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In vivo estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout

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Summary

We have previously described the cloning, sequencing and in vitro expression of a full-length rainbow trout estrogen receptor cDNA (rtER cDNA). This full cDNA randomly labelled was used to study the estrogen induction of hepatic rtER mRNA in correlation with vitellogenin (Vg) mRNA in different physiological situations. In this paper, we show that in the liver two mRNA species are under hormonal control and their level increases about 8-fold after estrogen stimulation. These two mRNAs are expressed and induced in the liver as early as the hatching stage in correlation with the expression of Vg mRNA. A long-term analysis of rtER mRNA after estradiol (E₂) injection shows a transient induction of the nuclear ER and its mRNA which recover to the basal level after 2 weeks. Nevertheless, a memory effect was observed on the expression of the Vg gene which does not appear to be directly related to the estrogen receptor level.

Introduction

The effect of steroids on their target tissues is mediated by hormone-specific receptor proteins, which, after associated with the steroid, acquire increased affinity for chromosomal binding sites. The hormone-receptor complex binds to specific sequences called hormone-responsive elements (HRE) which are generally localized upstream

from the hormone-sensitive genes. These receptor/genome interactions are able to selectively stimulate the transcription rate of specific genes. Steroid hormone receptors currently represent some of the best understood models for nuclear receptor proteins containing metal binding fingers (for reviews see Anderson, 1984; Yamamoto, 1985; Gorski et al., 1986; Green and Chambon, 1988). The identification of DNA sequences interacting with hormone receptors, cloning of receptors and transient expression of the receptor cDNA have increased our knowledge of the mechanism of action of the steroid receptors. However, the control of expression of the hormone receptor itself has received much less attention (Yamamoto,

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1985; Green and Chambon, 1986; Riegel et al., 1987; Barton and Shapiro, 1988).

In rainbow trout, as in other salmonids, there is no oviduct, and the liver is the main target organ for estrogens. In this organ, we have previously shown that vitellogenin gene expression is under estrogen control (Maitre et al., 1985; Le Guellec et al., 1988; Vaillant et al., 1988).

In *Xenopus* and chicken, the characteristics of vitellogenin (Vg) induction by estrogens are different if animals have been previously treated with estrogen. Secondary induction is defined by the disappearance of the lag period and the increase in the accumulation rate of Vg mRNA revealing that liver cells retain a 'memory' of the primary stimulation (Tata and Smith, 1979; Shapiro, 1982; Evans et al., 1987). This memory effect persists for as long as 6 months and is passively maintained through cell division (Burch and Evans, 1986). Different hypotheses have been proposed to explain this phenomenon, for example either the maintaining of a high level of estrogen receptors (Westley and Knowland, 1979; Tata et al., 1987) or the presence of an estrogen-binding protein (Hayward et al., 1982), but persistent modification of the structure of the vitellogenin gene has also been suggested (Tata and Baker, 1975; Gerber et al., 1981; Burch and Weintraub, 1983).

We have previously described the cloning, sequencing and in vitro expression of a full-length random trout estrogen receptor cDNA (rtER cDNA) (Pakdel et al., 1990). This full cDNA randomly labelled was used to study the estrogen induction of hepatic rtER mRNA in correlation with vitellogenin mRNA in different physiological situations.

Materials and methods

Animals and treatments

Male and female rainbow trout (*Oncorhynchus mykiss*) were supplied by a trout farm (Gournay-sur-Arond, Oise, France). Male trout were stimulated using a saline (NaCl 9‰) injection of estradiol (E_2) (0.5–1.5 mg/kg body weight). After hatching, the alvines were stimulated by the addition of E_2 to the water (1.5 μ M), left for 2 h and then transferred to E_2 -free water. Their livers were collected 24 h later.

RNA preparation

Total RNA was extracted using a modification of Auffray and Rougeon's technique (1980). Poly(A⁺) mRNA was obtained after affinity chromatography of the total RNA on oligo(dt) Trisacryl M (Aviv and Leder, 1972).

Slot blot analysis

Five to 10 μ g of total RNA was applied to a nylon membrane as described by Cheley and Anderson (1984). The amounts of rtER and Vg mRNA were obtained by comparison of slots with various amounts of M_{13} mp₁₉ containing rtER or Vg cDNA fragments.

Northern blot analysis

Poly(A⁺) RNA was subjected to 1% agarose gel electrophoresis in formaldehyde denaturing conditions, then transferred to a nylon membrane and hybridized in Thomas's conditions (1980).

Labelling of cDNA probe

The rtER cDNA insert (Pakdel et al., 1989) and the Vg cDNA insert (Le Guellec et al., 1988) were labelled with the random primer labelling kit (Amersham), in the presence of [α -³²P]dCTP (~800 Ci/mmol).

Nuclear ER assay

Nuclear extract preparation. The livers were homogenized in Tris-sucrose buffer (2.5 ml/g) (20 mM Tris-HCl pH 7.9, 5 mM CaCl₂, 3 mM MgCl₂, 330 mM sucrose, 10 mM monothioglycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF) using a polytron homogenizer (5 s) then a Potter homogenizer (1000 rpm, 20 plunges). After centrifugation (15 min, 1000 $\times$ g, 4°C), the pellet was washed once with homogenization buffer containing 0.5% Triton and twice without Triton, then nuclei were extracted with 10 mM Tris-HCl pH 7.5, 1.5 mM EDTA, 500 mM KCl, 10 mM monothioglycerol (1 ml/g of initial liver) and centrifuged (30 min, 90,000 $\times$ g, 4°C). Supernatants were used for nuclear ER determination.

Estradiol binding. Preliminary binding kinetics had shown that more than 90% of the specific binding was obtained after 12 h of incubation and that desteroidization was not necessary for the nuclear extracts. 0.2 ml of each nuclear extract

was incubated with 3 nM [^3H]E $_2$ (99 Ci/mmol) without (total binding) or with (non-specific binding) 300 nM E $_2$. After 12 h incubation at 4°C, free and bound hormones were separated by two successive dextran charcoal (0.1–1%) treatments (5 min at 0°C, centrifugation 5 min at 10,000 $\times$ g). Supernatant radioactivity was determined in 4 ml of Picofluor 30 and specific binding was calculated as the difference between total and non-specific binding.

Estradiol level determination. E $_2$ levels were determined using a radioimmunoassay (RIA) according to Terqui et al. (1973).

Results

In vivo estradiol induction of rtER mRNAs

A group of male rainbow trout, each weighing 250–350 g, was stimulated by a single intraperitoneal injection of 17 β -estradiol (0.5 mg/kg), a control group received the solvent only. The animals were sacrificed 24 h after treatment. 5–10 μg of liver total RNA or poly(A $^+$) RNA was quantified by slot or Northern blot analysis with rtER probe. The rtER mRNA level increases about 8-fold 24 h

after estrogen stimulation (Fig. 1A). In addition, Fig. 1B reveals that the two species (3.5 and 4.5 kb) of rtER mRNA are under hormonal control.

Detection and estradiol induction of rtER and Vg mRNA after hatching

These experiments were performed in order to study ontogenesis of rtER mRNA and sensitivity of rtER and Vg genes to estradiol. After hatching (fry weighing only 50–150 mg), estrogen induction was performed as indicated in Materials and Methods by E $_2$ addition to the water. 50–100 livers, according to age, were pooled for RNA preparation. RtER and Vg mRNAs were quantified by slot blot analysis of 5 μg of total RNA. As soon as the fish hatch, they have a basal level of ER mRNA in the liver comparable to that of either adult males or immature females (Fig. 2). This basal level increases during the first 2 weeks before recovering after 1 month. In addition, Fig. 2 shows that ER and Vg genes are always inducible by estrogen. After 24 h, the levels of ER and Vg mRNA in the liver cells are identical to those found in adult fish stimulated for the same period. Both mRNA species (3.5 and 4.5 kb) are also found and induced in the same proportion as in mature fish (Northern blot analysis, not shown).

Fig. 1. Estrogen induction of hepatic rtER mRNA. (A) Total RNA was isolated from control males (left-hand column) and 24 h E $_2$ (right-hand column) stimulated livers (500 μg E $_2$ /kg). The amounts of rtER mRNA were estimated as described in Materials and Methods after slot blot hybridization and autoradiography. Values are given as mean $\pm$ standard deviation ($n = 12$ for control males and $n = 8$ for estrogen treated males). Means were compared using Student's t -test ($P < 0.001$). (B) Northern blot analysis of poly(A) RNA from (C) control and (E $_2$) stimulated trouts. The markers used were a mixture of rRNA from rat (5.4, 2.1 kb), trout (4.6, 2.1 kb) and *Escherichia coli* (3.2, 1.7 kb).

In vivo long-term analysis of rtER mRNA stimulation

A group of male rainbow trout (600–900 g) was injected with E $_2$ (1.5 mg/kg). Blood and livers were collected at the times indicated in Fig. 3 and plasma, nuclear extracts and total RNA prepared according to Materials and Methods. rtER and Vg mRNA, nuclear ER and plasma E $_2$ levels were determined. Nuclear ER and its mRNA are induced in parallel a few hours after hormone administration (Fig. 3C and A). The levels increase until day 6 then recover to the basal value 3 weeks after hormonal injection. Fig. 3B shows the transient expression of Vg gene after E $_2$ administration. Vg mRNA induction follows that of rtER mRNA with a longer lag period. However, Vg mRNA accumulation is 1000 times greater and the decrease only starts after the second week. These results show the close relationship between the expressions of these two genes in the trout liver after E $_2$ treatment.

Memory effect after a second estradiol stimulation

As rtER mRNA and nuclear ER levels recover to basal values 3 weeks after E_2 treatment, we decided to investigate whether the Vg gene in rainbow trout was secondarily induced with a memory effect as described for Vg genes in *Xenopus* and chicken (Tata and Smith, 1979; Shapiro, 1982; Evans et al., 1987). Male rainbow trout (600–900 g) were first injected with E_2 (1.5 mg/kg) or with the same amount of solvent. A second injection was performed 1 month later with the same amount of E_2 or solvent. The fish

Fig. 2. Estradiol receptor ontogenesis in rainbow trout liver. At different periods after hatching, the alvines were stimulated with E_2 according to Materials and Methods. 50–100 livers were pooled for each RNA extraction. (A) At the indicated times rtER mRNA was determined as described in Materials and Methods by slot blot hybridization. Left-hand columns: control; right-hand columns: E_2 stimulated. (B) At the indicated times Vg mRNA was determined as described in Materials and Methods by slot blot hybridization. Left-hand columns: control; right-hand columns: E_2 stimulated.

were divided into four groups (Fig. 4): control animals injected with solvent at the first and second injection (I), animals injected with E_2 at the first injection and with solvent at the second injection (II), animals injected with solvent at the first injection and with E_2 at the second injection (III), and the last group treated with E_2 at the first and second injection (IV). For all animals, the livers were collected for RNA extraction 7 h after the second injection. The amounts of rtER and Vg mRNA were estimated after slot blot hybridization of 5 μ g total RNA with rtER and Vg probes and autoradiography. Fig. 4 shows that 1 month after the first injection the level of rtER mRNA decreased back to the basal level whereas Vg mRNA was undetectable (compare groups I and II in Fig. 4A and B). The animals which received a second estrogen injection have an increased rtER mRNA level which is, however, no higher than after a first injection (compare groups IV and III in Fig. 4A). On the other hand, Vg mRNA is much greater after a second estrogen injection (compare groups IV and III in Fig. 4B). Thus the Vg gene memory effect does not appear to be directly related to the ER level.

Discussion

rtER mRNA level determination after E_2 treatment shows that, in contrast to the inhibitory effect of glucocorticoids (Okret et al., 1986) and progesterone (Wei et al., 1988), estrogen induces 10–20 times the basal level of their receptor mRNA. Northern blot analysis reveals the presence of two mRNAs in the liver. The specific function of these two mRNAs has not yet been determined but some preliminary observations indicate that they both code for estrogen receptors: their stimulation by estradiol is parallel, they both hybridize, in stringent conditions, with probes containing DNA or hormone binding domains (data not shown). In other species, the presence of multiple steroid receptor mRNA has also been described: in *Xenopus* four ER mRNAs from 2.5 to 9 kb (Weiler et al., 1987), in rat and man, respectively two and three glucocorticoid receptor mRNAs from 4.8 to 7.1 kb (Hollenberg et al., 1985; Miesfeld et al., 1986). Progesterone receptor mRNA seems to be the more heterogeneous with

Fig. 3. Long-term analysis of rtER and Vg mRNA stimulation in vivo. Male rainbow trout (600–900 g) were injected with E_2 (1.5 mg/kg). The blood and livers were removed at the indicated times. Total RNA and nuclear extracts were prepared as described in Materials and Methods. rtER (A) and Vg (B) mRNA levels were determined as described in Materials and Methods by slot hybridization, the nuclear estrogen receptor levels in liver (C) were measured by incubation with 3 nM [3H] E_2 in the presence or absence of a 100-fold excess [3H] E_2 at 2°C for 12 h, and plasma E_2 levels (D) were measured by radioimmunoassay. Each determination is the mean of two independent experiments.

four messengers from 1.8 to 8 kb in the chicken (Conneely et al., 1986, 1987) and six from 2.5 to 11.4 kb in man (Misrahi et al., 1987; Wei et al., 1988). The origin and functions of these multiple steroid receptor mRNAs are not yet well known. However, recent works show that two forms of human progesterone receptor (hPR), differing in their N-terminal region and proceeding from two classes of hPR mRNAs, exhibit different functional properties (Kastner et al., 1990). Cloning of several steroid receptor's genes reveals that these mRNAs are likely to be transcribed from a unique gene (Huckaby et al., 1987; Ponglikitmongkol et al., 1988; Kuiper et al., 1989) and they might result from the use of different transcription initiation sites (Kastner et al., 1990) or of different polyadenylation sites (Hollenberg et al., 1985). They can also arise from a differential splicing of

the same primary transcript (Mitsushashi et al., 1988). In the rainbow trout, the higher molecular weight mRNA is only observed in the liver while the lower molecular weight mRNA is found in the hypothalamus immature male gonads and pituitary; in the latter tissue a shorter 1.4 kb mRNA is also observed (Pakdel et al., 1990).

The rtER gene is expressed in the liver as early as at hatching. The basal levels of rtER mRNA are very similar to those found in the adult, and on Northern blot (result not shown) the presence of the two rtER mRNAs was noted. rtER and Vg genes also seem to be fully induced as early as at hatching and our results show that the estrogen response arises independently of sexual maturation which occurs later during development (Van den Hurk et al., 1982). These results are in good agreement with previous studies on chicken yolk

Fig. 4. Memory effect after a second E_2 stimulation. Male rainbow trout (600–900 g) were injected with E_2 (1.5 mg/kg) or with the solvent. A second injection was performed 1 month after the first injection. The animals were divided into four groups. The livers were collected for RNA extraction 7 h after the second injection. (I) Control animals were injected with solvent at first and second injection. (II) Animals were injected with E_2 at first injection and with solvent at second injection. (III) Animals were injected with solvent at first injection and with E_2 at second injection. (IV) Animals were injected with E_2 at first and second injection. The amounts of rtER (A) and Vg (B) mRNA were estimated as described in Materials and Methods after slot blot hybridization and autoradiography. Values are given as mean $\pm$ standard deviation ($n = 4$). Four animals are used for each determination and value.

protein regulation (Elbrecht et al., 1984). However, in the rainbow trout, further studies of primary and secondary inductions are necessary to determine the kinetics of stimulation and to confirm that there is no variation in sensitivity of ER and Vg genes after hatching, in contrast to the results obtained with the chicken (Evans et al., 1987). Further studies are also necessary to determine if the presence of ER is sufficient during early embryogenesis for Vg gene induction or if another mechanism such as chromatin structure

modification is also necessary (Weisbord, 1982). In the *Xenopus* tadpole, for instance, ER has been observed in the liver before metamorphosis (stage 54); however, estrogens are only able to induce Vg synthesis during metamorphosis (stage 62) (May and Knowland, 1981; Riegel et al., 1987). In rainbow trout, these studies are not easy owing to the small size of the embryos, but in situ hybridization is a possible alternative.

Estrogen stimulation kinetics in the adult male rainbow trout show a transient induction of rtER mRNA and nuclear ER preceding Vg mRNA transcription. Vg mRNA accumulation continues until day 15 when the nuclear ER and ER mRNA have already recovered to basal levels. This may be explained by an increase in the Vg mRNA half-life. Comparison of the induction levels of the two genes shows that the Vg gene is expressed about 1000 times more under estrogen treatment than the ER gene, indicating the differential strength of the two promoters. In the rtER promoter, Le Pennec et al. (personal communication) have shown the presence of potential imperfect ERE which may explain the weak induction of the rtER gene compared to the Vg gene.

After E_2 induction, the nuclear receptor and rtER mRNA levels decrease and return to the basal value 2 or 3 weeks after E_2 treatment, which may be due to the gradual disappearance of E_2 following injection (Fig. 3D). A negative feedback, by binding and competition of free receptors to ERE when the E_2 concentration becomes too small, may be involved as it has been shown that free receptors can bind ERE without gene transcription activation (Kumar et al., 1987). Nevertheless, our results are different from those described in *Xenopus* (Barton and Shapiro, 1988). In this species, it has been shown that *Xenopus* estrogen receptor (XER) mRNA induction in the liver is permanent and stable for at least 4 months after E_2 treatment; then Vg gene activation is transient. After a first stimulation, the low level of endogenous estrogen is sufficient to maintain activation of the XER gene but not that of the Vg gene. This hypothesis is supported by experiments in liver cell culture where the induction of XER mRNA is maintained with 0.2 nM E_2 but Vg mRNA is not (Barton and Shapiro, 1988). In the rainbow trout, preliminary results with cultured

hepatocytes show that rtER mRNA is primarily induced with lower doses of estrogen (10^{-10} M) than Vg mRNA is (results not shown). Further experiments are necessary to determine if there is a specific sensitivity of the two genes after a first treatment by estrogens. However, while the level of rtER mRNA is not maintained and returns to the basal value 3 weeks after primary injection, we still observed a memory effect on the induction of Vg gene following the second injection. To explain this memory effect, we cannot invoke the presence of a higher level of receptors at the time of the second injection as in *Xenopus* or chicken liver (Westley and Knowland, 1979; Hayward et al., 1982; Tata et al., 1987). Persistent structure modification of the Vg gene, or the presence of additional transcription factors, is more likely to explain the phenomenon.

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