Blue gourami (*Trichogaster trichopterus*) gonadotropic β subunits (I and II) cDNA sequences and expression during oogenesis

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ABSTRACT

We have cloned two cDNAs from the pituitary gland of blue gourami (*Trichogaster trichopterus*), coding for the β subunits of the gonadotropin hormones GtH-I and GtH-II. The two cDNAs were sequenced and subjected to sequence analysis. We have found that the deduced amino acid sequences of both cDNAs were most similar to their striped bass counterparts. The β GtH-I subunits of blue gourami and striped bass shared 73% of their residues, and the β GtH-II subunits 84%.

The cloning of the cDNAs of β GtH-I and β GtH-II has enabled us to measure the expression of their respective mRNAs in the pituitaries of female blue gourami at different stages of the reproductive cycle. The highest levels of β GtH-I and β GtH-II mRNA were found in specimens classified as high vitellogenic and in females that were at the final stages of oocyte maturation.

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INTRODUCTION

Oogenesis in teleosts, as in other vertebrates, involves a complex interaction along the brain-pituitary-gonad axis. It is generally accepted that gonadotropin-releasing hormone (GnRH) controls the release of gonadotropin (GtH) from the pituitary gland, which, in turn, controls ovarian development (Sherwood *et al.* 1989, Swanson 1991). Oogenesis is induced by increasing levels of GtH, via the mediation of ovarian steroids, from early growth of primary oocytes to vitellogenesis and through maturation to ovulation.

GtH is a glycoprotein consisting of two subunits, α and β , which are non-covalently bound. There are two distinct GtHs in the pituitary gland of teleosts: GtH-I and GtH-II. These proteins share a common α subunit, but differ in their β subunits, which confer the immunological and biological specificity to each hormone. In salmonids, GtH-I and GtH-II were found to differ in their pattern of expression at different stages of the reproductive cycle (Nozaki *et al.* 1990a,b, Swanson 1991). GtH-I is expressed during oocyte vitellogenesis and

GtH-II is expressed at maturation and ovulation (Mal *et al.* 1989).

Most of the physiological studies of teleost GtHs have been conducted on GtH-II, which controls maturation and ovulation, while the function of GtH-I in fish has not been fully addressed (Elizur *et al.* 1996). It is believed that GtH-I regulates gametogenesis, since it promotes the production of 17 α -estradiol (Sekine *et al.* 1989). GtH-II, on the other hand, is known to be involved in oocyte maturation and ovulation (Nagahama 1994). This hormone is probably responsible for the final maturation of the oocytes (FOM), since it is more active than GtH-I in stimulating the release of the maturation-inducing factor 17 α ,20 β ,dihydroxy-4-pregnen-3-one produced by post-vitellogenic oocytes (Nagahama 1994).

The α and β subunits of teleost GtH-I and/or GtH-II are encoded by separate genes. Trinh *et al.* (1986) was the first to clone these genes in teleost fish (chinook salmon, *Oncorhynchus tshawytscha*). Since then, sequencing of α and β subunits of both GtH-I and/or GtH-II has been reported for several fish. β subunits of GtH-I and GtH-II have been

cloned and sequenced in chum salmon, *Onchorhynchus keta* (Itoh *et al.* 1988), masu salmon, *Onchorhynchus masou* (Kato *et al.* 1993), goldfish, *Carassius auratus* (Yoshiura *et al.* 1997), mummichog, *Fundulus heteroclitus* (Lin *et al.* 1992), European eel, *Anguilla anguilla* (Querast *et al.* 1990), baikal omul, *Coregonus aummalis migratorius* (Trofimova & Belikov 1994), and gilthead seabream, *Sparus aurata* (Elizur *et al.* 1996). The α and β subunits of GtH-I and/or GtH-II have been cloned and sequenced in common carp, *Cyprinus carpio* (Chang *et al.* 1993), chum salmon (Sekine *et al.* 1989), and Japanese eel, *Anguilla japonica* (Nagae *et al.* 1996). The $\alpha 1$ and $\alpha 2$ subunits of GtH have been cloned and sequenced in goldfish (Kobayashi *et al.* 1977) and the channel catfish, *Ictalurus punctatus* (Liu *et al.* 1997).

The synthesis and secretion of gonadotropins are regulated by positive and negative factors that act at the levels of brain, pituitary and gonads. Most studies that have described the changes in the level of gonadotropins during the reproductive cycle in teleosts relate to the secretion of gonadotropin, but not to their synthesis. The use of a molecular biological approach has made it possible to examine changes in the levels of the mRNA of the gonadotropins during the gonadal cycle. Changes in the expression of GtHs during the gonadal cycle, or under the influence of different hormones, have been studied in recent years in teleosts (Querast *et al.* 1991, Weil *et al.* 1995, Elizur *et al.* 1996, Nagae *et al.* 1996, Liu *et al.* 1997).

The blue gourami (*Trichogaster trichopterus*) belongs to the family Anabantidae, which contains 16 genera and about 50 species, distributed throughout most of southern Asia, India and Central Africa. This family belongs to the suborder Labyrinthici, which is characterized by the presence of an air-filled breathing cavity (the labyrinth), located above the gills under the operculum. The blue gourami is multi-spawning and male-dependent, with an asynchronic ovary development (Degani 1993a,b). Its hormone profile during oogenesis has been studied in detail in our laboratory (Degani & Boker 1992, Degani 1993a, Degani *et al.* 1994, 1997). Since each stage of its gonadal development can be controlled and examined separately in the laboratory, the blue gourami provides a useful model to study the role of GtH-I and GtH-II in the hormonal control of oogenesis and the expression of their genes during the gonadal cycle. The aim of the present study was to clone and sequence the cDNAs of the blue gourami β GtH-I and β GtH-II and to quantify the mRNA of these hormones, in order to understand the dynamics of the synthesis of GtH-I and GtH-II in the female

blue gourami during the gonadal cycle in fish. The initiation of mRNA synthesis is the first step in the regulation of gene expression and the quantification of mRNA is now commonly used to measure levels of gene expression (Swanson 1991, Dickey & Swanson 1995, Weil *et al.* 1995).

MATERIALS AND METHODS

Fish

Female blue gouramis (*T. trichopterus*), maintained and bred at MIGAL Laboratories in northern Israel, were used in this study. The fish were grown in containers measuring $2 \times 2 \times 0.5$ m, at a temperature of 27 °C, and under a light regime of 12 h light:12 h darkness. The fish were fed an artificial diet (45% protein, 7% fat) supplemented by live food (*Artemia salina*). The pituitaries were collected from 3-month-old females (3.5 ± 0.6 g), at various stages of gonadal development (Degani 1994).

cDNA cloning

The cloning of the cDNAs of β GtH-I and β GtH-II was carried out according to the rapid amplification of cDNA ends (RACE)-PCR protocol for the amplification of cDNA ends (Frohman 1990).

RNA extraction and cDNA synthesis Total RNA was extracted from 20 freshly excised pituitaries by means of the Rneasy total RNA kit (QIAGEN, Hilden, Germany). Usually, 40 ng/ μ l total RNA was obtained from each pituitary. The cDNA pools for both the 3' and 5' amplifications were synthesized as described by Frohman (1990), using an AMV reverse transcriptase (Promega, Madison, WI, USA). The primer for the synthesis of the 3' cDNA end consisted of an oligo dT (17 bases), linked to a unique 17 mer adapter (Table 1: dT₋₁₇ adapter). The 5' cDNA end synthesis was performed with specific primers, prepared in each case according to the sequence information previously obtained, by sequencing the 3' end of the cDNAs (Table 1: primers P2 and P7). The 5' cDNA pool was tailed with a poly A stretch, to allow the use of the dT₋₁₇ adapter and the adapter primers in both the cDNA synthesis and the 5' RACE-PCR which followed.

Amplification of the 3' and 5' ends The PCR primers which were used in the amplification step are listed in Table 1. The degenerate primers were designed according to conserved amino acid sequences which

TABLE 1. cDNA synthesis and PCR primers used in this study

Primer name	cDNA target	Use	Nucleotide sequence	Degeneracy (fold)	Direction in relation to the mRNA	Corresponding conserved amino acid sequence
P1	β GtH-I	3' RACE; PCR	5'-TG(CT)AA(CT)GG(ACGT)GT(ACGT)TGG	× 64	5'>3'	CNGVW
P2	β GtH-I	5' RACE; cDNA synthesis	5'-GCAGTACACTCGCAGTTTC		3'>5'	
P3	β GtH-I	5' RACE; PCR	5'-TTACTTCGTAGGTCCAGTCCC		3'>5'	
P4	β GtH-I	Complete cDNA cloning	5'-GTCTGTACAGATGTTTAGAGAG		5'>3'	
P5	β GtH-I	Complete cDNA cloning	5'-AACGTGGGATGAATGATGAGTG		3'>5'	
P6	β GtH-II	3' RACE; PCR	5'-TA(CT)CA(AG)CA(CT)GT(ACGT)TG(CT)AC	× 64	5'>3'	YQHVCT
P7	β GtH-II	5' RACE; cDNA synthesis	5'-CAGTCAGGAAACTCAAATGTC		3'>5'	
P8	β GtH-II	5' RACE; PCR	5'-TAGAACAAATCCCGGTATGTG		3'>5'	
P9	β GtH-II	Complete cDNA cloning	5'-CTGGCTAACCTGCCGCTGACAC		5'>3'	
P10	β GtH-II	Complete cDNA cloning	5'-TTGCTTTTGGTTTGCTGTGCAG		3'>5'	
dT ₁₇ -adapter Adapter	Universal	cDNA synthesis	5'-GACTCGAGTCGACATCGA(T) ₁₇			
	Universal	RACE; PCR	5'-GACTCGAGTCGACATCG			

were found in β GtH-I and β GtH-II. The PCR amplifications were carried out in 100 μ l reactions, using 2.5 units Taq polymerase (Promega), a suitable reaction buffer (Promega), 1.5 mM MgCl₂, 0.2 mM of each nucleotide, 25 pmol of each primer, 5 μ l cDNA and 20 μ l mineral oil. Cycling parameters were 3 min denaturation at 94 °C, followed by 35 cycles, comprising of 1 min denaturation at 94 °C, 1 min annealing at 54 °C and 1 min extension at 72 °C.

The 3' end of each cDNA was amplified with a degenerate primer, complementary to an internal region (Table 1: primers P1 and P6), and an adapter primer, which corresponded to the terminal adapter sequence of the cDNA. The 5' end of each cDNA was amplified with the adapter primer and a second cDNA-specific primer (P2 and P7), situated upstream from the primer which was used in the cDNA synthesis step.

Cloning and sequencing of PCR products The resulting amplified DNA was cloned into a pGEMT vector (Promega) and sequenced as double-stranded DNA. The sequencing was carried out by the Unit for Biological Services, Weizmann Institute. Three separate clones from the same pool were sequenced for each cDNA. All three independent clones of each cDNA end were obtained from the same cDNA pool.

Once the complete sequence information of the two cDNAs was obtained, we designed new pairs of PCR primers, each of them flanking the entire length of its corresponding cDNA (Table 1: primers P4, P5, P9, P10). These PCR primers were used to reclone the complete cDNA of β GtH-I and β GtH-II, using a DNA polymerase with a proof-reading capability and an error rate of 8.5×10^{-6} (Expand; Boehringer, Mannheim, Germany). The resulting PCR products were subjected to direct sequencing of both strands. The use of high fidelity polymerase and the direct sequencing of PCR products minimized the risk of introducing amplification errors into the sequence data. Comparison of the cDNA sequences from the two cloning steps revealed no differences. Each nucleotide of the three cDNAs was sequenced at least four times, twice on each strand.

Sequence assembly and analysis

The sequences of each cDNA were assembled with the aid of the GAP4 software package (Bonfield *et al.* 1995). Sequence analysis was carried out by using various softwares from the GCG package (Genetics Computer Group 1996). Multiple

sequence alignments were performed with the aid of PILEUP and converted into distance matrices by DISTANCES software, using the correction method for superimposed substitutions developed by Kimura (1983). Places in which the alignments contained many gaps were eliminated from the distance matrix. The resulting distance matrices were then used to create dendrograms by means of GROWTREE software, using the unweighted pair group method of arithmetic averages (UPGMA) for cluster analysis (Swofford *et al.* 1996).

Measurement of β GtH-I and β GtH-II mRNA levels by RT-PCR

We have measured the relative levels of both β GtH-I and β GtH-II mRNAs by reverse transcriptase (RT)-PCR, using the 18S rRNA as an internal standard. The RT-PCR method was employed because the very small size of the blue gourami pituitary gland precluded the use of Northern blotting which requires relatively large amounts of RNA (Kawasaki 1990). The 18S rRNA was used as an internal standard of the measurements, since it is an abundant RNA and its expression is considered stable (Ambion Technical Bulletins 1998, Amoureux *et al.* 1997). It has been observed that the levels of 18S rRNA are more uniform than those of other commonly used internal standards, such as β actin (Ambion Technical Bulletins). We have determined the uniformity of 18S rRNA expression throughout the reproductive cycle, by amplifying its cDNA, using cDNA pools prepared from females at different stages of ovary development. The amount of the resulting PCR products, normalized to 1 μ g total RNA, is shown in Fig. 1.

RT-PCR was conducted as described above for 3' RACE-PCR, except that a mixture of random decamers (Biotechnological Services, Weizmann Institute, Rehovot, Israel) was added to the RT reaction for the synthesis of the cDNA of the 18S rRNA. A separate cDNA pool was prepared from the pituitary of each sampled fish. In addition, small pieces of the ovary of each female were fixed in Bouin's fixative (5% glacial acetic acid, 25% formaldehyde, 75% saturated solution of picric acid) and processed for light microscopy, in order to determine the stage of ovary development. At least five female blue gouramis from each of the following stages of the reproductive cycle were used in this study: previtellogenesis (immature females), low and high vitellogenesis (mature non-reproductive females) and FOM (mature reproductive females). The procedure of Jackson *et al.* (1994) was used to induce mature females to reach FOM.

FIGURE 1. Amplification of 18S cDNA prepared from pituitaries of female blue gourami at different stages of the reproductive cycle. The different stages are: previtellogenic (PV), low vitellogenesis (LV), high vitellogenesis (HV) and maturation (MS). The amount of PCR products was normalized per concentration of total RNA ($\mu\text{g}/\text{ml}$), and is expressed in arbitrary units of intensity as defined by the densitometry software (PCBAS-2). Each histogram represents the average of three independent measurements (means $\pm$ s.e.).

Amplification of the cDNA of β GtH-I, β GtH-II and 18S rRNA Each amplification of either β GtH-I or β GtH-II cDNA was coupled to an amplification of the internal standard 18S rRNA. In each case, the 18S rRNA cDNA and the hormone's cDNA were amplified in separate tubes, since many attempts to use multiplex PCR resulted in a considerable interference between the two reactions. Amplification of the 18S rRNA cDNA in the same tube with either β GtH-I or β GtH-II cDNA caused a reduction in one or both PCR products. Such interference problems are not uncommon in multiplex PCR, as reported by Bercovich *et al.* (1997). We have carefully calibrated the concentration of each primer pair and the number of cycles for amplifications which were linearly dependent on the initial concentration of the target cDNA.

β GtH-I and β GtH-II cDNAs were amplified with gene specific primers (see Table 1) at a concentration of 6.25 pmol/reaction. The 18S rRNA cDNA was amplified with specific primers, at a concentration of 5 pmol/reaction, which were obtained from Ambion as part of its Quantum RNA kit. The reagents, except for the cDNA and the primers, were prepared as a batch for each set of reactions, to minimize variations. The cycling parameters were the same as those described for the RACE-PCR, but in a smaller volume (50 μl) and with only 30 cycles. The number of cycles was determined by using an increasing number of PCR cycles to amplify serial dilutions of the cDNA pool and then plotting the amount of PCR products as a function of the cDNA pool's dilution. Only 30 cycles produced enough PCR products for the semi-quantitative analysis, while maintaining linear

FIGURE 2. The linearity of the amplification of GtH-I and GtH-II cDNAs after 30 cycles. The cDNA pool for the amplification of β GtH-I (solid diamonds) was obtained from a female at high vitellogenesis. The cDNA pool for the amplification of β GtH-II (solid squares) was obtained from a female at the final maturation stage. The amount of PCR products is expressed in arbitrary units of intensity as defined by the densitometry software (PCBAS-2). The coefficients of linearity are: 0.9989 for GtH-I and 0.9946 for GtH-II. Each point is an average of five different reactions.

dependence on the concentration of the cDNA. This linear dependence was kept after 30 cycles even when amplifying cDNA obtained from females in high vitellogenesis or at maturation (Fig. 2).

Quantification of PCR products The PCR products (1/2 of the PCR reaction) were electrophoresed on 2% agarose gel at 120 mA for 45 min. The DNA was then stained by ethidium bromide (0.03 mg in 100 μl distillate water), and imaged with the Eagle Eye II Image analysis system (Stratagene, La Jolla, CA, USA). The gels were imaged at three different exposure times to prevent a possible saturation of the images. The resulting images were analyzed with the aid of the PCBAS-2 densitometry software (Raytest, Straubenhardt, Germany). The significance of the differences in the amount of PCR products was analyzed by Student's *t*-test (Parker 1986).

RESULTS

cDNA cloning and sequence analysis

The complete cDNAs of the β subunits of GtH-I and GtH-II were cloned by RACE-PCR. The 5'

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1  GTCTGTACAGATGTTTAGAGAGTAACAGGCAAAACCTGCAGCAGAGGTTCAACGAGACAA    60
61  CAGAGATTTACAGGCGTCTGTGCTGCACCCAAAGGATGCAGCTGGTTGTCATGGCAGCAG    120
      MetGlnLeuValValMetAlaAlaV
121  TGTTCGTCAGTGGCGGGGGTGGGACAGGGTTGCCGCTTCGGCTGTCATCTAACCAACATCA    180
      alLeuAlaValAlaGlyValGlyGlnGlyCysArgPheGlyCysHisLeuThrAsnIleS
181  GCTTCCCCGTAGACAGCTGTGGCATCACCGAGTTCATCTACACCACCATATGCGCAGGAC    240
      erPheProValAspSerCysGlyIleThrGluPheIleTyrThrThrIleCysAlaGlyH
241  ATTGCTACCACGAGGATCCAGTCTACATCGGCCACGATGACTGGGCTGAACAGAAAATCT    300
      isCysTyrHisGluAspProValTyrIleGlyHisAspAspTrpAlaGluGlnLysIleC
301  GTAACGGGGACTGGACCTACGAAGTAAACACCTACAAGGATGTCCGGTGGCTGTCACGT    360
      ysAsnGlyAspTrpThrTyrGluValLysHisLeuGlnGlyCysProValAlaValThrT
361  ACCCTGTTGCCAGAACTGCGAGTGTACTGCTTGTAAATGCAGGAAACACATACTGTGGTC    420
      yrProValAlaArgAsnCysGluCysThrAlaCysAsnAlaGlyAsnThrTyrCysGlyH
421  ACTTTCATGGATACATACCCAGCTGTCTGTGATTCAAAGGCACTCATCATTCATCCCAC    480
      isPheHisGlyTyrIleProSerCysLeu
481  GTTTACTTTGCATCTTTCTCTGATGGTACTAAAATAAACAGATATGTCTTAAAAAAAAA    540
541  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA    570

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B.

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1  CTGGCTAACCTGCCGCTGACACTAAAGAGGATGATGACTGTAGAAATAAGCAAAGTGTTT    60
      MetThrValGluIleSerLysValPhe
61  GTCCTAATGATGTTAAACCTCTTTCTGGGAGCTTCATCTTCCATTTGGTCTGTGGCTCCA    120
      ValLeuMetMetLeuAsnLeuPheLeuGlyAlaSerSerSerIleTrpSerValAlaPro
121  GCAGCAGCCTTCCAGCTGCCACCGTGTGAGCTCATCAACCAGACTGTCTCTCTGGAGAAG    180
      AlaAlaAlaPheGlnLeuProProCysGlnLeuIleAsnGlnThrValSerLeuGluLys
181  GAAGGCTGCCCCAGGTGTCACGCAGTGGAACGACAATCTGCAGTGGCCACTGCCTCACC    240
      GluGlyCysProArgCysHisAlaValGluThrThrIleCysSerGlyHisCysLeuThr
241  AAGGACCCAATCATCAAGATACCGTTCAGCAATGTGTACCAGCACGTGTGCACATACCGG    300
      LysAspProIleIleLysIleProPheSerAsnValTyrGlnHisValCysThrTyrArg
301  GATTTGTTCTATAAGACATTTGAGTTTCTGACTGTCTCTCTGGTGTGGACCCAGTCGTC    360
      AspLeuPheTyrLysThrPheGluPheProAspCysProProGlyValAspProValVal
361  ACCTACCCTGTGGCTCTGAGCTGCCACTGCAGCCGTTGTGTGATGGACACATCCGATTGC    420
      ThrTyrProValAlaLeuSerCysHisCysSerArgCysValMetAspThrSerAspCys
421  ACCTTCGAGAGTCTTCAGCCAGACTTCTGCATGAATGACATACCTTTCTACTACTAGTCT    480
      ThrPheGluSerLeuGlnProAspPheCysMetAsnAspIleProPheTyrTyr
481  GATGAAATAGCTGCACAGCAAACCAAAAGCAAAGTAAATAAATGAATTGTGACAAAAA    540
541  AAAAAAAAAAAAAA    552

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FIGURE 3. The nucleotide sequences of blue gourami β GtH-I (A) and β GtH-II (B). The amino acid sequence of each hormone appears in its three-letter code. The GenBank accession number of these sequences are AF157630 and AF157631 respectively.

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gth1      1  ~~~~~~M Q D . V V M . A . . A V L A V A G V G Q G C R F . . . G C H L T N . . I S F . . . . . E V D S C
gth2      1  M T V E I S K V F V L M M L N L F L G A S S S I W S V A . . . P A A A F Q L P P C Q L I N Q T V S L E K E G C P . . R C

gth1     36  . G I T E F I Y T T I C A G H C Y H E D P V Y . I G H D D . W A E Q K I C N . G D . . W . T Y E V K H L Q G C P . . V .
gth2     56  H A V . E . . . T T I C S G H C L T K D P I I K I P F S N V Y . . Q H V C T Y R D L F Y K T F E . . . F P D C E P G V D

gth1     86  . A V T Y P V A R N C E C T A C N A G N T Y . C G H F H G Y I . P S . C L ~~~~~~
gth2    107  P V V T Y P V A L S C H C S R C . V M D T S D C . T F E S . L Q P D F C M N D I P F Y Y

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FIGURE 4. A comparison of the amino acid sequences of β GtH-I (gth1) and β GtH-II (gth2). Identical amino acids are boxed in black whereas residues with similar physicochemical properties are boxed in gray.

and 3' ends of β GtH-I and β GtH-II cDNA were cloned separately, but with a short overlap, which allowed their proper joining into a single nucleotide sequence that spanned the entire coding region. The cDNA sequences of both β subunits, together with their deduced amino acid sequences, are shown in Fig. 3. A comparison of the two predicted polypeptides is shown in Fig. 4. There was only ~30% similarity between β GtH-I and β GtH-II showing that they are very distant from each other. Eleven out of twelve cysteines, which are probably involved in the formation of six disulfide bonds required for proper folding and interaction with the α subunit, represent about a third of the identical residues (Beebe *et al.* 1990).

We have found that blue gourami β GtH-I is most similar to its striped bass (*Morone saxatilis*) counterpart, with the two polypeptides sharing 73% of their residues. The lowest similarity was found between blue gourami β GtH-I and the β GtH-I of the goldfish, with only 44% of their residues identical. A dendrogram, which graphically represents the relationships among the various β subunits of GtH-I, is presented in Fig. 5A. Similarly, when the amino acid sequence of the blue gourami β GtH-II was compared with β GtH-II polypeptides from the same group of fish as in the former comparison, the highest similarity was found with striped bass β GtH-II, 84% of their residues being identical. The lowest similarity was found with the baikal omul β GtH-II (only 65% identical residues). The dendrogram which represents the relationships among the β GtH-II polypeptides is presented in Fig. 5B.

Variations in the levels of β GtH-I and β GtH-II mRNAs during the reproductive cycle

The amplification of β GtH-I cDNA produced a 306 bp product and that of β GtH-II cDNA a 284 bp product. The amplification of the internal standard (the cDNA of 18S rRNA) generated a 430 bp long product. The identity of each PCR product was confirmed by DNA sequencing.

Figure 6 shows an agarose gel electrophoresis of the three PCR products, which were taken from reactions with cDNAs from four different maturation stages.

The variations in the expression of the β GtH-I and β GtH-II genes at the mRNA level are presented in Fig. 7. Amplification of cDNA from previtellogenic fish resulted in almost undetectable levels of β GtH-I and β GtH-II specific products, which indicate very low levels of their transcripts. The expression of β GtH-I and β GtH-II rose at the stage of low vitellogenesis, as manifest by the increased amount of PCR products.

There was a significant increase ($P < 0.05$, t -test) in the levels of β GtH-I and β GtH-II expression in high vitellogenesis and final maturation as compared with the levels which were detected during pre- and low vitellogenesis. On the other hand, there was no significant difference in the levels of β GtH-I and β GtH-II transcripts measured during high vitellogenesis and final maturation ($P > 0.05$, t -test).

DISCUSSION

The deduced amino acid sequences of β GtH-I, β GtH-II of *T. trichopterus* were compared with similar polypeptides from several other teleosts by multiple sequence alignments and cluster analysis. This analysis revealed that both β GtH-I and β GtH-II of the blue gourami were most similar to their counterparts from the striped bass (Hassin *et al.* 1995) (Fig. 5). Both of these species belong to the order Perciformes, the largest order of teleosts, with 251 families, and therefore this sequence similarity agrees well with known systematics. It is interesting to note that, in agreement with previous findings (Elizur *et al.* 1996), our comparison clearly shows that the amino acid sequences of β GtH-I have changed more rapidly and show a lower degree of similarity than the corresponding amino acid sequences of β GtH-II (Fig. 5). The rapid divergence of β GtH-I, compared with β GtH-II,

FIGURE 5. Dendrograms which graphically show the relations among various fish β GtH-I (A) or β GtH-II (B). The dendrograms were created with the method of UPGMA from similarity matrices which were produced from the corresponding sequence alignments. The scale bar is a measure of the estimated number of amino acid substitutions per 100 residues which are found when the sequences are compared in a pairwise fashion.

has already been described in other teleost species (Kato *et al.* 1993).

It is reasonable to assume that an inference of phylogenetic relations based on only one hormone-coding cDNA would have questionable reliability. However, analyses of two different polypeptide hormones from the blue gourami are presented

here, and both are consistent with its classification. The results of the cluster analysis, which positioned the blue gourami close to other members of the order Perciformes, can therefore be regarded as reliable.

In the present study, the temporal differences in the biosynthesis of β GtH-I and β GtH-II during gonadal development were determined by studying the changes in their respective mRNAs. GtH-I transcripts were first detected during low vitellogenesis and an accentuated increase occurred during high vitellogenesis and maturation. The participation of GtH-I in vitellogenesis of the teleost has been reported previously (Tyler *et al.* 1991, 1997). The presence of GtH-I transcripts in the pituitary glands of low vitellogenic females was expected since the deposition of vitellogenin had already started in the most developed oocytes (Degani 1993a). The process of vitellogenesis continues until 30% to 40% of the oocytes are at the post-vitellogenic stage. An increase in the level of GtH-I transcripts in previtellogenic (Weil *et al.* 1995) and

FIGURE 6. Gel electrophoresis of 18S cDNA together with either GtH-I (lanes 1–4) or GtH-II (lanes 5–8) PCR products. Each lane shows amplification of cDNAs prepared from the pituitary of a single female. Young females (lanes 1 and 5), previtellogenic females (lanes 2 and 5), high vitellogenic females (lanes 3 and 7), and females at FOM (lanes 4 and 8). (SM=size marker).

FIGURE 7. Variations in (A) β GtH-I and (B) β GtH-II transcript levels during the gonadal cycle: previtellogenic (PV), low vitellogenesis (LV), high vitellogenesis (HV), and maturation (MS). Each histogram represents the average of five independent measurements (means $\pm$ S.E.).

vitellogenic teleosts (Naito *et al.* 1991) has been described previously.

The level of mRNA of β GtH-I also remains high in reproductive females during the final stage of oocyte maturation. The blue gourami is a multi-spawner with an asynchronic type of ovary development. The same female can spawn four to five times consecutively with 2- to 3-day pauses between the spawnings (unpublished results, K Jackson & G Degani). Therefore, vitellogenic

oocytes should be found in the ovary of maturing and post-spawned blue gourami females.

A similar pattern of gene expression has been found for GtH-II. The levels of its β subunit transcript were undetectable in immature females, rose in low vitellogenic fish and showed a sharp increase during high vitellogenesis. The high level of β GtH-II transcript remained high at the stage of FOM. An increase in the level of β GtH-II mRNA around maturation and spawning has already been

described (Naito *et al.* 1991, Weil *et al.* 1995, Nagae *et al.* 1996). A similar pattern was observed for GtH-II hormone expression in the blue gourami. The beginning of GtH-II expression is low in vitellogenic fish and it is present in high levels in the pituitaries of high vitellogenic females. However, the hormone is released to the blood only during FOM (Degani *et al.* 1997).

It has been suggested that estrogens and androgens stimulate GtH-II synthesis in the pituitary (Querat *et al.* 1991, Huggard *et al.* 1996, Nagae *et al.* 1996). Dickey & Swanson (1995) found that in immature coho salmon injected with estradiol there was an increase in the steady-state mRNA levels of β GtH-II but not β GtH-I. The presence of a steroid response element in the promoter for the β GtH-II subunit gene has already been described in salmonids (Xiong *et al.* 1994). We have found an increase in the level of β GtH-II mRNA in the pituitaries of low vitellogenic females, which may be the result of the rise in the levels of estradiol in low vitellogenic females (Degani 1990). The steady high levels of β GtH-II mRNA in high vitellogenesis and during FOM may be a consequence of the synthesis and release of other regulatory factors, such as GnRH, since at this time there is a decrease in the level of estradiol (Degani 1990). GnRH affects GtH biosynthesis and was shown to cause an increase in the levels of α and β GtH-II mRNAs (Khakoo *et al.* 1994).

In the blue gourami, both GtH-I and GtH-II RNA levels increase during the advancement of the gonadal cycle. This pattern is similar to that found in goldfish (Yoshiura *et al.* 1997) and gilt-head seabream (*Sparus aurata*) (Meiri *et al.* 1995, Elizur *et al.* 1996). Both are multi-spawners with group-synchronic or asynchronic type of ovary development. This pattern differs from that found in salmonids, where GtH-I is expressed during vitellogenesis and GtH-II during late vitellogenesis and FOM, as was found in rainbow trout (Naito *et al.* 1991, Weil *et al.* 1995), an annual spawner with a synchronous type of ovary. The difference in the pattern of GtH-I and GtH-II gene expression is probably related to the different types of gonadal cycles: multi-spawners versus annual spawners.

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