Novel expression of the stanniocalcin gene in fish

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Abstract

It is currently accepted that the fish stanniocalcin (STC) gene is expressed exclusively in the corpuscles of Stannius (CS), unique endocrine glands on the kidneys of bony fishes. In this study, we have re-examined the pattern of fish STC gene expression in the light of the recent evidence for widespread expression of the gene in mammals. Surprisingly, we found by Northern blotting that the fish gene was also expressed in the kidneys and gonads, in addition to the CS glands. Moreover, Southern blotting of RT-PCR products revealed STC mRNA transcripts in all tissues assayed, including brain, heart, gill, muscle and intestine. In situ hybridization studies using digoxigenin-labeled riboprobes localized STC mRNA to chondrocytes, and both mature and developing nephritic tubules. Immunocytochemical staining indicated that the STC protein was widespread in cells of the gill, kidney, brain, eye, pseudobranch and skin. We also characterized the salmon STC gene, establishing that it was comprised of five exons as opposed to four in mammals. A single transcription start site was identified by primer extension 99 bp upstream of the start codon. This is the first evidence of STC gene expression in fish tissues other than the CS glands and suggests that, as in mammals, fish STC operates via both local and endocrine pathways. 


Introduction

Stanniocalcin (STC) is a homodimeric glycoprotein hormone with an integral role in calcium and phosphate homeostasis in fishes (So & Fenwick 1977, Lafeber & Perry 1988, Wagner 1994, Hulova & Kawauchi 1999). STC was originally identified in endocrine glands known as the corpuscles of Stannius (CS), which were discovered in 1839 (Stannius 1839). The CS are unique to bony fish, and in salmonids are morphologically distinguishable as cream colored bodies situated midway along the ventral surface of the kidneys. STC in fish functions to prevent hypercalcemia and is released into the bloodstream in response to elevations in serum Ca^{2+}. In gills and gut, the hormone inhibits Ca^{2+} uptake, thereby lowering Ca^{2+} entry into the extracellular compartment (Fontaine 1972, Fenwick & So 1974, Sundell et al. 1992), whereas in kidney it stimulates phosphate reabsorption in order to chelate excess Ca^{2+} in the serum (Lu et al. 1994).

Up until the mid 1990s, it was believed that STC was produced uniquely in fish corpuscles of Stannius. It was only recently established that STC was not only present in mammals, but also that the mammalian hormone was much more widely expressed than in fishes. In mammals the highest levels of STC gene expression occur in the ovary, prostate, kidney and thymus, with lower levels detectable in numerous other tissues (Wagner et al. 1995, 1997, Olsen et al. 1996, Varghese et al. 1998). The complete spectrum of STC actions in mammals has yet to be established, but as is the case in fishes it appears to be involved in mineral metabolism. Mammalian STC promotes phosphate reabsorption in both intestine and kidney, while inhibiting intestinal calcium uptake (Olsen et al. 1996, Wagner et al. 1997, Madsen et al. 1998) both of which could serve to lower serum ionic calcium levels. In addition, STC expression is upregulated in neurons during cerebral ischemia (Zhang et al. 2000), in MDCK cells during hypertonic stress (Sheikh-Hamad et al. 2000), in human endothelial cells during differentiation (Kahn et al. 2000) and during mammalian development (Stasko & Wagner 2001). Interestingly, STC only circulates in mammals during pregnancy (Deol et al. 2000), suggesting that many of the reported effects are likely brought about by local hormone signaling through autocrine or paracrine pathways. This study is a re-examination of STC production in fish in the light of what is now known of the hormone in mammals. We have characterized the fish STC gene and established that, as in mammals, the fish STC gene is expressed in a multitude of different tissues, suggesting that STC signals via local pathways in fish in addition to its well characterized endocrine route of hormone delivery.
Materials and Methods

Tissues and reagents

All chemicals and solvents used were of reagent or molecular biology grade as appropriate to the procedures employed. Salmon STC was purified from the CS glands as previously described (Wagner et al. 1986). A polyclonal antiserum to salmon STC was used for the immunological procedures described below. This antiserum has already been characterized as being highly specific for fish STC by Western blotting, immunocytochemistry and radio-immunooassay (Wagner et al. 1995). Rainbow trout of mixed sex and age (0·1–2 kg) were obtained from a local hatchery (Rainbow Springs, Thorndale, ON, Canada). Animals were killed by severing the spinal cord, and dissected tissues were either frozen directly on dry ice or fixed overnight in 4% paraformaldehyde containing 0·15 M NaCl and buffered to pH 7·4 with 0·01 M sodium phosphate. Fixed tissue was embedded in paraffin, sections were cut at a thickness of 6 µm and mounted on frosted glass microscope slides (Fisher, Canada).

Genomic library screening

Salmon STC genomic clones were isolated from a sockeye salmon genomic library prepared in Lambda Fix vector (Stratagene, La Jolla, CA, USA) which was kindly provided by Dr Bob Devlin, Department of Fisheries and Oceans, West Vancouver, BC, Canada. The library was plated and screened with 32P-labeled salmon STC cDNA probes under high stringency conditions using established methods (Wagner et al. 1992). A total of 5 × 1010 plaque forming units were plated, transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) and hybridized with a random primer (Life Technologies, USA) [γ-32P]dATP (3000 Ci/mmol) labeled STC cDNA in 5 × SSC (0·75 M NaCl/0·075 M sodium citrate), 40% formamide, 1 × Denhardt’s solution (0·02% each of Ficoll, polyvinylpyrrolidone and BSA), 0·02 M Tris – HCl, pH 7·6, 0·1% SDS at 42 °C, with final filter washes in 0·5 × SSC, 0·1% SDS at 55 °C. Positive clones were purified to homogeneity, subcloned into pBSII (Stratagene) or pGEM vectors (Promega, Madison, WI, USA) and analyzed by restriction enzyme digestion and Southern blot hybridization using sub-fragments of the salmon STC cDNA to identify exon-containing gene fragments. These fragments were then subcloned and sequenced using the T7 Sequenase v2·0 kit (Amersham, Arlington Heights, IL, USA) and an Applied Biosystems automated sequenator.

Primer extension

Total RNA was prepared with Trizol or by the single step guanidium method of Chomczynski and Sacchi (1987). Poly(A)+ RNA was isolated from total RNA by column chromatography, using oligo(dT) cellulose, following the manufacturer’s instructions (Life Technologies, Burlington, ON, Canada). Purified poly(A)+ RNA was solubilized in deionized formamide, quantified spectrophotometrically, and stored at −76 °C. For primer extension, 0·1 µg of a 19 base oligonucleotide (5’ ATGGGATAGGTTGGACAGG 3’), complementary to bp −44 to −29 of the salmon STC cDNA was end-labeled with [γ-32P]ATP (50 µCi) using 10 units T4 polynucleotide kinase (Promega) at 37 °C for 45 min. The labeled probe was separated from unincorporated isotope using a MERmaid kit (Bio/Can Scientific, Burlington, ON, Canada). The labeled probe (5 × 105 c.p.m.) was annealed to 1 µg poly(A)+ RNA from muscle, 10 µg CS total RNA, and 25 µg yeast tRNA (Life Technologies) as a negative control, or for 90 min at 65 °C in 1 × hybridization buffer (0·15 M KCl, 0·01 M Tris–HCl, pH 8·3, 0·001 M EDTA). The reaction was gradually cooled to 42 °C over 30 min, and primer extension was performed with 20 U Superscript II reverse transcriptase (Life Technologies) for 60 min at 42 °C in 30 µl extension buffer (0·023 M Tris–Cl, pH 8·3, 10 mM MgCl2, 5 mM dithiothreitol (DTT), 2·25 µg actinomycin D, 150 mM dNTP mix). Reactions were stopped by the addition of 155 µl stop buffer (0·1 µg/µl yeast tRNA, 0·2 M sodium acetate, pH 4·8, 0·01 M Tris–Cl, pH 8·0, 0·005 M EDTA), followed by 0·5 µl of 10 mg/ml RNase and then incubated at 37 °C for 20 min. After extraction, cDNAs were resuspended in 7 µl sequencing reaction stop buffer (95% formamide, 20 mM EDTA, 0·05% bromophenol blue, 0·05% xylene cyanol) and resolved on a denaturing 6% polyacrylamide gel. Sequencing reactions using the same 19 bp oligonucleotide primer were performed on a salmon STC genomic clone containing exon 1 and 4 kb of 5’ flanking DNA using a 70770 Sequenase version 2·0 kit (USB, Cleveland, OH, USA), and loaded in lanes adjacent to the primer extension reactions for estimation of product size. The gel was dried and autoradiography was carried out at −76 °C with an intensifying screen and Reflection NEF- 495 film (NEN Research Products, USA) for 50–250 h.

Southern and Northern blot analysis

For Southern blot analysis, genomic DNA was isolated from the testes of Chinook and Coho salmon, rainbow trout and Arctic char (sockeye salmon was unavailable). Tissue was pulverized in liquid nitrogen and digested overnight (37 °C) in a solution of proteinase K (100 µg/ml in 0·1 M Tris, 0·1 M NaCl, 5 mM EDTA, 1% SDS, pH 8·0) at a concentration of 0·5 g tissue/ml. Following a treatment with RNase A (200 µg/ml for 15 min at 65 °C), the digest was extracted twice with phenol/chloroform (1:1) and chloroform/isoamyl alcohol (25:1). Aliquots of DNA (15 µg) were digested overnight with XbaI,
EcoRI and PstI. The digests were then subjected to electrophoresis in 0.8% agarose gels and blotted onto nitrocellulose (Amersham Life Sciences, UK). Prior to hybridization with radiolabeled probes, genomic blots were subjected to a 2-h period of pre-hybridization at 65 °C in 6×6×SCP (where 1× SCP consisted of 0.1 M NaCl, 0.03 M dibasic sodium phosphate and 1 mM EDTA, pH 6.2), 1% sarkosyl, 100 µg/ml salmon sperm DNA and 1× Denhardt’s solution. Blots were then hybridized overnight at 65 °C in the same solution containing 10% dextran sulfate and random primed, 32P-labeled salmon STC cDNA probes (pure insert; 2×106 c.p.m./ml). Blots were then washed 2×15 min in 6×6×SCP containing 1% sarkosyl at room temperature, 1×15 min in 1×SCP, 1% sarkosyl at 65 °C, air dried and exposed to X-ray film.

Northern blotting was performed using standard methods (Wagner et al. 1992). Poly(A)+ RNA was subjected to electrophoresis in 1% agarose/formaldehyde gels at concentrations of 5 µg/lane respectively (except CS where 0.1 µg/lane) and blotted onto Hybond-N nitrocellulose (Amersham Life Sciences, Oakville, ON, Canada). Blots were then UV-crosslinked (Stratagene Stratalinker). Following a 2-h pre-hybridization period (under the same conditions used for hybridization with probes), blots were probed overnight with a randomly primed 32P-labeled fish STC cDNA (2×106 c.p.m./ml) under conditions of high stringency (0.1 M NaCl, 0.08 M Tris–HCl, 0.004 M EDTA, pH 7.8, 10× Denhardt’s solution, 0.1% sodium pyrophosphate, 0.025 M sodium phosphate, pH 6.9 and 0.2% SDS; 65 °C). Blots were washed for 4×15 min in 2× SSC/0.1% SDS at room temperature, followed by 2×30 min in 0.1× SSC/0.1% SDS at 65 °C. Blots were then exposed to X-ray film (Eastman Kodak Co., USA) with an intensifying screen at −76 °C for 24–96 h.

Histological studies

In situ hybridization was performed to localize sites of STC gene expression in specific tissues. Hybridization was performed on 6 µm tissue sections mounted on Superfrost slides (Fisher, Irvine, CA, USA) with digoxigenin-UTP labeled (Roche, Laval, QC, Canada) sense and antisense riboprobes prepared by in vitro transcription of a salmon STC cDNA as described previously (Sterba et al. 1993).

A polyclonal antiserum against salmon STC was used for immunocytochemical localization of STC protein on adjacent tissue sections, using the Avidin–Biotin peroxidase method (Vector Laboratories Inc., Burlingame, CA, USA). Serial sections were sequentially dewaxed in xylene, rehydrated in descending concentrations of ethanol, incubated with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity, and blocked with normal goat serum to prevent non-specific binding. Slides were then incubated overnight with the STC antiserum at 1:1000 dilution at 4 °C. Control sections were incubated with pre-immune serum at the same dilution. The next day, slides were washed in 1× PBS and incubated for 2 h at room temperature with a biotinylated goat anti-rabbit antibody (Vector Laboratories Inc.), then washed with 1× PBS and incubated for another 2 h at room temperature with Avidin–Biotin peroxidase linked complex. The sites of antigen–antibody binding were visualized by adding 3,3’-diaminobenzidine tetrachloride (Sigma Chemical Co., St Louis, MO, USA) as the chromogen. Sections were counterstained with hematoxylin, dehydrated, then mounted with Micromount mounting media (Surgipath, Winnipeg, MB, Canada). Controls included the application of non-immune rabbit serum (NRS) and primary antiserum preabsorbed with STC (30 µg/ml) in lieu of primary antiserum alone.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was carried out on 10 µg total RNA isolated from fresh rainbow trout tissues using Trizol, as described earlier. To prepare the template for the reverse transcription reaction, RNA was added to 1 µg oligo dT, and incubated for 10 min at 70 °C. The reaction was placed on ice for 5 min to anneal the primer to the template. First strand synthesis was performed by adding the primer/template preparation to reverse transcriptase buffer at a final concentration of 50 mM Tris–HCl (pH 8.3 at room temperature), 75 mM KCl, 3 mM MgCl2, 0.01 M DTT, 0.5 M deoxyribonucleotide triphosphates (dNTPs), and 10 U/µl Superscript II reverse transcriptase (Life Technologies) in a total volume of 40 µl. The solution was mixed, quick spun, overlaid with 40 µl paraffin oil and incubated for 90 min at 43 °C. Following first strand synthesis reverse transcription, the reaction was incubated at 95 °C for 5 min to destroy the Superscript II enzyme.

Amplification of the cDNA was performed by adding 1 µl of the RT reaction to the PCR buffer in a final concentration of 10 mM Tris–HCl, pH 8.8; 50 mM KCl; 0.8% Nonidet P40, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 M 5’ primer, 0.2 mM 3’ primer in a total volume of 100 µl. The 5’ (CCT GTC CAA CCT ATC CCA TCG) and 3’ (TGT GTG TCA GTG TGC TGA) PCR primers (Life Technologies) were designed to amplify a 971 bp fragment of cDNA containing the entire coding region, 34 bp of the 5’ UTR, and 151 bp of the 3’ UTR spanning four introns, ensuring that any contaminating DNA, if amplified, would be a different size (~1500 bp) than the expected 1000 bp product. A hot start strategy was employed by adding 2.5 U normal recombinant Taq polymerase (MBI Fermentas, Burlington, ON, Canada) to the above PCR mix during the first cycle at 70 °C. The cycle profile consisted of 40 cycles of 95 °C for 30 s (5 min for initial denaturing), 60 °C annealing for 30 s, and 72 °C extension for 1 min (10 min final extension). RT-PCR products were subjected to agarose gel electrophoresis and

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Southern blotting as described above with the following changes. The prehybridization buffer and wash buffer for RT-PCR Southern contained SSC (sodium chloride, sodium citrate) rather than SCP (sodium citrate, sodium phosphate) and the blots were probed with a $^{32}$P-labeled cDNA spanning exons 1 and 2 instead of the full length cDNA used for genomic blots.

Results

Evidence for STC gene expression in multiple fish tissues

Northern blotting of poly(A$^+$) RNA revealed that STC was expressed in testes, ovary and anterior kidney in addition to the CS glands (Fig. 1). The principal message was estimated to be ~2 kb in length, with a second larger transcript of approximately 3 kb. The levels of expression in these other tissues were estimated to be some 100-fold lower than in the CS glands. Southern blot analysis of RT-PCR products confirmed the expression of STC in the kidney and gonads and also revealed low levels of transcription in all tissues assayed (Fig. 2). The expected product of ~1 kb was present in all tissue RNAs, while two smaller bands of ~600 and ~300 bp were evident in all tissues, excluding the liver and gill filament, which were at the limits of detection. These RT-PCR smaller products were also not observed in the CS and ovary lanes due to prior gel purification. The 1 kb product was confirmed as STC by DNA sequence analysis (results not shown).

Primer extension

The transcription start site of the fish STC gene was ascertained by primer extension analysis using RNA from salmon corpuscles of Stannius and skeletal muscle (Fig. 3A). Using a primer located 43 bp 5' to the initiator methionine, one cDNA product was obtained from CS RNA, yielding a 5' UTR of 99 nucleotides (Fig. 3B). Primer extension experiments utilizing muscle RNA and yeast tRNA yielded no products.

Salmonid STC gene structure

Two full length, salmon STC genomic clones were isolated, ranging in size from 13.0–13.3 kb. The sockeye STC gene locus spanned ~4 kb and was flanked by ~6.5 kb of 5' flanking and ~3 kb of 3' flanking DNA (Fig. 4). The fish gene consisted of five exons as follows: exon 1 encoded the 5' UTR (99 bp) and the first 39 residues of preproSTC (118 bp), followed by exon 2 (48 residues/143 bp), exon 3 (36 residues/108 bp), exon 4 (35 residues/104 bp) and exon 5 which encoded the last 98 amino acids (295 bp) as well as 1035 bp of the 3' UTR. These exons were separated by introns of 266bp (intron I), 122 bp (intron II), 294 bp (intron III) and 165 bp (intron IV) in length. In comparison with Coho salmon STC, there was 99.5% nucleotide identity, and 100% amino acid identity. A restriction map of the STC gene is provided in Fig. 5, denoting exon/intron boundaries and UTRs.
Figure 3  Primer extension analysis of trout CS total RNA, indicating the presence of a single transcriptional start site 99 bp upstream of the translational start site. A γ-32P end-labeled 19 bp oligonucleotide complementary to a portion of the 5′ untranslated region of salmon STC was used to perform primer extension on CS total RNA. Primer extension with yeast tRNA and trout muscle served as negative controls. (A) The size of primer extension products was determined by comparison with the adjacent sequencing reaction performed using the same primer. A single 75 nucleotide (nt) cDNA was produced corresponding to a transcription start site 99 bp upstream of the translation start site. (B) Nucleotide sequence of the region upstream of the first STC exon in rainbow trout. The translation initiation site is position +1. The transcription start site as determined by primer extension, is indicated by an arrow at position −99, and the entire 5′ UTR is italicized. Two putative TATA box elements are shown enclosed in boxes. The oligonucleotide primer is bold and underlined, and the amino acid coding sequence is capitalized.
1 atgctagaaagtgtgaaaaacggagttaatatgtattttgtcataaggtgtgtgataattctctgactctcactgtaatttac
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81 aagccagacattttgtactagtcgacgagccagagcagagtaccggaggtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgaatgg
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161 atcagttctctgctgactatctactttctacctgtctctgtgtctctgtgtcaagggataaggtgatcttctttt
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Comparative analysis of fish and mammalian STC genes

A comparative alignment of exons comprising the mouse and fish STC genes is shown in Fig. 6. Here it can be seen that exons 1 and 2 in both species are highly conserved in terms of size. However mammalian exon 3 has been subdivided into two exons in fish, exons 3 and 4, such that exon 5 in fish is synonymous with exon 4 in mammals. Histological studies

STC gene expression was clearly evident in discrete cell types in both kidney and cartilage. In the kidney, the level of gene expression was generally low yet detectable in proximal tubules, distal tubules and collecting duct segments of the nephron. Much higher levels, however, were evident in the cells of newly developing tubules.

Figure 4 (opposite and above) Sockeye salmon gene sequence. The gene consists of 5 exons that are indicated in bold, coding for 256 residues. The non-protein coding region of exon 1 is shown as deduced by primer extension. The 5' and 3' flanking regions are italicized, and the numbering of amino acids is based on the sequence of Coho salmon STC. Residue 34 (arrow) corresponds to the N-terminus of mature STC, and residues 62–64 (underlined) comprise the glycosylation consensus sequence. The termination codon (TAG) is indicated by an asterisk.

Figure 5 Restriction map of fish STC gene. Unique restriction sites are indicated above the gene, shown in black blocks representing exons. The gene contains 23 unique restriction sites. The 3' UTR is shown in grey, and the 5' flanking and intronic sequence in white. Exon and base pair numbering is given below the gene.
Figure 6  Deduced amino acid sequence alignment of salmonid and mouse STC. Alignment was performed using the Baylor College of Medicine's optimal global alignment with no short-cuts. Both sequences share nearly identical splicing boundaries, with the exception of the 3rd exon in the mouse gene, which in fish is encoded by 2 exons. Amino acid sequences show 48.7% identity (71% homology), the mouse and fish proteins consisting of 248 and 257 amino acids respectively. Also of note is the remarkable consistency of half cysteine residues which have been attributed to the dimeric nature of STC. The start of each exon is denoted by arrows above the sequence, and each exon is denoted by labels above the sequence in italicized, bold, or underlined text.

Figure 7 (opposite) In situ hybridization and immunolocalization of STC mRNA and protein. Panels A and C are in situ hybridization (ISH) of rainbow trout mRNA using digoxigenin-labeled riboprobes (2 ng/μl); panels B, D, E, F, G and H are sections which were immunocytochemically (ICC) stained for STC protein (1:1000 dilution of primary antibody, counterstained with hematoxylin); NRS controls are provided at the top of each panel bordered in black. (A, B) Kidney. (C, D) Cranial cartilage. (E) Skin and brain. (F) Pseudobranch. (G) Eye. (H) Gill. Abbreviations: ba, basal layer; ch, chondrocyte; cl, chloride cell; co, connective tissue; dl, dendritic layer; dt, developing tubule; ep, epithelial cell; gl, granular layer; in, inner nuclear layer; ip, inner plexiform layer; mu, mucus gland; nf, nerve fiber layer; op, outer plexiform layer; pl, pilaster cell; pl, pigment epithelium; pr, proximal tubule; pt, proximal tubule; r c, rods and cones; 1, primary lamella; 2, secondary lamella.


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stained specific and granular layers of the brain (Fig. 7E) as well as nerve localized gene expression. For example, both the dendritic (Fig. 7 G), and gill (Fig. 7H), but little evidence of the brain and skin (Fig. 7E), pseudobranch (Fig. 7F), eye little evidence of gene expression. For instance, there was in situ hybridization on these tissues was insufficiently evident and it was much more apparent in the surrounding connective tissue (Fig. 7D). In contrast to the stage in development. In chondrocytes, irSTC protein was only weakly evident and it was much more apparent in the surrounding connective tissue (Fig. 7D). In contrast to the surrounding connective tissue (Fig. 7D). In contrast to the in situ data, some tissues contained high levels of irSTC but little evidence of gene expression. For example, there was relatively intense immunostaining in specific cell types in the brain and skin (Fig. 7E), pseudobranch (Fig. 7F), eye (Fig. 7 G), and gill (Fig. 7H), but little evidence of localized gene expression. For example, both the dendritic and granular layers of the brain (Fig. 7E) as well as nerve fiber and inner plexiform layers of the eye (Fig. 7 G) also stained specifically for STC protein. However, correlative in situ hybridization on these tissues was insufficiently sensitive to reveal the cellular sources of hormone.

Discussion

In this report we have provided the first characterization of a fish STC gene, identified the transcription start site and characterized the pattern of gene expression in a closely related species, the rainbow trout. Our reasons for embarking on this study were twofold: first, to compare and contrast the fish and mammalian genes and secondly, to revisit the issue of STC gene expression in fishes in light of the wider distribution of its mammalian counterpart. Our findings have revealed differences in gene structure and similarities in the pattern of expression, and may have important implications in the physiology of STC in fish.

The most notable structural features of the fish STC gene were its markedly smaller size and greater number of exons in comparison with the mammalian gene. In contrast to the human and mouse genes which span some 12 kb (Varghese et al. 1998), the fish gene is only 3-9 kb in length. This is principally due to it having much less intronic DNA (0-8 vs 8-6 kb) as well as a shorter 3′ UTR (1 vs 2 kb). More interesting, perhaps, was the discovery that the fish gene is composed of five exons as opposed to four in mammals. Discounting the amino acid sequence differences between species, the first two exons and the last exon in both species were nearly the same size and encoded the same regions of the protein. However, it is now apparent that exon 3 in mammals arose from the fusion of two exons, exons 3 and 4 in fish. Precisely when fusion of these exons took place is not known, but perhaps it coincided with the transition of vertebrates from aquatic to terrestrial life (i.e. during the evolution of amphibians) when bone supplanted the aquatic environment as the principal source of readily available calcium. The advantage of an additional exon is that it would allow for greater latitude in the creation of alternatively spliced transcripts. Indeed, there are multiple lower molecular forms of STC in fish (Wagner 1993). However, while RT-PCR amplified additional products that were smaller than that predicted by the fish cDNA sequence (Wagner et al. 1992), they likely arose from primer annealing, or reading errors in the reverse transcriptase (Superscript II) and Taq polymerase (normal recombinant Taq, Fermentas) employed, as none of them proved to be genuine transcripts upon DNA sequence analysis. Furthermore, Northern analysis has revealed little if any evidence for alternatively spliced transcripts in fish. Mammals, on the other hand, generate two STC transcripts of 2 and 4 kb, presumably through the use of different polyadenylation signals resulting in different 3′ UTRs (Varghese et al. 1998). Thus the existence of alternatively spliced transcripts of STC in fish remains to be proven.

Perhaps the most notable aspect of our findings is that they overturned a long-held dogma about STC in fishes, namely that the hormone is produced solely in CS cells. Previous attempts to identify STC gene expression in other fish tissues proved unsuccessful but clearly our data indicate that the STC gene in fish is much more widely expressed than was once accepted. Whereas the CS glands have by far the highest level of gene expression (over 100-fold greater than any other tissue), STC mRNA was also evident in kidney, ovary and testis, and by RT-PCR in most other tissues. This wide distribution in fish mirrors the mammalian expression pattern, suggesting that the local role of STC in mammals first evolved in fish. In the case of kidney, where STC regulates proximal tubular phosphate transport (Lu et al. 1994), the level of expression was highest in the anterior region or head kidney. At the cellular level, low levels of STC mRNA and higher levels of irSTC protein were evident in most nephron segments (proximal I and II, distal tubule and collecting duct), similar to that found in mammals (Haddad et al. 1996, Wong et al. 1998, Yoshiko & Maeda 1998). However, the highest levels of expression were observed in the cells of newly developing nephron segments, which is somewhat reminiscent of mammalian development where STC gene and protein activity are abundant in both metanephrogenic mesenchyme cells and developing ureteric bud (Stasko & Wagner 2001). Therefore, locally produced STC may have a role that is fundamental to the process of nephrogenesis in all vertebrates.

The evidence for equivalent levels of STC mRNA in male and female gonad suggests that, as in mammals, fish STC has a role in reproduction. Precisely where the gene is expressed in fish gonads and what its role might be are currently under investigation. A major difference between fishes and mammals, however, is that gonadal
gene expression in mammals is overtly sexually dimorphic, with high mRNA levels in ovary and virtually undetectable levels in testis (Varghese et al. 1998). STC mRNA was also readily detectable in mature chondrocytes, which agrees with the findings in mammalian bone, where STC gene expression is evident in both chondrocytes and osteoblasts, but not osteoclasts (Yoshiko et al. 1999). On the other hand, we observed only weak associated staining for STC protein in chondrocytes, which also agrees with the mammalian findings where irSTC is much more evident in pre–hypertropic chondrocytes and then diminishes as they undergo hypertrophy (jiang et al. 2000). Conversely, in the brain, skin, gill, eye and pseudobranch, STC gene expression was only detectable by RT-PCR whereas staining for STC protein was clear-cut and discretely localized. The pseudobranch and skin both contain mitochondrial rich cells believed to be involved in ion transport (Philpott 1980, Fenwick 1987, McCormick et al. 1992), suggesting that STC may play a role in these tissues similar to its well-characterized actions on gill, intestinal and renal calcium/phosphate transport. In the gill, however, irSTC appeared to be localized to cells of the respiratory epithelium, rather than to chloride cells, which is surprising given that the chloride cells are predominately involved in regulating mineral transport (Perry & Flik 1988). Thus, in the gill locally produced STC may have actions on ion transport that are unrelated to those of the CS–derived hormone. Lastly, there was also evidence of STC in both the brain and eye. The presence of STC-like immunoreactivity has previously been reported in the pituitary, neurohypophysis, and preoptic lobe of Coho salmon (Fraser et al. 1991). Mammals also have high levels of STC in neurons (Zhang et al. 1998), and the hormone is reported to increase phosphate transport in a human neural crest–derived cell line as a protective hormone is reported to increase phosphate transport in a high levels of STC in neurons (Zhang et al. 1998), and the hormone is reported to increase phosphate transport in a human neural crest–derived cell line as a protective mechanism against calcium toxicity during ischemic injury (Zhang et al. 2000). Therefore, it may be that in the fish brain, ocular nerve fibers and the inner plexiform layer, STC also has a role in the regulated transport of calcium and/or phosphate.

In summary, we have cloned and characterized the fish STC gene and provided evidence that it is much more widely expressed than the literature has suggested to date. The fish gene is considerably smaller than the mammalian gene and is comprised of five exons as opposed to four in mammals. The nature of the STC produced in other fish tissues, the signaling pathways involved and the potential for alternative splicing are but a few of the questions raised by this study that warrant further investigation.

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References


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