Transgenic rainbow trout expressed sGnRH-antisense RNA under the control of sGnRH promoter of Atlantic salmon

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ABSTRACT

A recombinant vector containing antisense DNA complementary to Atlantic salmon (*Salmo salar*) sGnRH cDNA driven by specific promoter Pab derived from a corresponding sGnRH gene was introduced into rainbow trout (*Oncorhynchus mykiss*) eggs. This resulted in transgenic animals that had integrated one copy of the transgene into their genome and transmitted it through the germline. Antisense-sGnRH mRNA (AS) was expressed mainly in the brain of transgenic AS(+) fish. Levels of sGnRH endogenous mRNA in the brain were lower in 11-month-old AS(+) fish compared with nontransgenic AS(−) individuals from the same F2 progeny. sGnRH levels significantly decreased in the pituitary of trans-

genic males and females around the maturation period and in the brain of AS(+) immature females compared with controls. No reliable statistical difference was found in the levels of FSH and LH between AS(+) and AS(−) groups either in immature or mature fish. The majority of transgenic fish reached maturity at the same time as did nontransgenic individuals, although the maturation of AS(+) animals seemed to be more asynchronous. For the first time, the influence of antisense messengers on endogenous mRNA in transgenic fish and the corresponding protein is described.

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INTRODUCTION

A certain number of farmed fish species such as salmon, common carp, tilapia, seabream and rainbow trout (Chen *et al.* 1996, Iyengar *et al.* 1996, Maclean 1998) are routinely used for gene transfer experiments. Besides the commonly used reporter genes *lacZ*, *cat* or *gfp*, several functional genes have been used to produce transgenic fish, such as the growth hormone (GH) gene in salmon (Du *et al.* 1992, Devlin *et al.* 1994), carp (Chen *et al.* 1993) and tilapia (Martinez *et al.* 1996); and the antifreeze protein gene (AFP) in goldfish (Wang *et al.* 1995) and salmon (Hew *et al.* 1992). Expression of foreign genes may significantly change the physiological characteristics of transgenic fish (Mori & Devlin 1999, Pitkanen *et al.* 1999). Although the majority

of transgenic farmed fish have been raised indoors, escape remains possible and is considered as a risk to the environment. The goal of biosafety through biological containment may be achieved by means of induced sterility in transgenic fish lines.

It is known that hypogonadal mice, bearing a deletion in the GAP region of the gonadotropin-releasing hormone (GnRH) gene, have infantile reproductive systems and levels of gonadotropins lower than normal, causing complete sterility in this line (Mason *et al.* 1986). In fish, as in other vertebrates, GnRH is the main neuromediator controlling the gonadotropic function of the brain–pituitary–gonadal axis (Breton *et al.* 1992). Two forms of this decapeptide have been identified in salmonids, the salmon GnRH (sGnRH) and the chicken GnRH-II (cGnRH-II), which are both

FIGURE 1. Schematic representation of Pab-sGnRH-antisense construct used to produce transgenic rainbow trout. Positions of restriction sites are in brackets. AS1 and UpGnRH are specific primers.

present in the brain and gonads of rainbow trout (Von Schalburg *et al.* 1999b). Salmon GnRH is considered to be the gonadotropin releaser and plays a key role in sexual maturation in salmonids (Breton *et al.* 1992). There are two different genes for sGnRH in rainbow trout: sGnRH gene-1 and gene-2 encoding the same sGnRH peptide (Ferriere *et al.* 1999, Von Schalburg & Sherwood 1999). We hypothesised that *in vivo* blocking of sGnRH production would probably induce sterility in fish, as it does in hypogonadal mice, and that gametogenesis in these former animals may be induced by a prolonged treatment with gonadotropins (Singh *et al.* 1995).

The partial inhibition of target gene expression, at either the mRNA or protein level, by complementary antisense mRNA in transgenic organisms has been shown in numerous works (for reviews see: Sokol & Murray 1996, Erikson 1999). However, in transgenic animals, the inhibitory effect of antisense RNA is seen to vary from complete failure (Munir *et al.* 1990) to a 50% reduction in the level of target protein (Matsumoto *et al.* 1993, 1995). There are several mechanisms by which antisense RNA may exert its effects (Denhardt 1992), but the main inhibition of endogenous RNA is due to its destabilisation by antisense transcripts (Scherzinger *et al.* 1992, Jiang *et al.* 1994).

The objectives of the present study were to try to antagonise the *in vivo* production of sGnRH in a transgenic line of rainbow trout through the transfer of a sGnRH-antisense construction driven by a specific salmon sGnRH Pab promoter (Klungland *et al.* 1992b, Husebye *et al.* 1997). We hypothesised that antisense sGnRH mRNA transcripts may interact with prepro-sGnRH messengers and lead to a decrease in sGnRH production if the expression occurred in the sGnRH-producing cells. Inhibition of gametogenesis, if such an inhibition occurred, would be restored by injections of pituitary

extracts as demonstrated in immature rainbow trout (Upadhyay 1977).

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from the Drennec INRA fish farm (Sizun, France). Before handling, fish were deeply anaesthetised in fresh water with 0.03% 2-phenoxy-ethanol. The eggs from newly ovulated females were fertilised and given microinjections of linear DNA fragment (see below). Transgenic fish and their progenies were maintained at 12 °C under artificial light-darkness conditions, mimicking the annual photoperiodic variations.

The gene constructs

The 'Pab-sGnRH-antisense' construct used in this study (H Husebye & P Alestrom, unpublished data) contains a 2.5 kb sGnRH Pab promoter (Klungland *et al.* 1992b), a 180 bp fragment of the SV40 late 16s/19s splice donor and an SD/SA acceptor signal (Okayama & Berg 1992) fused to the inverted sGnRH cDNA 151 bp fragment, containing the last 8 bp of exon I, and the entire exon II (Klungland *et al.* 1992a), followed by the SV40 polyadenylation signal (Fig. 1). There are a total of 16 bp mismatches between the sGnRH-antisense and trout sGnRH-I cDNA, and 4 bp mismatches between the sGnRH-antisense and trout sGnRH-II cDNA (Ferriere *et al.* 1999, Von Schalburg & Sherwood 1999) (Fig. 2). The 'Pab-lacZ' construct (Husebye *et al.* 1997) had the same regulatory sequences as Pab-sGnRH-antisense, which surrounded 3.5 kb of the LacZ coding sequence.

Plasmid DNA was digested with KpnI and SalI and fragments of approximately 3 kb for

rtGnRHI TTCTCTCAGCATTTGGTCGTATGGGTGGCTACCTGGAGGAAGAAGAAGTGTGTGGGGAGCTGGAGGCCACCATCAGG -3'
..........*.....*.....*.....*.....*
GnRH-as gagagagtctgtgaccagcataccgacccgatggacctccttctcttcacatcccctcgacctccggttgtagttc -5'
..........*.....*.....*.....*.....*
rtGnRHII CTCCTCTCAGCACTGGTCGTATGGCTGGCTACCTGGAGGAAGAAGAAGTGTGTGGGGAGCTGGAGGCCACCATCAAG -3'

FIGURE 2. Comparative alignment of sGnRH-antisense transgene sequences written as complementary sequences in sense direction with rainbow trout (rt) sGnRH-I and sGnRH-II brain cDNA (Ferriere *et al.* 1999). Mismatches in the complementary sequences are indicated by stars. The UpGnRH primer is underlined. Start codon and sGnRH-decapeptide coding sequences are boxed.

Pab-sGnRH-antisense and 6.3 kb for Pab-lacZ were recovered from the gel and purified prior to microinjection.

Production of transgenic rainbow trout

The linear DNA fragment was microinjected into freshly fertilised trout eggs (approximately 10^6 copies per egg), using the two-step method developed by Chourrout *et al.* (1986). Microinjected eggs were incubated at 12 °C.

Transgenic embryos raised from eggs micro-injected with Pab-lacZ construct were analysed for β -galactosidase (β -gal) expression at the age of 57 days postfertilisation. The Pab-lacZ insert was detected by PCR with specific primers in the trunk of fish prior to immunostaining. Pab-lacZ stable transgenic lines were not produced.

Five-month-old fish raised from eggs micro-injected with Pab-sGnRH-antisense construct were screened for the presence of transgenes by PCR. Fish containing the insert were kept. The sex of Pab-sGnRH-antisense immature fish was determined at 7–9 months by measurement of plasma concentrations of 11-ketotestosterone using RIA (Le Bail *et al.* 1983), as there are no phenotypic differences in the sexes before maturity in trout. Precocious spermatogenesis was induced in F0 (founder) and F1 males after repeated intraperitoneal injections of pituitary extract in 0.9% NaCl (3 mg/kg body weight, twice a week) (Upadhyay 1977). Males carrying the transgene in sperm were crossed with wild-type females in order to produce following generations. Integration of a transgene insert into the genome was confirmed by Southern-blot hybridisation on F1 fish and their F2 progenies. F2 Pab-sGnRH-antisense transgenic families were

produced by mating the same wild type female with two F1 transgenic males with the identical transgene integration hybridisation pattern. Both families had the same genotype at the transgenic locus, and were thus considered to be identical transgenic lines.

Extraction and analysis of DNA

For rapid screening using PCR, genomic DNA was extracted according to Estoup *et al.* (1996) from small pieces of fin incubated for 1–3 h at 55 °C in 0.5 ml 10% Chelex100 resin (Biorad, Marne la Coquette, France) containing 250 µg/ml of proteinase K. Proteinase K was then inactivated for 10 min at 95 °C. Samples were centrifuged at 12 000 *g* for 1 min. Two microlitres of each sample were amplified in 12.5 µl standard PCR mix, using 125 nM of specific primers for sGnRH-antisense and 0.65 unit AmpliTaq Perkin Elmer (Foster City, CA, USA) per reaction. Thirty five to forty PCR cycles were performed in a PTC-100 thermocycler (MJ Research Inc., Watertown, Massachusetts, USA), each cycle containing 30 s denaturation at 94 °C, 30 s annealing at 60 °C and 30 s amplification at 72 °C.

For Southern-blot hybridisation, genomic DNA was extracted from 50 µl blood or 200 mg muscle lysed in 4 ml TNES-urea buffer with 200 µg/ml of proteinase K (Tewari *et al.* 1992). DNA was extracted twice with phenol-chloroform, then ethanol precipitated and dissolved in sterile water. 10 µg DNA were digested with restriction enzymes, run on a 0.9% agarose gel at 50 V for 20 to 24 h and then transferred onto HybondN⁺ membranes according to the manufacturer's protocol (Amersham, Les Illes, France). Hybridisation with

a ^{32}P -labeled probe was performed for 16 h at 65 °C in 0.5 M phosphate buffer pH 7.4, 7% SDS and 50 µg/ml calf thymus DNA. The membrane was washed in $2 \times \text{SSC}$, 0.5% SDS, then in $1 \times \text{SSC}$, 0.1% SDS, and twice in $0.2 \times \text{SSC}$, 0.1% SDS at 60 °C and exposed on a Kodak film at -80°C .

RNA preparation and analysis

Total RNA was prepared from brains and other tissues of individual fish using the 'RNA-Insta-Pure' extraction kit (Eurogentec, Angeres, France) or total RNA isolation reagent (Trisol, Gibco, Gaithersburg, USA). When 60- and 90-day-old fish were analysed, RNA was extracted individually from the whole heads. DNase treatment of total RNA was performed using 1 unit RQ1-DNase (Promega, Charbonnières, France) per 1 µg RNA at 37 °C for 15 min. RNA was re-extracted with phenol-chloroform, ethanol precipitated and dissolved in sterile water. Total RNA (0.5–2 µg) was reverse transcribed with oligo-dT primer and 200 units MMLV-Reverse-Transcriptase per reaction according to the manufacturer's protocol (Gibco). Template cDNAs were amplified using 2.5 units Taq DNA-polymerase (Perkin Elmer applied system) and 500 nM primers specific for rainbow trout β -actin, sGnRH-I, sGnRH-II and sGnRH-antisense mRNA in a final volume of 50 µl, containing $1 \times \text{PCR}$ buffer (PE), 1.5 mM MgCl_2 and 200 µM dNTPs.

Development of a semi-quantitative RT-PCR analysis

Determination of the linear range of amplification was performed experimentally by varying the number of cycles and the quantity of RNA taken for reverse transcription. PCR conditions were as follows: after an initial denaturing step at 94 °C for 3 min, the subsequent cycles consisted of denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s and elongation at 72 °C for 30 s, for a number of cycles determined experimentally in order to be in the linear range of amplification for each gene of interest. The final incubation was performed at 72 °C for 10 min. Each PCR reaction mixture was subjected to electrophoresis in 2.5% (w/v) agarose gel stained with ethidium bromide and photographed under UV illumination with an image analysis system (Gel-Doc1000, Bio-Rad). The relative concentration of DNA was determined using the Molecular Analyst/PC software (Bio-Rad), and expressed as ratios of the gene specific signal (sGnRH-I or sGnRH-II) versus the β -actin standard.

Primers used in RT-PCR analysis:

rainbow trout β -actin (319 bp fragment amplified):
 5'-AAAGACCCTGAGTTCATCATGC
 5'-ACCCTAATCACCTCTGACCC
 rainbow trout sGnRH-I (189 bp product amplified):
 5'-TCAGGTCACTTTCTCTCAGCA
 5'-TCTGGCATTCGTTTCTTCGAG
 rainbow trout sGnRH-II (193 bp product amplified):
 5'-GGTAGCGCAGGTCACGC
 5'-GGGGCATCCATTTCTTTCAAT
 sGnRH-antisense (236 bp product amplified):
 5'-TGCTCGAGGAAGTGAAGAAACC-3'
 5'-GGCTACCTGGAGGGAAGAG-3'
 lacZ (765 bp product amplified):
 5'-TTTGATGGCGTTAACTCGGCG
 5'-AGCGGATGGTTCGGATAATGC

Northern blot

PolyA mRNA was prepared by using the poly-ATtract mRNA isolation system III (Promega). Six brains from 11-month-old fish were pooled in each group. mRNA (5 µg) was size fractionated in a 1% agarose-formaldehyde gel and transferred to Hybond N⁺ membrane as described by the manufacturer's protocol (Amersham). Hybridisation with ^{32}P -labelled sGnRH-antisense and β -actin DNA probes was performed as described above.

Immunocytochemistry

Embryos obtained from eggs microinjected with Pab-lacZ DNA were collected 57 days post-fertilisation. The trunks were used to extract the genomic DNA and the heads were fixed in 0.1 M phosphate buffer (PB) pH 7.4 with 4% paraformaldehyde for 12 h at 4 °C, washed 3 times with PB, then incubated for 12 h in PB with 20% sucrose. Samples were embedded in O.C.T. compound (Miles, Elkhart, IN, USA), frozen at 20 °C and cut into 20 µm serial sections. Sections were maintained on TESPA-treated slides and washed twice in PBS pH 7.4. Double immuno-staining was performed by incubating the slides overnight with specific GnRH-antiserum (rabbit anti-sGnRH at a dilution of 1/200) and anti- β -galactosidase monoclonal antibodies (at a dilution of 1/500, Promega) in PBS containing 0.1% Triton X1100 at 4 °C. Sheep anti-mouse FITC-conjugated antibodies (Sigma) were used for β -galactosidase detection and donkey-anti-rabbit TRITC-conjugated antibodies (Jackson) were used to visualise GnRH-producing cells. Sections were observed using a fluorescent microscope equipped with the appropriate filters.

FIGURE 3. GnRH and β -gal expression in Pab-lacZ transgenic rainbow trout: GnRH and β -gal immunoreactive cells in the ganglion of the cribriform bone (gCB) in 57-day-old transgenic fish. Double immunostaining was performed on the same section. Arrows indicate cells in which GnRH (red fluorescence) and β -gal (green fluorescence) are colocalised. Bar represents 10 μ m.

Determination of sGnRH concentrations in brain and pituitary

Extraction of GnRH from pituitaries and brains was performed according to Kah *et al.* (1994). In juvenile fish (60- and 90-days-old), GnRH was extracted from the whole skulls of 16 fish from each family. Tissues from each fish were homogenised in 2M acetic acid, sonicated for 30 s on ice, then heated for 10 min at 80 °C. The samples were then centrifuged for 20 min at 12 000 *g*. The supernatants were lyophilised and reconstituted in BSA-free EIA-buffer (250 μ l for one pituitary and 2 ml for one brain) and subjected to ELISA as described by Kah *et al.* (1994). The specific sGnRH antiserum was used at a 1:20 000 dilution, and the conjugated sGnRH-acetylcholinesterase (Spibio, Massy, France) was used as a tracer.

FSH and LH measurements

Blood was sampled from a caudal vessel and plasma was separated by centrifugation at 2000 *g* for 10 min and kept frozen until assayed. Levels of gonadotropins, Luteinizing hormone (LH) and follicle stimulating hormone (FSH), were measured in 50 μ l plasma using specific radioimmunoassays (Govoroun *et al.* 1998).

Protein assays

Pituitary and brain extracts (in BSA-free EIA-buffer) were analysed for protein content using the BCA protein assay kit (PIERCE, Rockford, IL, USA).

Statistical analyses

Data are expressed as means $\pm$ S.E.M. Results were analysed using Student's *t*-test. The differences were considered significant at $P < 0.05$.

RESULTS

Expression of lacZ driven by Pab-promoter in F0 transgenic rainbow trout

In order to prove that the tissue-specific expression was directed by the Pab-promoter, we analysed several juvenile fish, obtained from eggs micro-injected with Pab-lacZ fragment, at 57 days post-fertilization. It was assumed that the GnRH-immunoreactive (GnRH-ir) cells that express β -gal would be observable even though there are only several dozen sGnRH neurones in the trout brain at this stage of development. As was expected, a few cells were β -gal positive in fish showing the presence of the lacZ transgene in their genomic DNA by PCR analysis, but no such cells were observed in control animals. By double-immunochemistry, the colocalisation of sGnRH and β -galactosidase was detected in 57-day-old fish in the same cells of the ganglion of the cribriform bone (gCB), where most of the GnRH-ir neurones were concentrated at this stage of development (Fig. 3).

Generation of a rainbow trout Pab-sGnRH-antisense transgenic line

F2 Pab-sGnRH-antisense transgenic families were produced as described in Fig. 4. Briefly, the

FIGURE 4. Production and analysis of 'Pab-sGnRH-antisense' rainbow trout populations: time schedule schematic presentation. F0 fish were often mosaic, they were considered as transgenic (AS(+)) if transgene was detected in at least one tissue. Only one F0 male (male 3) retained the sGnRH-antisense transgene in its sperm, and it became a founder for the F1 generation. The same wild type (wt) female A was crossed with F1 brothers 6 and 15, identical at transgenic locus. Juveniles from both F2 families were analysed at 60- and 90-days-old for sGnRH levels in the brain. About 160 fish from F2 family 6A were analysed at 11-months-old for several parameters. Forty AS(+) and 52 non transgenic (AS(-)) males from F2 family 15A were analysed at 15-months-old for spermiation. Sixteen AS(+) and 14 AS(-) females from F2 family 15A were analysed for several physiological parameters at 20 months of age.

microinjection of Pab-sGnRH-antisense DNA fragment into rainbow trout eggs was performed in December 1995. In June 1996, DNA samples from the blood and fins of 56 fish were analysed for the

presence of the transgene. After PCR analysis, 15 females and 5 males proved to be positive for the transgene in at least one tissue. Following hormonal induction of precocious spermatogenesis beginning

FIGURE 5. Southern-blot hybridisation of genomic DNA of Pab-sGnRH-antisense transgenic fish with 'AS1-UpGnRH' 236 bp fragment as a probe. PvuII-digested DNA (10 µg) was loaded per line. Lanes 1–2: F1 founders 6 and 15; lanes 3–5: individual fish from 6A family; lane c: control nontransgenic fish. Transgene is marked by the arrowhead.

in September 1996, one male was found to carry the gene of interest in its sperm. It was crossed with a wild type female in November 1996.

Ten point five per cent of progeny were found to possess the transgene (30 positive fish of the 286 analysed). This confirmed the mosaic integration of introduced construct in F0 founder. Two groups of 25 F1 off-spring issued from the same F1 family, one positive, retaining the transgene (group AS(+)), the other one, negative, not retaining the transgene (group AS(–)) were reared in the same tank. There were 19 females and 6 males among the positive fish and 14 females and 11 males in the negative group. Four out of six AS(+) males started natural spermiation in November 1997, whereas all negative males did so. The 2 AS(+) F1 nonspermiating males were still nonspermiating 1.5 months later. They finally started spermiation after a 1 week treatment with pituitary extract (mid-January 1998). Two F2 Pab-sGnRH-antisense families, 6A and 15A, were produced in April 1998 by crossing these males with the same wild type female. Both F1 founders had the same transgene integration locus which was characterised by an identical hybridisation pattern

FIGURE 6. Detection of sGnRH-antisense, sGnRH-II and β -actin transcripts in brains of 11-month-old transgenic Pab-sGnRH-antisense F2 individuals by RT-PCR. Total RNA (1 µg) was converted to cDNA; 0.1 part of cDNA was subjected to 27 PCR cycles with specific primers for sGnRH-II and β -actin and to 35 cycles for sGnRH-antisense. The PCR products were visualised by Southern-blot hybridisation with sGnRH and β -actin DNA probes.

transmitted without modification to their F2 progenies (Fig. 5). Both F2 families showed a transgene incidence close to 50% with a frequency of 51.8% in the 6A family (114/220) and 52.2% in the 15A family (92/176). Taken together, these results allowed us to consider the 6A and 15A F2 transgenic families to be heterozygous, stable lines identical at the Pab-sGnRH-antisense transgene locus.

Analysis of sGnRH-antisense transgene expression

Antisense sGnRH RNA was detected using the high-sensitivity RT-PCR method. Using specific primers for sGnRH-antisense (Fig. 1), a fragment of expected size (236 bp) was amplified only in AS(+) individuals of both sexes at 11 months of age, while the presence of β -actin and sGnRH messengers were detected in all examined fish (Fig. 6). Expression of sGnRH-antisense was also detected in the brain of all transgenic fish analysed at 60 and 90 days post-fertilisation (data not shown).

Northern-blot hybridisation of brain mRNA with sGnRH-antisense cDNA as a probe revealed an approximately 650 bp long transcript which corresponds to host sGnRH mRNA in the two groups. In AS(+) samples, a smear band of higher molecular weight was detected that might be sGnRH-antisense RNA (Fig. 7). A detailed analysis of GnRH-antisense expression in tissues other than brain was not carried out in transgenic fish, but from RT-PCR analysis we detected sGnRH-antisense transcripts in the gonads and gills, but not

FIGURE 7. Northern-blot hybridisation of mRNA from the brain of 11-month-old transgenic Pab-sGnRH-antisense F2 males (M) and females (F). PolyA mRNA (5 µg) was loaded per line. Hybridisation with sGnRH-antisense (236 bp fragment) and β-actin DNA probes was performed.

in the muscles, pituitary, kidney or intestine of individual immature AS(+) fish (Fig. 8).

Expression of sGnRH-I and sGnRH-II mRNA in the brain of transgenic and nontransgenic fish was compared using a RT-PCR technique, thus allowing the semi-quantitative determination of their relative abundance in correlation with β-actin mRNA expression. The linear range of the reaction was defined as being the PCR period during which amplification efficiency was at its maximum and remained constant over a number of cycles. To obtain meaningful results, the relative RT-PCR reaction had to be terminated and the products quantified when all the reactions were in the linear range of amplification. Experiments were performed to determine the number of cycles which produced quantifiable signals within a linear range of amplification. Figure 9 shows that the linear range of amplification was obtained between cycles 26 and 32, for all amplified products. Moreover, all PCR

FIGURE 8. RT-PCR analysis of sGnRH-antisense expression in different tissues of Pab-sGnRH-antisense transgenic fish. Forty PCR cycles using specific primers were performed using 1 µg transcribed DNAase-treated RNA. br, brain; mus, muscle; gl, gills; pit, pituitary; kid, kidney; ov, ovary; tes, testis; int, intestine.

FIGURE 9. Establishment of relative RT-PCR analysis. Determination of the linear range of amplification of sGnRH-I, sGnRH-II and β-actin cDNA. Total RNA (0.5 µg) from the forebrain of vitellogenic rainbow trout was used for reverse transcription with oligo-dT primers. cDNA was then amplified as described in Materials and Methods and PCR products were subjected to electrophoresis and subsequently quantified.

reactions remained in the linear range between cycles 24 and 28, even if the RNA concentration for RT reactions increased (data not shown). Twenty-seven cycles of PCR were carried out using 1 µg transcribed RNA in the subsequent analysis.

There was a decrease in the sGnRH/β-actin mRNA ratio in 11-month-old F2 AS(+) groups. However, this ratio was significantly lower only in AS(+) females for sGnRH-I mRNA, and in AS(+) males for sGnRH-II messengers (Fig. 10).

Physiological studies

AS(+) and AS(−) groups of F2 fish were compared for endocrinological parameters in relation to the gonadotropic function of the brain–pituitary complex (GnRH, LH and FSH).

Brain and pituitary sGnRH

Brain and pituitary sGnRH contents were measured in 60- and 90-day-old fish in both 6A and 15A F2 transgenic families that showed no difference in weight or size of juveniles. There was a nonsignificant difference in sGnRH brain and pituitary content between transgenic and non transgenic fish. The mean sGnRH content in the brain and pituitary of 60-day-old AS(+) fish was 359 ± 98 pg/animal versus 430 ± 87 pg/animal in AS(−) individuals. In 90-day-old fish, the brain and pituitary content of AS(+) fish was 1153 ± 89 pg sGnRH per fish versus 1085 ± 104 pg in AS(−) fish.

FIGURE 10. Expression of sGnRH-I and sGnRH-II mRNA in the brain of 11-month-old Pab-sGnRH-antisense transgenic rainbow trout. Six individual fish were analysed in each group. Total RNA (0.5 µg) was reverse transcribed followed by 27 PCR cycles. The ratio of optical density absorbency of amplified sGnRH-I and -II to β-actin products was calculated. *The differences were considered significant at $P > 0.05$.

Later, AS(+) and AS(-) 11-month-old males and females from the 6A family (about 80 individuals in each group) were compared. Pituitary sGnRH content decreased significantly in AS(+) males, but not in females (Table 1). Conversely, the sGnRH concentration in the brain was significantly lower in AS(+) females, but not in AS(+) males. The same results were observed in F1 immature AS(+) females (data not shown). Further analysis of sGnRH levels in vitellogenic 20-month-old females from the 15A family showed a significant decrease in the sGnRH pituitary concentration in both AS(+) fish and mature 11-month-old AS(+) males (Table 1).

Plasma FSH and LH

Mean plasma levels of FSH were not significantly different between the AS(+) and AS(-) groups, regardless of the age of the fish (Table 1). LH plasma levels did not significantly decrease in AS(+) fish (Table 1), although there was a great variability between individual AS(+) females. LH levels ranged from a minimal value of 1.2 ng/ml to a maximal value of 30.9 ng/ml. Values were more homogenous in AS(-) fish, with concentrations ranging from 1.1 ng/ml to a maximum of 5.12 ng/ml.

Gonadal development and maturation

No significant difference was found either in mean body weight or gonado-somatic index (GSI) between AS(+) and AS(-) groups in 11-month-old

fish (Table 1). The sex ratio was also comparable. In both 11-month-old groups there was a high variability in maturation stages among males, ranging from stage I (only spermatogonia are present) up to stage V (spermatozoa are already formed). At 15 months of age, the majority of males were already sexually mature. Among the 40 AS(+) males, 31 spermiated (77.5%), and 41 out of the 52 males gave sperm simultaneously in the AS(-) group (78.8%). At the same age, females remained immature or were just beginning vitellogenesis ($GSI < 0.5$). Maturation of females from this family was further analysed when they reached 20 months of age in December 1999. The mean GSI was significantly lower in AS(+) females, being in the order of $5.99 \pm 1.34\%$ (minimal and maximal values being 0.05% and 14.97% respectively) than in AS(-) females, for which the mean GSI was $10.02 \pm 0.99\%$ (minimum and maximum values being 7.03% and 15.74% respectively). In AS(+) females, all stages of maturation from immature to ovulation were found, whereas AS(-) females were more synchronous, all fish being vitellogenic.

In summary, physiological studies revealed that: (1) sGnRH levels significantly decreased in the pituitary of transgenic males and females around the maturation period.

(2) LH and FSH levels were not significantly different between transgenic and non transgenic fish, regardless of sex and stage of maturity, although there was a greater variability among positive fish.

(3) There was no difference in the percentage of spermiating males in either group at 15 months.

(4) There was a high variability in the maturation stages (from immature to ovulated) of AS(+) females at 20 months, all AS(-) being fully vitellogenic animals.

DISCUSSION

Pab-directed expression of inserted genes in transgenic trout

Fused Pa and Pb promoter regions (Pab) from the sGnRH gene of Atlantic salmon (Klungland *et al.* 1992a) used in this study conveyed specific expression in sGnRH producing cells. It has already been shown that this promoter directs the specific transient expression of lacZ in neurone-like cells in the anterior forebrain of zebrafish embryos (Husebye *et al.* 1997). We showed that the same construct microinjected into rainbow trout eggs gave a weak but specific expression in GnRH-ir cells in gCB of transgenic fish 57 days post-fertilisation. At this stage of salmonid development,

TABLE 1. Comparative physiological analysis of transgenic AS(+) and nontransgenic AS(−) fish from ‘Pab-sGnRH-antisense’ F2 transgenic families

Parameters	Males 11-months-old		Females 11-months-old		Females 20-months-old	
	AS(−) (n=43)	AS(+) (n=39)	(AS(−) (n=34)	AS(+) (n=41)	AS(−) (n=14)	AS(+) (n=16)
Weight (g)	187 ± 33	180 ± 27	162 ± 31	165 ± 39	1096 ± 56	905 ± 49
GSI (%)	0.28 ± 0.27	0.25 ± 0.20	0.13 ± 0.02	0.13 ± 0.03	10.02 ± 0.99	5.99 ± 1.34
sGnRH in brain (pg/μg protein)	2.36 ± 1.59	2.60 ± 1.89	3.30 ± 0.21	2.54 ± 1.25	6.87 ± 0.53	7.43 ± 0.75
sGnRH in pituitary (pg/pituitary)	416.3 ± 35.2	271 ± 24.9	298.3 ± 33.9	257 ± 17.4	3112 ± 379	2223 ± 247
FSH, plasma level (ng/ml)	3.08 ± 0.31	2.99 ± 0.32	3.11 ± 0.50	3.73 ± 1.10	7.38 ± 1.51	8.48 ± 1.74
LH, plasma level (ng/ml)	0.48 ± 0.17	0.41 ± 0.18	0.33 ± 0.03	0.35 ± 0.03	2.68 ± 0.31	5.26 ± 2.28

Values considered to be significantly different (*P*<0.05) are in **bold**.

the GnRH-expressing cells (about 100 per brain) are mainly concentrated in a relatively limited area including olfactory placode, gCB and rostral olfactory bulbs prior to their migration throughout the brain (Parhar *et al.* 1995, authors’ unpublished observations). During ontogenesis, the functionality of GnRH-expressing neurones does not change, thus the expression of marker genes in sGnRH-ir cells of juvenile fish allowed us to conclude that the salmon Pab-promoter in rainbow trout was functional. The fact that few cells, and not in all transgenic animals, were found to express β-galactosidase may be due to the mosaicism of F0 transgenics (not all the cells retained the transgene in F0 fish), as well as to the effect of variegation of lacZ expression, a phenomenon that has already been described in detail in the F2 lines of transgenic trout (Amoros 1998). These last transgenic fish expressed β-galactosidase driven by the 2.4 kb promoter of the salmon prolactin (PRL) gene only in PRL-producing cells of the pituitary. Expression of lacZ varied significantly between individuals of the same progeny but also between the PRL-expressing cells of the same pituitary. Not all of the cells that should normally express lacZ were observed to do so. In the present study, the salmon Pab-promoter may similarly not have targeted all the sGnRH-expressing cells, and this may also explain the mosaicism of transgene expression.

In Pab-sGnRH-antisense F2 transgenic fish, transgene expression was detected at different stages, beginning in 60-day-old fish. The fact that expression of sGnRH-antisense RNA was observed in AS(+) fish up to F2 progeny demonstrates that the Pab-sGnRH-antisense transgene is functional

and integrated into active chromatin in a stable manner. It was transmitted without modification through the germ line. The transgene integration pattern analysed by Southern-blot hybridisation revealed the presence of a single copy of Pab-sGnRH-antisense DNA fragment in the transgenic lines (Fig. 4). There was only one additive band appearing in transgenic DNA compared with controls. Its size (approximately 8 kb) did not correspond to those of several copies integrated into concatameres. The other bands that hybridised with the same probe (sGnRH-antisense DNA) corresponded to endogenous sGnRH genes and could be a reference for estimating the number of integrated copies of sGnRH-antisense transgene. The mendelian ratio of transgene transmission:non transmission in F2 (about 1:1) also supported a single integration site.

Expression of Pab-sGnRH-antisense construct was found to occur in greater amounts and with greater regularity in the brains of AS(+) fish, although expression was also detected in other tissues. The Atlantic salmon Pab-promoter used in this study had a high degree of homology with the rainbow trout sGnRH-II gene promoter, especially in its downstream region (Von Schalburg & Sherwood 1999). However, this promoter fragment lacks several regulatory sequences present in trout sGnRH-1 and sGnRH-2 genes. The sGnRH gene-2 is found expressed in the ovary and testis in salmonids (Von Schalburg & Sherwood 1999, Von Schalburg *et al.* 1999a). The presence of GnRH transcripts in tissues other than the brain and gonads have also been shown to occur in mammals (Maier *et al.* 1992, Kottler 1997) and Cichlidae (White & Fernald 1998). Thus, the expression of

transgenes driven by Pab promoters could potentially be detected in tissues other than the brain. The possibility of a rearrangement of the transgene flanking ends due to integration events cannot be excluded. Position effect itself may also influence expression. These factors, in addition to the relatively short size of the salmon Pab-promoter, can lead to an aberrant expression of the transgene in other tissues of transgenic trout, as was detected in our study.

More than 30 PCR cycles were needed to visualise the amplified band of GnRH-antisense cDNA in brain by ethidium bromide staining. The amplification was linear up to 42 cycles (data not shown), whereas only 27 PCR cycles were necessary to visualise the amplified bands of sGnRH-I and -II cDNA in the linear zone of the reaction. These data indirectly indicate that antisense RNA expression was less abundant than was endogenous sGnRH mRNA. In order to directly visualise the sGnRH sense and antisense transcripts in the brain of transgenic fish, Northern-blot analysis was performed. The DNA probe which was used for hybridisation (AS1-UpGnRH 236 bp fragment) detected both transgenic antisense and endogenous sGnRH transcripts with a better specificity for sGnRH-antisense (100% homology, 236 bp of complementary sequence) than for rainbow trout sGnRH mRNA (50 bp of complementary sequence with three and one mismatches for sGnRH-I and -II respectively). The antisense RNA was only present in the brain of AS(+) males and females, whereas sGnRH messengers were also present in AS(−) fish, and their size (650–700 bp) corresponded to that reported previously (Bailhache *et al.* 1994, Ashihara *et al.* 1995). An additional smear band of approximately 900 bp was only detected in AS(+) fish and could be the sGnRH-antisense transcript. Degradation of the sense/antisense RNA duplex may explain the smear of corresponding bands visualised in this study, as well as the relative decrease in the corresponding endogenous transcripts. However, the same results demonstrated that not all antisense RNA is implicated in 'sense-antisense' interactions, otherwise it would not be possible to detect it due to a rapid degradation of these complexes. Nevertheless, there was more sGnRH mRNA in AS(−) than in AS(+) fish, in comparison to β -actin gene expression, especially in females. This was confirmed by relative RT-PCR analysis that showed a lower level of sGnRH-I mRNA in females (Figs 6 and 10) and sGnRH-II in males (Fig. 10) at the age of 11 months. These results show the specific expression of Pab-sGnRH-antisense construct in the brain of transgenic trout and its influence on endogenous sGnRH transcripts.

sGnRH-I and -II mRNA are differently distributed in the brain of rainbow trout. Based on recent data from early vitellogenic females (GSI<1%), sGnRH-I mRNA is predominantly expressed in the olfactory bulbs and telencephalon, whereas sGnRH-II mRNA is the most abundant form in the preoptic area and hypothalamus (F Ferrière, unpublished observation). However, in teleosts, projection of sGnRH fibres into the pituitary has been shown to originate from preoptic and hypothalamic neurones (Anglade *et al.* 1993, Parhar *et al.* 1998). This is in agreement with the findings of the present study whereby sGnRH-I mRNA was less abundant in the brains of 11-month-old AS(+) immature females, whereas the sGnRH-II form decreased in maturing AS(+) males.

Physiological effect of sGnRH-antisense expression

The comparative physiological characteristics of transgenic and control animals are given in Table 1. Under the experimental breeding conditions of the present study, trout reach maturity at 1 year of age for males and at 2 years for females. In fish brain and pituitary, the GnRH levels remain low in juveniles, increase in adults (Rosenblum *et al.* 1994) and peak when fish reach maturity. This is the case in the brown trout (Breton *et al.* 1986), goldfish (Yu *et al.* 1987) and roach (Breton *et al.* 1988). In the absence of a hypothalamo–pituitary portal system, sGnRH during this period accumulates mainly in the pituitary, the contents of which are higher than in the brain (Breton *et al.* 1986, 1988). This indicates an active synthesis of sGnRH prior to maturity. The mechanisms which control sGnRH synthesis are not fully understood. However, in immature rainbow trout, testosterone and estradiol-17 β induce a rapid and highly significant increase in sGnRH that is much greater in the pituitary than in the brain (Breton & Sambroni 1996), although there are no sGnRH secretory cells in the pituitary. Due to the anatomical characteristics of the fish hypothalamo–pituitary complex, it has been hypothesised that steroids stimulate sGnRH synthesis in brain neurones. sGnRH is not accumulated in sGnRH-producing perikaria but in peptidergic terminals of the neurohypophysis. Thus, an increase in pituitary sGnRH can reflect sGnRH synthesis, whereas a decrease in sGnRH pituitary content can be the result either of a decreasing sGnRH synthesis or of sGnRH release from peptidergic endings (Breton & Sambroni 1996).

In this study, at 11-months, both AS(+) and AS(−) females were still immature, whereas nearly all

males were close to maturity. At 20 months, AS(–) females were undergoing vitellogenesis and AS(+) varied from immature to mature. This could explain the discrepancy observed in the GnRH content of the brain and pituitary during these two different periods. In immature female goldfish, the sGnRH contents are low both in the brain and the pituitary with a very low rate of sGnRH synthesis (Rosenblum *et al.* 1994). In 11-month-old immature females (GSI=0.13 ± 0.03) transgene expression did not modify pituitary sGnRH content, but significantly decreased that of the brain. In mature 11-month-old males and 20-month-old females, in which sGnRH synthesis was more active and the release of sGnRH in the pituitary was also activated, the presence of the transgene was accompanied by a significant decrease in the pituitary sGnRH content, which may have accumulated in the gland at that stage. This could be the result of an inhibition of sGnRH synthesis in AS(+) individuals. This is underlined by the fact that in 11-month-old males the main effect of the transgene expression was a decrease in sGnRH-II transcripts in the brain, which were shown to be the main form synthesised in the area from which the sGnRH projected into the pituitary. In summary, the expression of the transgene in both males and females seems to result in a decrease in pituitary sGnRH content following a possible inhibition of sGnRH synthesis at stages during which this process is very active.

There was no significant correlation between the decreased level of sGnRH, and either the level of FSH, or of LH in the blood of both transgenic and nontransgenic fish. The fact that FSH and LH levels did not change in AS(+) fish may signify that the decrease in sGnRH in the pituitary was not great enough to have a direct effect on *in vivo* secretion of gonadotropins. This could also support the idea of a putative role of sGnRH expression in the gonads as an additional determining factor of sexual maturation in rainbow trout.

The great variability in maturation levels in AS(+) females at 20 months of age would tend to suggest the putative role of sGnRH in the synchronisation of egg development. In addition, the important role of GnRH as a paracrine regulator of ovarian functions, in particular in the reinitiating of oocyte meiosis, has recently been demonstrated in teleosts (Nabissi *et al.* 1997, 2000). We observed the expression of sGnRH-antisense mRNA in the gonads of transgenic fish. Thus, the effect of sGnRH-antisense cannot be excluded at the gonadal level; however, the expression of a sGnRH-antisense transgene was not sufficient to generate a more pronounced effect on the process of sexual maturation in this line of transgenic trout.

CONCLUSIONS

The present study has demonstrated that the expression of complementary sGnRH-antisense RNA from the single copy of an integrated transgene driven by a sGnRH promoter from a cognate species may act at the level of sGnRH mRNA and decrease sGnRH levels in the brain and pituitary of transgenic rainbow trout. The induced down-regulation of sGnRH did not decrease FSH or LH concentrations, nor did it induce sterility in transgenic fish. However, some significant disturbances of gametogenesis, which were more pronounced in females, were noticed in the transgenic fish line.

The use of promoter-driven antisense RNA could be a useful approach to inhibit GnRH synthesis in fish. The use of highly appropriate regulatory sequences from the same species, enhancer elements and 100%-matched complement RNA may prove to be absolutely necessary in order to obtain the satisfactory level of GnRH inhibition.

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