Identification and Estrogen Induction of Two Estrogen Receptors (ER) Messenger Ribonucleic Acids in the Rainbow Trout Liver: Sequence Homology with other ERs

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The estrogen-binding region of the cDNA for chicken ER reveals a mRNA of 3.5 kilobases (kb) in rainbow trout liver. The level of this messenger, which is very low in the liver of naive male animals, can be increased by estrogen stimulation. With this chicken probe, we have isolated a clone from a λ gt_{10} trout liver cDNA library. The partial cDNA sequence, which encompasses most of the coding region, shows two domains of striking amino acid homology with human, avian, and Xenopus estrogen receptors (ERs) (DNA binding region: 90%, Hormone binding region: 60%). With this specific probe rainbow trout ER, we detected another messenger (4.5 kb) that is less expressed than the 3.5 kb messenger. The kinetics of stimulation of the two messengers is compared with the kinetics of accumulation of vitellogenin mRNA after E_2 administration. This report constitutes the first identification of ER mRNA from a fish. (Molecular Endocrinology 3: 44–51, 1989)

INTRODUCTION

In the teleost liver, as in the liver of other oviparous fishes, egg-yolk protein synthesis is under the control of estrogen. It is assumed that estrogen acts through an hepatic estrogen receptor (ER). In avians (1, 2), amphibians (3, 4), reptiles (5), and fishes (6, 7) hepatic ERs have been characterized to varying degrees and shown to be necessary for the induction of vitellogenin. The ER has been purified from several species—human, rat, chicken, and Xenopus—and the respective cDNAs have been cloned (8–12). The ER has been divided into six domains labeled A–F (10). The putative DNA-binding domain (region C) is characterized by its high cystein and basic amino acid content. This region is 100% homologous in chicken, man, and rat, and it shows also a high homology with the human glucocorticoid receptor and the product of the v-erbA gene (9, 10, 13, 14). The gene products probably belong to a large family of potentially oncogenic hormone receptors (15). The aim of our study is to characterize the gene of the trout ER. Fishes are the first vertebrates to evolve, and the comparison of sequence homologies of rainbow trout ER (rtER) with other species could yield interesting results: the evolutionary distance between the fishes and other species is important; furthermore, the rainbow trout lives in a cold environment (5–16 C) and is not temperature regulated. Comparison of the ER sequence of several species shows that the hormone-binding domain is very highly conserved (90% homology), whereas this domain appears more variable (15–30% homology) in the other gene products of the family. Therefore, a cDNA probe corresponding to this part of the ER sequence is probably the most specific for screening a heterologous cDNA library. We describe here the cloning and partial sequencing of the rtER cDNA and the estrogen induction of the corresponding mRNA.

0888-8809/89/0044-0051$02.00/0
Molecular Endocrinology

RESULTS

Identification of the Putative Estrogen mRNA in Rainbow Trout Liver and Correlation of Expression with Vitellogenin (Vg) mRNA

Northern analysis of trout liver and hen oviduct poly(A^+) RNA with the chicken probe, in Thomas's standard conditions (Fig. 1A), shows that only the hen oviduct RNA displays a band at 7.5 kilobases (kb). When the

stringency of the hybridization medium was lowered (Fig. 1B: 30% formamide), autoradiographic signals from several trout RNAs are obtained. However, there is no absolute means of identifying those corresponding to the ER mRNA. Several different hybridization conditions were tested, and the best results were obtained at 65 C in the absence of formamide (see *Materials and Methods*). Under these conditions (Fig. 2A), a unique band of approximately 3.5 kb in trout liver RNA is observed, whereas the specific band in hen oviduct RNA is still seen at 7.5 kb (Fig. 2A, lane 1). The level of the trout messenger is very low in the male unstimulated animal but increases with estrogen stimulation (Fig. 2A, lane 4). In vitellogenic females, the level is quite identical to that of the stimulated male (compare lanes 4 with 5 and 6 on Fig. 2A). Hybridization of the same filter with the Vg probe shows that Vg mRNA is present only in the females and in treated male fish (Fig. 2B) in which the level of the 3.5 kb mRNA is high. These preliminary studies suggest that the 3.5 kb messenger may be the putative ER mRNA in trout liver.

Identification of the rtER cDNA Clone and Sequence Homology with Other ERs

Since rtER protein is present at only a few hundred molecules per cell in control male rainbow trout liver cells, we prepared a λ gt_{10} library from vitellogenic females, in which the ER concentration per cell is about 8 to 10 times higher (6). Five × 10^5 recombinants were obtained and amplified in *Escherichia coli* hf1 (BNN91 strain). This cDNA library was screened with the estrogen-binding region (700 nucleotides) of the chicken ER cDNA. Four × 10^4 recombinants were analyzed first and several positive clones were detected. One of them (λ rtER), which hybridized with the 3.5-kb mRNA previously observed, was chosen for further analysis. The insert was found to be 2700 base pairs (bp) in length, and its 5′-end showed a very much stronger hybridization with the chicken probe than the 3′-end. The insert was subcloned in Bluescript plasmid rtER (PrtER) and, after mapping (data not shown), the 5′-end of the cDNA was inserted in M_{13} mp_{18} and mp_{19} vectors for sequencing. The 5′-end of the insert contains an open reading frame. Figure 3 shows the 1278 nucleotides of this 5′-end coding for 425 amino acids.

A polyadenylation signal and a poly(A) tail (result not shown) are present at the 3′-end of the 2.7 kb fragment. Therefore, the 3′-noncoding sequence of this rtER messenger is only 1400 nucleotides. Amino acid sequence comparison (with a LGBC Macintosh program) of the PrtER (Fig. 4) with other ER sequences: human ER (hER) (8) chicken ER (cER) (10), and *Xenopus ER* (xER) (12) shows a high homology in the DNA and estrogen-binding regions. A window of four residues was used and a dot plotted for a match of four or more identical residues. The program therefore generates a diagonal line where any homology between the two sequences is found. Figure 5 shows that the homologies found between rainbow trout and the other species are similar to those found between hER and cER. According to the nomenclature of Krust *et al.* (10) the cDNA we have isolated contains only the C, D, E, and F regions of the trout ER. The comparative primary structures of the four ERs are represented with dashes (–). This structure shows that the sequence of clone PrtER starts from the ninth amino acid of the C region, which is the most conserved domain (>90%) of the trout receptor. Of the

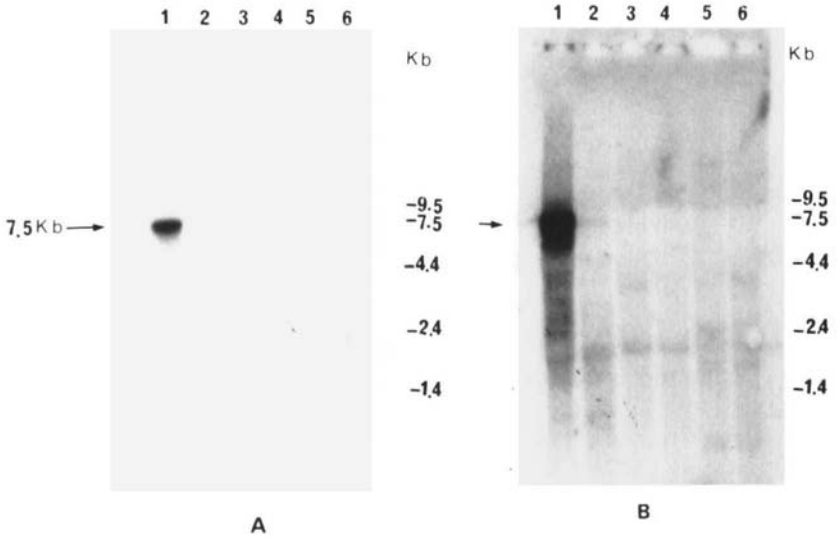

Fig. 1. Northern Blot Analysis

Poly(A^+) RNA were prepared from trout liver, chicken liver, and oviduct as described in *Materials and Methods*. Ten micrograms of poly(A^+) RNA from chicken oviduct and liver (respectively, lanes 1, 2) and 10 μg poly(A^+) RNA from trout liver of female, 24-h E_2-stimulated female, naive male, and 24-h E_2-stimulated male (respectively; lanes 3, 4, 5, 6) were separated on a denaturing glyoxal agarose gel, transferred to a nylon membrane, and hybridized with the chicken probe in Thomas's standard conditions (A), or in 30% formamide (B). The size of cER mRNA is estimated with the RNA ladder from BRL (Gaithersburg, MD).

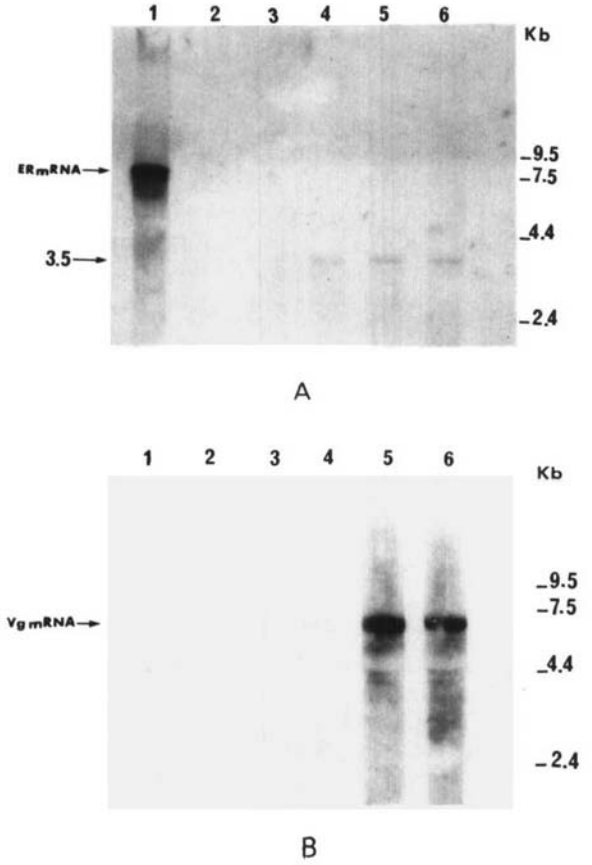

Fig. 2. Characterization of Putative mRNA rtER and Correlation with Vg mRNA in Liver

Lane 1, Two micrograms of poly(A^+) RNA from chicken oviduct, or 20 μg poly(A^+) RNA from trout liver, (lanes 2, 3) two different naive males, (lane 4) 24-h E_2-stimulated male, (lanes 5, 6) two different females were separated on a denaturing glyoxal agarose gel, transferred to a nylon membrane, and hybridized with the chicken probe at 65 C (A) and autoradiographied for 11 days at −70 C, or hybridized with Vg probe in Thomas's condition (B) and autoradiographied about 20 min.

74 amino acids in this region, 69 and 68 are identical with those of xER and cER DNA binding regions, respectively. As in the other species, we found a high number of cysteins (eight residues) and basic amino acids (Arg, lys). All these residues are remarkably conserved except the lysine residue number 226 of xER and cER or 231 of hER, which is replaced by an arginine residue in the rainbow trout.

Another very well conserved region in the other species is the hormone-binding domain, which is less conserved in rainbow trout (rtER/xER, 58%; rtER/cER, 62%; rtER/hER, 60%). Nevertheless, this very long region (>250 amino acids) shows two areas (from amino acid 329–450; and from amino acid 486–546) where the homology is higher (71% and 83%, respectively). For the two other regions (D and F), the homology between species is less important—especially in the rainbow trout, in which the comparison gives a homology between 14% and 22%. Figure 6 gives the hydropathic index of the C, D, E, and F domains in the different species. The profiles indicate that there is also a good conservation of the protein structure, with a highly hydrophilic C region and a relatively hydrophobic E region.

Identification and Estradiol (E_2) Stimulation of Two ER Messengers in the Liver of Rainbow Trout

Using the PrtER cDNA insert, we analyzed the characteristics of the corresponding trout mRNAs. Male rainbow trout were injected with E_2 (500 μg/kg BW). The livers were removed and frozen in liquid nitrogen according to the time course of Fig. 7A. Poly(A^+) RNA was prepared from 500 μg total RNA and submitted to Northern analysis (16). Figure 7A shows that the 3.5-kb mRNA, which was previously detected with the chicken probe, is induced by estrogen. In addition, a second messenger (4.5 kb) can also be detected with this probe. The level of this 4.5 kb messenger is lower than that of the 3.5 kb, but their inductions follow parallel time courses. After E_2 administration, the level of both messengers increases for 12 h. The beginning of induction is observed after 1–2 h. By comparison, (Fig. 7B), the induction of Vg mRNA starts only 5–6 h after hormone injection.

DISCUSSION

The preliminary results of hybridization of the chicken probe with trout liver poly(A^+) RNA under high stringency conditions shows no specific mRNA; whereas, under the same conditions, chicken oviduct and liver poly(A^+) RNA display a 7.5-kb specific band. This result shows that homology between cER and trout ER mRNA is probably less conserved than in other studied species: cross-hybridization between human and chicken was previously observed in the same condition (13). In contrast, low-stringency hybridization results in the appearance of additional bands in trout liver by Northern analysis but there was no absolute means of identifying a specific ER mRNA. Under intermediate conditions of stringency, we succeeded in showing a putative ER mRNA in trout liver whose physiological characteristics are in agreement with the properties of ERs (low level in the naive male, induction with E_2). We have isolated and partially sequenced a cDNA clone, corresponding to the rtER mRNA, that represents the first steroid hormone receptor to be sequenced from a fish. The comparison between the sequences of the rtER and the ER of three other species shows that the homology is not conserved in an homogeneous way all along the molecules, but that two domains are highly conserved (region C and E corresponding to DNA and hormone binding, respectively). These two functional domains, which are absolutely necessary for full acti-

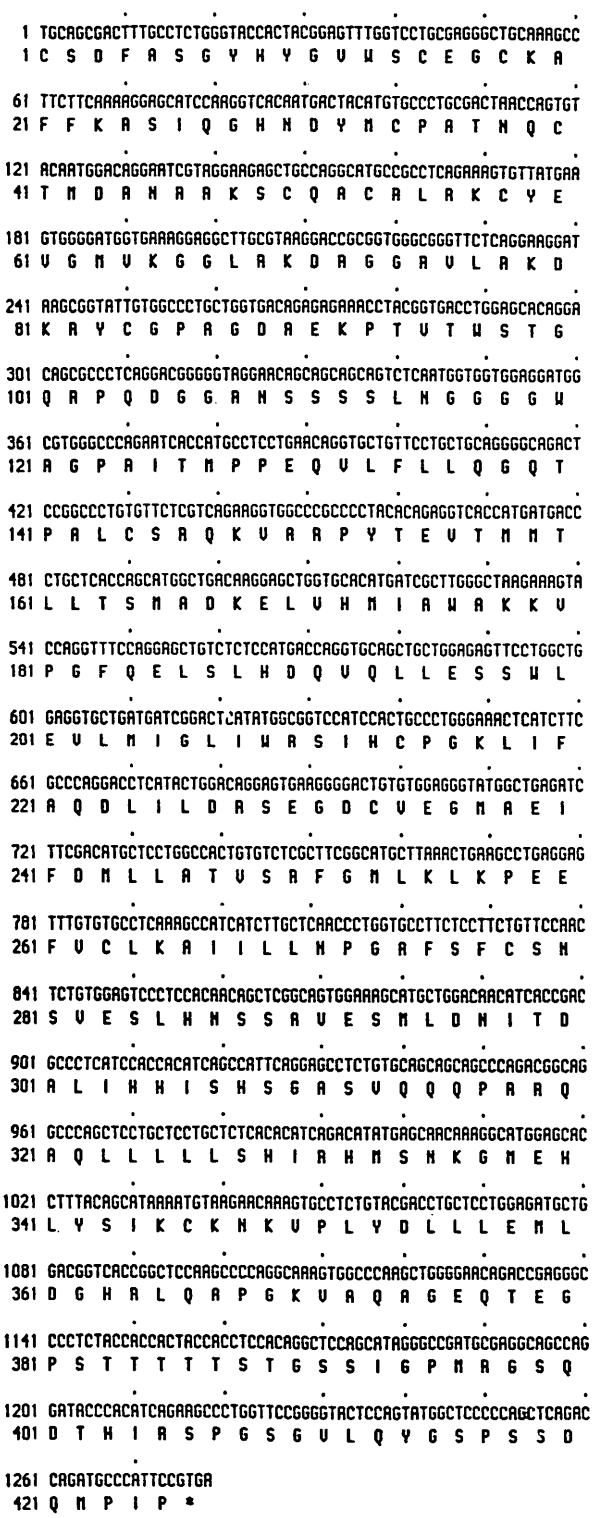

Fig. 3. Nucleotide Sequence and Predicted Amino Acid Sequence of the 5′-PrtER Insert

The *numbers on the left* designate the position of the nucleotides and amino acids starting with an open reading frame.

vation of transcription and which have been very well characterized by mutational analyses and transient expression (17), are present in rtER. The DNA binding domain is the most highly conserved domain in other species (>98%) and in rainbow trout (92%). This region shows a distribution of cysteine and basic amino acid residues identical to that of other species and also contains the consensus sequence established by Weiler *et al.* (12) for the DNA binding regions of hormone receptors. These characteristics are related to the zinc finger structure proposed for 5S gene transcription factor TF HI A (11, 18). Last, the results of hydropathicity plots show that this domain is highly hydrophilic, as are all the DNA binding domains of the steroid receptors.

The hormone binding domain (region E) is less well conserved in rainbow trout, (60%) although this domain does contain two smaller areas with greater homology (70% and 80%). The hydropathicity plots show that this domain is relatively hydrophobic, as in other species.

We have previously shown that the relative affinity of diethylstilbestrol for ER in trout liver is about five times lower than E_2 itself (7), whereas DES is considered to be the strongest competitor for ER in other species. We have also shown that diethylstilbestrol gave only 65% of Vg mRNA induction in primary trout hepatocytes culture when compared to E_2 (19). These results are in agreement with the differences observed in the structure of the hormone-binding domain of the rainbow trout. The variation of homology may be related to the fact that rainbow trout lives in cold water, which could change the dissociation constant of the hormone-protein complex. In this respect it will be interesting to investigate whether the sequence of hormone-binding domain is different in tropical fishes.

The most important difference between rtER and that of other species is perhaps the size of the mRNA. In addition, two inducible mRNAs were detected in the liver of rainbow trout. The kinetics of stimulation with estrogen shows that the time course of induction is very similar for the two mRNAs; this suggests that they are both ER mRNAs. We also observe a delay of several hours between the kinetics of the rtER mRNAs and the Vg mRNA. This result is in agreement with previous work (20), in which the authors show that the time course of the appearance of xER protein precedes the kinetics of accumulation of Vg mRNA.

It is possible that the two rtER mRNAs are transcribed from the same gene and that the size difference is due to polyadenylation signals. But there is also the possibility that these two messengers are transcribed from different genes and the ratio of the expression from the two genes is under the control of additional factors. We have investigated the presence of ER messenger in five different species of salmon (unpublished results); in all these species the two messengers are present, but their proportion varies from one species to another. It will be also interesting to analyze whether the expression of the two messengers varies during rainbow trout development.

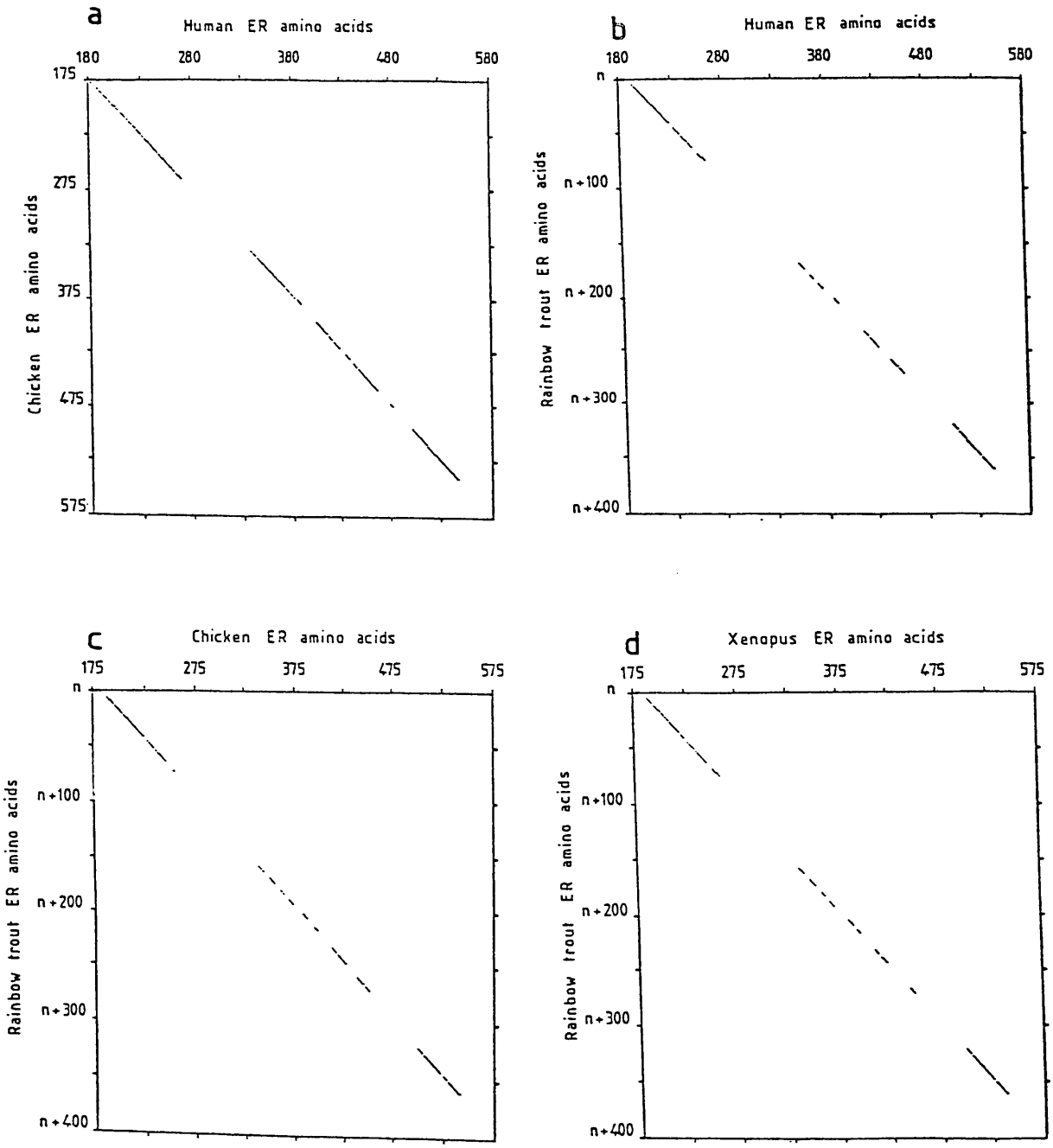

Fig. 4. Comparison of the Amino Acid Sequences of rtER Clone with C, D, E, F Regions of hER, cER, and xER

The amino acid sequences were compared by a matrix analysis that used a window of four residues and a dot plotted for a match of four or more identical residues. The program (LGBC Macintosh) therefore generates a diagonal line where any homology between the two sequences is found. a, Comparison of the amino acid sequences between cER and hER. b, Comparison of the amino acid sequences between rtER and hER. c, Comparison of the amino acid sequences between rtER and cER. d, Comparison of the amino acid sequences between rtER and xER.

Therefore, the difference in the size of the mRNA for rtER compared with that of other species is only a difference in the noncoding region. In other species, the size of the 3′-noncoding region is more than 4000 nucleotides, whereas in rainbow trout this sequence is only 1400 nucleotides for the 3.5 kb messenger, which is the most abundant. This difference is sufficient, therefore, to explain the smaller size of the rtER mRNA. The comparison of the sequences in the 3′-noncoding region shows that there is no significative homology (data not shown); this implies, therefore, that this portion of the gene was not subjected to evolutionary pressure with regard to the function of the receptor. On the other hand, this sequence is perhaps very important for

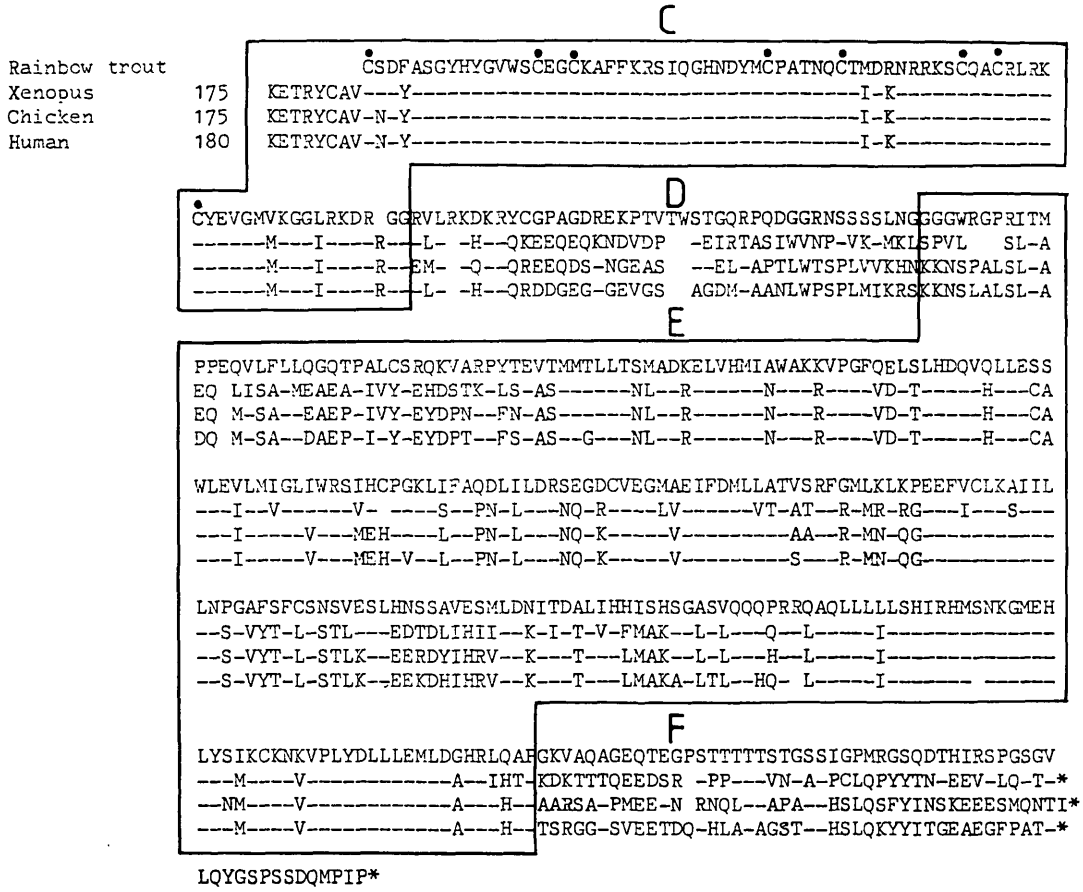

Fig. 5. Alignment of the Amino Acid Sequences of the C, D, E and F Domains of the rtER, xER, cER, and hER

The PrtER sequence was positioned to yield the maximum homology between those sequences. The identical amino acids between PrtER clone and these ER are shown by *dashes* (–). In region C the cysteine residues are indicated by *black spots* (●).

mRNA translation and stability of the messenger, as suggested by recent work (21).

MATERIALS AND METHODS

Animals and Treatments

Male and female rainbow trout (*Salmo gairdnerii*) weighing 250 g were supplied by a trout farm (Gournay-Sur-Aronde, Oise, France), and kept in recycled water (22). The hens were supplied by a local farm (Les Clariaux, Amanlis, Ille-et-Vilaine, France). Male rainbow trout were stimulated using a saline (NaCl, 0.9%) injection of E_2 (0.5 mg/kg BW).

Poly(A$^+$) RNA Preparation and Northern Analysis

Trout livers and hen oviducts were removed and immediately frozen in liquid nitrogen. They were stored at −80 C until use. Total RNA was extracted with a modification of the Auffray and Rougeon technique (23). The poly(A$^+$) RNA were obtained after chromatography of the total RNA on oligo(dT)Tris acryl M (I.B.F.) (24) and were subjected to 1% agarose (type I low EEO, Sigma Chemical Co, St. Louis, MO) gel electrophoresis in denaturing conditions (16, 25). The RNA was transferred to nylon membrane (Pall Biodyne) essentially as described by Thomas (26).

Labeling of the cDNA Probes

Chicken Probe The 2.1 kb chicken oviduct cDNA clone containing the complete open reading frame of ER (10) was kindly provided by Chambon [Laboratoire de Genetique Moleculaire des eucaryotes du C.N.R.S., Strasbourg, France]. We subcloned a 0.7 kb *Hin*dIII fragment containing estrogen binding domain in the SpL vector, which was previously dephosphorylated. The *insert* was labeled by nick translation (Amersham Kit N 5000) using α^{32}P dCTP 800 Ci/mmol (Amersham, UK).

Rainbow Trout Probe The 2.7 rtER cDNA insert was labeled with the random primer labelling kit (Boehringer).

Vg Probe The plasmid PSG Vg 509 (27), containing a 2.2 kb fragment of cDNA from the terminal 3′-end rainbow trout Vg mRNA, was labeled by nick translation.

Hybridizations

According to the text, Thomas's standard conditions (26) were also used to hybridize with the cER, rtER, and Vg probes.

Specific hybridization conditions were used to show the putative rtER mRNA and to screen the λ gt_{10} library with the cER probe. The filters were prehybridized (6× SSC, 1× Denhardt's solution, 10 mM NaH_2PO_4 pH 7, 0.1% sodium dodecyl sulfate (SDS), heat denatured yeast tRNA, 50 μg/ml) for 6 h at 65 C and hybridized (6× SSC, 5× Denhardt's solution, 10 mM NaH_2PO_4, pH 7, 0.1% SDS, heat-denatured yeast tRNA 50 μg/ml) containing nick translated and heat-denatured probe

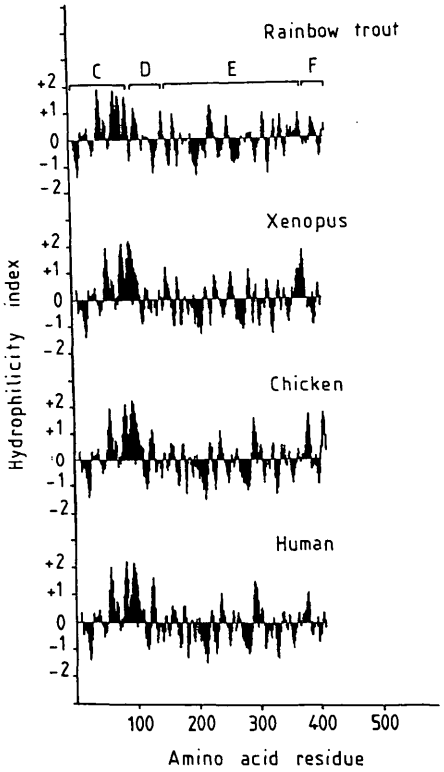

Fig. 6. Hydropathic Profiles of the C, D, E and F Regions of Four ER Protein

The profiles for rtER, xER, cER, and hER were displayed by computer using the algorithm of Kyte and Doolittle (31) with a window of seven amino acids; positive and negative values on the ordinate indicate hydrophilic and hydrophobic degrees, respectively. The four regions (C–F) are indicated at the *top of the panel*.

for 20 h at 65 C. After washing at 68 C four times, 30 min each, in 6× SSC, 10 mM NaH_2PO_4, pH 7, 0.1% SDS followed by two times, 30 min each at 68 C in 1× SSC, 0.1 M NaH_2PO_4, pH 8.4, 0.5% SDS, the membranes were autoradiographied at −70 C.

Preparation and Screening of Trout Liver cDNA Library

One microgram of mRNA poly(A^+) from a female trout liver was used for double stranded ds cDNA synthesis. The ds cDNA was synthesized by the method of Gubler and Hoffman (28) with Amersham's cDNA synthesis system.

*Eco*RI sites in cDNA were methylated by *Eco*RI methylase (Biolabs, Beverly, MA) and the cDNA was ligated to the *Eco*RI linker (Amersham), then digested with *Eco*RI, and fractionated by gel filtration on a sepharose 4B (Pharmacia, Piscataway, NJ), column. The largest fractions were pooled, ligated to λ gt_{10} vector (29) at a molar ratio of 1:1 (cDNA :λ phage DNA), and packaged *in vitro* (Promega Biotec's packaging system, Madison, WI).

The phages were plated onto *E. coli* hfl (BNN9 strain); 5×10^5 recombinants were obtained and amplified. Four × 10^4 recombinants were plated onto *E. coli* hfl, with 2000–3000 phages/8.5-cm-diameter Petri dish. Duplicate filters (Hybond-N, Amersham) were screened with the chicken probe (10^6 cpm/filter) in the hybridization conditions described above.

Several positive clones were detected, identified, and characterized by hybridization with Northern blot of mRNA poly(A^+) from trout liver.

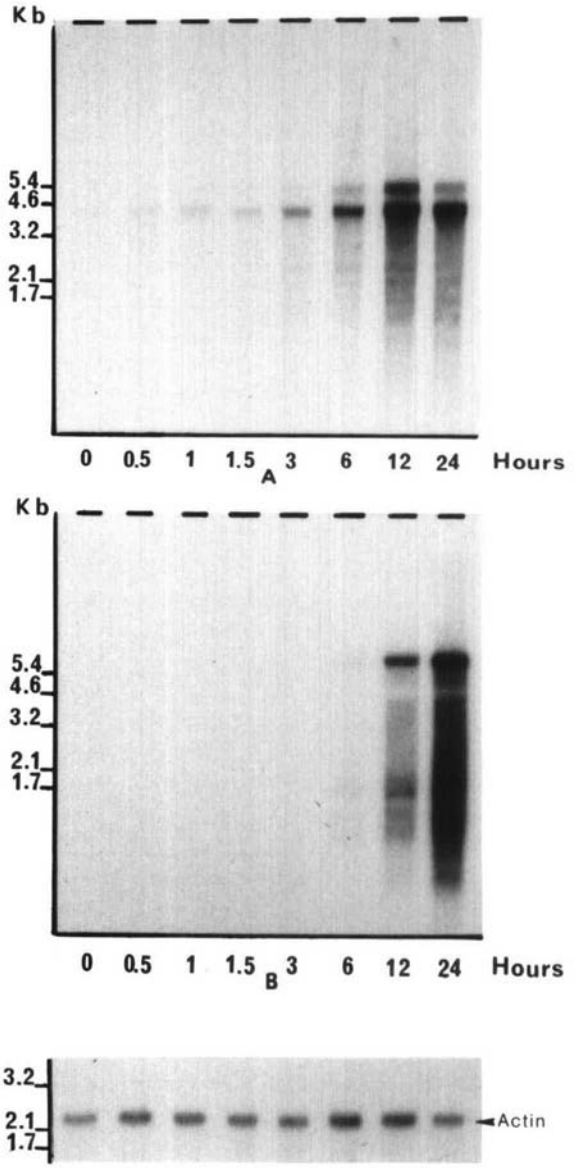

Fig. 7. Estrogen Regulation of rtER mRNAs Level and Correlation with Vg mRNA in Trout Liver

Male rainbow trouts (250 g each) were injected by E_2 (500 μg/kg BW) at time 0 h. At the indicated times (0.5, 1, 1.5, 3, 6, 12, 24 h) after injection, the animals were killed and the livers were removed, poly(A^+) RNA was prepared from 500 μg total RNA and separated on a denaturing formaldehyde agarose gel, transferred to a nylon membrane, and hybridized in Thomas's condition with rtER probe (A) and autoradiographied about 1 day, or with Vg probe (B) and autoradiographied about 4 h. Hybridization of the membrane with actin probe reveal equivalent amount of poly(A^+) RNA in each lane.

Sequencing

One clone (λ rtER) containing a 2.7-kb insert hybridized with a 3.5-kb messenger in Northern blot. The insert of λ rtER clone was subcloned into the *Eco*RI site of Bluescript (Genofit) plasmid (PrtER) and mapped. The 5′-regions were subcloned into the $M_{13}mp_{18}$ and $M_{13}mp_{19}$ cloning vectors and sequenced by the dideoxy sequencing method (30) on both strands of the DNA with Sequenase Kit (USB).

Acknowledgments

We are extremely grateful to Prof. P. Chambon and Prof. J. P. Le Pennec for the gift of the avian ER clone and to Dr. B. Osborne for reviewing the manuscript. The authors thank Mme. Becouarn for typing the manuscript.

Received August 2, 1988. Accepted October 2, 1988.

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This work was supported by the Groupement de Cooperation Scientifique Bases Biologiques de l'Aquaculture.

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