PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 75 (2000) 291–298

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Expression of cytochrome $P450_{11\beta}$ (11β-hydroxylase) gene during gonadal sex differentiation and spermatogenesis in rainbow trout, *Oncorhynchus mykiss*

Shaojun Liu [a,1], Marina Govoroun [a], Helena D'Cotta [b], Marie-José Ricordel [a], Jean-Jacques Lareyre [a], Oonagh Marie McMeel [c], Terry Smith [c], Yoshitaka Nagahama [d], Yann Guiguen [a,*]

[a] *Station INRA SCRIBE, Equipe Sexualite et Reproduction, Campus de Beaulieu, 35042 Rennes Cedex, France*
[b] *CIRAD-EMVT Campus de Beaulieu, 35042 Rennes Cedex, France*
[c] *National Diagnostic Centre (NDC), National University of Ireland, University Road, Galway, Ireland*
[d] *Laboratory of Reproductive Biology, National Institute for Basic Biology, Okazaki 444, Japan*

Accepted 20 October 2000

Abstract

Androgens and especially 11-oxygenated androgens are known to be potent masculinizing steroids in fish. As a first step to study their physiological implication in gonadal sex differentiation in fish, we cloned a testicular cytochrome $P450_{11\beta}$ (11β-hydroxylase) cDNA in the rainbow trout, *Oncorhynchus mykiss*. We isolated a 1882 bp $P450_{11\beta}$ cDNA (rt11βH2, AF217273) which contains an open reading frame encoding a 552 putative amino acids protein. This sequence was highly homologous (98% in nucleotides and 96.5% in amino acids) to another rainbow trout $P450_{11\beta}$ sequence (AF179894) and also to a Japanese eel $P450_{11\beta}$ (68% in amino acids). Northern blot analysis detected a single transcript of 2 kb which was highly expressed in the testis (stage II) and to a lesser degree in the anterior kidney (containing the interrenal tissue). No signal was detected in the posterior kidney, brain, liver, skin, intestine and heart. In the testis this transcript was highly expressed at the beginning of spermatogenesis (stages I and II), followed by a decrease during late spermatogenesis (stages III to V). By semi-quantitative reverse transcription polymerase chain reaction, $P450_{11\beta}$ expression during gonadal differentiation was estimated to be at least 100 times higher in male than in female gonads. This difference was first detected at 55 days post-fertilization (dpf), i.e. 3 weeks before the first sign of histological sex differentiation, and was sustained long after differentiation (127 dpf). Specific $P450_{11\beta}$ gene expression was also demonstrated before testis differentiation (around 50 dpf) using virtual Northern blot, with no expression detected in female differentiating gonads. From these results, and also based on the already known actions of 11-oxygenated androgens in testicular differentiation in fish, it is now suggested that $P450_{11\beta}$ gene expression is a key factor for the testicular differentiation in rainbow trout.

Keywords: Fish; Steroids; 11-Oxygenated androgens; Testis; Interrenal

1. Introduction

Steroids have been shown to play a crucial role in non-mammalian vertebrate gonadal sex differentiation in fish [1], amphibian [2], reptiles [3,4] and birds [5,6]. According to Yamamoto [7], androgens in fish would act as andro-inducers driving testis differentiation and estrogens as gyno-inducers driving ovarian differentiation. This model was primarily based on the masculinizing and feminizing effects of androgens and estrogens,

Abbreviations: bp, base pair; 11βH, 11β-hydroxylase; dpf, days post fertilization; kb, kilobase; RT-PCR, reverse transcription polymerase chain reaction.

* Corresponding author. Tel.: +33-2-23485009; fax: +33-2-23485020.

E-mail address: guiguen@beaulieu.rennes.inra.fr (Y. Guiguen).

[1] Present address: Department of Biology, Hunan Normal University, Changsha, Hunan, People's Republic of China.

0960-0760/01/$ - see front matter
PII: S0960-0760(00)00186-2

respectively, when administrated during the sex differentiation period. A physiological role for estrogens in ovarian differentiation has been suggested by the masculinizing effects of some aromatase inhibitors [8,9] and by the detection of an aromatase gene expression before and during ovarian differentiation [9]. On the other hand, the respective implication of androgens in testicular differentiation is much less detailed and their physiological involvement remains to be proven [10].

With regards to androgens, the most potent ones in fish have been shown to be 11-oxygenated [11]. Their actions are clearly related with male secondary sexual characters' development, spermatogenesis and male reproductive behavior [11]. Also, they have been shown to be the most potent natural androgens acting on sex differentiation [10].

The first enzyme involved in the 11-oxygenated androgen synthesis from precursors such as testosterone or androstenedione is 11β-hydroxylase, cytochrome $P450_{11\beta}$ [12]. In higher vertebrates this enzyme is mainly involved in the synthesis of mineralocorticoids and glucocorticoids in the adrenal cortex [13]. In fish, this enzyme is found in the testis as well, and the testicular expression of a $P450_{11\beta}$ (11β-hydroxylase) gene has been found in the Japanese eel [14]. We now report the cloning of a rainbow trout, *Oncorhynchus mykiss*, testicular $P450_{11\beta}$ (11β-hydroxylase) cDNA sequence, slightly divergent from a recently reported homologous sequence, and characterize its expression during gonadal sex differentiation and spermatogenesis.

2. Materials and methods

2.1. Animals and samplings

All-female and all-male populations of rainbow trout have been obtained by fertilization of normal eggs with sperm from XX and YY males, respectively, as previously described [9]. For the semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) assay, 200–400 gonads were isolated under the dissecting microscope at each sampling date. Ten serial samplings were performed for both the all-male and all-female populations at 1-week intervals starting at the onset of first feeding (55 dpf). Gonads were immediately frozen in liquid nitrogen and pooled in 2-ml eppendorf tubes according to their origin and sampling date. They were stored at −70°C until PolyA+ RNA extraction. For the virtual Northern analysis gonads were sampled in all-male (1830 gonads) and all-female (1860 gonads) populations between 49 and 63 dpf, i.e. during the 2 weeks following swim-up.

One and 2-year-old male rainbow trout, either immature or in the first gametogenesis were used and kept in recycled freshwater at 12–15°C under natural photoperiod. Fish were anesthetized with 0.3% phenoxyethanol and killed by a blow to the head. Testis was quickly collected and a small piece of tissue was fixed in Bouin's Hollande fixative for histological analysis. The rest of the gonads were quickly frozen in liquid nitrogen and stored at −70°C until RNA extraction. Stages of rainbow trout spermatogenesis were determined by histological analysis according to Billard and Escraffre [15]. Stage I corresponds to immature fish, stages II and III to early spermatogenesis, stages IV and V to mid-spermatogenesis.

2.2. RNA extractions

Total RNA was isolated from frozen gonads (−70°C) using the TRIzol reagent (Gibco) and resuspended in 50 μl DEPC treated water. The concentration of RNA was determined by spectrophotometry. PolyA+ RNA was isolated either directly from liquid nitrogen frozen gonads using oligo$(dT)_{25}$ coupled to magnetic beads (Dynabeads®, Dynal, Oslo, Norway) as previously described [9], or following total RNA purification with Quiagen mRNA purification kit (Quiagen).

2.3. Cloning of rainbow trout 11β-hydroxylase cDNA

A homologous PCR fragment was obtained in rainbow trout by using the degenerate primers designed for Japanese eel 11β-hydroxylase cloning [14] with some slight modifications (primers designed for the cloning of eel $P450_{11\beta}$ = amino acid regions sense FELARNP: 5′-TTY GAR YTN MGN AAY CC-3′; antisense NYHIPAG: 5′-CC NGC NGG DAT RTG RTA RTT-3′). The corresponding amino acids in the rainbow trout sequence are underlined in Fig. 1. This primer combination was used in RT-PCR on cDNA synthesized from rainbow trout testis mRNA. Reverse transcription and RT-PCR conditions were as previously described [9]. Following PCR (cycling conditions = 94°C 30 s, 55°C 30 s, 72°C 30 s, for 40 cycles), the PCR product was separated on a 1.5% agarose gel and the expected size fragment was excised from the gel, purified using geneclean (BIO101) and then subcloned into Pmosblue TA cloning vector (Amersham). Following confirmation of identity by sequencing, this fragment was then used to screen a λ ZAP (stratagene) rainbow trout cDNA testis library (1.5×10^6 initial recombinants, mean insert size = 1.7 kb) under stringent hybridization conditions as previously described [16]. Positive clones were finally sequenced on both strands using an ABI prism 310 automatic sequencer (Perkin–Elmer).

2.4. Semi-quantification of gene expression by RT-PCR

Semi-quantification by RT-PCR was performed as previously described [9]. Oligonucleotides used as primers in PCR amplification were: 5′-T GAC GCC CAC AAG GCC CTG C-3′ (sense primer, underlined in Fig. 1) and 5′-GTG AGT TCA TTG AGA TTA CCT G-3′, (antisense primer, underlined in Fig. 1). In order to work in the exponential range of amplification, the number of cycles was defined for each set of primers in a preliminary experiment using our standard conditions. Cycle number was then set at 20 for β-actin and 30 for $P450_{11\beta}$. After PCR amplification, 1 μl of each PCR reaction including appropriate controls (water only, and genomic DNA) was dotted on nylon membrane (Hybon N+, Amersham). The membrane was then denatured (3 min) and neutralized (5 min) by capillary transfer with denaturation solution (NaOH

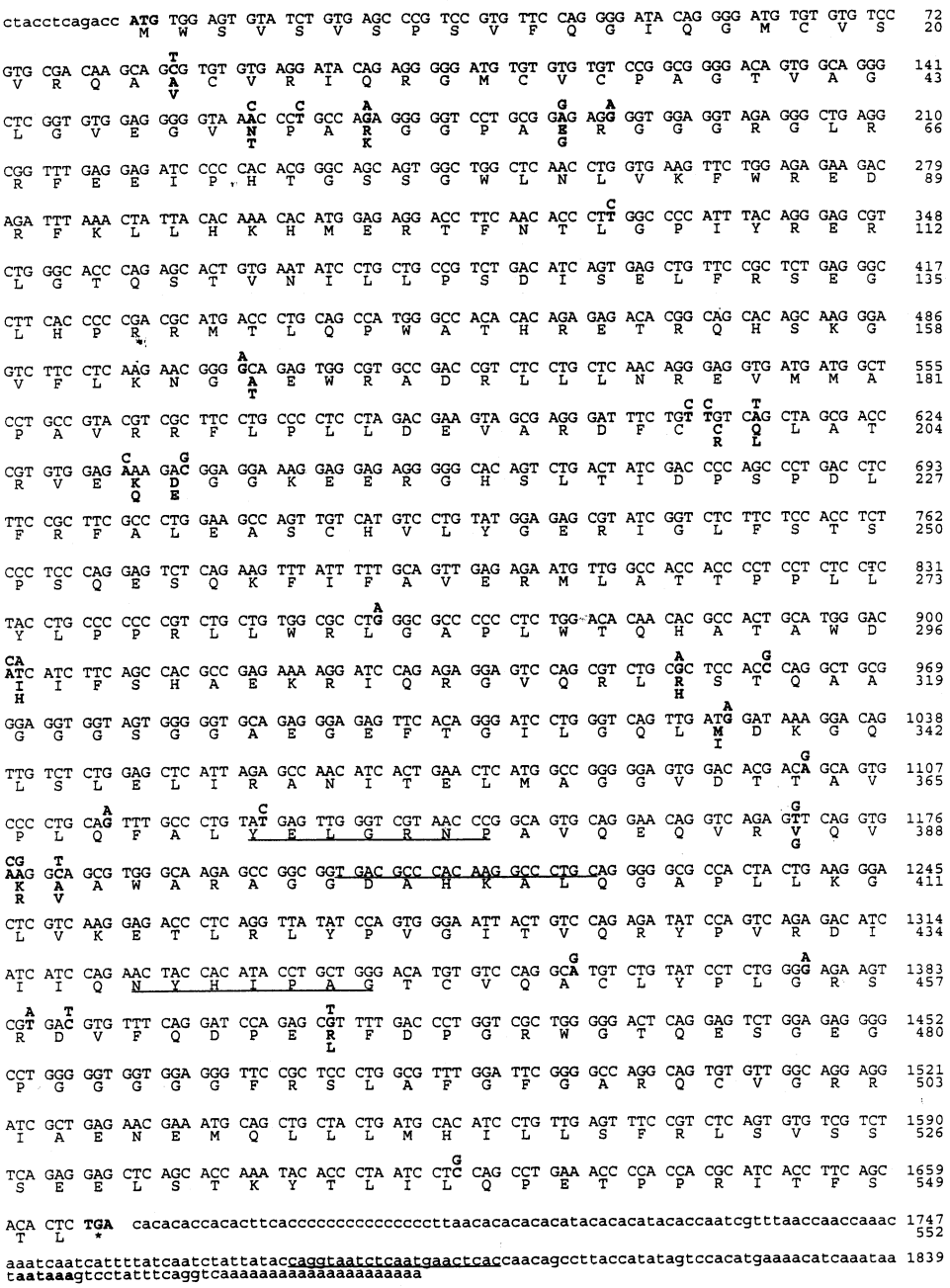

Fig. 1. Sequence of the rainbow trout $P450_{11\beta}$ (rt11βH2) with the corresponding translation in amino acids (bottom lines). Differences between rt11βH2 and the previously described rainbow trout $P450_{11\beta}$ (rt11βH1, AF179894) are shown in bold for nucleic acids (top lines) and amino acids (bottom lines). Amino acids are numbered relative to the first in-frame methionine codon and the amino acid regions used for the design of degenerate primers are underlined. The start codon (ATG), stop codon (TGA) and the polyadenylation signal (AATAAA) are in bold type, and the nucleic acid sequences of primers used for semi-quantification by PCR are underlined.

0.5 M, NaCl 1.5 M) and neutralization solution (Tris–HCl 0.5 M, NaCl 1.5 M, pH 7), and finally rinsed quickly in 2 × SSC, before UV light cross-linking. Membranes were then hybridized with the corresponding probe, i.e. rt11βH2 or β-actin, labeled by random priming with [α-32]-dCTP following the multiprime DNA labeling systems protocol (Amersham). Hybridization was carried out overnight at 65°C, in 5 × SSC, 5 × Denhart's, 0.5% SDS and 20 μg/ml of denatured calf thymus DNA. Membranes were washed to high stringency in 0.1 × SSC/0.1% SDS at 65°C and then quantitatively analyzed using an instant imager (Packard). Data were expressed as logarithms of 11β-hydroxylase/β-actin ratios. With these PCR conditions no amplification was detected either with water or with genomic DNA control. Only one single band of the expected size was detected in PCR reactions.

2.5. Northern blot and virtual Northern blot analysis

Northern blots were essentially performed as previously described [17], except that calf thymus DNA was used instead of salmon sperm DNA. In addition, the ULTRAhyb™ solution (Ambion) was used when the hybridization was carried out with rt11βH2 as a probe. 20 μg of total RNA was loaded in each lane. Northern blots were reprobed with a 28S cDNA to standardize the loaded RNA samples. The hybridization signal corresponding to the 11β-hydroxylase mRNA and 28S rRNA was measured using an instant imager (Packard).

Because of the very small amount of available tissue, classical techniques such as Northern blot analyses were not used on differentiating gonads, thus the more sensitive technique of virtual Northern blot has been chosen instead as it has been proven to be perfectly sound for quantification purposes [18,19]. Virtual Northern blots were performed as previously described [9]. Briefly, first strand cDNA synthesis was carried out using 1 μg total RNA. The RNA was reverse transcribed with a modified oligo dT primer in the presence of the SMART™ oligonucleotide (SMART™ PCR cDNA Synthesis Kit, Clontech). This was carried out simultaneously for both male and female RNA using 200 units of superscript II RNaseH (Gibco BRL) in a 10 μl reaction. PCR amplification was then carried out using 2 μl of the first strand cDNA in a total PCR reaction volume of 100 μl at 95°C for 1 min followed by 15 cycles of 95°C for 15 s, 68°C for 5 min. Primers for amplification were as described in the manufacturer's instructions. These 100 μl PCR reactions were precipitated (50 μl of ammonium acetate 4 M, and 375 μl ethanol 95%), resuspended in 10 μl water, and then loaded on a 1% TBE/agarose gel. After migration of the samples, the gel was denatured, neutralized and DNA transferred to nylon membrane (Hybond-N, Amersham) by Southern blotting in 20 × SSC. DNA was fixed to the membrane by baking at 80°C for 2 h. The membrane was then hybridized overnight as described for the Northern blot using a [α-32]-dCTP labeled rt11βH2 cDNA probe. The membrane was washed in 0.2 × SSC, 0.1% SDS at 65°C. The probe was removed from the membrane by incubation for 15 min in 0.1% SDS at 95°C. To confirm loading of cDNA samples, the membrane was then reprobed with rainbow trout β-actin under the same hybridization and washing conditions.

3. Results

3.1. Rainbow trout 11β-hydroxylase cloning and sequence comparisons

Using degenerate primers designed in conserved regions of known 11β-hydroxylase cDNAs, a PCR fragment corresponding to a rainbow trout $P450_{11\beta}$ was isolated. This fragment was then used as a probe to screen a rainbow trout testis cDNA library. The sequence obtained after this screening is shown in Fig. 1. This 1882 bp sequence contains an open reading frame encoding a 552 putative amino acid protein (rt11βH2, AF217273) and was found to be a close homologue of another, recently released, rainbow trout 11β-hydroxylase sequence (rt11βH1, accession number AF179894). The overall homology between rt11βH1 and rt11βH2 is 98% for the cDNA and 96.5% at the amino acid level in the same region (see differences in Fig. 1). Besides, rt11βH2 also includes a complete 193 bp untranslated 3′ end (without the polyA tail), including a polyadenylation signal located 15 bases upstream of the polyA tail. Clustaw amino acids sequence comparison with already characterized 11β-hydroxylase sequences i.e. Japanese eel, *Anguilla japonica* [14] and bullfrog, *Rana catesbeiana* (D10984) shows, respectively, 68 and 46% homology (data not shown).

3.2. Rainbow trout 11β-hydroxylase gene expression

By Northern blot analysis we looked at the expression of $P450_{11\beta}$ in different adult tissues (Fig. 2). A single 2-kb size transcript was only detected in the testis (stage II) and the anterior kidney. Expression was a few times less active in the anterior kidney than in the testis. No signal was detected in the posterior kidney, brain, liver, skin, intestine and heart. In the testis (Fig. 3), the 2-kb transcript was highly expressed at the beginning of spermatogenesis (stages I and II), followed by a decrease during the late spermatogenesis (stages III to V).

$P450_{11\beta}$ gene expression was also analyzed by semi-quantitative RT-PCR during the process of gonadal differentiation. $P450_{11\beta}$ was found to be differentially

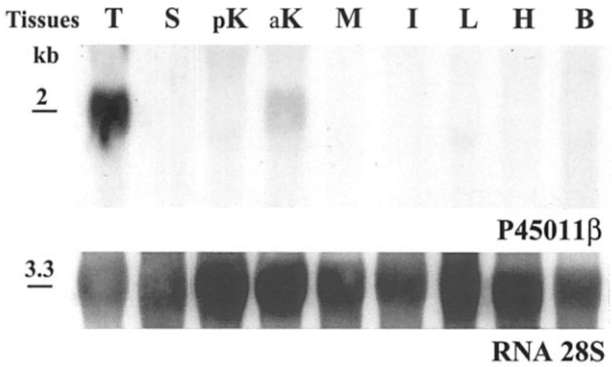

Fig. 2. Northern blot analysis of $P450_{11\beta}$ mRNA (top panel) and 28S rRNA (bottom panel) in different adult rainbow trout tissues (T = testis, S = skin, pK = posterior kidney, aK = anterior kidney, M = muscle, I = intestine, L = liver, H = heart, B = brain). Transcript sizes are indicated on the left of the figure.

expressed according to the sex genotype at a very early stage (55 dpf) and was found to be at least 100 times more expressed in male than in female gonads (Fig. 4). This sex dimorphic expression was sustained until the last sampling date (127 dpf). This differential expression was confirmed by virtual Northern blot (Fig. 5), which showed a single band of the expected size (2 kb) before gonadal differentiation only in genetic males

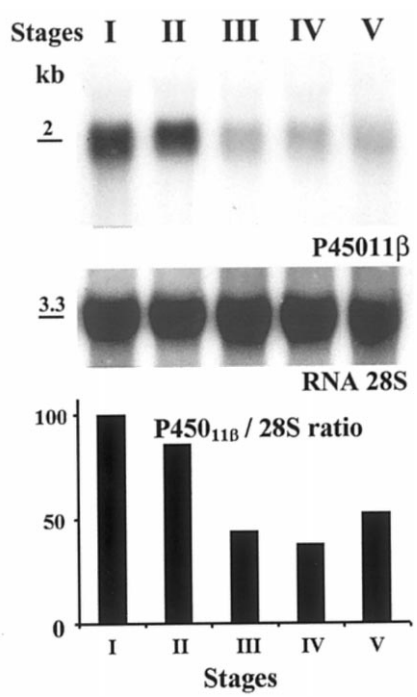

Fig. 3. Northern blot analysis of $P450_{11\beta}$ mRNA (top panel) and 28S rRNA (bottom panel) at different stages (I to V) of rainbow trout testicular gametogenesis. Transcript sizes are indicated on the left of the figure. The ratio of $P450_{11\beta}$ over 28S rRNA is shown (represented as a percentage of the highest ratio) on the figure below to allow an accurate quantification of the $P450_{11\beta}$ mRNA evolution during spermatogenesis.

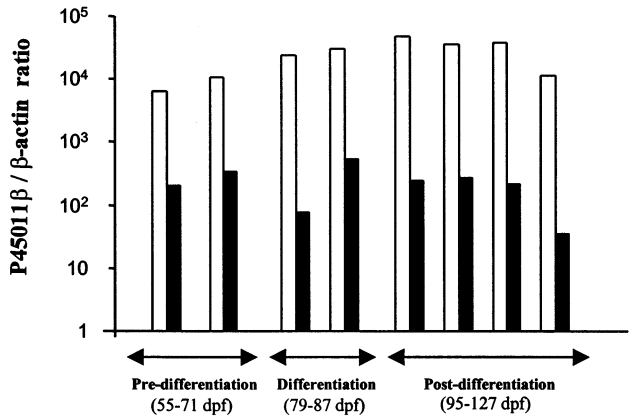

Fig. 4. Semi-quantification by RT-PCR of $P450_{11\beta}$ gene expression before, during and after histological sex differentiation. Data are logarithmic and represented as a $P450_{11\beta}$ ratio over β-actin with an arbitrary scale. Black bars represent females and open bars males. dpf = days post-fertilization.

(sampled between 49 and 63 dpf). No signal could be detected in female gonads at the same stage while loading homogeneity between male and female samples was confirmed by β-actin hybridization.

4. Discussion

We reported in this study the molecular cloning of a full-length rainbow trout $P450_{11\beta}$ cDNA from a testicular library. Its identity is supported by a good overall homology (68% in amino acids in the coding region) with the already known fish sequence (Japanese eel, [14]). Homologies with other $P450_{11\beta}$ sequences from frog or mammals are around 40–45% in amino acids which is similar to the result obtained when Japanese eel was compared with these sequences [14]. Moreover,

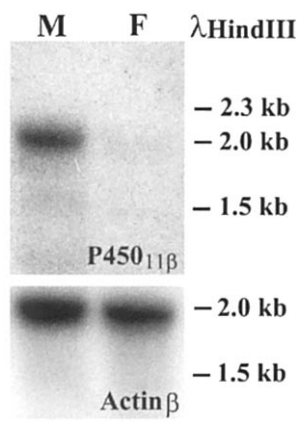

Fig. 5. Virtual Northern blot analysis of $P450_{11\beta}$ (top panel) and β-actin (bottom panel) gene expression in rainbow trout differentiating gonads from an all-male (M) and all-female (F) populations sampled between 49 and 63 dpf. λ *Hin*dIII DNA size ladder is indicated on the right of the figure.

another highly homologous cDNA sequence encoding a second rainbow trout $P450_{11\beta}$ has been recently released (Kusakabe et al., unpublished results, accession number AF179894). Interestingly, in our hands, the PCR product initially obtained, was identical with this unpublished sequence but the small differences (four nucleotide changes over 180 bp between the PCR product and the cDNA from the testis library) were considered as PCR errors at that time. This high homology could indicate that these two sequences are recently duplicated genes since fish are thought to have undergone one or even more genome duplication events [20]. This gene duplication coupled with the high level of homology existing between these two sequences lead to technical problems in that discrimination of the two cDNA in hybridization studies e.g. Northern blot or virtual Northern is not possible. This is further complicated by the lack of comparative sequence information for either the 3′ or 5′ untranslated regions, which are generally thought to be more divergent than the coding sequence. The fact that only one single size signal was detected both in Northern and in virtual Northern would indicate that either both transcripts are of the same size (published sequence size is 1710 bp for 11βH1 and 1880 bp for 11βH2) or that only one gene was expressed. However, since both transcripts were found in the same mRNA population used for producing the initial PCR fragment and for the cDNA library construction both genes were probably expressed, at least in the testis. Whether they have a differential tissue and/or stages expression pattern remains unknown and more accurate studies are needed, using gene specific regions like the 3′ end of 11βH1. These regions may be sufficiently divergent to facilitate either specific hybridization or identification of a discriminatory primer site. Concerning tissue expression, this or these gene(s) were found to be highly expressed in the testis in agreement with the high 11β-hydroxylase activity reported in fish testis [11,21]. However, a significant $P450_{11\beta}$ expression has also been found in the rainbow trout anterior kidney as previously shown in Japanese eel $P450_{11\beta}$ [22]. The fish anterior kidney (also called 'head kidney') contains the interrenal tissue which is the homologue of the adrenal cortex in mammals and 11β-hydroxylase enzyme activity has been localized in the anterior kidney of various salmonid species [23,24]. In agreement with our results no activity was found in any tissue other than in the testis and the anterior kidney. In the Japanese eel, $P450_{11\beta}$ has no aldosterone synthesis activity and thus is thought to be involved mainly in cortisol biosynthesis [22]. However, it has also been hypothesized that this interrenal 11β-hydroxylase activity may even produce significant amounts of circulating 11-oxygenated androgens or steroid substrates that could be important in respect to the sex differentiation process [25,26]. Concerning the testis, the highest expression of $P450_{11\beta}$ occurred in trout at the very beginning of spermatogenesis (stages I and II) and decreased by half during subsequent stages (III to V) which correspond to the initiation of testicular meiosis [15]. This is particularly surprising in regards to the literature on plasma or gonad levels of 11-oxygenated androgens (mainly 11-ketotestosterone), which are reported to be low during early spermatogenesis and peak at spermiation in salmonids (reviewed in Ref. [11]). Either, this increase in transcription of the $P450_{11\beta}$ gene is posterior to the stages that we studied (i.e. stage VI and onwards), or there is another limiting step such as the availability of substrate, the translation of the enzyme, its activation, etc. However it should be also noticed that the somatic cells where the $P450_{11\beta}$ gene is supposed to be expressed are considerably diluted by germinal cells during the late stages of spermatogenesis. This high dilution factor may also be an explanation for the apparent gene expression decrease during rainbow trout spermatogenesis.

The involvement of androgens in male fish sex differentiation has been mainly inferred from results obtained with in vivo steroid treatments, and it is generally accepted that 11-oxygenated androgens are the naturally active steroids acting on gonadal differentiation in fish [1]. Our results clearly demonstrate that $P450_{11\beta}$ gene is already expressed before and during testicular differentiation. Consistent with that finding, it has been shown that the 11β-hydroxylase enzyme activity is detected (by the identification of 11-oxygenated metabolites) during or just after testis differentiation in the tilapia, *Oreochromis niloticus* [10], and in the rainbow trout [1,27,28]. In the catfish *Clarias gariepinus* [29], it has also been reported, as unpublished data, that 11-oxygenated androgens were male specific and synthesized during testis differentiation in larvae. In common carp, 11-oxygenated androgens are the major metabolites in the male differentiating gonads, whereas in females, estrogens are the major metabolites while no 11-oxygenated androgens are produced. In XX males homozygous for a masculinizing factor called 'mas', both estrogens and 11-oxygenated androgens are produced and animals differentiate into the male phenotype. It would therefore appear that the precocious synthesis of 11-oxygenated androgens in the $XX_{(mas/mas)}$ animals directs male sex inversion [30].

This male-specific expression of $P450_{11\beta}$ in the differentiating rainbow trout testis is however much lower than the $P450_{aro}$ (aromatase) female-specific expression found in differentiating ovaries [9]. Roughly ten times more concentrated sample was needed to get the same hybridization signal in virtual Northern than those found for $P450_{aro}$ in the ovary. This could mean that a higher estrogen production is needed for ovarian differentiation than levels of 11-oxygenated androgen production for testis differentiation. This may be related to

the higher estrogens doses required for feminization than those of androgens required for masculinization. In salmonids for instance 20 mg/kg of food of 17β-estradiol and only 3 mg/kg of methyltestosterone are needed [31,32]. In this respect, it should also be noticed that natural 11-oxygenated androgens have been shown to be much more efficient than testosterone in fish masculinization protocols [33–36].

In conclusion, we were able to demonstrate for the first time that a $P450_{11\beta}$ gene is expressed exclusively in the testis before and during gonadal differentiation of rainbow trout. However, the $P450_{11\beta}$ gene duality encountered in this species needs to be further investigated by analyzing each gene (11βH1 and 11βH2) expression in more detail.

Acknowledgements

This work and fellowships to M. Govoroun, H. D'Cotta and O. McMeel were supported by an European Community Project (PL 97-3796). We thank Dr. Maurice Loir and Dr. Alexis Fostier for critical reading of the manuscript.

References

[1] J.F. Baroiller, Y. Guiguen, A. Fostier, Endocrine and environmental aspects of sex differentiation in fish, Cell Mol. Life Sci. 55 (1999) 910–931.
[2] T.B. Hayes, Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms, J. Exp. Zool. 281 (1998) 373–399.
[3] M.H. Bogart, Sex determination: a hypothesis based on steroid ratios, J. Theor. Biol. 128 (1987) 349–357.
[4] C. Pieau, M. Dorizzi, N. Richard-Mercier, Temperature-dependent sex determination and gonadal differentiation in reptiles, Cell Mol. Life Sci. 55 (1999) 887–900.
[5] K. Shimada, Gene expression of steroidogenic enzymes in chicken embryonic gonads, J. Exp. Zool. 281 (1998) 450–456.
[6] M. Clinton, L.C. Haines, An overview of factors influencing sex determination and gonadal development in birds, Cell Mol. Life Sci. 55 (1999) 876–886.
[7] T. Yamamoto, Sex differentiation, in: W.S. Hoar, D.J. Randall (Eds.), Fish Physiology, Academic Press, New York, 1969, pp. 117–175.
[8] F. Piferrer, S. Zanuy, M. Carrillo, I.I. Solar, R.H. Devlin, E.M. Donaldson, Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males, J. Exp. Zool. 270 (1994) 255–262.
[9] Y. Guiguen, J.F. Baroiller, M.J. Ricordel, K. Iseki, O.M. Mcmeel, S.A. Martin, A. Fostier, Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (*Oncorhynchus mykiss*) and a tilapia (*Oreochromis niloticus*), Mol. Reprod. Dev. 54 (1999) 154–162.
[10] J.F. Baroiller, Y. Guiguen, K. Iseki, A. Fostier, Physiological role of androgens on gonadal sex differentiation in two teleost fish, *Oncorhynchus mykiss* and *Oreochromis niloticus*, J. Exp. Zool. 281 (1998) 506–507.
[11] B. Borg, Androgens in teleost fishes, Comp. Biochem. Physiol. 109C (1994) 219–245.
[12] D.E. Kime, "Classical" and "non-classical" reproductive steroids in fish, Rev. Fish Biol. Fisheries 3 (1993) 160–180.
[13] W.L. Miller, Molecular biology of steroid hormone synthesis, Endocr. Rev. 9 (1988) 295–318.
[14] J.Q. Jiang, T. Kobayashi, W. Ge, H. Kobayashi, M. Tanaka, M. Okamoto, Y. Nonaka, Y. Nagahama, Fish testicular 11beta-hydroxylase: cDNA cloning and mRNA expression during spermatogenesis, FEBS Lett. 397 (1996) 250–252.
[15] R. Billard, A.M. Escraffre, Identification des stades de la spermatogenèse de la truite fario d'après la morphologie des gonades et la spermiation, Bull. Fr. Pisci. 256 (1975) 111–118.
[16] J. Sambrook, E.F. Fritsh, T. Maniatis, Molecular Cloning: a Laboratory Manual, second ed., Cold Spring Harbor, New York, 1989.
[17] J.J. Lareyre, G.Q. Zhao, S. Kasper, R.J. Matusik, D.E. Ong, M.C. Orgebin-Crist, Molecular cloning and hormonal regulation of a murine epididymal retinoic acid binding protein mRNA, Endocrinology 139 (1998) 2971–2981.
[18] W.O. Endege, K.E. Steinmann, L.A. Boardman, S.N. Thibodeau, R. Schlegel, Representative cDNA libraries and their utility in gene expression profiling, Biotechniques 26 (1999) 542–550.
[19] O. Franz, I. Bruchhaus, T. Roeder, Verification of differential gene transcription using virtual northern blotting, Nucleic Acids Res. 27 (1999) e3.
[20] A. Meyer, M. Schartl, Gene and genome duplications in vertebrates: the one-to-four (-to eight in fish) rule and the evolution of novel gene functions, Curr. Opin. Cell Biol. 11 (1999) 699–704.
[21] A. Fostier, B. Jalabert, R. Billard, B. Breton, Y. Zohar, The gonadal steroids, in: W.S. Hoar, D.J. Randall, E.M. Donaldson (Eds.), Fish Physiology, Academic Press, New York, 1983, pp. 277–372.
[22] J.Q. Jiang, G. Young, T. Kobayashi, Y. Nagahama, Eel (*Anguilla japonica*) testis 11beta-hydroxylase gene is expressed in interrenal tissue and its product lacks aldosterone synthesizing activity, Mol. Cell Endocrinol. 146 (1998) 207–211.
[23] D.R. Idler, H.C. MacNab, The biosynthesis of 11-ketotestosterone and 11β-hydroxytestosterone by atlantic salmon tissue in vitro, Can. J. Zool. 45 (1967) 581–589.
[24] R. Arai, H. Tajima, N.I. Tamaoki, In vitro transformation of steroids by the head kidney, the body kidney, and the corpuscles of stannius of the rainbow trout (*Salmo gairdneri*), Gen. Comp. Endocrinol. 12 (1969) 99–109.
[25] R. van den Hurk, W.R. Leeman, Increase of steroid-producing cells in interrenal tissue and masculinization of gonads after long-term treatment of juvenile rainbow trout with cyanoketone, Cell Tissue Res. 237 (1984) 285–289.
[26] R. van den Hurk, P.G. van Oordt, Effects of natural androgens and corticosteroids on gonad differentiation in the rainbow trout, *Salmo gairdneri*, Gen. Comp. Endocrinol. 57 (1985) 216–222.
[27] R. van den Hurk, G.A. Slof, F.A. Schurer, Gonadal sex differentiation in rainbow trout, *Salmo gairdneri*, with special reference to the effects of steroi hormones and N,N-dimethylformamide, Gen. Comp. Endocrinol. 40 (1980) 323.
[28] R. van den Hurk, J.G. Lambert, J. Peute, Steroidogenesis in the gonads of rainbow trout fry (*Salmo gairdneri*) before and after the onset of gonadal sex differentiation, Reprod. Nutr. Dev. 22 (1982) 413–425.
[29] R. van den Hurk, C.J.J. Richter, J. Janssen-Dommerholt, Effects of 17α-methyltestosterone and 11β-hydroxyandrostenedione on gonad differentiation in the african catfish, *Clarias gariepinus*, Aquaculture 83 (1989) 179–191.

[30] J. Komen, J.G.D. Lambert, C.J.J. Richter, H.J.T. Goos, Endocrine control of sex differentiation in XX female, and in XY and XX male common carp (*Cyprinus carpio*, L.), in: F. Goetz, P. Thomas (Eds.), Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish, Austin, Texas, 2–8 July 1995, p. 383.
[31] R. Johnstone, T.H. Simpson, A.F. Youngson, Sex reversal in salmonid culture, Aquaculture 13 (1978) 115–134.
[32] G.A. Hunter, E.M. Donaldson, Hormonal sex control and its application to fish culture, Fish Physiol. Vol IX, Academic Press, New York, 1983, pp. 223–303.
[33] T.O. Hishida, N. Kawamoto, Androgenic and male-inducing effects of 11-ketotestosterone on a teleost, the medaka (*Oryzias latipes*), J. Exp. Zool. 173 (1970) 279–283.
[34] R. Arai, Androgenic effects of 11-ketotestosterone on some sexual characteristics in the teleost, *Oryzias latipes*, Annot. Zool. Jap. 40 (1967) 1–5.
[35] H. Takahashi, Masculinization of the gonad of juvenile guppy, *Poecilia reticulata*, induced by 11-ketotestosterone, Bull. Fac. Fish. Hokkaido. Univ. 26 (1975) 11–22.
[36] F. Piferrer, I.J. Baker, E.M. Donaldson, Effects of natural, synthetic, aromatizable, and nonaromatizable androgens in inducing male sex differentiation in genotypic female chinook salmon (*Oncorhynchus tshawytscha*), Gen. Comp. Endocrinol. 91 (1993) 59–65.