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Detection and Assessment of Androgenic Potency of Endocrine-Disrupting Chemicals Using Three-Spined Stickleback, *Gasterosteus aculeatus*

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The male three-spined stickleback (*Gasterosteus aculeatus*) produces a glue protein named "spiggin" that is used as a cementing substance for building its nest. The synthesis of spiggin is under the control of androgenic stimulation. Therefore, spiggin is an effective biomarker of any androgenic activity displayed by environmental chemicals, similarly to the use of vitellogenin as an estrogenic biomarker. The aim of this study was to establish a quantification system for spiggin mRNA to develop a highly sensitive system for evaluating environmental androgens. In this process, two different types of cDNA encoding spiggin (SPG-1 and SPG-2) were isolated. They closely resemble each other in primary structure and features. In addition, the transcriptions of both spiggin gene were induced by only androgenic stimulation in a receptor-mediated manner. These findings suggest the multiplicity albeit specificity of spiggin in the stickleback. The quantification system for spiggin mRNA was established using a real-time RT-PCR technique. This system enables accurate quantification within a wide range of spiggin mRNA from 10^1 to 10^6 copies.

1. Introduction

Recently, it has been suggested that some chemical compounds, including natural steroids, industrial contaminants, insecticides and herbicides have endocrine activity in both humans and wildlife. Many of these substances, so-called endocrine-disrupting chemicals (EDCs), display agonistic or antagonistic effects to sex hormones (estrogen and androgen) acting via their receptors. Sex hormones control many physiological phenomena, but are particularly important in sex determination, gonadal sex differentiation, gametogenesis and sexual behavior. Therefore, EDCs detected at significant concentrations in the aquatic environment, pose a significant threat to the normal repro-

ductive capability and have been implicated in a number of reproductive and developmental abnormalities in fish and other vertebrates.[1,2]

Until recently, research on EDCs has concentrated on environmental estrogens and their physiological effects, but hardly on environmental androgens. Numerous *in vivo* and *in vitro* detection and evaluation systems for estrogenic stimulation have been prepared, reflecting the existence of a large number of estrogenic compounds. For example, vitellogenin (VTG), an egg yolk precursor protein in oviparous animals, is a female-specific protein, and its synthesis in the liver is specifically controlled by estrogenic stimulation. In male, VTG synthesis is easily inducible by exogenous estrogen treatment.[3] Therefore, the serum VTG concentration of male animals is an effective biomarker for environmental estrogens and has been used extensively particularly in fish.[3,4,5] The use of VTG as a biomarker of environmental estrogen enables an extremely rapid and precise analysis at the molecular level.[5,6,7,8] However, such an effective molecular biomarker for evaluating environmental androgen has not yet been identified.

The three-spined stickleback (*Gasterosteus aculeatus*) shows a unique reproductive strategy. In the male, the kidney undergoes hypertrophy during the breeding season and produces a glue protein, spiggin, which is used in building a nest that females enter for spawning.[9,10] The production of this protein is strongly regulated by androgenic stimulation. Females do not produce this protein naturally, but produce it after androgen treatment.[11,12] Therefore, spiggin induction in female stickleback is an excellent biomarker of androgenic and anti-androgenic chemicals.

The aim of this study was to establish a quantification system for spiggin mRNA, and develop a highly sensitive test for environmental androgens. In this process, multiple spiggin mRNAs including novel type and previously cloned type[11] were isolated from the male kidney cDNA library. Here, we introduce a newly established spiggin mRNA quantification system, including the cloning and expression analyses of multiple spiggin mRNAs.

2. Spiggin cDNA Cloning

Using mRNA isolated from the kidney of laboratory-bred mature male stickleback (collected in Cefas Laboratory, UK), RT-PCR was performed with a set of primer selected from a previously reported spiggin sequence.[11] The products were electrophoresed in a 5% acrylamide gel, and two different PCR fragments were obtained. These fragments were subcloned into the plasmid vector, and sequenced. Figure 1 shows the deduced amino acid sequence of the present cloned two PCR fragments and its comparison with that of the previously reported spiggin cDNA

```
                                                                    50
SPG-I  IVQPFKGESYYVRSDCPFKLTSFNVNRGEYSVTIRRGHNGLLVQVEIIIN
spgα   ..................................................
SPG-II .................Q.R.I..T..QK.................K....

                                                                   100
       KVTTLLRNGHILVQNK--------------------SVSLPYDHTYQHI
       ......Q.....................................
       ......Q...VV.K..KFELRQKTGLGHRHQWRRQRLK.TH.CNRK....

                                                                   150
       FKYGIYTRLRSSLLPFTVTWHNVHGGINSLWVTLESELCTDMCGLCGKQN
       ..................................................
       ......N..K......S......Y............K.N.TTS.......

              161
       VAGHRDELIRD
       ...........
       I..........
```

Fig. 1. Nucleotide sequences of PCR fragments of SPG-1 and SPG-2. The previously reported spgα sequence was also aligned for comparison.

(spgα).[11] One fragment (422 bp, designated SPG-1) is almost identical (except for one point mutation) with the previously reported spgα sequence,[11] but the other (485 bp, designated SPG-2) has an additional 63 nucleotides. However, the nucleotide sequence of the SPG-2 fragment shows a high homology (80%) with that of SPG-1. This high similarity suggests that both molecules are essential components of the glue protein used for nest building. We attempted to obtain full-length cDNAs encoding SPG-1 and SPG-2 from the kidney cDNA library using these fragments as probes.

From kidney cDNA library screening, only one full-length cDNA (2261 bp) containing complete SPG-2 ORF (621 amino acids) was isolated. On the other hand, three different full-length SPG-1 cDNAs were isolated (i.e., SPG-1A (2253 bp), 1B (2897 bp) and 1C (3792 bp)), although these three cDNAs encode completely the same ORF (616 amino acids). The three SPG-1 cDNAs differed in the length of 3'-UTR (Fig. 2). Therefore, these appear to be transcripts derived from the same gene, by alternative splicing and the selection of polyadenylation signals. The sequence of these cDNAs have been registered with the GenBank database (Accession Nos. AB243101 (SPG-1A), AB243102 (SPG-1B), AB243103 (SPG-1C), and AB243104 (SPG-2)).

SPG-1 and SPG-2 are similar deduced primary protein structures, and the amino acid identity of SPG-1 with SPG-2 is 83%. They also have common structural features to fulfill their function as a water-insoluble glue. Both are extremely hydrophobic, a feature that is indispensable for building a nest under the water. Another similarity is the multiple N-glycosylation sites, which are also related to their the adhesive function. SPG-I and SPG-II have seven and nine N-linked glycosylation sites (motif of Asp-X (except for Pro)-Ser or Thr), respectively (Fig. 3).

More recently, four different spiggin genes have been identified from the Japanese three-spined stickleback, and multiple transcripts derived from the same spiggin gene were observed.[13] Jones *et al*.[11] also reported the existence of alternative splicing variants from one spiggin gene in sticklebacks caught in Sweden. As described above, splicing variants of one spiggin gene (i.e., SPG-1A, SPG-1B and SPG-1C) were also detected in this study. However, the manner of spiggin mRNA splicing differs among these three studies including this study. It is suggested that regional differences (Japan, Sweden and United Kingdom) form the basis of the diversification of spiggin production. A various spiggin molecules (four different spiggin molecules), as reported by Kawahara and Nishida,[13] are important for the understanding of stickleback reproductive physiology. An extensive and worldwide study of spiggin production including the identification of multiple spiggin components is necessary to elucidate the diversity of spiggin molecules.

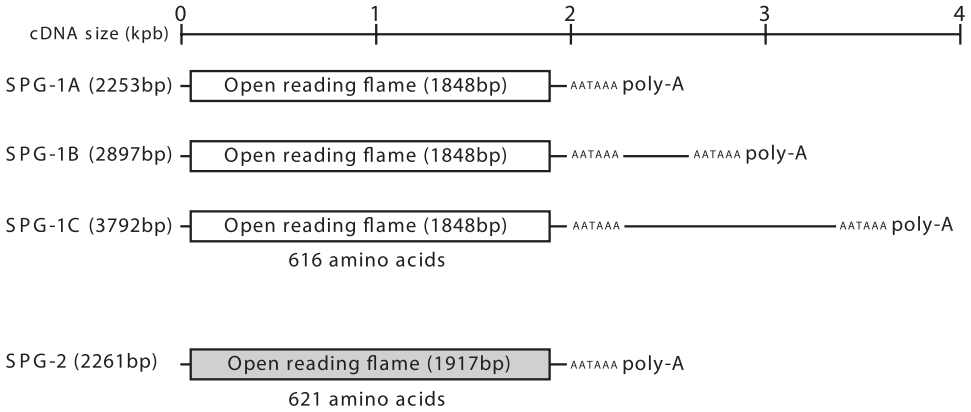

Fig. 2. Primary structure of cDNAs encoding SPG-1 (1A, 1B and 1C) and SPG-2.

```
SPG-1 MTTQRRILAFSLSLASVFGTVELLKTKETQTYTCRTFGSGIVQPFQGESYYVRSDCPFTL  60
SPG-2 .....W....CF.........K.F..N..................K..........Q.R.

      TSFNVNQGEYSVTIRRGHNGLLVQVEIVLNNVTTLLQDGHVVVKNKS------------- 120
      I..T...K.................K.II.K......N.........KFELRQKTGLGHRH

      --------VSDPCDQKYQHIFKYGIYTRLKSSLLPFSVTWHSVCGGINSLWVTLESELNT 180
      QWRRQRLK.TH..NR...........N.............N.Y...........K..... 

      TTSGLCGKQNVAGHRDELIRDSKLHDHKCKTSSPVLQKNDICRQFFQEIKDCVQYNNSHY 240
      ..........I..................N.........................*.....

      QRLCEENIFGFENSQSVFCPFFKEFASQCNQSTINRFWRHLTKCAEPRCPGDLIYREKGP 300
      ............N..............*..R......G....EK...........N..

      AVIPSCSNPKPPPFYQELTESCACPEGNVLNNGAKGYRCIPWSSCSCEFAGKSYRNGEIR 360
      ..........L..............K..............N.................

      RSKCHSCTCHGVVWRCSENFCRRRCVIEGPFVTTFDGKQYVLPQKCSYVASKGPNWRIRI 420
      ....................H..............K....Y..A.......S.....

      HFSRKGLYLRKVVVRFSEELFVFKENKVLWNGQEITKFHQFQENCNAKIYWVSSTFLQVH 480
      ..............LPG..I..RK......W....Q......T.............. 

      TTSGFFFQIQLSPEIQLFIDAPD--NSNYKIKGLCGNSNNNTTDDFTTKSGVIENSAEPF 540
      ....L..............NQSK.DD..E.......R*........ET.FFP..VQL.

      ALSWSLGNCQGNIPTNCTKREYENYAHEKCAVLNQPTEVFAQGHPHIPTDYYYKACIQRI 600
      ....IM.........T.....N..........K...G..S....R.............

      CNSGRSQKEALCIGLASYAKACAGVGVVIGDWKKNMGCA                     639
      ....................................
```

Fig. 3. Comparison of deduced amino acid sequences of SPG-1 and SPG-2. The putative N-linked glycosylation sites are boxed. Dots denote amino acids identical to those in SPG-1. Dashes are introduced for maximal fitting of alignment.

3. Hormonal Induction of Spiggin mRNA

Female sticklebacks (three fish in a 4-L glass aquarium) were treated with various steroids (i.e., 17β-estradiol (E_2), progesterone (P), hydrocortisone (HC), 5α-dihydrotestosterone (DHT) and 17α-methyltestosterone (MT)) at 10 μg/L for one week. Only the vehicle solvent (ethanol) was added to the control group. Water was changed every day and an appropriate quantity of each steroid was added. Kidney samples were collected after one week of treatment, and the mRNA extracted from the kidney was used for Northern blot analysis. The sequence of the hybridization probe showed a high homology with those of SPG-1 and SPG-2; whose mRNAs were probably recognized in this analysis.

Spiggin mRNA was detected only with the DHT and MT treatments. No positive signal was observed in nonandrogenic steroids (Fig. 4). MT was more effective in spiggin gene transcription than DHT. This result indicates that the relative androgenic activity of testing substances is reflected on the transcriptional level of the spiggin gene. The present result confirms that spiggin is a quantitative biomarker of androgenic activity. This induction of spiggin gene transcription by MT was completely abolished by the cotreatment with an excess concentration of flutamide, an androgen antagonist (data not shown). This result confirms that the induction of spiggin gene transcription by androgens is activated in a receptor-mediated manner.

From the result of the Northern blot analysis, two positive bands of spiggin mRNA were recognized (Fig. 4). This result supports the multiplicity of spiggin (including the existence of splicing variants). However, it remains unknown which spiggin sequences correspond to the two bands. For detailed study, it may be necessary to individually detect SPG-1 and SPG-2 mRNAs.

4. Sensitive Detection of Spiggin mRNA

To develop a sensitive and precise quantification method for spiggin mRNA, a real-time quantitative RT-PCR technique was employed. A set of primers for real-time quantitative RT-PCR was selected from the complete nucleotide sequences of SPG-1 and SPG-2 to amplify both types of spiggin mRNA simultaneously. In addition, genome DNAs encoding both spiggins were sequenced to determine effective primers for the specific amplification of reverse-transcribed spiggin cDNA, not the genomic DNA encoding spiggins contaminating total RNA samples. Figure 5 shows the relationship between Ct value (threshold cycle) and the copy number of the synthesized spiggin DNA standard for the presently established real-time quantitative RT-PCR technique. There is a strong correlation between Ct value and the original copy number of the spiggin DNA standard. In addition, the system enables accurate quantification within a wide range of spiggin mRNA from 10^1 to 10^6 copies.

A trial run for testing the spiggin quantification system established above was performed using various kidney RNA samples. Kidney total RNA samples were extracted from mature males, immature females and immature females treated with androgen (DHT and MT) for one week at 10 µg/L. Using these RNA samples, spiggin mRNA was quantified. As shown in Fig. 6, there is a clear difference in spiggin mRNA concentration between the samples. Almost the same results were obtained by the Northern blot analysis (Fig. 4). Thus, the presently established quantification system has great advantages in terms of accuracy and convenience over Northern blot analysis.

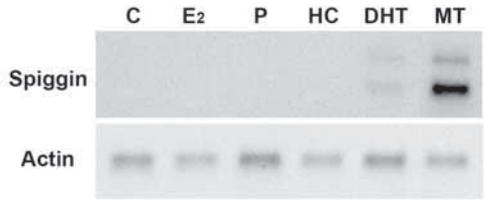

Fig. 4. Northern blot analysis of spiggin mRNA isolated from kidneys of immature females treated with various steroids. Actin was also included for positive control. C: control (vehicle treatment); E_2: 17β-estradiol; P: progesterone; HC: hydrocortisone; DHT: 5α-dihydrotestosterone and MT: 17α-methyltestosterone.

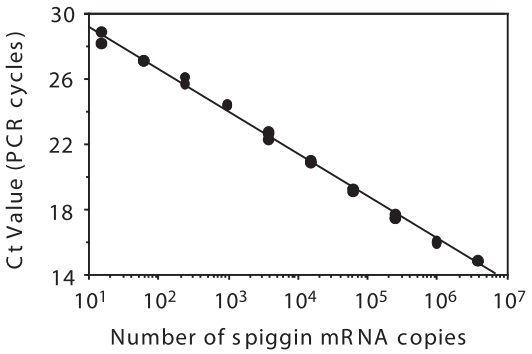

Fig. 5. Standard of spiggin mRNA quantification using real-time RT-PCR technique.

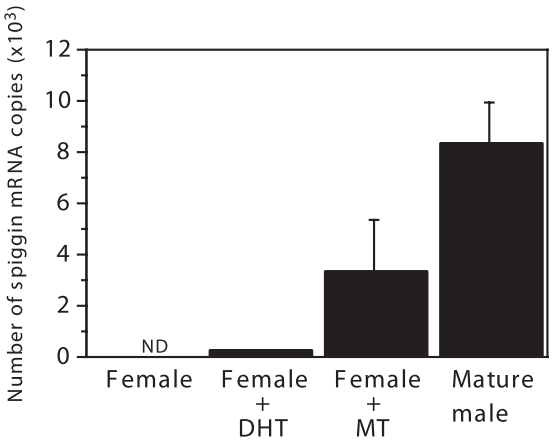

Fig. 6. Trial quantification of spiggin mRNA in various samples.

5. Conclusions

In this study, a sensitive quantification system for spiggin mRNAs based on a real-time RT-PCR technique was developed. This system enables accurate quantification within a wide range of spiggin mRNA from 10^1 to 10^6 copies and is a useful tool for evaluating the androgenic potency of environmental androgens using *in vivo* exposure systems. Using this system, we plan to evaluate the androgenic potency of various chemicals.

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