Evolution of oogenesis: the receptor for vitellogenin from the rainbow trout

Blandine Davail,[1,2,*] Farzad Pakdel,[1,†] Hideaki Bujo,[3,§] Luciane Maria Perazzolo,[*] Marianne Waclawek,[§] Wolfgang Johann Schneider,[§] and Françoise Le Menn[*]

Biology of Fish Reproduction,[*] UA INRA, University Bordeaux I, F-33405 Talence, France; Molecular Endocrinology of Reproduction,[†] UPRES-A 6026, C.N.R.S., University Rennes I, F-35042 Rennes, France; and Department of Molecular Genetics,[§] University and Biocenter Vienna, A-1030 Vienna, Austria

Abstract **Receptors that transport vitellogenin (VTG) into oocytes are of vital importance to egg-laying species because they mediate a key step in oocyte development. Here we describe the cloning of the first piscine oocyte-specific receptor cDNA, i.e., that encoding the VTG receptor from the rainbow trout (*Oncorhynchus mykiss*). The receptor, a 826-residue type-I membrane protein, is a member of the low density lipoprotein receptor (LDLR) superfamily. It closely resembles the mammalian so-called very low density lipoprotein receptors, in that its aminoterminal ligand binding domain consists of a cluster of 8 cysteine-rich repeats. The short intracellular portion contains the internalization signal typical for the LDLR superfamily, Phe-Glu-Asn-Pro-Val-Tyr. Notably, the receptor lacks a domain with a high density of potential O-glycosylation sites often found in somatic cell-specific members of the LDLR family. A specific transcript of 3.9 kb is abundant in ovary, but undetectable in muscle and heart, which are the major sites of expression of very low density lipoprotein receptors in mammals. In vitro translation of the full-length cDNA produced a 97-kDa protein, and transient expression in COS-1 cells showed that the cDNA encodes a protein of the same size that binds vitellogenin in ligand blots. As revealed by in situ hybridization, transcripts are present in previtellogenic oocytes, indicating that production of receptor protein precedes the phase of yolk deposition. Our results in fish, together with those in birds (Bujo, H., et al. 1994. *EMBO J.* 13: 5165–5175) suggest that vitellogenesis provides a prime model for the study of ligand/receptor systems designed to sustain reproduction.—**Davail, B., F. Pakdel, H. Bujo, L. M. Perazzolo, M. Waclawek, W. J. Schneider, and F. Le Menn. **Evolution of oogenesis: the receptor for vitellogenin from the rainbow trout. *J. Lipid. Res.* 1998.** 39: **1929–1937.**

Supplementary key words oocyte • receptor • vitellogenin • lipoproteins • yolk • fish

Oocyte growth in oviparous species is dependent on the uptake of nutrients and their storage as yolk, whose constituents are subsequently used by the embryo during early stages of its development. Among the major yolk components, lipoproteins are predominant. For instance, vitellogenin (VTG), a complex glycophospholipoprotein synthesized by the liver under estrogenic induction, is transported via the bloodstream to the ovary, where it is sequestered by growing oocytes in cell-specific fashion. Based on results of in vivo tracer studies in fish (1, 2), VTG makes its way through multiple cellular and extracellular layers surrounding the oocyte from the capillary network dispatching blood at the periphery of the follicle. After penetrating through the basal lamina and between the granulosa cells, VTG reaches the oolemma by passing along the oocyte microvillosities into channels in the zona radiata, and is finally internalized by a selective mechanism via specific cell-surface receptors involving coated pits and coated vesicles (3). Proteins with high affinity for VTG have been identified and characterized on oocyte membranes from chicken (4), *Xenopus* (5), and several fish species (6–11).

Detailed studies in the chicken have demonstrated that a 95-kDa protein binds not only VTG but also very low density lipoprotein (VLDL), triglyceride-rich particles of hepatic origin (12, 13). Indeed, molecular characterization of the chicken oocyte protein revealed extensive homology between it (14) and the mammalian so-called VLDL receptors (VLDLR) (15, 16), a branch of the low density lipoprotein receptor (LDLR) superfamily (14). Elements characteristic of this gene family are *i*) clusters of cysteine-rich repeats constituting the ligand-binding domain; *ii*) epidermal growth factor-precursor repeats, also containing six cysteines each; *iii*) usually 5 consensus tetrapeptide motifs, Phe/Tyr-Trp-Xaa-Asp, within subdo-

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoprotein; VLDLR, VLDL receptor VTG, vitellogenin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; LDLR, low density lipoprotein receptor; EGF, epidermal growth factor.

[1]These authors contributed equally to the study.

[2]To whom correspondence should be addressed.

[3]Present address: Second Department of Internal Medicine, Chiba University School of Medicine, Chiba 260, Japan.

mains of 50 residues each (17); *iv*) a transmembrane domain anchoring the receptor in the plasma membrane; and *v*) a consensus peptide in the cytoplasmic domain, Phe-Glu-Asn-Pro-Xaa-Tyr, that mediates the internalization of the receptor via coated pits (18). In order to provide further information on the evolutionary aspects of VTG receptors and to shed first light on regulation of vitellogenesis in fish, we now report the molecular characterization and expression of the VTG receptor from the rainbow trout, *Oncorhynchus mykiss*.

MATERIALS AND METHODS

Preparation of mRNA and identification of rainbow trout VTG receptor cDNA by RT-PCR

Total RNA was extracted from various tissues and from ovaries of adult rainbow trout using a modification of the technique described by Auffray and Rougeon (19). Ovaries were in the slow developmental stage and contained early vitellogenic follicles (mean diameter, 1.2 mm) (20). Poly A(+)-RNA was purified by Dynabeads oligo (dT) (DYNAL) according to the manufacturer's protocol. To obtain a rainbow trout VTG receptor cDNA fragment for use as probe in cDNA library screening, reverse transcription-polymerase chain reaction (RT-PCR) was performed with two degenerate primers, (A), 5′-AT(CT)(CG)A(AG)TG GCC(CT)AA(CT)GG(CAGT)AT-3′, and (B), 5′-TA(GC)AC(GA) GG(GA)TT(GA)TC(GA)AA(AG)TT-3′, which are based on conserved sequences in the epidermal growth factor (EGF) precursor homology domain and cytoplasmic domain, respectively, of rabbit VLDLR (15) and chicken VLDL/VTGR (14). Single-stranded cDNA was synthesized from 5 μg of poly A(+)-RNA using SuperScript reverse transcriptase (Gibco-BRL) and random primers. Amplification of cDNA was performed with primers A and B using the geneAmp PCR kit (Perkin-Elmer) on a Perkin-Elmer Thermal Cycler 480. PCR parameters were 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 30 cycles. Amplified products were separated by agarose gel electrophoresis, and products of about 800 bp were excised and purified. Products were subcloned into the pGEM-T vector (Promega). Several clones were isolated and sequenced using Sequenase (US Biochemical). Clone BL8, which encoded a sequence (635 bp open reading frame) highly homologous to VLDL and LDL receptors, was used as a probe for screening a rainbow trout ovary cDNA library.

Construction and screening of a cDNA library

Two μg of Poly A(+)-RNA isolated from ovaries containing vitellogenic follicles with a mean diameter of 1.2 mm were used to prepare double-stranded cDNA as described previously (21) with Amersham's cDNA synthesis system. The cDNA was ligated to the EcoRI/NotI linker (Pharmacia) and then phosphorylated by polynucleotide kinase for 45 min at 37°C. Double-stranded cDNA was fractionated by gel filtration on a Sepharose 4B column (Pharmacia). The largest fractions were pooled and ligated to λgt10 vector, which had been dephosphorylated and digested with EcoRI (Stratagene) at a molar ratio of 1:1 (cDNA:lambda phage DNA). The ligation products were packaged in vitro as recommended by the manufacturer's protocol (Promega Biotec's packaging system). In order to clone the full-length rainbow trout VTG receptor cDNA, approximately 2.5×10^5 recombinant phages were screened by plaque hybridization using ^{32}P-labeled BL8 under high stringency hybridization conditions (22). Several positive clones were obtained, and the clone VTGR4kb, containing a 3940 bp cDNA fragment, was purified, subcloned into the EcoRI site of pBluescript II SK (Stratagene), and sequenced on both strands. Nucleotide sequences were analyzed by the Genejockey computer program.

Expression of rainbow trout VTGR in COS-1 cells and in vitro translation

The 3940 bp cDNA fragment was subcloned into the EcoRI site of the mammalian expression vector pCMV5 (Invitrogen). The resulting plasmid pCMV-VTGR4kb was used for transient expression. COS-1 (African green monkey kidney cells derived from SV40-transformed CV1 cells) cells were maintained in Dulbecco's minimal essential medium (Sigma) containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum. The cells were transfected using the DEAE-dextran method. They were seeded in 10-cm dishes at a density of one million cells/plate. After 36–44 h, the medium was removed and the cells were transfected with either 10 μg of CMV-VTGR4kb expression vector or the control plasmid (pCMV5). After 48 h at 37°C, cells were washed with cold PBS and harvested. Cell extracts were prepared and used for ligand blotting as described previously (14).

The in vitro translation reaction was performed using 1 μg of pBluescript II SK plasmid containing the entire VTGR cDNA (3940 bp) and T3 RNA polymerase in a rabbit reticulocyte lysate (methionine depleted) with 740 kBq [^{35}]S-methionine. Reactions were performed at 30°C for 60–120 min as recommended by the supplier (TNT Reticulocyte Lysate System from Promega Biotec). Translation products of synthetic mRNA were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Ligand preparation, electrophoresis and ligand blotting

Vitellogenin was obtained from plasma of estrogen-treated male rainbow trout. Purification and radio-iodination were performed as previously described (10) to specific activities of 300 cpm/ng protein. One-dimensional SDS-PAGE (7.5% acrylamide) was performed in the absence of reducing agents as previously described (23) using a minigel system (Mini-Protean II, Bio-Rad). Electrophoresis was performed at 130 V for 90 min at room temperature. Prestained high range molecular weight standards (Bio-Rad) were used. Electrophoretic transfer of the protein to PVDF membranes (Immobilon, Millipore) was performed in transfer buffer (25 mm Tris, 192 mm glycine, 20% methanol, pH 8.3) at 100 V for 1 h using a Mini Trans-Blot system (Bio-Rad). Ligand blotting with [^{125}]I-labeled VTG was carried out with 5% (w/v) non-fat dry milk as blocking agent in buffer containing 25 mm Tris-HCl (pH 7.8), 50 mm NaCl, and 2 mm $CaCl_2$. Autoradiograms were obtained by exposing the dried membrane to Kodak X-OMAT film for the indicated time at −70°C.

Northern blot analysis

Poly A(+)-RNAs were denatured at 65°C for 15 min in 50% formamide, 7% formaldehyde, 0.5 mm EDTA and 10 mm NaH_2PO_4, pH 6.5. The samples were electrophoresed in a 1% agarose gel containing 9% formaldehyde and 10 mm NaH_2PO_4, pH 6.5, for 1 h at 70 V, then transferred onto a nylon membrane (Biodyne Pall Industrie). The membrane was prehybridized (5 h at 42°C) in hybridization buffer (40% formamide, 5 × SSC, 50 mm NaH_2PO_4 (pH 6.5), 5 × Denhardt's, 0.1% SDS, 100 μg/ml yeast tRNA) and hybridized (16 h at 42°C) in hybridization buffer containing [^{32}]P-labeled probe (Rediprime DNA labeling kit, Amersham). The full-length VTGR cDNA was radiolabeled and used as probe. Four washings in 2 × SSC and 0.1% SDS were performed at 42°C for 15 min and four washings in 0.3 × SSC and 0.1% SDS were performed at 45°C for 30 min. Hybridization with a trout actin probe was performed under identical conditions (24).

In situ hybridization

Paraffin sections were dewaxed in toluene and rehydrated in graded ethanol solutions, subjected to digestion with proteinase K (2 μg/ml) for 15 min at 37°C and 0.2N HCl for 5 min at 23°C. The sections were hybridized overnight at 55°C with [^{35}]S-labelled probes (10^6 cpm/ 50 ml) in hybridization buffer. Slides were then treated according to the method described by Le Moine and Bloch (25). For autoradiographic visualization, slides were dipped into Ilford K5 emulsion and exposed for 20 days before developing. The slides were stained with 0.25% toluidine blue in 0.5% sodium tetraborate, before dehydration and mounting.

Probe synthesis

^{35}S-labeled antisense and sense cRNA probes were prepared by in vitro transcription from a 700 bp fragment of the VTGR4kb cDNA. The transcription was performed on 100 ng of linearized plasmid in the presence of ^{35}S-labeled UTP (NEN, >3.7 10^4 GBq/mmol) and T3 or T7 RNA polymerases. After alkaline hydrolysis, 250 bp cRNA fragments were obtained. The probes were then purified on G50 Sephadex and precipitated in 3 m sodium acetate (pH 5.2)–absolute ethanol 1:25, (vol/vol). Probes were stored at −20°C until use.

RESULTS

Cloning of the trout oocyte VTGR

Two degenerate oligonucleotides according to consensus nucleotide sequences in the chicken VLDL/VTGR (14) and rabbit VLDLR (15) were used as primers in RT-PCR to amplify cDNAs prepared from Poly A(+)-RNA of trout ovary. Two products were obtained, subcloned into a plasmid vector, and sequenced. Analysis of the clones, BL8 (635 bp) and BL13 (740 bp), showed that they were identical with the exception of an internal additional stretch of 105 bp in BL13. The deduced amino acid sequences clearly identified the cDNAs as specifying a novel LDLR family member. There was 69% identity with the chicken VLDL/VTGR and 53% with the rabbit VLDLR. In addition, the extra 105 bp sequence in BL13 corresponded to a serine- and threonine-rich stretch typical of the so-called O-linked sugar domains in other (V)LDL receptors. Moreover, Northern blot analysis with BL8 as probe showed hybridizing transcripts (~3.9 kb) in trout ovary, but not in muscle or heart (not shown, but cf. Fig. 3 below), as expected for a receptor involved in oocytic VTG uptake. These characteristics strongly suggested that BL8 corresponded to the rainbow trout's oocyte-specific VTG receptor.

Thus, BL8 was used as probe for screening our trout ovary cDNA library. Of the four positive clones obtained, clone VTGR4kb was the largest (insert size, 3940 bp) and was further analyzed. Its nucleotide sequence (**Fig. 1**) contained an open reading frame of 2544 bp encoding 847 amino acid residues, 48 bp of the 5′- noncoding region and a 1348-bp 3′-untranslated region with a polyadenylation signal, AATAAA. A single ATG codon in the 5′ part of the open reading frame presumably codes for the initiating methionine. According to the rules of von Heijne (26), we assigned the mature protein's aminoterminus to a glycine following a 22-residue presumed signal sequence (Fig. 1). A second hydrophobic region at amino acid residues 745–766 was tentatively identified as the transmembrane domain by structural analogy with LDLR family members. All of the features typical of LDLR family members were clearly discernible in the newly cloned protein (**Fig. 2**): the ligand binding domain at the N-terminus, containing eight repeats with six cysteines each; the epidermal growth factor (EGF) precursor homology domain with three cysteine-rich repeats named A, B, C; and a cytoplasmic region containing the peptide Phe-Asp-Asn-Pro-Val-Tyr (FDNPVY), required for receptor internalization via coated pits (18).

The molecular mass of the mature receptor protein, calculated according to its deduced amino acid sequence, is 91,254 Da. Compared to other VLDLRs and VTGRs, the primary sequence of the trout VTGR is 68%, 72%, and 70% identical to those of the corresponding gene products from Xenopus, chicken, and rabbit, respectively. Similar degrees of identity exist between the sequences in the ligand binding domain (71–75%), the EGF precursor homology domain (68–73%), and in the transmembrane region (64–91%), respectively. The FDNPVY sequence in the cytoplasmic domain (where overall identity is 71–76%) is completely conserved between trout, *Xenopus*, chicken, and rabbit VTG/VLDLRs. Our trout VTGR clone did not contain an O-linked sugar domain, i.e., it represents the oocyte-specific transcript (27), in agreement with being isolated from an ovarian cDNA library.

To further investigate the tissue distribution of VTGR expression in fish, we used the full-length trout VTGR4 cDNA as probe in Northern blots using polyA(+)-RNA isolated from trout vitellogenic ovary (28), muscle, heart, and intestine. **Figure 3A** shows that a hybridizing mRNA of the expected size (~3.9 kb) is found in the ovary, but not in trout heart or in muscle, which are the major sites of expression of VLDLR in mammals (15, 29), or in intestine. Figure 3B shows the result of a control hybridization of the same RNAs with a trout actin probe (24).

In vitro translation and expression of the trout VTGR in COS-1 cells

To determine the authenticity of the isolated cDNA with that of the trout VTGR, we first performed in vitro translation to produce ^{35}S-methionine-labeled protein. A rabbit reticulocyte lysate driven by the VTGR4 cDNA revealed the synthesis of a protein with an apparent molecular mass of 97 kDa in SDS-PAGE under non-reducing conditions (**Fig. 4**, left panel). The size of the identified band is in good agreement with that calculated from the deduced sequence, as well as with that of the chicken VLDL/VTGR (95 kDa) expressed in COS-7 cells (14). Likely, the protein is identical to protein(s) previously identified by ligand blotting with VTG in trout ovarian membrane extracts, reported to have M_rs of ~110 kDa (10, 30).

Second, extracts of COS-1 cells transfected with the expression vector containing the entire VTGR cDNA (CMV-VTGR4kb, see Materials and Methods) or with vector alone were subjected to SDS-PAGE under non-reducing conditions, blotted onto nitrocellulose membranes, and the blots were incubated with purified trout ^{125}I-labeled

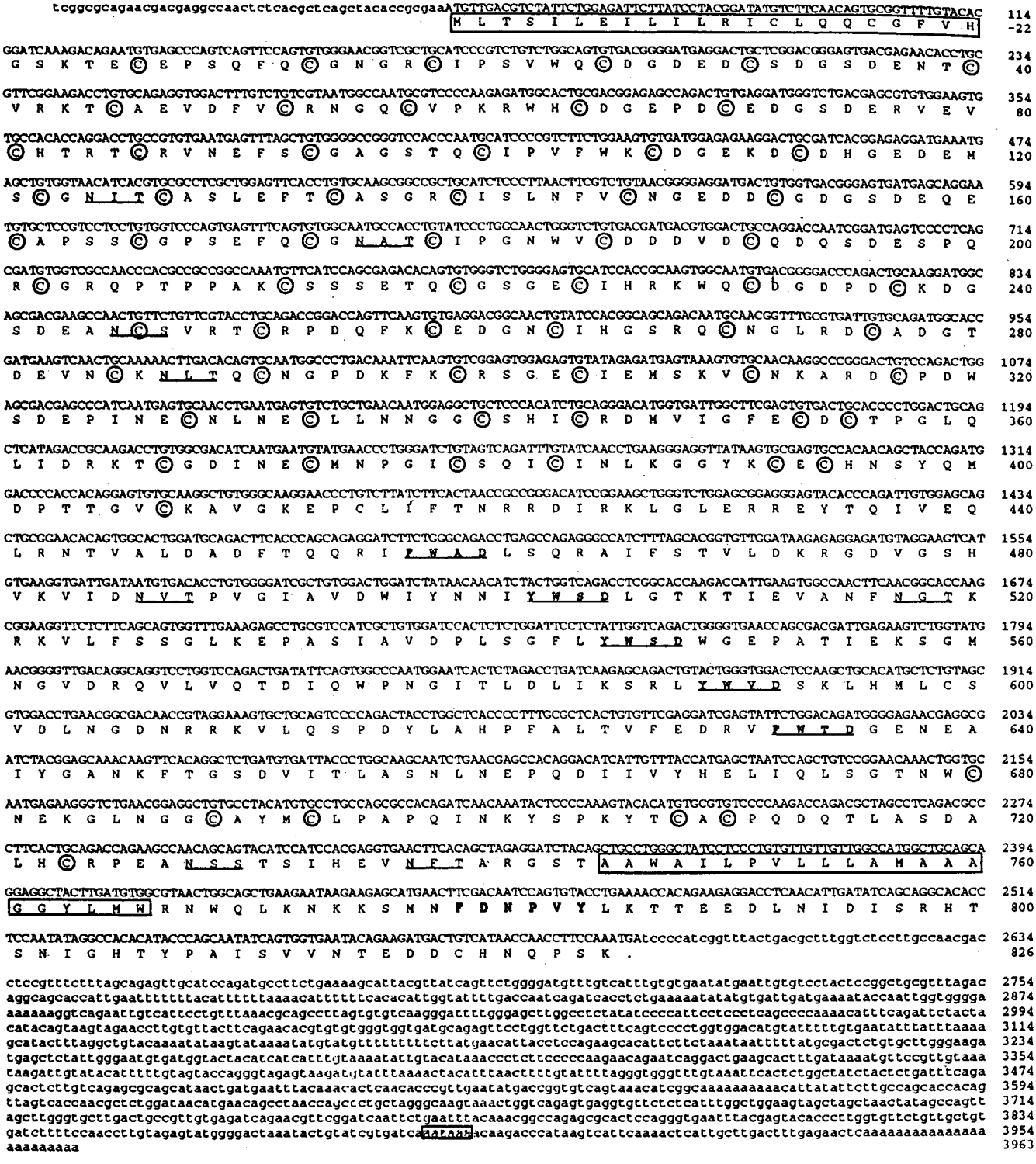

Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA encoding the trout VTG receptor. Nucleotide residues are numbered in the 5′ to 3′ direction. The numbering of the amino acid sequence starts from the first aa of the mature receptor; negative numbers refer to the signal sequence (boxed at the N-terminus). Cysteine residues are circled, the potential N-glycosylation sites are underlined, F/YWXD-repeats are indicated in bold letters and underlined, the transmembrane sequence (near the C-terminus) is boxed, and the FDNPVY-internalization signal is indicated by bold letters. The polyadenylation signal is boxed; the nucleotide sequences of untranslated regions are in lower case.

VTG (Fig. 4, right panel, TB). Several proteins became labeled in both control and transfected cells, one of which had a mobility corresponding to ~95 kDa; however, a protein migrating slightly slower than this band was clearly stained only in the transfected cells. Inasmuch as the binding of ^{125}I-labeled VTG to all of these bands was competed by an excess of unlabeled VTG (Fig. 4, right panel, NSB), and COS-7 cells contain an endogenous 95 kDa protein

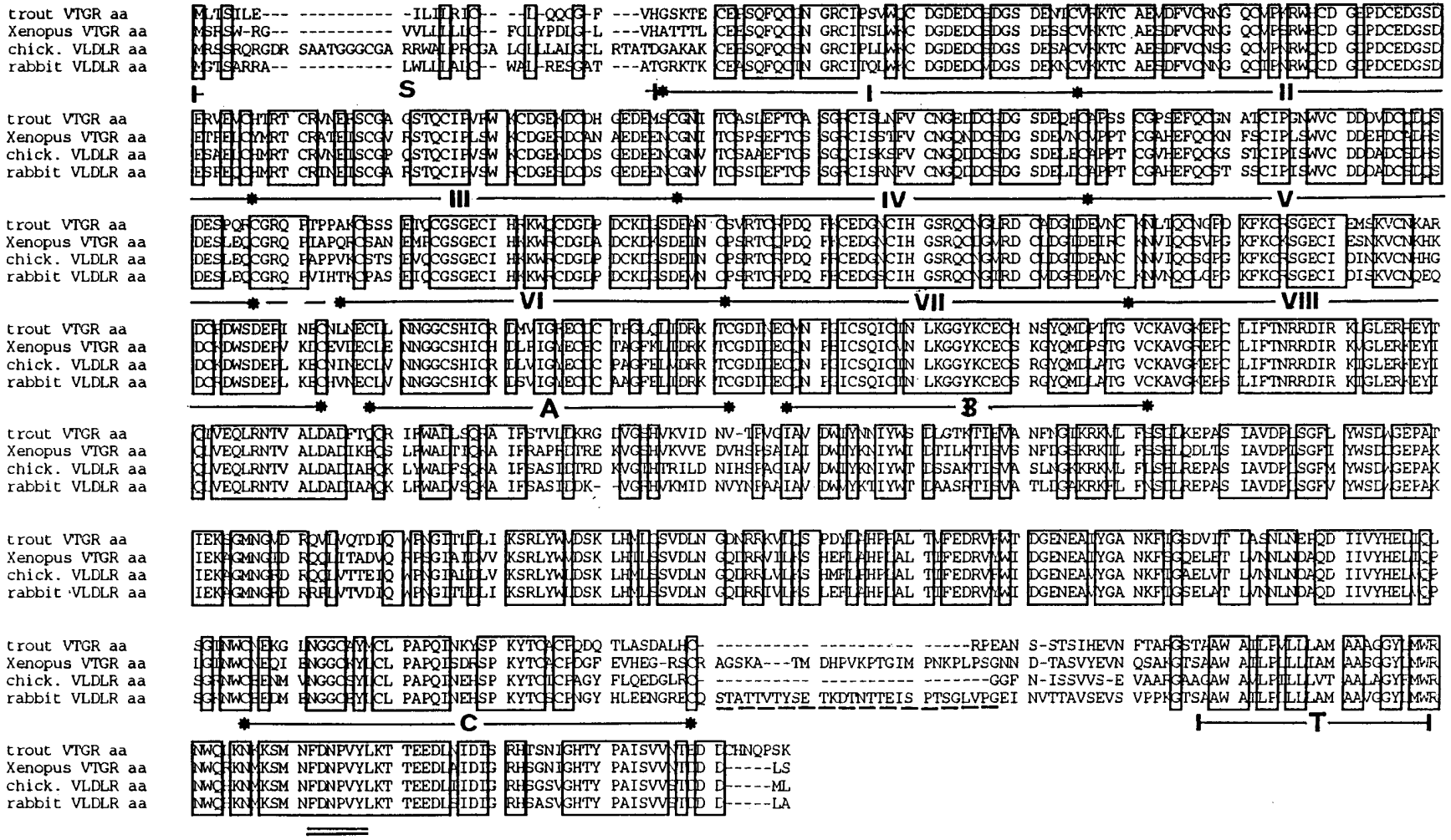

Fig. 2. Comparison of the amino acid sequences of the VTG receptors from trout, *Xenopus* (33), chicken (VLDL/VTGR, 14), with that of the rabbit VLDL receptor (15). The numbering starts at the initiating methionine. Identical amino acids are boxed. The signal sequence is noted by an S. The following cysteine-rich repeats within the ligand-binding domain, indicated by thin lines, are numbered from I to VIII with a spacer sequence between repeat V and VI, indicated by a short dashed line. The cysteine-rich repeats within the EGF-precursor homology domain are designated A, B, and C. The clustered O-linked sugar region of the rabbit VLDL receptor is marked with a broken underline, the transmembrane region is indicated by a T, and the totally conserved FDNPVY internalization signal is underlined twice.

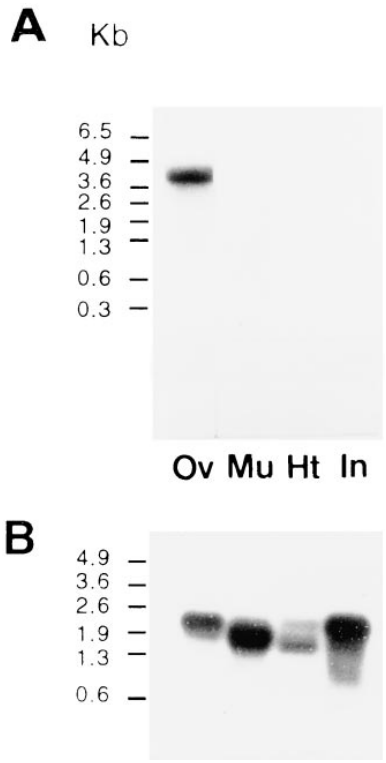

Fig. 3. Characterization of putative VTGR mRNA by Northern blot analysis. A: Poly A(+)-RNA (15 μg per lane) isolated from rainbow trout ovary (Ov), muscle (Mu), heart (Ht), and intestine (In) were denatured and separated by electrophoresis on a 1% agarose gel. Poly A(+)-RNA were transferred to a nylon membrane and hybridized with ^{32}P-labeled VTGR4kb probe as described in Materials and Methods; autoradiography was for 5 days at −70°C. B: The same poly A(+)-RNA preparations (3 μg per lane) were analyzed as in (A), except that a ^{32}P-labeled trout actin probe (24) was used for hybridization; autoradiography was for 2 h.

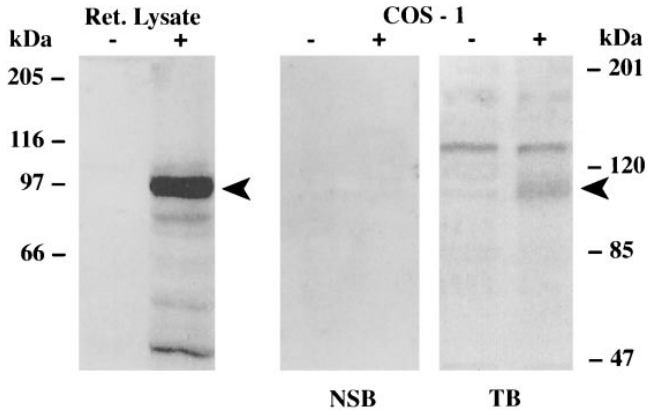

Fig. 4. In vitro translation and expression of VTGR in COS-1 cells. Left panel: one microgram of plasmid containing the entire VTGR cDNA (+) or not (−) was used with rabbit reticulocyte lysate in the presence of [^{35}]S-methionine as described in Materials and Methods. An aliquot (10 μl) of the in vitro translation mix was subjected to electrophoresis on a 10% SDS-polyacrylamide gel and autoradiography for 2 days. The arrow indicates the position of the 97 kDa in vitro translation product. Right panel: COS-1 cells were transiently transfected with expression vector containing the entire VTGR cDNA (+) or not (−) as described in Materials and Methods. The cell extracts (200 μg protein/lane) were subjected to SDS-PAGE under non-reducing conditions and transferred to nitrocellulose membrane. Ligand blotting was performed with ^{125}I-labeled trout VTG (10^5 cpm/ml) alone (TB) or in the presence of an excess of non-labeled VTG (NSB); autoradiography was for 3 days. The arrow indicates the position of the 97 kDa VTGR. Molecular masses in kilodaltons (kDa) are also indicated.

known to bind both chicken VTG and VLDL (14), the additional bands likely represent simian VLDLR family members endogeneously expressed in COS-1 cells. In this context, the equivalence of chicken and trout VTG in terms of receptor binding properties has been demonstrated previously (6). Likewise, one could consider that such protein might exist in COS-1 cells. The additional band in the transfected cells that bound VTG had an apparent molecular mass of 97 kDa. These data demonstrate that the isolated trout cDNA encodes a bona-fide VTGR.

Localization of VTGR mRNA in ovarian follicles

^{35}S-labeled sense and antisense cRNA probes, prepared by in vitro transcription from a 700 bp fragment of the gene encoding for the VTGR, were used to perform in situ hybridization on trout ovarian sections. **Figure 5** (panels A and B) shows that VTGR mRNA appears to be confined to the cytoplasm of previtellogenic oocytes (150–200 mm diameter). No specific signals are present in the oocyte nucleus and in surrounding cell layers (theca and granulosa). In stage II of oocyte development, which precedes the beginning of internalization of VTG into the oocyte, a very strong and evenly distributed signal is observed throughout the cytoplasm. On the contrary, there is no more detectable signal in the larger, fully vitellogenic oocytes (2 mm diameter; stage IV). Thus, during the phase of rapid ovarian development, there seems to be little or no transcription of the trout VTGR gene in oocytes in which VTG uptake (i.e., VTGR protein level) is at a maximum (28). Figure 5C shows the results of a typical hybridization experiment with the sense (control) cRNA probe; background staining amounts to less than 2% of the specific signal.

DISCUSSION

We describe the cloning, sequencing, expression, and transcript localization of the first piscine receptor for VTG uptake into oocytes. The VTGR of rainbow trout, *Oncorhynchus mykiss*, is a cell-specific lipoprotein receptor clearly belonging to the VLDLR branch of the LDLR superfamily (14–16, 29, 31–33). Its molecular mass according to the cDNA-deduced amino acid sequence is ~91 kDa, which was confirmed by expression in COS-1 cells followed by ligand blotting with ^{125}I-labeled-VTG and by in vitro translation in a reticulocyte lysate system. However, in previous ligand blotting studies performed in rainbow trout, not one but several follicle-membrane proteins have been demonstrated to show affinity for VTG (10, 30). Upon close inspection of these data, the most specific binding was associated with a 110/113 kDa protein, and both groups concluded that the larger proteins they had observed rep-

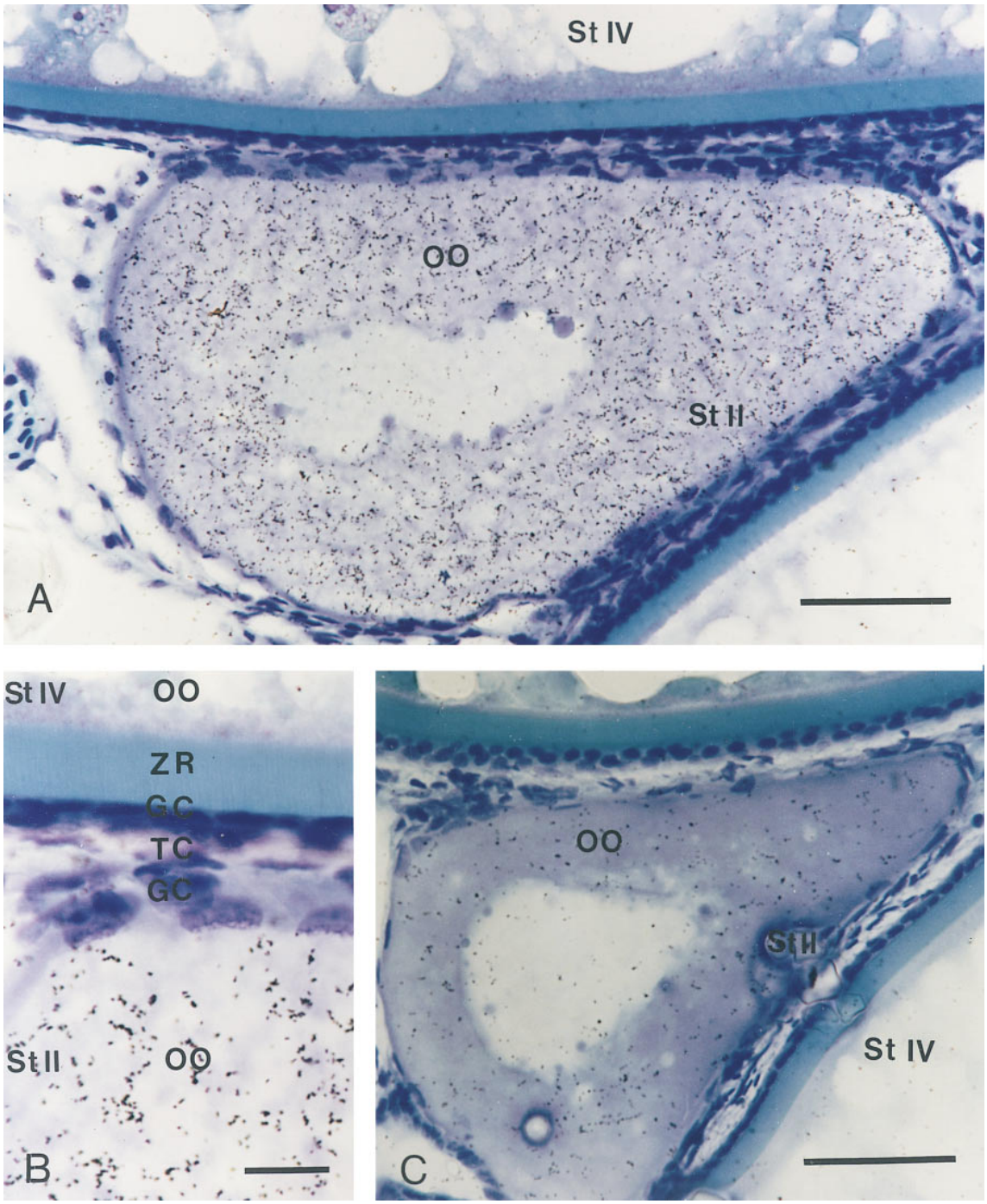

Fig. 5. In situ hybridization of VTGR transcripts in trout ovarian follicles. Ovaries were sampled from 24-month-old rainbow trout. Paraffin sections were subjected to in situ hybridization with an antisense (A and B) and a sense (C) ^{35}S-labeled cRNA probe, respectively. Exposure time was 20 days. Sections A and C contain previtellogenic oocytes of 200 μm (A) and 150 μm (C) diameter at the plurinucleolar stage (St II) with surrounding cell layers and portions of fully vitellogenic oocytes (St IV, 2 mm diameter). Panel B: at higher magnification, hybridizing transcripts are seen to be restricted to the cytoplasm of the smaller oocyte (cf. panel A). The cytoplasm of the stage II oocyte (OO) is distinctively labeled, whereas the stage IV oocyte (panel A, top) and peripheral granulosa cells (GC) and theca cells (TC) are not. Bars: 50 μm (A and C) and 10 μm (B), respectively.

resented SDS-resistant aggregates of the smaller receptors (10, 30). This interpretation is compatible with our present findings on the cloning and expression of the trout VTGR.

Furthermore, early studies directed at the identification of VTGRs used ligand blotting with homologous as well as heterologous VTGs, and revealed that VTGRs of different species share numerous properties. First, in most oviparous vertebrates studied, VTGRs were found to be single polypeptides, with molecular masses of 95 kDa in chicken (12) and Japanese quail (34), ~100 kDa in coho salmon (6), ~100 kDa in sea bass (11), and ~115 kDa in *Xenopus* (5). Moreover, VTGRs are known to bind heterologous

ligands, in that follicular membrane proteins from chicken, frog, and fish can specifically bind VTG from fish, frog, or chicken, respectively (6); the chicken VTGR binds quail VTG and vice versa (34). These results suggest that VTGRs share common binding sites for VTGs, a notion strengthened by our current results. Furthermore, some studies demonstrated immunological relationships between VTGRs from chicken, quail, frog, and salmon (6, 34). Here we reveal that the trout VTG receptor's structure is indeed essentially identical to that of the VTGRs of chicken (14) and *Xenopus* (35). Interestingly, the degree of conservation between VTGRs is greater than that among LDLRs. Thus, although the modes of follicle growth and oocyte maturation vary greatly between species, rapid, synchronized oocyte growth lasts 1 month in the winter strain of rainbow trout, whereas chicken oocytes grow hierarchically during a 1 wk final rapid phase, the key players in oocyte growth and yolk formation have been conserved during evolution.

In this context, the isolation of a cDNA coding for the trout oocyte VTGR has been useful for the identification of the site of synthesis of this important receptor. In situ hybridization experiments clearly demonstrated that transcripts are exclusively localized to oocytes, and are not found in somatic cells within the ovary. The absence of transcripts from oocytes in the most rapid growth phase, i.e., when receptor-mediated uptake of VTG occurs at maximal rates (28), was surprising at first. However, we have previously observed that levels of transcript and of VTGR protein in the chicken ovary are very high in the ovaries of immature hens, i.e., animals that do not yet produce fully grown oocytes (36), and transcript levels drop during the final growth phase of the female germ cells. These common findings suggest that during rapid growth and vitellogenesis the oocytes rely on VTGR previously synthesized during early previtellogenic stages of oocyte development.

Finally, we are now in a position to gain better understanding of the regulation of VTGR gene expression, which is not only of scientific interest but, at least in fish, also of potential economical impact. In addition, vitellogenesis has been proposed as a biomarker system for estrogenic contamination of the aquatic environment (37, 38); we are now able to analyze one of the key marker elements at the molecular genetic level.

We thank Romana Kukina for excellent technical assistance. This work was supported by grants from the Austrian Science Foundation (F-0608, to W. J. S.), from the French Foreign Office (to B. D.), from the CNPq-Brazil (to L. M. P.), and from the European Community (FEDER 5b). We are grateful to the firm "Salmonidés d'Aquitaine/Viviers de France" for having provided the fish.

Manuscript received 18 August 1997, in revised form 20 January 1998, and in re-revised form 28 May 1998.

REFERENCES

1. Busson-Mabillot, S. 1984. Endosomes transfer yolk proteins to lysosomes in the vitellogenic oocyte of the trout. *Biol. Cell.* **51:** 53–66.
2. Selman, K., and R. Wallace. 1989. Cellular aspects of oocyte growth in teleosts. *Zool. Sci.* **6:** 211–231.
3. Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russel, and W.J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* **1:** 1–39.
4. Stifani, S., R. George, and W. J. Schneider. 1988. Solubilization and characterization of the chicken oocyte vitellogenin receptor. *Biochem. J.* **250:** 467–475.
5. Stifani, S., J. Nimpf, and W. J. Schneider. 1990. Vitellogenesis in *Xenopus laevis* and chicken: cognate ligands and oocyte receptors. *J. Biol. Chem.* **265:** 882–888.
6. Stifani, S., F. Le Menn, J. Nunez Rodriguez, and W. J. Schneider. 1990. Regulation of oogenesis: the piscine receptor for vitellogenin. *Biochim. Biophys. Acta.* **1045:** 271–279.
7. Le Menn, F., and J. Nunez Rodriguez. 1991. Receptors mediate endocytosis of VTG in fish follicle. *In* Proceedings of Fourth Int. Symp. Reprod. Physiol. Fish. A. P. Scott, J. P. Sumpter, D. E. Kime, and M. S. Rolfe, editors. Fish Symposium 91, Sheffield, U.K. 300–302.
8. Lancaster, P., and C. R. Tyler. 1994. Developmental expression and modulation of the vitellogenin receptor in ovarian follicles of the rainbow trout. *Oncorhynchus mykiss. J. Exp. Zool.* **269:** 458–466.
9. Harel, M., A. Tandler, G. W. Kissil, and S. W. Applebaum. 1995. The role of broodstock dietary protein in vitellogenin synthesis and oocyte development, and its effects on reproductive performance and egg quality in gilthead seabream *Sparus auratus*. *In* Proceedings of Fifth Int. Symp. Reprod. Physiol. Fish. F. W. Goetz and P. Thomas, editors. Fish Symposium Austin 95, Austin, TX. 105–107.
10. Nunez Rodriguez, J., E. Bon, and F. Le Menn. 1996. Vitellogenin receptors during vitellogenesis in the rainbow trout *Oncorhynchus mykiss. J. Exp. Zool.* **274:** 163–170.
11. Mananos, E., J. Nunez Rodriguez, F. Le Menn, S. Zanuy, and M. Carillo. 1997. Identification of vitellogenin receptors in the ovary of a teleost fish, the Mediterranean sea bass (*Dicentrarchus labrax*). *Reprod. Nutr. Dev.* **37:** 51–61.
12. Stifani, S., D. L. Barber, J. Nimpf, and W. J. Schneider. 1990. A single chicken oocyte plasma membrane protein mediates uptake of very low density lipoprotein and vitellogenin. *Proc. Natl. Acad. Sci. USA.* **87:** 1955–1959.
13. Barber, D. L., E. J. Sanders, R. Aebersold, and W. J. Schneider. 1991. The receptor for yolk lipoprotein deposition in the chicken oocyte. *J. Biol. Chem.* **266:** 18761–18770.
14. Bujo, H., M. Hermann, M.O. Kaderli, L. Jacobsen, S. Sugawara, J. Nimpf, T. Yamamoto, and W. J. Schneider. 1994. Chicken oocyte growth is mediated by an eight ligand binding repeat member of the LDL receptor family. *EMBO J.* **13:** 5165–5175.
15. Takahashi, S., S. Kawarabayasi, T. Nakai, J. Sakai, and T. Yamamoto. 1992. Rabbit low density lipoprotein receptor-like protein with distinct ligand specificity. *Proc. Natl. Acad. Sci. USA.* **89:** 9252–9256.
16. Sakai, J., A. Hashino, S. Takahashi, Y. Miura, H. Ishii, H. Suzuki, Y. Kawarabayasi, and T. Yamamoto. 1994. Structure, chromosome location and expression of the human very low density lipoprotein receptor gene. *J. Biol. Chem.* **269:** 2173–2182.
17. Davis, C. G., J. L. Goldstein, T. C. Sudhof, R. G. W. Anderson, D. W. Russel, and M. S. Brown. 1987. Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region. *Nature.* **326:** 760–765.
18. Chen, W. J., J. L. Goldstein, and M. S. Brown. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* **265:** 3116–3123.
19. Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* **107:** 303–314.
20. Bon, E., and F. Le Menn. 1994. Effect on vitellogenesis of shortening the reproductive cycle of rainbow trout. *In* Proceedings of the 17th Conference of European Comp. Endocrinol., Cordoba, Spain.
21. Gubler, U., and B. J. Hoffman. 1983. A simple and very efficient method for generating cDNA library. *Gene.* **25:** 263–269.
22. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* **77:** 5201–5205.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
24. Pakdel, F., C. Leguellec, C. Vaillant, M. Le Roux, and Y. Valotaire.

1989. Identification and estrogen induction of two estrogen receptors (ER) messenger ribonucleic acids in the rainbow trout liver: sequence homology with other ERs. *Mol. Endocrinol.* **3:** 44–51.
25. Le Moine, C., and Bloch, B. 1996. Expression of the D_3 dopamine receptor in peptidergic neurons of the nucleus accumbens: comparison with the D_1 and D_2 dopamine receptors. *Neuroscience.* **73:** 131–143.
26. Von Heijne, G. V. 1983. Patterns of amino acids near signal-sequence cleavage sites. *Eur. J. Biochem.* **133:** 17–21.
27. Bujo, H., K. A. Lindstedt, M. Hermann, L. M. Dalmau, J. Nimpf, and W. J. Schneider. 1995. Chicken oocytes and somatic cells express different splice variants of a multifunctional receptor. *J. Biol. Chem.* **270:** 23546–23551.
28. Bon, E., U. Barbe, J. Nunez Rodriguez, B. Cuisset, C. Pelissero, J. P. Sumpter, and F. Le Menn. 1997. Plasma vitellogenin levels during the annual reproductive cycle of female rainbow trout (*Oncorhynchus mykiss*): establishment and validation of an ELISA. *Comp. Biochem. Physiol.* **117 B:** 75–84.
29. Webb, J. C., D. D. Patel, M. D. Jones, B. L. Knight, and A. K. Soutar. 1994. Characterization and tissue-specific expression of the human "VLDL receptor" mRNA. *Hum. Mol. Genet.* **3:** 531–537.
30. Tyler, C. R., and K. Lubberink. 1996. Identification of four ovarian receptor proteins that bind vitellogenin but not other homologous plasma lipoproteins in the rainbow trout, *Oncorhynchus mykiss*. *J. Comp. Physiol. B.* **166:** 11–20.
31. George, R., D. L. Barber, and W. J. Schneider. 1987. Characterization of the chicken oocyte receptor for low and very low density lipoproteins. *J. Biol. Chem.* **262:** 16838–16847.
32. Jokinen, E. V., K. T. Landschulz, K. L. Wyne, Y. K. Ho, P. K. Frykman, and H. H. Hobbs. 1994. Regulation of the very low density lipoprotein receptor by thyroid hormone in rat skeletal muscle. *J. Biol. Chem.* **265:** 26411–26418.
33. Jingami, H. and T. Yamamoto. 1995. The VLDL receptor: wayward brother of the LDL receptor. *Curr. Opin. Lipidol.* **6:** 104–108.
34. Elkin, R. G., I. MacLachlan, M. Hermann, and W. J. Schneider. 1995. Characterization of the Japanese quail oocyte receptor for very low density lipoprotein and vitellogenin. *J. Nutr.* **125:** 1258–1266.
35. Okabayashi, K., H. Shoji, T. Nakamura, O. Hashimoto, M. Asashima, and H. Sugino. 1996. cDNA cloning and expression of the *Xenopus laevis* vitellogenin receptor. *Biochem. Biophys. Res. Commun.* **224:** 406–413.
36. Bujo, H., T. Yamamoto, K. Hayashi, M. Hermann, J. Nimpf, and W. J. Schneider. 1995. Mutant oocytic low density lipoprotein receptor gene family member causes atherosclerosis and female sterility. *Proc. Natl. Acad. Sci. USA.* **92:** 9905–9909.
37. Sumpter, J. P., and S. Jobling. 1995. Vitellogenesis as a biomaker for estrogenic contamination of the aquatic environment. *Environ Health Perspect.* **103** (Suppl 7): 173–178.
38. Sumpter, J. P. 1995. Feminized responses in fish to environmental estrogens.*Toxicol Lett.* **82–83:** 737–742.