	1.20	0.92	0.38	0.51	0.39	0.34
tski2 / tβ-a3	0.53	0.75	0.35	0.47	0.41	0.39
tski1 / tski2	2.25	1.22	1.08	1.08	0.94	0.86

Fig. 4. RNase protection assay for tilapia t*ski*1 and t*ski*2 transcripts in (**A**) different tissues from adult fish, and (**B**) different developmental stages. The fragment sizes of t*ski*1 and t*ski*2 mRNAs protected by t*ski*1- and t*ski*2-specific antisense RNAs were 453 and 244 bp, respectively. The protection fragments' size of tilapia β-actin serving as internal control were 197 and 163 bp. H, heart; I, intestine; M, skeletal muscle; S, stomach; B, brain; E, eye; G, gill; O, ovary; T, testis; Oc, oocytes; Ha, freshly hatched embryos (stage 15); La, larva at stage 23 (yolk absorption); lanes 1, 2, and 4 cm, fry with 1, 2, and 4 cm body lengths, respectively. Quantified expression levels of *ski* gene (see Materials and Methods) are presented in tables under the figure as t*ski*1 over β-actin (tski1/β-actin), t*ski*2 over β-actin (tski2/β-actin) and t*ski*1 over t*ski*2 (tski1/ tski2).

fish have two distinct c-*ski* genes. These two genes have their own expression patterns of both in tissues and during development.

Southern blot analysis of tilapia genomic DNA showed that the restriction patterns for the t*ski*1 and t*ski*2 probes were not identical. The t*ski*1 and t*ski*2 probes share common DNA sequences at both ends, corresponding to exon 1 and exons 6 and 7 of c-*ski*, but vary in the middle segment. The positive bands of the same M_r (Fig. 3A) may thus be accounted for by the fact that the two probes may hybridize with a common fragment, although each probe would also hybridize with its own specific bands. The different restriction fragment profiles suggest that there might be two independent c-*ski* genes. This hypothesis is supported by the PCR results: PCR products of 688 and 305 bp were generated from

each individual fish when t*ski*1- and t*ski*2-specific primers were respectively employed. All of this evidence strongly suggests that two independent but non-allelic c-*ski* genes exist in the tilapia genome. Although Northern blot analysis has commonly been used to study the expression and distribution of the c-*ski* gene in many previous studies, cross-hybridization may prevent this method from being sensitive enough to discriminate between two similar c-*ski* mRNAs. We therefore used an RNase protection assay with a class-specific RNA probe. The two classes of tilapia t*ski* mRNAs are differentially expressed in some tissues and at certain developmental stages. Thus, like *Xenopus* X*ski*1, which is preferentially expressed in the female gonads of *Xenopus* (Sleeman and Laskey, 1993), tilapia t*ski*1 is expressed at a high level in the ovary, but at a low level in the testes. Surprisingly, however, we found that the tilapia t*ski*2 transcript was extremely rare in the ovary, but absolutely predominant in the testes, an observation that has not previously been reported. In fact, the tilapia t*ski*1 is maternally inherited, and in this respect it is similar to X*ski*1 transcripts, which are maternally regulated during early development (Sleeman and Laskey, 1993). Thus as the tilapia embryos develop from oocytes to 4-cm fry and the t*ski*2 transcripts increase, the tsk1 transcripts decline, presumably because of the degradation of the maternally-inherited stockpile of t*ski*1 mRNA. It should therefore be very worthwhile to investigate what roles tilapia TSki1, an ovary-dominant class, and TSki2, a testis-dominant class, might play in gonad development.

Grimes et al. (1993) reported that in any cell a complete absence of c-*ski* expression is unlikely or extremely rare. Namciu et al (1995) demonstrated that in mouse embryos at 12.5 days of gestation c-*ski* was expressed at a high level in the skeletal muscle. On the other hand, some reports stated that c-*ski* is widely expressed in many different tissues, but they were nevertheless unable to detect either chicken or *Xenopus* c-*ski* expression within skeletal muscle (Sleeman and Laskey, 1993; Engert et al., 1995). The present study demonstrated that either t*ski*1 or t*ski*2 gene expression is extremely rare in the skeletal muscle of adult tilapia. Tilapia c-*ski* transcript can also be detected in the heart, which is consistent with Claycomb and Lanson's (1987) report that c-*ski* was expressed in rodent cardiac tissue.

Nagase et al. (1993) reported a three tandem repeat of 25 AA at positions 571–645 in human c-Ski. Sleeman and Laskey (1993) stated that *Xenopus* c-Ski contained a novel sequence of four contiguous 25-mer repeat elements at positions 527–664 which form a pattern of five hydrophobic, one acidic and two basic residues. Meanwhile, Zheng et al. (1997) demonstrated that chicken c-Ski contains five imperfect tandem repeats of 25-mer and formed hydrophobic "bottoms." They proposed that the core consensus of the repeat motif was LXXELEXLR. In this study, however, we found that actually the last three 25-mer repeats (located at AA 535–593 in tilapia TSki1) are quite well-conserved in the c-Ski proteins of humans, chickens, *Xenopus,* and fish. This fact strongly suggests that LXXELEXXR would more accurately characterize the core consensus sequence of the tandem repeat element at the C-terminus of c-Ski. In this context it is also interesting to note that compared to TSki2, the AA composition of TSki1 is much less similar to the previously known c-Ski proteins. In addition, TSki1 is the only c-Ski protein that lacks the AA 509–529 segment that appears in TSki2. Thus, in TSki1, the first sequence of the five 25-mer tandem repeats is not perfectly conserved.

Based on a computer analysis of the predicted secondary structures of tilapia TSki, we found that the C-terminus (AA 522–659 for TSki1 and AA 539–714 for TSki2) of both classes of TSki encompasses an amphipathic α-helical domain, which has a tendency to form a coiled-coil structure. These are characteristics that are conserved in human, chicken, and *Xenopus* c-Ski sequences, presumably because they are structurally significant and functionally important in Ski proteins. At the C-terminal leucine zipper domain of the Ski protein, there is a periodically arranged AA element, LALELL, which is responsible for dimerization (Nagase et al., 1993) and is also essential for cooperation with the NF1 family of transcription factors (Tarapore et al., 1997). Tilapia TSki1 and TSki2 display a LALELL array at the C-terminus, located in precisely the same position reported for other c-Ski proteins. Because this heptad repeat is well-conserved among Ski proteins, it is highly likely that this sequence may also play an important role in forming the coiled-coil structure of fish TSki polypeptides.

Although t*ski*2 mRNA contains four separate AUGs within the 370-bp segment of the predicted mRNA 5′-leader, three of these, AUG-1, AUG-2, and AUG-3, located at nucleotide 50, 165, and 253, would terminate after translation of only 20, 19, and 40 residues, respectively. Thus, we propose that tilapia TSki2 starts at the AUG located at nucleotide 370. t*ski*1 mRNA, meanwhile, contains eight AUGs within a 540-bp segment of the predicted untranslated region, but again, the reading frame beginning with five of these eight AUGs would give ORF of only a few AA. This leaves AUG-2, AUG-3, and AUG-8, which might plausibly terminate t*ski*1 cDNA. These AUGs all keep the same ORF and share the same stop codon. Since authentic tilapia TSki1 has not yet been purified, the first AA cannot be conclusively determined. However, we propose that the initiation codon of t*ski*1 mRNA is more likely located at nucleotide 541 (AUG-8), as evidenced from the weight matrix analysis (Bishop, 1994). In this analysis, the score of the AUG-2 sequence (GATTCAATG) was −0.56. The corresponding score for AUG-3 (AGACTGAATG) was 0.72, while the AUG-8 sequence (GACAACATG) scored highest with +6.85. Furthermore, a comparison of the known Ski proteins of humans (Nomura et al., 1989), *Xenopus* (Sleeman and Laskey, 1993) and chickens (Sutrave and Hughes,

1989), also suggested that the initiation codon most likely starts at AUG-8.

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