Transcriptional regulation of expression of the rainbow trout albumin gene by estrogen

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ABSTRACT

Estrogens modulate the expression of many liver-specific genes in oviparous species. For instance, expression of the estrogen receptor and vitellogenin genes is strongly up-regulated by estradiol in rainbow trout liver. Using hepatocyte primary cultures, we demonstrate that trout albumin (Alb) gene is also regulated by this hormone. Indeed, treatment of hepatocytes with 1 µM estradiol led, after 24 h, to a dramatic decrease in Alb mRNA level. To investigate the mechanism of this down-regulation, run-off experiments were performed and mRNA half-lives were determined in the presence and absence of estradiol. The results show that the down-regulation of Alb mRNA expression by estrogens occurs only at the transcriptional level.

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INTRODUCTION

In all oviparous vertebrates, liver is one of the main target organs for estradiol (E2). For this reason, this organ is often used as a model to study regulation of expression of estrogen-dependent genes. Recently we developed an aggregate primary culture in which rainbow trout hepatocytes were able to maintain stable and specific gene expression over a period of 1 month (Flouriot et al. 1993, 1995, Cravedi et al. 1996). This culture system allowed us to study mechanisms involved in the E2 regulation of two estrogen-dependent genes: vitellogenin, which codes for the precursor of egg yolk proteins, and estrogen receptor (ER), the product of which is a ligand-inducible transcription factor. We demonstrated that the up-regulation by E2 of these two genes was the result of both an increase in transcriptional activity and mRNA stabilization (Flouriot et al. 1996). Similar mechanisms of vitellogenin gene regulation by E2 have also been described in Xenopus and chicken (Wiskocil et al. 1980, Searle & Tata 1981, Brock & Shapiro 1983, Perlman et al. 1984), but no mechanistic studies of the regulation of the ER gene in these species have been carried out. E2 has many roles in oviparous species and many of these are mediated by the control of the expression of genes in the liver. For instance, in addition to the E2 regulation of vitellogenin synthesis, E2 induction of several oocyte membrane proteins has also been reported in rainbow trout (Hyllner et al. 1994). Although most examples concern E2 up-regulation, it is interesting to note that, in parallel, E2 down-regulation occurs in the same organ. In Xenopus, several studies show that E2 is able to down-regulate the production of albumin (Alb), a serum protein synthesized in the liver (Wolffe et al. 1985, Riegel et al. 1986, Selcer & Palmer 1995). This down-regulation in Xenopus is mediated by a decrease in gene transcriptional activity and destabilization of its mRNA. In the present paper, using hepatocyte culture, we show that the rainbow trout Alb gene is also down-regulated by estrogens. The mechanism is, however, different from that of the Xenopus gene, since a decrease was only seen in transcriptional activity.

MATERIALS AND METHODS

Animals and cell culture

Male rainbow trout (Oncorhynchus mykiss) weighing 250–750 g were supplied by a trout farm
(Gournay-Sur-Aronde, Oise, France), and kept in recycled water. The liver was dissociated by collagenase perfusion as described by Seglen (1973) with modifications for trout (Maitre et al. 1986). Briefly, the trout was stunned and the abdominal cavity opened to cannulate the vena porta. The liver was first washed with 400 ml calcium-free solution containing 11·76 mM HEPES, 160·8 mM NaCl, 3·15 mM KCl and 0·3 mM Na2HPO4, pH 7·65, at 18°C and then perfused with 250 ml of the dissociating solution containing 10 mM HEPES, 136·9 mM NaCl, 2·68 mM KCl, 0·28 mM the dissociating solution containing 10 mM HEPES, pH 7·65, at 18°C and then perfused with 250 ml of the dissociating solution containing 10 mM HEPES, 136·9 mM NaCl, 2·68 mM KCl, 0·28 mM Na2HPO4, pH 7·65, at 18°C and then perfused with 250 ml of the dissociating solution containing 10 mM HEPES, 136·9 mM NaCl, 2·68 mM KCl, 0·28 mM Na2HPO4, 6·67 mM CaCl2 and 260 mg/l collagenase. Finally the liver was removed and cells were dispersed in a Petri dish containing culture medium. The cell suspension was filtered through gauze and the cell pellet was collected by centrifugation (at 50 g for 5 min) at room temperature. The cell pellet was washed 3 times in culture medium. At this stage, most cells in the pellet were hepatocytes. Cell viability was about 95% as determined by the trypan blue exclusion test. The cell pellet was resuspended and diluted to 4 × 10^6 cells/ml in Dulbecco’s modified Eagle’s medium nutrient mixture/Ham’s F12 (1:1 mixture, with L-glutamine and 15 mM HEPES, without phenol red) supplemented with 15 mM TES, 12 mM NaHCO3, 1% (v/v) antibiotic mixture (penicillin; streptomycin; amphotericin B) (Sigma) and 2% (v/v) serum substitute (Ultroser SF). Cells in suspension were plated in untreated plastic Petri dishes (Falcon, Meylan, France). Aggregates were obtained by constant gyratory shaking at 70 r.p.m. at 18°C. After 3 days, when aggregates had started to form, the culture medium was changed every 2 days. All estrogen treatments were carried out after 8 days of culture, when the aggregation process was over and gene expression was stable (Flouriot et al. 1993).

Isolation of RNA and blot hybridization

Total RNA was prepared using the LiCl/urea method (Auffray & Rougeon 1980) with some modifications as previously described (Searle & Tata 1981). Total RNA (2–10 µg) samples were either electrophoresed in a 1:5% agarose gel containing formaldehyde and then transferred to nylon membrane (Biodyne Pall, Pall Industry, St Germain en Laye, France) or spotted on to nylon membranes, using a Bio-Rad slot-blot apparatus, as described by Cheley & Anderson (1984). The membranes were prehybridized in a solution containing 50% (v/v) formamide, 5 × SSC, 5 × Denhardt’s solution, 50 mM NaH2PO4, pH 6·5, 100 µg/ml calf thymus DNA and 0·1% (w/v) SDS for 6 h at 42°C. Hybridization was carried out in the following mixture: 50% (v/v) formamide, 5 × SSC, 1 × Denhardt’s solution 20 mM NaH2PO4, pH 6·5, 50 µg/ml calf thymus DNA and 0·1% (w/v) SDS containing heat-denatured cDNA for 15 h at 42°C. The membrane was washed 3 times for 10 min in 2 × SSC/0·1% SDS, twice for 20 min at 55°C in 0·1 × SSC/0·1% SDS, and layered on Whatman 3MM paper wetted with the latter solution, covered with Saran wrap and autoradiographed.

The specific probes (Alb, ER, actin) used in these hybridizations have been described previously (Pakdel et al. 1990, Byrnes & Gannon 1990). All cDNAs were labeled using the random primer method with [α-32P]dCTP (3000 Ci/mmol).

Nuclear run-off analysis

Nuclei from trout hepatocytes were prepared as described by Gorski et al. (1986) with the modifications introduced by Sierra (1990). Briefly, cells were homogenized in homogenization buffer (2 M sucrose, 10 mM HEPES, pH 7·6, 15 mM KCl, 1 mM EDTA, pH 8, 10% glycerol, 0·15 mM spermine, 0·5 mM spermidine, 0·5 mM phenylmethylsulphonyl fluoride (PMSF), 14 µg aprotinin/ml, 1 mM benzamidine, 0·5 µg leupeptin/ml and 0·5 µg pepstatin/ml) containing low-fat milk (1%), using 10 strokes of a Potter homogenizer (K 14164-Bioblock, Bioblock Scientific, Illkirch, France). Homogenized cells were then loaded on the top of 10 ml homogenization buffer in SW27 tubes and centrifuged for 1 h at 120 000 g at 0°C. The nuclear pellet was washed and resuspended in nuclear buffer (20 mM Tris, 72 mM NaCl, 0·5 mM EDTA, 0·85 mM dithiothreitol, 0·125 mM PMSF, pH 7·9) and 50% glycerol (at a concentration of 2 × 107 nuclei/100 µl), and stored at −70°C before utilization.

As previously described (Schibler et al. 1983), the run-off transcription assay was performed at 26°C for 30 min in 87 mM Tris–HCl, pH 7·9, containing 25 mM NaCl, 0·4 mM EDTA, 0·05 mM PMSF, 0·9 mM dithiothreitol, 300 mM (NH4)2SO4, 1 mg/ml heparin, 6·6 mM MnCl2, 20 U RNAsin, 200 µCi [α-32P]UTP (800 Ci/mmol) and 1 mM each GTP, ATP and CTP. The RNA was extracted as previously described (Groudine et al. 1981) and hybridized in the presence of 5 µg plasmid Bluescript with or without the various cDNAs (i.e. Alb, ER, actin). These plasmids were denatured and placed on Hybond-N membrane using a slot-blot apparatus. The DNA was cross-linked to the filter with UV light and baked at 80°C. The membrane was prehybridized and hybridized at
42 °C for 72 h ($5 \times 10^6$ c.p.m., labeled RNA/membrane in 3 ml). Prehybridization and hybridization conditions were as described previously for slot-blot analysis. The autoradiographs were analysed using a densitometer.

RESULTS

Expression of Alb mRNA in primary culture of rainbow trout hepatocytes

We have previously shown that rainbow trout hepatocytes cultured in aggregates are able to survive and perform specific liver functions such as vitellogenin synthesis over a 1 month period (Flouriot et al. 1993). In order to check that expression of Alb mRNA, another liver-specific marker, was also maintained in aggregate hepatocyte culture, Northern and slot-blot experiments were performed using Alb cDNA from Atlantic salmon as a probe (Byrnes & Gannon 1990). Northern blot hybridization analysis of total RNA extracted from freshly isolated trout hepatocytes showed that Alb mRNA was abundantly expressed (Fig. 1A). In agreement with previous results in Atlantic salmon, two major bands at 2·3 and 1·7 kb were identified.

Over a 1 month period, the time course of Alb mRNA expression in culture decreased during the first 6 days to become stable at 10% of the initial value for the rest of the culture time (3 weeks at least) (Fig. 1B). The decrease in Alb mRNA expression corresponded to the period of hepatocyte aggregation. This period has also been previously characterized by unstable expression of ER and vitellogenin genes (Flouriot et al. 1993). However, after this transient decrease, the period showing stable expression was long enough to perform experiments on the regulation of Alb gene expression. Therefore, in the following experiments, hepatocyte treatments were carried out after 8 days of culture.

Effect of different hormones on Alb mRNA accumulation in rainbow trout hepatocytes

As previously shown, Alb mRNA expression is significantly reduced in hepatocyte culture. This decrease may be due to the lack of an important serum hormone in the culture medium. To investigate this, after 8 days of culture, hepatocytes were treated for 5 days with one of the following hormones: insulin-like growth factor-I, prolactin,
growth hormone, tri-iodothyronine, testosterone, progesterone, dexamethasone or E2. Figure 2 shows that none of these hormones was able to restore Alb expression to that detected in freshly isolated trout hepatocytes. The only effect on Alb gene expression was a significant decrease in its mRNA level during E2 treatment. In the same experiment, we also measured ER mRNA expression in order to verify hepatocyte sensitivity. In previous studies, we demonstrated that hepatocyte treatment with E2 led to rapid ER mRNA induction. This induction was observed after 3 h of E2 treatment. In comparison, the level of Alb mRNA remained unchanged during the first day of E2 treatment and only started to decrease after 24 h (Fig. 3). This relatively long lag period suggests that the effect of E2 on Alb gene expression may be either an indirect consequence of hepatocyte E2 stimulation, such as intensive use of the ribosome machinery for vitellogenin synthesis, or a more direct E2 effect on the transcriptional activity of the Alb gene and/or its mRNA turnover.

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Estrogen regulates transcriptional but not post-transcriptional Alb gene expression

As transcriptional and/or post-transcriptional regulation may be implicated in the decrease in Alb mRNA level after E2 treatment, we determined the respective influence of each mechanism. Run-off experiments were performed using hepatocyte nuclei prepared from cells treated with 1 µM E2 for increasing time periods. The hormone was renewed every 24 h in the culture medium. The transcriptional activities of the Alb gene were related to the previously demonstrated constant actin gene activity (Flouriot et al. 1996). Figure 4 shows the variation in Alb gene transcriptional activity observed during E2 treatment. The rate of transcription of the Alb gene decreased dramatically during the first 3 h and then remained stable at a very low level (10% of the initial value) for the rest of the treatment. During the same experiment, induction of ER gene transcriptional activity was obtained as expected. Inhibition of Alb gene transcription may itself explain the down-regulation of Alb mRNA amount if the Alb mRNA half-life was long enough to account for the lag period and the slow Alb mRNA decrease observed during estrogen treatment. To investigate this possibility, Alb mRNA stability was determined in the presence and absence of E2 using actinomycin D to block hepatocyte transcriptional activity. The actinomycin D concentration used (0.4 µg/ml culture medium) was demonstrated to be the minimum able to completely inhibit ER and vitellogenin induction with 1 µM E2 (data not shown). After 8 days of culture, hepatocytes were maintained with or without 1 µM E2 for 48 h and then treated with actinomycin D. The levels of Alb mRNA were determined in both experiments at different times after actinomycin D treatment. Figure 5 shows there was no significant change in Alb mRNA half-life after E2 treatment. In the same experiment, ER mRNA turnover was, however, modified by E2 as expected.

DISCUSSION

Along with vitellogenin, albumin is one of the most abundant serum proteins synthesized during vitellogenesis and secreted by the liver in oviparous vertebrates. Northern blot analysis of rainbow trout hepatocyte mRNAs shows the presence of two abundant Alb mRNA isoforms. A similar result was reported for Atlantic salmon (Byrnes & Gannon 1990) and Xenopus (May et al. 1983). These isoforms might proceed from different genes, from alternative splicing and promoter usage or from the utilization of different polyadenylation signals. In
Actin gene transcription rate as an internal control for transcription at each time point was normalized by the quantified using densitometry. The rate of Alb gene transcription assay (as described in Materials and Methods) were hybridized to the specific cDNAs (Alb, ER and actin) cross-linked to the hybond-N membranes. The resultant autoradiographs were presented. The [32P]UTP-labeled mRNAs made from the run-off transcription assay (as described in Materials and Methods) were hybridized to the specific cDNAs (Alb, ER and actin) cross-linked to the hybond-N membranes. The resultant autoradiographs were quantified using densitometry. The rate of Alb gene transcription at each time point was normalized by the actin gene transcription rate as an internal control for possible differences in incorporation of [32P]UTP into nuclei. The background obtained by hybridization of a Bluescript plasmid was subtracted. Values are presented as the percentage of the rate of Alb gene transcription detected at the beginning of the treatment. Variations in the rate of ER gene transcription are also presented.

In aggregated hepatocytes, the kinetics of Alb gene expression show significant variations. During the first few days corresponding to aggregate formation, a large decrease in Alb mRNA level is observed. However, after 5–6 days, stabilization occurs, and Alb mRNA level remains stable for at least 1 month. We have previously shown that the expression of other liver-specific genes is also stably maintained during this last period (Flouriot et al. 1993, 1995, Cravedi et al. 1996). It should be noted that a decrease in Alb gene expression after liver dissociation and hepatocyte culture has also been reported for Xenopus and rat (Wolfe et al. 1984, Fraslin et al. 1985, Jackson & Shapiro 1986), and the reasons for this difference in Alb gene expression in vivo and in vitro are not yet known.

In rainbow trout, Alb gene expression was not modified when cultured hepatocytes were treated for several days with insulin-like growth factor-I, growth hormone, prolactin, tri-iodothyronine, progestins, androgens and glucocorticoids, whereas in Xenopus, dexamethasone induces a 10-fold increase in Alb mRNA level in culture (Jackson & Shapiro 1986). Only E2 was able to modify Alb gene expression in trout, a large decrease in Alb mRNA level being observed after 5 days of treatment. Several studies performed in Xenopus (Wolfe et al. 1985, Riegel et al. 1986) and more recently in turtle (Selcer & Palmer 1995) showed a similar inhibition by E2 of Alb gene expression in oviparous liver. However, our results differ from those obtained in Xenopus with regard to the mechanisms involved in this inhibition. The transcription rate and mRNA half-life determination carried out in the presence or absence of E2 demonstrated that E2 causes a very rapid decrease in Alb gene transcriptional activity in trout liver, whereas it does not affect Alb mRNA turnover. The inhibitory effect of E2 on Alb mRNA accumulation is only detected several days after the treatment because of the long half-life of Alb mRNA (36 h). Hybridization of the same RNA samples with a specific ER probe shows that regulation of ER gene expression by E2 occurs at both the transcriptional and post-transcriptional level, as previously demonstrated (Flouriot et al. 1996). Reduction of transcription of the Xenopus Alb gene has also been reported, but in this species the main mechanism involved in the down-regulation of Alb gene expression is mRNA destabilization (Kazmaier et al. 1985, Wolfe et al. 1985, Riegel et al. 1986, 1987). This Alb mRNA destabilization is mediated by a polysomal endoribonuclease which is under estrogenic control (Pastori et al. 1991, Dompenciel et al. 1995). The cleavage site of this enzyme is an overlapping repeat sequence (APyrUGA) localized in the 3′ untranslated region of Alb mRNA (Dompenciel et al. 1995). The lack of such a sequence in the 3′ untranslated region of Alb mRNA in salmonids (Byrnes & Gannon 1990) may explain why Alb mRNA turnover is not regulated by estrogens in this species. A direct result of the absence of trout Alb mRNA destabilization by E2 is that the down-regulation of the Alb gene in the trout is slower than in other species. As it is likely that the repression of the Alb gene by estrogen is due to competition between vitellogenin and other E2-inducible genes for RNA

**FIGURE 4.** Effect of E2 on the transcription rate of the Alb gene. After 8 days of culture, hepatocytes were treated with 1 μM E2, and hormonal treatment was renewed every 24 h. At different times (0 to 48 h), cells were harvested and nuclei were prepared. The [32P]UTP-labeled mRNAs made from the run-off transcription assay (as described in Materials and Methods) were hybridized to the specific cDNAs (Alb, ER and actin) cross-linked to the hybond-N membranes. The resultant autoradiographs were presented. The background obtained by hybridization of a Bluescript plasmid was subtracted. Values are presented as the percentage of the rate of Alb gene transcription detected at the beginning of the treatment. Variations in the rate of ER gene transcription are also presented.

Xenopus, several genes have been identified (May et al. 1983), suggesting differential regulation of these genes. In rainbow trout, additional studies are needed to elucidate the functional significance of these Alb mRNA isoforms.

In aggregated hepatocytes, the kinetics of Alb gene expression show significant variations. During the first few days corresponding to aggregate formation, a large decrease in Alb mRNA level is observed. However, after 5–6 days, stabilization occurs, and Alb mRNA level remains stable for at least 1 month. We have previously shown that the expression of other liver-specific genes is also stably maintained during this last period (Flouriot et al. 1993, 1995, Cravedi et al. 1996). It should be noted that a decrease in Alb gene expression after liver dissociation and hepatocyte culture...
polymerase, transcription factors or translational machinery, a potential physiological consequence of the slow down-regulation of the trout Alb gene may be the relative delay in vitellogenin synthesis also observed in this species.

In conclusion, the present study demonstrates that estrogens regulate Alb gene expression in the liver of rainbow trout only through the repression of transcriptional activity. In contrast with Xenopus, no post-transcriptional regulation by E2 was observed. Further work is needed to determine the detailed events at the molecular level involved in this down-regulation.

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