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Abstract

cDNA clones encoding the European eel (*Anguilla anguilla* L.) prolactin were isolated from a pituitary cDNA library constructed in γgt10, using a rainbow trout Prl cDNA fragment as a probe. Four different inserts were subcloned into the pGEM 3Z plasmid after PCR amplification. The 1082 bp-long nucleotide sequence revealed an open reading frame of 627 bp encoding a 24 amino acid-long signal peptide followed by a 185 amino acid-long mature protein. Comparison studies showed 60–70% homology with other known teleost fish prolactins and 30–45% with non-teleost fish, amphibian, reptilian, avian and mammalian prolactins. In situ hybridization studies using labelled prolactin RNA probe showed a strong signal in the rostral pars distalis of the pituitary gland. We next examined the physiological regulation of this prolactin synthesis in vivo using Northern blot analysis and prolactin cDNA probe labelled by random priming. The pituitary prolactin mRNA level was markedly decreased 3 weeks after transfer of eels from freshwater to sea water. Implants of thyroid hormones left for up to three weeks were ineffective on prolactin mRNA. Estradiol administered as implant, alone or in combination with 500 μg testosterone, was also unable to significantly alter the pituitary mRNA level for prolactin in the freshwater silver eels whatever the dose used (20–500 μg) and whatever the duration of treatment (from 4 days to 10 weeks).

Key words: Prolactin; Osmoregulation (Eel)

1. Introduction

Prolactin is a pituitary polypeptide hormone belonging to a family of hormones that includes growth hormone and placental lactogen (Nicoll et al., 1986), and the newly discovered somatolactin (Ono et al., 1990; Rand-Weaver et al., 1991). The close structural relationships between these hormones has led to the assumption that they have evolved from a common ancestral gene. The primary structure of prolactin has been characterized in all vertebrates classes. In teleosts, the amino acid sequence of prolactin has been determined in different species of tilapias (Yamagushi et al., 1988; Rentier-Delrue et al., 1989), salmonids (Yasuda et al., 1986; Song et al., 1988; Mercier et al., 1989), cyprinids (Yasuda et al., 1987; Chao et al., 1988; Chang et al., 1992), and in the catfish (Watanabe et al., 1992). In tilapias, two different forms of prolactin, a 20 kDa and a 24 kDa proteins have been identified, each showing substantial differences in their amino acid sequences. These proteins are encoded by two distinct genes (Rentier-Delrue et al., 1989). In the Japanese eel, also, two different variants of prolactin were purified. Although these two forms were slightly different in amino acid composition, their partial amino acid sequences were identical (Suzuki et al., 1991) leaving the question of the number of genes encoding prolactin in the eel still open.

Prolactin is a pituitary hormone involved in a wide variety of functions in fish, such as osmoregulation, reproduction, growth and development (Clarke and Bern, 1980). The best documented of these functions, and certainly the most important one, is osmoregulation (Hirano, 1986). If several different functions are indeed fulfilled by prolactin in a given species, its expression and secretion should be subjected to multifactorial regulation. Owing to its original biological cycle, the eel could be an interesting model for the study of these regulations. A first metamorphosis, oc-

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curring in sea water, transforms the leptocephali (eel larvae) into young (glass) eels, allowing them to invade the continental fresh or brackish waters. Then, after a growth phase, the eels (the yellow stage eels) are subjected to a second metamorphosis that pre-accommodate them (then at the silver stage) to sea water and to their reproductive migration.

In this paper, we describe the cloning of the European eel prolactin and a preliminary study of the potential effects of some factors on the steady-state prolactin mRNA levels: the salinity of the water as a factor affecting the osmoregulation; thyroid hormones, as hormones involved in development; and finally, estradiol and testosterone as hormones of reproduction.

2. Materials and methods

2.1. Prolactin mRNA cloning and sequencing

Isolation of mRNA and construction of the eel (*Anguilla anguilla* L.) pituitary cDNA library in γgt10 vector have been previously described (Quérat et al., 1990). A rainbow trout prolactin cDNA fragment of 740 bp encompassing the coding region, kindly provided by Dr F. Rentier Delerue, was used as a probe for library screening. 25 000 pfu were screened according to methods previously described (Quérat et al., 1990). Four recombinant phages were purified by the plate lysate method (Sambrook et al., 1989) and the inserts were amplified by PCR using primers corresponding to sequences flanking the cloning *Eco*RI site of the phage. The complete inserts or their *Hin*dIII restricted fragments were then subcloned in pGEM 3Z vector (Promega Biotech). Sequences were determined using the Sequenase 2.0 sequencing system (USB) after alkali denaturation. Sequence analysis and comparisons were performed using the CITI2 programs (Dessen et al., 1990).

2.2. In situ hybridization

Two freshwater female silver eels were anaesthetized in MS 222 (Sandoz) and perfused intracardiacally for 5 min with 2% paraformaldehyde (PFA) in 100 mM phosphate-buffered saline (PBS). The pituitary was removed with the attached brain, rinsed in 2% PFA, 100 mM PBS, and placed in 20% (w/v) sucrose, 100 mM PBS, for 6 h at 4°C. The tissue block was then frozen in isopentane cooled on dry ice. Serial sections of 15 μm were cut using a Leitz cryostat, thaw-mounted onto gelatin-coated slides, and stored at −20°C until hybridization. The anti-sense and sense labelled single-stranded RNA probes were synthesized using [α^{35}S]UTP (> 1000 Ci/mmol; Amersham) and SP6 RNA polymerase (Promega Biotech) according to the manufacturer's recommendations from two linearized pGEM 3Z vectors containing a 500 μg *Hin*dIII-*Hin*dIII fragment of the prolactin cDNA (encompassing the coding region) in both orientations. The sections were thawed at room temperature, fixed in 2% PFA, 100 mM PBS for 15 min, immediately rinsed twice in PBS and twice in 4 × SSPE (SSPE: 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM Na_2 EDTA), 1 × Denhardt's solution (0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone and 0.02% (w/v) bovine serum albumin). Some sections were treated with RNAse A (10 μg/ml in 100 mM PBS at 37°C for 30 min) just after the postfixation in PFA. The sections were dehydrated through serial dilutions (60–100%) of ethanol, delipidated in chloroform, partially rehydrated in 100% and 95% ethanol, and air-dried. 40 μl of hybridization buffer (50% formamide, 4 × SSPE, 1 × Denhardt's solution, 1% sarkosyl, 10% dextran sulfate, 500 μg/ml yeast tRNA, 500 μg/ml calf thymus DNA, and 50 mM dithiothreitol) containing 1×10^6 cpm of labelled probe was distributed on each slide, and covered with a coverslip. Hybridization was carried out overnight at 46°C. Following incubation, coverslips were removed in 2 × SSPE, 20 mM β-mercaptoethanol. The slides were washed 2 × 15 min in 1 × SSPE, 20 mM β-mercaptoethanol at 45°C, treated with RNAse A (10 μg/ml in 1 × SSPE at 37°C for 30 min), and washed again 2 × 15 min in 1 × SSPE, 20 mM β-mercaptoethanol at 45°C. The sections were finally dehydrated through serial dilutions (60–100%) of ethanol containing 0.3 M ammonium acetate, air-dried, and covered with Hyperfilm beta-max X-ray film (Amersham) for 24 h. After development of the film, sections were dipped in Kodak NTB3 emulsion (diluted 1:1 with 300 mM ammonium acetate) and exposed for three days. The slides were then developed (Kodak D19), fixed and counterstained with hematoxylin and eosin, and examined using bright and dark-field microscopy.

2.3. Treatments

Freshwater female silver eels weighing 250–300 g were caught in ponds in Northern France and kept in tanks in running freshwater. Six eels were accommodated for 2 days in 50% artificial sea water (Wiegand GMBH)(0.5 volume in freshwater) then transferred to full strength artificial sea water. The water was kept at 13–15°C in a closed system, filtered on charcoal, aerated, and renewed twice a week. For implantation, triiodothyronine and thyroxine (Sigma) were first dissolved in NaOH 0.1 N at a concentration of 40 μg/ml and then diluted in ethanol to 10 μg/ml and mixed with 10 volumes of coconut oil (Sigma) at 25°C. The steroid hormones, testosterone and estradiol (Sigma), were directly dissolved in ethanol (10–50 μg/ml) be-

fore being mixed with 10 volumes of coconut oil. One ml of hormone/ethanol/oil or ethanol/oil preparation (vehicle) was injected in the perivisceral cavity. The implanted eels were divided into groups depending on the nature of treatment and kept in separate tanks of running freshwater at 13–15°C. Eels were killed by decapitation and the pituitary glands were immediatly collected, occasionally pooled, and kept in liquid nitrogen. Blood samples were collected in heparinized tubes and the plasma separated by centrifugation and kept at −20°C. Triiodothyronine and thyroxine, and testosterone and estradiol were radioimmunoassayed as previously described (Leloup and De Luze, 1980; Quérat et al., 1987).

2.4. RNA extraction and Northern blot experiments.

Total cytoplasmic RNA was extracted from individual or pools of two to four pituitary glands, quantified by measuring the absorbance at 260 nm and submitted to electrophoresis through a 1.2% (w/v) agarose gel containing 6% formaldehyde as previously described (Quérat et al., 1991a). Transfer to nylon membranes (Hybond N, Amersham) was achieved by capillary blotting (Sambrook et al., 1989). Filters were baked for 2 h at 80°C. Prehybridization, hybridization and washings were performed as previously described (Quérat et al., 1991a). The complete eel prolactin cDNA and a 1.5 kb rainbow trout β-actin cDNA fragment (kindly provided by Pr Y. Valotaire) were labelled using a Multiprime labelling system (Amersham) with [α^{32}P]dCTP (3000 Ci/mmol; Amersham). Between two hybridization experiments, the blots were dehybridized by incubating 3 h in 15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS at 75°C. Signals on autoradiograms (Kodak X-OMAT films, Eastman Kodak) were quantified by scanning densitometry and the results for the prolactin mRNA level were corrected for recovery by dividing either by the actin mRNA level or by the amount of total RNA loaded into the gel. Statistical analysis was performed using Student's *t*-test.

2.5. Southern blotting

Preparation of genomic eel DNA, electrophoresis, transfer to nylon membranes (Hybond N, Amersham), hybridization and washing were performed as previously described (Quérat et al., 1990), using the complete eel prolactin cDNA labelled as for the Northern blot analysis.

3. Results

3.1. Sequence determination and analysis

Four different phage inserts were subcloned after PCR amplification using primers corresponding to se-

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  GAGTGAGAG ATG GCT CAG CGA TTT AAA GGA AGA AGC CTC TTC TTG ACA GCT CTG CTG TGT    60
            M   A   Q   R   F   K   G   R   S   L   F   L   T   A   L   L   C    -8
CTG GCG AGC CAG GGG TAC GCG GTG GGA CTC GGC GAC ATG CTG GAA CGG GCG TCG CAG CTG   120
 L   A   S   Q   G   Y   A   V   G   L   G   D   M   L   E   R   A   S   Q   L    13
TCC GAC AAG CTT CAC TCT CTC AGC ACG TCC CTG ACC AAC GAC CTG GAC ACC CAT TTC CCT   180
 S   D   K   L   H   S   L   S   T   S   L   T   N   D   L   D   T   H   F   P    33
CCG ATG GGG AAG ATC CTG ATG CCA CGC CCC TCC ATG TGC CAC ACG GCT TCC CTC CAA ACC   240
 P   M   G   K   I   L   M   P   R   P   S   M   C   H   T   A   S   L   Q   T    53
CCT CAC GAC AAG GAC CAG GCT TTG AGA GTG CCG GAA TCA GAG CTG CTG TCC ATC GCC CGC   300
 P   H   D   K   D   Q   A   L   R   V   P   E   S   E   L   L   S   I   A   R    73
GCG CTC CTG CTG TCC TGG AAC GAT CCC CTG CTC CTG CTC GCC TCC GAG GCG CCC ACG CTG   360
 A   L   L   L   S   W   N   D   P   L   L   L   L   A   S   E   A   P   T   L    93
TCC CAT CCG CAG AAC GGC GCC ATC TAC AGC AAA ACA AGG GAA CTG CAG GAC CAG TCC AAC   420
 S   H   P   Q   N   G   A   I   Y   S   K   T   R   E   L   Q   D   Q   S   N   113
AGC CTG AGC TCT GGG CTG GAC AGG CTG ATT CAC AAG ATT GGC TCC TCC TCC AAG GCC CTC   480
 S   L   S   S   G   L   D   R   L   I   H   K   I   G   S   S   S   K   A   L   133
TCC CCC CTC CCC CTC CAA GGC GGC GAC CTC GGC AGC GAC AAG AAC TCC CGC CTC ATC AAC   540
 S   P   L   P   L   Q   G   G   D   L   G   S   D   K   N   S   R   L   I   N   153
TTC TAC TTC CTG CTG TCC TGT TTC CGT CGG GAC TCC CAC AAA ATC GAC AAC TTT CTC AAG   600
 F   Y   F   L   L   S   C   F   R   R   D   S   H   K   I   D   N   F   L   K   173
CTC CTC CGT TGC CGG GCA GCC AAG CAG GAC CGA TGC TAA ACTGGGGGGGGGGATATTCTAATTTGGG   666
 L   L   R   C   R   A   A   K   Q   D   R   C   *                               185
CTACAGTATAAAAAATAAGCTTATTTTTTTATTCTTTTTAGAAATGTTCATCGAATCACAGACCTGGATTCAGCCCCAT   745
TCCTAATACTTAATTTATAATTTTGTACTATGCCTTATTGTACACACTTAAAAGAACATATGCTACACAAAATTGACAT   824
ATCAGAAAACTTGAAAAAGGAAGGGATTTTAATCCGCACTCATTCAAATATTTGCAACCTGTGTCTGCAATACTGCAAT   903
AACATCAACATAACTTATGCAAATTACTCTAAACTGTCTAAAGACACCTTTAAGGTCTAAAATTGTTGAGGACCGTCA   982
CCATTTAATTATCAACCCAGATCGCTGAACTACAATTTTGTTTTATTCTTGGAAACTACTGTTGCTACACAAATGACAA  1061
TAAAATTCTATCTTGCATAACpoly(A)                                                      1082
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Fig. 1. Nucleotide and deduced amino acid sequences of the European eel prolactin. The presented nucleotide sequence was reconstructed from 4 different clones. The nucleotides that vary from one clone to another are underlined. Those that are absent in certain clones are indicated in italic. The modification of the nucleotide A (at position 33) to S, led to a change in the encoded amino acid R (−17 in the signal peptide) to S. The position of the cleavage site of the signal peptide was inferred from the partial amino acid sequence of the purified Japanese eel prolactin (Suzuki et al., 1991). The polyadenylation signal is twice underlined.

quences flanking the *Eco*RI cloning site. The figure 1 represents the nucleotide and deduced amino acid sequence reconstructed from the partial sequences of the 4 phage inserts. Each part has been sequenced on at least two different phage inserts (3 for the coding region). Several differences were seen between the inserts; most of them concerned punctual, silent mutations. One led to the change of the arginine at the eighth position (−17) of the signal peptide to a serine. Three segments of 2, 5 and 7 nucleotides of the 3′ untranslated region were absent in one or several clones. The 4 inserts ended within 12 to 17 nucleotides following the polyadenylation signal AATAAA. Southern blot experiments showed only one signal when genomic DNA was restricted with *Bam*HI (25 kb), *Eco*RV (15 kb), *Eco*RI (9 kb), and *Bgl*II (7 kb), and two signals when digested with *Pst*I (3.2 and 2.5 kb), and *Hin*dIII (2.2 and 2.3 kb), these two latter enzymes presenting 1 and 2 restriction sites in the full length cDNA respectively (data not shown).

The position of the cleavage of the signal peptide was inferred from the N-terminal sequence of the Japanese eel prolactin (Suzuki et al., 1991). The sequence of the 185 amino acid-long mature protein was compared to other known prolactin sequences (Fig. 2). The eel prolactin shows a 60–70% homology score with other teleostean fish prolactins (except with the 177 amino acid-long tilapia prolactin), 42% with sturgeon prolactin, 33–36% with lungfish, amphibian, reptilian and avian prolactins, and between 30–32% homology with mammalian prolactins.

3.2. *In situ hybridization*

Transversal sections of the pituitary gland show a strong signal in the anterior part when hybridized with

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          10        20        30        40        50        60        70        80        90       100       110
ANG  ----------VGLGDMLERASQLSDKLHSLSTSLTNDLDTHFPPMGKILMPRPS-MCHTASLQTPHDKDQALRVPESELLSIARALLLSWNDPLLLLASEAPTLS-
ONK  ----------I..S.LM.....R..........K...S......RVM.....-....S.....K..E...K.S.N..I.L..S...A........S......P-
CYP  ----------...N.L.....................S....V.RVM.....-....S...V.N......K...DP...L..S...A.S...A..S...SS.A-
CAT  ----------.N.N.L.D........M.....S..SSV.GK..-...-....S...I.N......S...S....LV.S..MA.S...A..S...TS.P-
TI24 ----------.PINEL......H.........T..QE..S....I.RVI....A-....S.....I......Q.S..D.M.L..S..QA.S...VV.S.S.S..P-
TI20 ----------.PIN.LIY....Q.....A...M..QE.GSEAF.IDRV.A------...S.....T..E...Q.S..D...L..S..QA.S...EV.S.STNV.P-
ACI  SPLC--GGLGCPPPIL.S.L....T...SR.....RTV.AG..P..S.L---.K....SL...S..A.DEN.E...TLQQEQ...LIMS..R..TP..MF.VR..QS.PP
PRO  LPICANGSTNCHQ-IP.D.LF.FVVK.AHRI...TSDMF.EF.ERYAQ-.RGFIS.AINN...S..T..E..E..QKFHHDD..RLVMKV.R.......Q.V..V.QG--
RAN  QPICPNGGTNC-Q-IPTSALFD..VK..HYI....SEMF.EF.ER.T.-.RRFLAKSGIS...S..N..E..E..RQIQHED..NLVLKV.R......VHMV..V-QD--
XEN  LPICPDGGTNC-Q-MST.ALCDK.VK..HYI....SEMF.EF.ER.T.-SRRFLAKSIMS...S..N..E.RE..QQIQHED..NLVMRV.K.......HMVG.V-QD--
CHE  LPICPSGSVGC-Q-.S.ENLFD..VK..HYI....SEMF.EF.ERYAQ-.RGFLTKAING...S..T..E..E..QQIHHED..NLVLGV.R.......H.V..V-QS--
CRO  LPICPSGSVNC-Q-.S..ELFD..VK..HYI.F..SEMF.EF.ERYAQ-.RGFITKAVNG......T..E..E..QQIHHED..NLVLGV.R.......H.VT.V-QR--
GAL  LPICPIGSVNC-Q-.S..ELFD..VK..HYI.Y..SEIF.EF.ERYAQ-.RGFITKAVNG...S..T..E..E..QQIHHED..NLVVGV.R......IH....V-QR--
MEL  LPICSSGSVNC-Q-.S..ELFD..VR..HYI.F..SEIF.EF.ERYAQ-.RGFITKAVNG...S..T..E..E.TQQIHHE...NLILGV.R......IH....V-QR--
RAT  LPVCSGG--DC-Q-TP.PELFD.VVM..HYI.T.Y.DMFIEF.KQYVQ-DREFIAKAIND.P.S..A..E..E..QK..PEV..NLILS.VH......FQ.ITGLG-G--
SUS  LPICPSGAVNC-Q-.S.R.LFD..VI..HYI.N..SEMF.EF.KRYAQ-.RGFITKAINS...S..S..E..E..QQIHHEV..NLILRV.R......YH.VT.V-RG--
BOS  TPVCPNGPGNC-Q-.S.R.LFD..VMV.HYI.D..SEMF.EF.KRYAQ-..GFITMALNS...S..P..E..E..QQTHHEV.M.LILG..R......YH.VT.V-RG--
HOM  LPICPGGAARC-Q-.T.R.LFD..VV..HYI.N..SEMFSEF.KRYTH-.RGFITKAINS...S..A..E..E..QQMNQKDF..LIVSI.R...E..YH.VT.V-RG--

         120       130       140       150       160       170       180       190       200       210  length % identity
ANG  -HPQNGAIYSKTRELQDQSNSLSSGLDRLIHKIGSSSKA-------LSPLPLQGGD-LGS-DKNSRLINFYFLLSCFRRDSHKIDNFLKLLRCRAAKQ--DRC  185      100
ONK  -..S..D.S..I.....Y.K..GD...IMVN.M.P..QY--------I.SI.FK...-..N-..T......H..M..........S...V.....T.IRPET.  187       71
CYP  -..ERNT.D...K...ENI...GA..EHVFQ.MD...DN---------..S..FYTNS-..E-..T...V..H.............S...V......-.KRPEM.  187       68
CAT  -..ERNS.N........HT...GA..E..GR.M...PES---------..S..FNSNS-..Q-.NI...V..H.............S...V.......MLPEM.  187       67
TI24 -...AQSS.FN.IQ.M.QY.K..KD...V.SS.M..PAQ.--------ITS..YR..TN..H-..ITK....N.....L........S...V.......MQPEM.  188       60
TI20 -YSAQSTLSKTIQKM.EH.KD.KD...I.SS.M.PAAQT--------ITS..FIETNEI.Q-..ITK.------------------S...V......NMQPQV.  177       43
ACI  N.SLS.SLSWQ.A..S-..QK.AK..ETILNRFDP.AAHKASFGNADD-.WKGGASDFP.S.RK...L..............S..........---.ENGG.  204       42
PRO  IGEAP.T.LW.VT.VE..TKQ.IE.MEKILSRMHPNGLDNEVLS------.WP-MPGAMHAG.G-.K.FAL.N..H......F...SY.......L--FHEGG.  200       35
RAN  IREAPDT.LW..V.VEE.TKR.LE.ME.I.GR.QPGDLENEIYS-----PWPGPAS--IPG.E....FA..N..H.L.........Y....K..L--IHEGN.  198      35.5
XEN  IREAPDT.LW..V.VEE.TKR.LE.MEKIVGR.HPFDLENDVNS-----.WSGPPA-AQSA.E....FG..N..H.L.........Y....K..L--IHDSN.  199       34
CHE  IKEAPDT.L-.AV.IEE.DKR.LE.MEKIVGQVHPGEIENELYS-----PWSGLPS-LQQV.ED...FA..N..H.L.........Y....K..L--IHDND.  198       36
CRO  IKEAPDT.LW.AV.IEE.NKR.LE.MEKI.GRVQPGDTGNEVYS-----RWSGLPS-LQLA.ED...FA..N..H.G.........Y....K..L--IHDSN.  199       34
GAL  IKEAPDT.LW.AV.IEE.NKR.LE.MEKIVGRVH.GHAGNEIYS-----HWDGLPS-LQLA.ED...FA..N..H.H.........Y..V.K..L--IHDSN.  199       34
MEL  IKEAPDT.LW.AV.IEE.NKR.LE.MEKIVGR.H.GDAGNEVFS-----QWDGLPS-LQLA.ED...FA..N..H.L.........Y..V.K..L--IHDNN.  199       34
RAT  I.EAPD..I.RAK.IEE.NKR.LE.IEKI.SQAYPEA.GNEIYL-----VWSQLPS-LQGV.EE.KDLA..NNIR.L......V..Y..F...QI--VHKNN.  197       29
SUS  MQEAPD..L.RAI.IEEENKR.LE.MEKIVGQVHPGI.ENEVYS-----VWSGLPS-LQMA.EDT..FA..N..H.L.........Y....K..I--IYNSN.  199      32.5
BOS  MKGAPD..L.RAI.IEEENKR.LE.MEMIFGQVIPGA.ETEPYP-----VWSGLPS-LQTK.EDA.YSA..N..H.L....S...TY....N..I--IYNNN.  199       32
HOM  MQEAPE..L..AV.IEE.TKR.LE.MELIVSQVHPET.ENEIYP-----VWSGLPS-LQMA.EE...SAY.N..H.L.........Y....K..I--IHNNN.  199       33
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Fig. 2. Alignment of the sequences of prolactin from different representatives of vertebrates with the European eel (Ang) sequence. Dashes indicate deletions introduced for best alignment. Dots represent residues identical to the eel sequence. The percentages of homology with the eel sequence are indicated at the end of the sequences. The references used are: Cyp (*Cyprinus carpio*), Yasuda et al., 1987; Cat (*Ictalurus punctatus*), Watanabe et al., 1992; Ti24 and Ti20 (*Oreochromis mossembicus* 24 K and 20 K variants), Yamagushi et al., 1988); Onk (*Oncorhynchus keta*), Song et al., 1988; Aci (*Acipenser gueldenstaedti*) Noso et al., 1993); Pro (*Protopterus* sp.), Noso et al., in press; Ran (*Rana catesbeiana*) Takahashi et al., 1990; Xen (*Xenopus laevis*), Buckbinder L. and Brown D.D., unpublished, appeared in EMBL under L07620; Cro (*Crocodylus novaeguineae*), Noso et al., 1992); Gal (*Gallus gallus domesticus*), Hanks et al., 1989; Mel (*Meleagris gallopavo*), Karatzas et al., 1990; Rat (*Rattus norvegicus*), Cooke et al., 1980; Sus (*Sus scrofa*), Li et al., 1976; Bos (*Bos taurus*), Miller et al., 1981; Hom (*Homo sapiens*) Cooke et al., 1981.

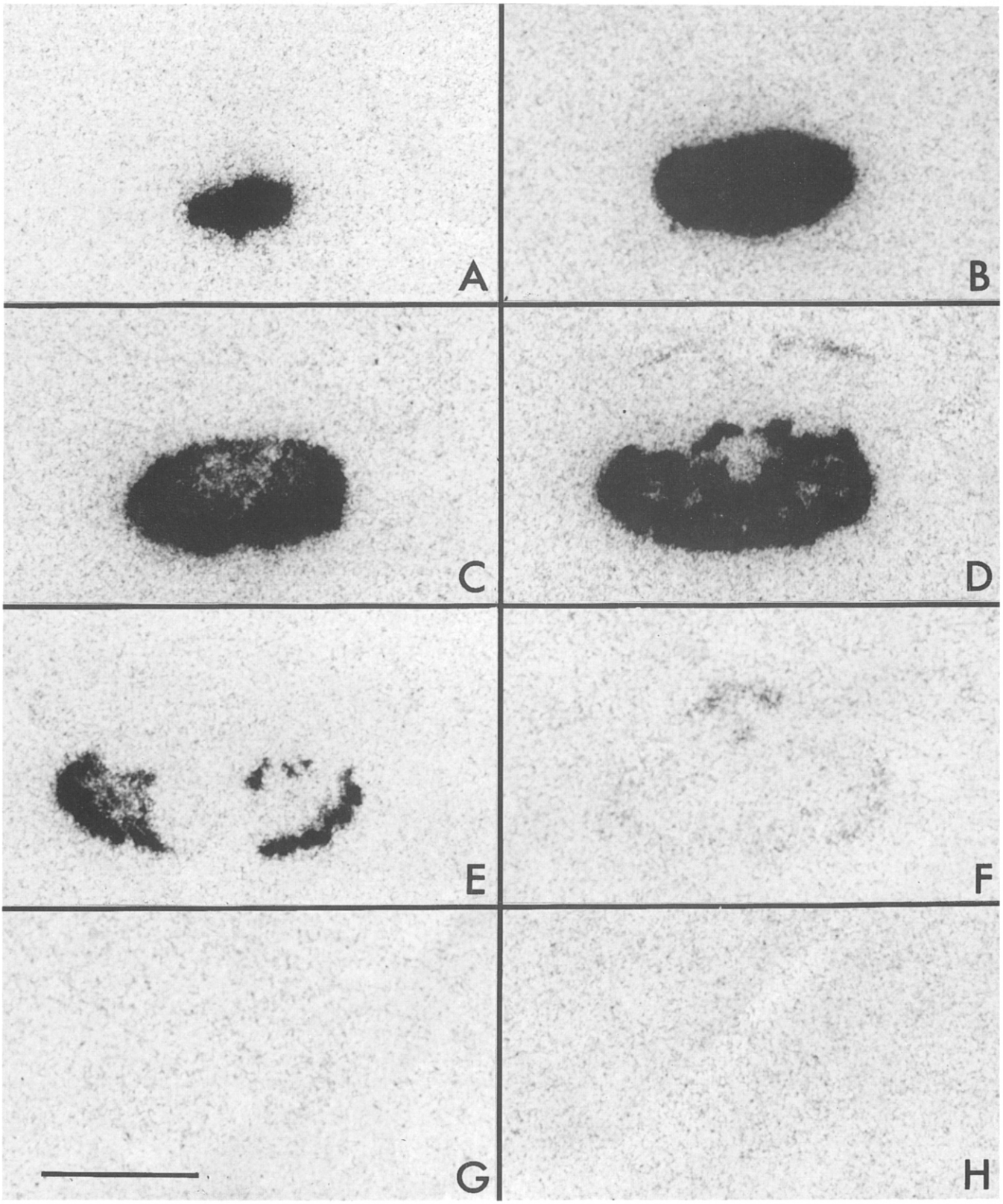

Fig. 3. Autoradiographs of selected transversal sections of the pituitary gland of freshwater silver eel, rostral to caudal (from A to F; G and H are in the same region as C), hybridized either with the anti-sense prolactin RNA probe (A–G) or with the control sense prolactin RNA probe (H). Section G was treated with RNAse A prior to the hybridization. Exposure time: 24 h. Scale bar = 1 mm.

the anti-sense prolactin RNA probe (Fig. 3A–C). When sections were taken from the region of the pituitary stalk, the signal is restricted to the periphery (Fig. 3D,E). No signal is detected in the posterior part of the pituitary gland (Fig. 3F). The specificity of the hybridization was confirmed by using the sense RNA probe (Fig. 3H) or following pre-treatment with RNAse A prior to hybridization with the anti-sense probe (Fig. 3G) on anterior part sections (i.e. corresponding to the region of section B) where no signal is detected. Fig. 4 shows a bright-field micrograph of a section adjacent to section E of Fig. 3, hybridized with the prolactin anti-sense RNA probe. Grain-containing cells are organized in clusters of 50–100 cells.

3.3. Regulation of the eel prolactin pituitary mRNA level in vivo

Action of accommodation to sea water. Three weeks after the transfer of silver eels from freshwater to sea water, the mRNA levels of prolactin in the pituitary was lowered by approximately 5-fold ($p < 0.01$) (Fig. 5 and Table 1).

Absence of action of the thyroid hormones. No effect was observed on the mRNA levels of prolactin in the pituitary 3 weeks after implantation of 500 μg of either triiodothyronine or thyroxine (Table 2). Plasma levels of triiodothyronine (2.9 ± 0.2 ng/ml in controls) reached 94 ± 16 ng/ml in the 500 μg triiodothyronine treated eels, and thyroxine was increased from 5.9 ± 1.5 to 94 ± 45 ng/ml when implanted with thyroxine, without important modification of the triiodothyronine plasma level (5.6 ± 1.2 ng/ml).

Absence of action of estradiol and testosterone. No significant effect of estradiol was observed whatever the dose used (between 20 and 500 μg) neither in the presence nor in the absence of testosterone at 500 μg, 2 weeks after implantation (Fig. 6). A significant difference ($p < 0.05$) was, however, observed between eels treated with 100 μg of estradiol and those receiving testosterone in combination with this dose of estradiol. Plasma levels of estradiol (0.22 ± 0.03 ng/ml in controls) reached 8.2 ± 0.3, 34 ± 1, and 84 ± 7 ng/ml in the 20, 100, and 500 μg estradiol-treated eels, respectively. Testosterone plasma levels (0.20 ± 0.06 ng/ml in controls) reached 60–90 ng/ml in the 500 μg testosterone treated eels, except with the highest dose of estradiol in which testosterone plasma level was only 29 ± 4 ng/ml after 2 weeks. Three other experiments also showed that neither estradiol alone or in combination with testosterone were effective in significantly altering (enhancing or lowering) prolactin mRNA levels 2 or 3 weeks after implantation of 500 μg of each steroid (data not shown).

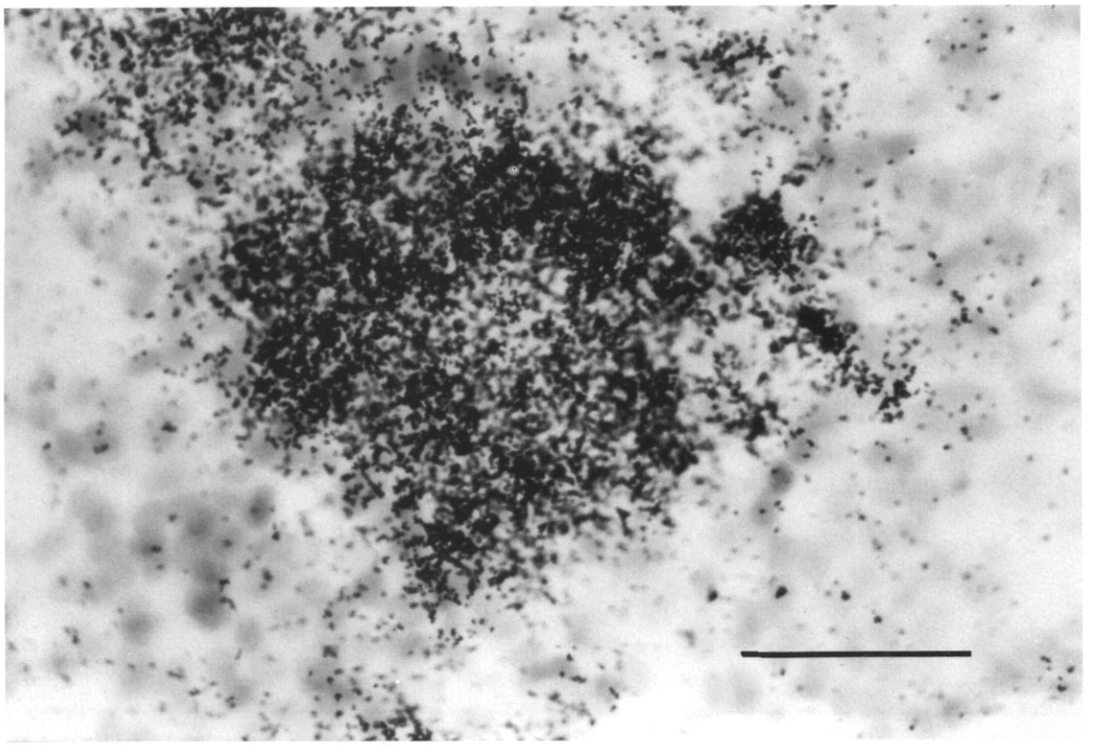

Fig. 4. Bright field micrograph of a section located in the rostral part of the pituitary gland (adjacent to section E of Fig. 3), hybridized with the anti-sense prolactin RNA probe. Exposure time: 3 days. Scale bar = 25 μm.

Table 1
Effect of accommodation to sea water for 3 weeks on the pituitary mRNA level for prolactin

Nature of treatment	Freshwater	Sea water
Prolactin mRNA/actin mRNA	1.0±0.2	0.2±0.04 *

Prolactin and actin mRNA levels were measured following Northern blot hybridization analysis (see Fig. 5) by scanning densitometry. The value of 1.0 was given to the control. Data are expressed as means± SEM of 6 individual values. * $P < 0.01$.

Table 2
Effect of a 3 weeks treatment with triiodothyronine or thyroxine on the pituitary mRNA level for prolactin

Nature of treatment	0	Triiodo-thyronine	Thyroxine
Prolactin mRNA/actin mRNA	1.0±0.15	1.2±0.35	0.75±0.2

mRNA levels were measured following Northern blot hybridization analysis by scanning densitometry. The value of 1.0 was given to the control. Data are expressed as means±SEM of 6 individual values.

Time-course experiments with estradiol (500 μg) in the presence of testosterone(500 μg) failed to show any positive or negative correlation from day 0 to 10 weeks after implantation (Table 3). In this latter experiment, the values obtained for the pools of 5 pituitary glands were in the range of the values obtained using pools of

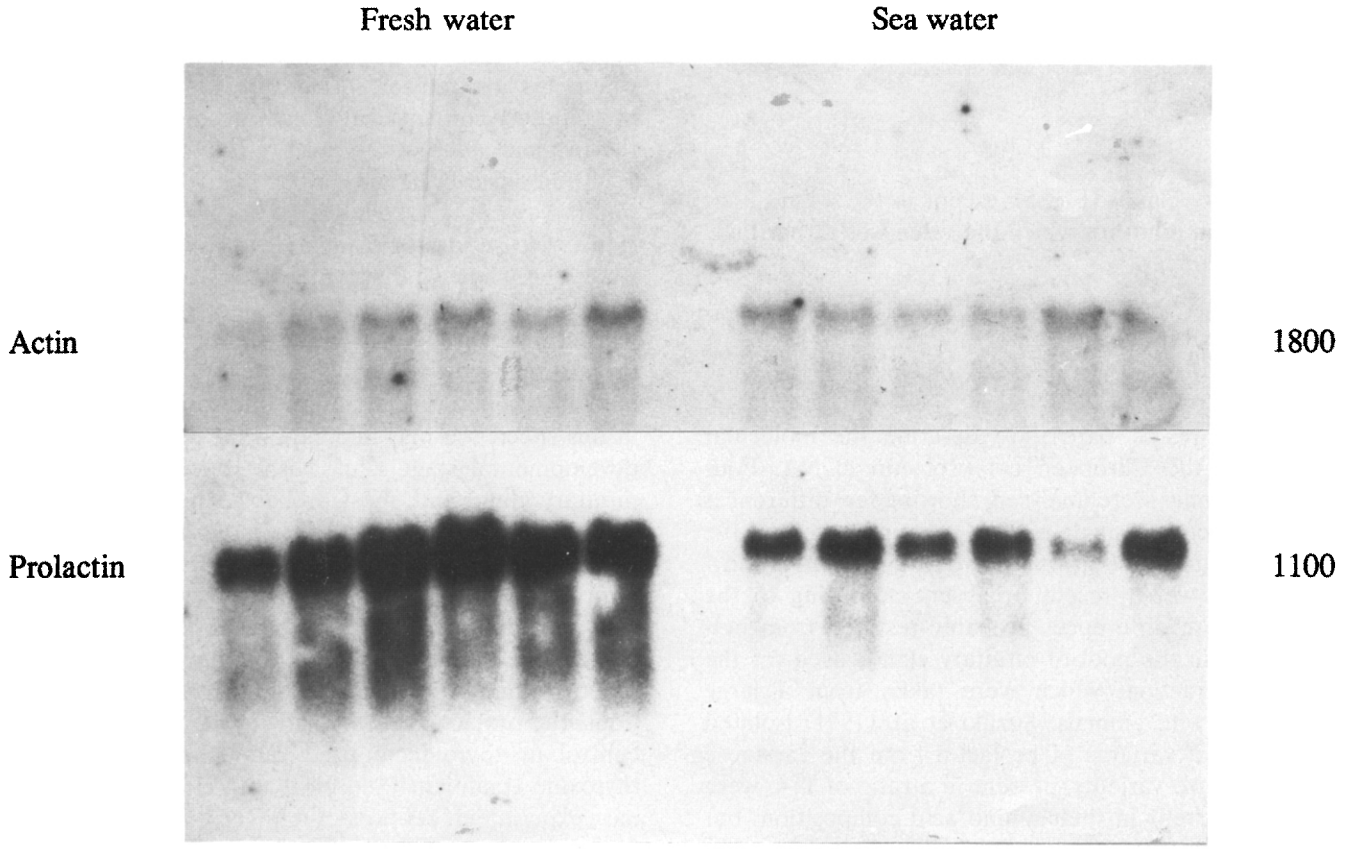

Fig. 5. Northern blot analysis of pituitary cytoplasmic RNA. 5 to 10 μg total cytoplasmic RNA extracted from eels kept in freshwater (left panel) or accommodated to sea water for 3 weeks (right panel) were electrophoresed through a formaldehyde containing agarose gel and transferred to nylon membrane. The filter was successively hybridized with the eel prolactin cDNA probe (lower lane), and a trout actin cDNA probe (upper lane).

Table 3
Time course effect of treatment with estradiol + testosterone on the pituitary mRNA level for prolactin

Duration of testosterone + estradiol treatment	0	4 days	2 weeks	4 weeks	6 weeks	10 weeks
Prolactin mRNA/actin mRNA	1.00	1.70	0.95	0.70	1.20	1.40

Eels were killed at different time after implantation of 500 μg each of testosterone and estradiol. mRNA levels were measured following Northern blot hybridization analysis by scanning densitometry. The value of 1.0 was given to the control. Data represent pools of 5 pituitary glands.

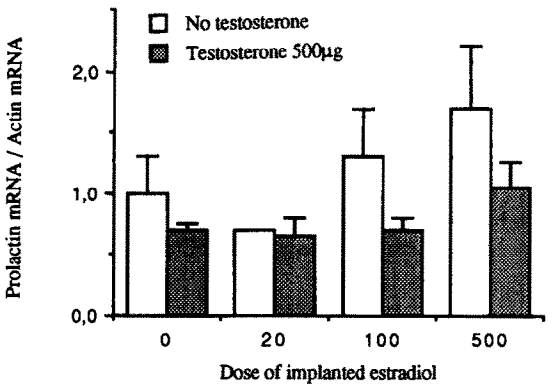

Fig. 6. Effect of different doses of estradiol together with or without testosterone, on pituitary mRNA level for prolactin. Estradiol and testosterone were administred as implants in coconut oil. Animals were killed two weeks after implantation. Prolactin and actin mRNA levels were measured as indicated in the legend of table 1. Data are expressed as means ± SEM of three pools of two pituitary glands, except for the 20 µg estradiol (without testosterone) point for which two pools, with identical values, were available.

two pituitary glands (Fig. 5), except in the 4 days after implantation point for which the value was rather high.

4. Discussion

In the present paper, we describe the molecular cloning of the European eel prolactin cDNA. Four different clones were analysed, showing few differences (all four encode a single mature protein), except in the 3′ untranslated portion where two fragments of 5 and 7 nucleotides were present or absent according to the clones. These differences probably resulted from heterogeneity in the pool of pituitary glands used for the mRNA extraction, which were taken from a large number of wild animals. Suzuki et al. (1991) isolated two different variants of prolactin from the Japanese eel. These two variants, present in a ratio of 1:4, were slightly different in their amino acid composition, but equipotent in biological activity. The first 57 amino acids sequenced for those two Japanese eel prolactins were identical and show only one difference with the European eel sequence (glycine instead of proline at position 54). In the European eel, only four different clones were sequenced and the possibility that another, rarer form, is present, cannot be excluded. However, the pattern of the Southern blot experiment is rather in favour of the presence of only one gene per haploid genome.

Comparison studies show that as for the other teleostean hormones (and also the holostean hormone: Dores et al., 1993), the eel prolactin lacks the N-terminal disulphide bond that is present in all other vertebrates including the chondrostean sturgeon and the dipnoid lungfish. Highest homology scores are found with teleostean hormones (except with the short variant of tilapia), then in a decreasing order, with the sturgeon, a large group encompassing the lungfish, the amphibian reptilian and avian, and finally with the mammalian hormones.

The hybridization signal obtained with the prolactin RNA probe corresponds to the localisation in the rostral pars distalis as determined by immunocytochemistry using anti-eel prolactin serum in the Japanese eel pituitary gland (Suzuki et al., 1991).

Prolactin is believed to be the hormone responsible for the control of osmoregulation in freshwater fish (cf. Clarke and Bern, 1980; Hirano, 1986). Transfer of euryhaline and stenohaline fish from sea water to hypoosmotic water induces a marked stimulation of prolactin secretion (cf. Nishioka et al., 1988). Inversely, when eels are transferred from freswater to sea water, the pituitary content of prolactin declines, returning to the original value after 8 weeks (Hall and Chadwick, 1978). In our experiment, prolactin mRNA levels in the pituitary were markedly reduced three weeks after transfer to sea water compared to the levels in freshwater eels, showing that synthesis as well as secretion is reduced in response to hyperosmolarity. In the eel, prolactin release in vitro was shown to be inversely proportional to the osmolarity of the incubation medium (Ingleton et al., 1973), indicating that at least in this species (it may depend on the species or on the developmental stage – cf. Nishioka et al., 1988), the pituitary gland and, most probably, the prolactin cells are directly controlled by the osmolarity of the blood.

There is still no evidence for a role of thyroid hormones on prolactin synthesis or secretion in fish. In rats, the prolactin mRNA levels were enhanced compared to controls, 72 h after administration of triiodothyronine (Franklyn et al., 1987). In other conditions, the prolactin mRNA activity was stimulated over control or thyroidectomized rats 1 week after daily thyroxine treatment (Seo et al., 1979). In our preliminary experiment, eel prolactin mRNA levels were unaffected by 3 weeks treatment with thyroid hormone implants that increased circulating levels more that 20-fold. These treatments did, however, lower pituitary mRNA level for α- and β-thyrotropin subunits (Salmon and Quérat, unpublished data), indicating that the doses and duration of treatment were compatible with physiological actions. However, these conditions might be inadequate for modulating prolactin gene expression. The first metamorphosis of the eel occurs in sea water, where the prolactin gene expression is expected to be low. A stimulatory action of the thyroid hormones on prolactin gene expression at the climax of this first metamorphosis, when the young eels are

preparing to enter freshwater would be an advantage. It would thus be interesting to test the action of thyroid hormones in eels kept in sea water.

Another aspect of prolactin regulation is the contribution of the sexual steroids. Estradiol has been shown (i) to activate prolactin cells in *Gillichtys mirabilis* (Nagahama et al., 1975) and in the European eel (Olivereau and Olivereau, 1979; Olivereau et al., 1986), (ii) to stimulate prolactin secretion from *Oerochromis mossambica* pituitary tissue culture (Barry and Grau, 1986), (iii) to stimulate prolactin synthesis but not release in the same species in similar conditions (Wigham et al., 1977), and (iv) to stimulate prolactin secretion without altering its synthesis in *Clarias batrachus* (Singh and Singh, 1981). These results show that the mechanism of action of estradiol on the prolactin gene expression might be complex and could depend on the species or the physiological state of the fish. In the experiments presented in this paper, there was no evidence that estradiol altered the eel prolactin mRNA level, whatever the dose used and the duration of treatment. These treatments were, however, adequate for selectively stimulating the pituitary mRNA levels for α- and β-subunits of the type 2 gonadotropin (Quérat et al., 1991a,b). The co-implantation of testosterone together with estradiol strongly potentiated the effect of estradiol on the gonadotropin mRNA levels, but did not modify prolactin mRNA levels. The lack of effect of estradiol on the prolactin pituitary mRNA levels was also reported in trout by Le Goff et al. (1992). In this latter case, the absence of a direct action of estradiol was attributed to a lack of discernible estradiol receptors in the prolactin cells as evidenced by in situ hybridization using a trout estradiol receptor RNA probe. Our results are in contrast with those previously reported in eels by Olivereau and coworkers (see above) in which an increased activity of the eel prolactin cells following estradiol treatment in vivo was observed using histological techniques. This increased activity could possibly solely reflect an activated secretion, independent of synthesis. Consequently, taken together, these results, tend to indicate that even if estradiol could stimulate the eel prolactin release from the prolactin cells, it does not stimulate de novo synthesis. Moreover, the gonadal maturation of the eel, with the expected increase in the gonadal steroid hormones, occurs during the reproductive migration across the ocean. If, as commonly admitted (see above), prolactin is implicated in osmoregulation in freshwater, but not in sea water, then levels of this hormones must be kept low during the reproductive migration of the eel. A stimulatory effect of gonadal steroids on prolactin production would be detrimental under these circumstances. Our results are then compatible with the physiological interpretation of the role of prolactin in osmoregulatory accommodation.

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