Short communication

Growth differences and growth hormone expression in male and female European eels \([\textit{Anguilla anguilla (L.)}]\)

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Abstract

In this study, we examined the growth differences of males and females following a sex reversion, and the growth hormone (GH) expression variation between sexes of European eels \([\textit{Anguilla anguilla (L.)}]\). A high percentage of females (88%) was found in the group fed with estradiol 17\(\beta\) compared to the control group (comprised of only 6% female eels), which was defined as the male population. Significant differences between growth rate and size were found following 480 days of growth, whereby the males reached 60\(\pm\)4.3 g (means\(\pm\)SE) in size and the females 73.4\(\pm\)5.9 g (SE); after 600 days, the males reached 114.1\(\pm\)4.3 and the females 171\(\pm\)11.7 g (SE). A cDNA coding for the complete growth hormone of the European eel \(A.\) anguilla (eeGH) was cloned by RACE PCR using several sets of degenerate oligonucleotides. The eeGH cDNA coding region is 627 bp long. A sequence comparison of eeGH with \(A.\) japonica GH (jeGH) cDNA showed a 98% identical base. Comparison of the deduced amino acid sequence revealed 99% identical residues, meaning that a difference exists in only two of the 209 residues. In both cases, the differing residues in the eeGH amino acid sequence are lysine. We measured the mRNA levels of growth hormone in the pituitaries of male and female eels growing at different rates. A significantly higher expression of eeGH was found in the female eels in comparison to the males. These results show that different levels of GH transcription eeGH can explain the growth rate differences between male and female European eels.

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1. Introduction

Growth hormone (GH) is a pituitary hormone that regulates development and somatic growth in vertebrates (Perez-Sanchez, 2000). It is a single chain polypeptide having two intramolecular disulfide bonds. The major mediator of GH function is the insulin-like growth factor I (IGF-I), which is structurally related to insulin and similarly contains three intramolecular disulfide bonds (Moriyama et al., 2000).

In seeking genetic factors affecting growth rate, the growth hormone (GH) is naturally considered a primary candidate. This 22-kDa single chain polypeptide, together with prolactin, placental lactogen, and somatomatolactin, forms a family of related polypeptide hormones whose sequences seem to have evolved from a common ancestor (Niall et al., 1971). GH has been studied extensively and its cDNA nucleotide sequences are available for many teleosts (Chao et al., 1989; Funkenstein et al., 1991; Mahmoud et al., 1996; Nicoll et al., 1987; Rentier-Delrue et al., 1989; Saito et al., 1988; Tanaka et al., 1995; and others).

Several investigators have studied the GH of eels. Kishida et al. (1987) isolated two forms of growth hormone secreted in vitro from the pituitary of Japanese eel \((A.\) japonica). The cDNA of the Japanese eel's GH (jeGH) was cloned by Saito et al. (1988), and it codes for a precursor of 209 amino acids, including a 19 amino acid putative signal peptide. The deduced amino acid sequence was identical to that determined for the jeGH polypeptide. GH stimulators in European eel could be linked to the many roles that GH plays in various physiological functions such as growth, metab-
The purpose of the present research was to clone and sequence the cDNA of European eel GH and use the data to measure GH expression in males and females.

2. Material and methods

2.1. Fish

European glass eels (Anguilla anguilla) caught during their migration in the spring of 1999 were purchased from the UK. The eels were transferred to Israel and had an average length of 7 cm and average weight of 0.5 g. They were maintained indoors in containers (1 m³) for six months at the MIGAL—Galilee Technological Center in northern Israel, as previously described (Degani and Levanon, 1983). For dry-feed training, they were fed with a commercial eel diet for approximately one month (Trovit, Hendrix, Italy: protein 42.5%, fats 8%, and ash 11.5%), combined with decreasing amounts of raw cod to adapt the eel to a commercial diet. The elvers were fed six days a week, five times a day, at about 3–4% of their body weight.

The elvers were kept at 23–26 °C, with 2–3 daily changes of total water volume and gentle aeration. The study was made on two groups of elvers: the first was trained to feed on a commercial diet and represented the control male population; the second was fed on an experimental diet including estradiol in order to produce a female population. The study commenced in April 1999; the final sample was taken 600 days later.

Table 1
The different primers used in this study

<table>
<thead>
<tr>
<th>Use</th>
<th>Nucleotide sequence</th>
<th>Direction in relation to the mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>3' RACE: PCR</td>
<td>5'-TAATGAGGTTGTAGGTG</td>
<td>5' &gt; 3'</td>
</tr>
<tr>
<td>5' RACE: cDNA synthesis</td>
<td>5'-AGGTCCCAAACATCAAG</td>
<td>3' &gt; 5'</td>
</tr>
<tr>
<td>5' RACE: PCR</td>
<td>5'-TGAGTGAGTGGGACTGAG</td>
<td>3' &gt; 5'</td>
</tr>
<tr>
<td>Complete cDNA cloning</td>
<td>5'-AACCGGAGATGATGGCGATG</td>
<td>5' &gt; 3'</td>
</tr>
<tr>
<td>cDNA synthesis</td>
<td>5'-GACTCGAGTTCGACATCGA(T)17</td>
<td>3' &gt; 5'</td>
</tr>
<tr>
<td>RACE PCR</td>
<td>5'-GACTCGAGTTCGACATCG</td>
<td></td>
</tr>
</tbody>
</table>

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from freshly excised pituitaries using the Rneasy Total RNA Kit (Qiagen-GR). Usually, 30 ng/ml of total RNA was obtained from each pituitary. The cDNA pools for both the 3' and 5' amplifications were synthesized using an AMV reverse transcriptase (Promega, USA), as described by Frohman (1990). The primers used in this study for the synthesis of the 3' cDNA and the 5' are shown in Table 1. Amplification of the 3' and 5' ends was carried out, as previously described (Jackson et al., 1999). An analysis of the cDNA sequences was made using the GAP4 software package (Bonfield et al., 1995). The GH mRNA levels were measured using RT-PCR, as previously described (Jackson et al., 1999). GH cDNA amplification was coupled with the amplification of the internal standard 18S rRNA cDNA, which was chosen due to its abundance and stable expression (Amoureux et al., 1997).

2.3. Statistical analysis

The differences between the means of mRNA GH levels were analyzed by ANOVA followed by the Student’s t test (Parker, 1986), with the level of significance in different groups set at p < 0.05.

3. Results

A high percentage of females (88%) was found in the group fed with E_2 compared to the control group (comprising only 6% females) defined as the male population.

The growth of males and females is show in Fig. 1. Significant differences (p < 0.05 ANOVA and t test) between growth rate and size were found following 480
days of growth, whereby the males reached 60 ± 4.3 (g ± SE) and the females 73.4 ± 5.9 (g ± SE); after 600 days, the males reached 114.1 ± 4.3 (g ± SE) and the females 171 ± 11.7 (g ± SE) (Fig. 1).

3.1. cDNA cloning and sequence analysis

The complete cDNA of the European eel GH (eeGH) was cloned by RACE PCR. The 5' and 3' ends of the eeGH cDNA were cloned separately, but with a short overlap, enabling proper joining into a single nucleotide sequence spanning the entire coding region. The coding region of eeGH cDNA is 627 bp long and is identical to that of the jeGH cDNA (Fig. 2). A sequence comparison of eeGH to jeGH cDNAs revealed a 98% identical base (Fig. 2). A comparison of the deduced amino acid sequence of eeGH and jeGH polypeptide revealed 99% identical residues, indicating a difference in only two of the 209 residues (Fig. 2). In both cases, the differing residues in the eeGH amino acid sequence are lysine.

3.2. European eel GH mRNA transcription in females (fast-growing) and males (slow-growing)

The cloned GH cDNA was used to measure variations in the GH mRNA levels in the pituitary gland of females grown from glass eels. The measurements were made using RT-PCR, as described above (Fig. 3). The amplification of GH cDNA produced a product 862 bp in length. The product generated by amplification of the internal standard (the cDNA of 18S rRNA) was 430 bp long. The identities of the PCR products were confirmed by DNA sequencing.

The expression of the eeGH gene at the mRNA level was evaluated by first measuring the amount of specific

Fig. 1. The growth of male and female (g ± SE) eels. Histology of European eel gonads. Female (A) Note the presence of small vesicles (black set) and larger vesicles (white set) along the periphery of the oocyte. Male (B) Histology of testis showing empty lobuli. A detail of a lobulus showing the presence of gametogenic cells at different developmental stages is shown (inset) Hematoxylin and Eosin.

Fig. 2. (A) cDNA and deduced amino acid sequences of European eel growth hormone. (B) Comparison of the amino acid sequences of growth hormones from European (A. anguilla) and Japanese (A. japonica) eels. Different amino acids are marked with a white background. The four cysteines that are known to form disulfide bonds are marked with asterisks.

Fig. 3. European eel GH mRNA expression in females and males.

PCR product (GH cDNA) and then dividing this value by the amount of amplified internal standard. This normalization step was intended to eliminate the effect
of random changes in the experimental setup. The results are presented in Fig. 3. The content of eeGH mRNA in males was significantly lower than that in females (ANOVA, $t$ test; $p < 0.05$).

### 4. Discussion

The results of this study on gender determination indicate that E$_2$ treatment affect a high percentage of females. These results are supported by previous papers on eel gender determination, which found high feminization rates following estrogen treatments. Under aquacultural conditions, the majority developed male gonads (Egusa, 1979; Tesch, 1977). Under laboratory conditions in the present study, 94% of the eels exhibited male gonadal development. We found that a diet supplemented with estrogen in the early stages of development affected female gonadal development and growth, as previously described (see Andersen et al., 1996; Degani and Kushnirov, 1992; Grandi et al., 2000).

As in most GH teleosts, four cysteine residues were found in eeGH. In comparison, five cysteine residues were described in goldfish (Law et al., 1996) and other cyprinids (Chang et al., 1992) (Fig. 4). These cysteine residues are involved in the formation of two disulfite bonds in a pattern analogous to the “big loop” “little loop” found in the human growth hormone (Vestling et al., 1991), which plays an important role in determining its biological activity (Lewis et al., 1980). A putative glycosylation site was found in bgGH. The existence of this site is a common feature of many GH teleosts. It was shown that N-linked glycosylation could serve as a signal for protein transport to the cell surface (Guan et al., 1985).

Measurement of eeGH mRNA expression showed that its relative level was 67% higher in females. This level continued to increase after the females reached gender determination and maturation faster than the males during gonadal development (Tesch, 1977). GH was shown to stimulate the growth of eels. The bovine growth hormone caused a significant increase in the growth rate of American eels (A. rostrata) (Degani and Gallagher, 1985). Kaiya et al. (2003) revealed that ghrelin is present in the Japanese eel (A. japonica) stomach and has the ability to stimulate the secretion of GH from fish pituitaries. In several fish species, IGF-I gene expression in the liver is more responsive to GH administration than other tissues (Duguay et al., 1994).

Recent studies on GH functions have confirmed other physiological effects in addition to those of growth promotion that are well established. Among these effects, the participation of GH in reproduction has been studied in several teleosts using different approaches, including gene expression. GH mRNA was detected shortly after hatching in Sparus aurata (Funkenstein and Cohen, 1996) and Oncorhynchus mykiss (Yang et al., 1999) before the organogenesis of the pituitary gland. In adult animals, GH mRNA was detected outside the pituitary gland in several sites, including the ovary (Yang et al., 1999).

Several studies have suggested that GH may play a role in female gonadal development (Holloway et al., 1999; Stacey et al., 1984; Young and Ball, 1983). Degani et al. (1996, 1998) found that GH did not change in the pituitary with a growth in steroid level, but plasma GH was increased, correlating to the levels of testosterone and estradiol 17β. Most of the papers studied the relationship between GH E$_2$ and vitellogenin levels in eels (see for review Peters et al., 2001). The native and recombinant growth hormones from mammalian and fish species promote the estrogenic induction of vitellogenin.
synthesis by cultured eel (*A. anguilla*) hepatocytes. The estradiol catabolic parameters were not affected by the growth hormone. The human and trout recombinant insulin-like growth factors did not promote vitellogenin synthesis induced by estradiol (Peters et al., 1998; Peyton et al., 1998).

It is possible that the difference in GH mRNA levels found in male and female European eels may be affected by various gender developments and sex hormones. Kumar et al. (2003) found that GnRHa implanted into the maturing masu salmon did not increase GH mRNA levels during the pre-spawning period. Furthermore, the GH mRNA levels in GnRHa-implanted males tended to be lower than those in control males during the pre-spawning season. Kumar et al. (2003) indicated that GnRH does not enhance in vivo transcription of GH gene during the pre-spawning season, but significantly increases in stages long before sexual maturation in association with the activation of sGnRH genes during the growth stages. In this study, low mRNA GH expression may be correlated to less secretion in males than in females but this hypothesis requires more detailed study.

Gender determination in European eels is very complex (Yamazaki, 1983). Yang and Chen (2003) found that in the pituitary gland of rainbow trout (*Oncorhyncus mykiss*), the level of somatolatin-like protein (rtSLP) mRNA in male fish is twofold higher than that in female fish. This might play a role in regulating the reproductive maturation in rainbow trout. The main difference between sexes is the steroid level in the plasma and the female gender determinations affected by E2 (Degani and Kushnirov, 1992). The high female GH eel transcription revealed in this study may be explained by the effect of E2, which developed female steroids in eels. In goldfish, increasing levels of the sex steroid estradiol raised the plasma levels of GH (Canosa et al., 2002). The mechanism of gender effects on GH transcription is less clear. It seems that the variation of GH transcription is correlated to steroids differing between the sexes. Zou et al. (1997) investigated the effects of E2 on growth/GH production in gonad-intact female goldfish. They suggest that this sex steroid may enhance GH synthesis at the post-transcriptional or translational level. However, E2 implants, even if they increased pituitary GH content and plasma GH levels, did not change pituitary GH mRNA levels. It seems that E2 may affect the secretion of GH on translation but not on transcription. In the study by Gomez et al. (1999), GH transcripts in rainbow trout increased moderately throughout most of the gametogenesis process, and more dramatically at sperminination and during the periovulatory period.

In conclusion, it is reasonable to assume that lower levels of eCGH mRNA were found in the slower growing males than in females, which could explain the effect of GH and its effect on eel growth.

References


