The preparation and characterization of vitellogenin messenger RNA from rainbow trout (*Salmo gairdneri*)

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(Received 29 July 1983/Accepted 22 August 1983)

Agarose-gel electrophoresis of polyadenylated RNA from livers of oestrogen-treated male rainbow trout revealed a major high- M_r species (7200 nucleotides), which is absent from the polyadenylated RNA isolated from hormonally unstimulated male trout liver. Translation *in vitro* of the RNA from oestrogen-treated males in a mRNA-dependent rabbit reticulocyte lysate produced a protein (M_r 200 000) that could be immunoprecipitated with antibodies against trout serum vitellogenin, but no immunoprecipitable protein was synthesized with RNA from control animals. DNA complementary to the RNA from oestrogen-stimulated and control male trout liver was synthesized and back-hybridized, with R_{ot} of 3.8×10^{-2} and $1 \times 10^{-1} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$ for RNA from hormone-treated and control animals respectively. The 9% increase in the abundant mRNA after oestrogen stimulation is due to the induction of vitellogenin mRNA.

In all egg-laying vertebrates, the egg yolk proteins are synthesized in the liver as a large precursor termed vitellogenin (M_r 200 000–250 000), which, after its uptake in the oocyte, is cleaved into lipovitellin and phosvitin (Clemens, 1974; Gruber *et al.*, 1976; Tata & Smith, 1979; Wahli *et al.*, 1981). Vitellogenin synthesis in vertebrates is regulated by oestrogen, and a most useful feature of this system is the ability of the hormone to activate reversibly the transcription of normally quiescent genes in the liver of male animals. This induction *de novo* in males has established vitellogenesis as an important model for the study of eukaryotic gene expression. It has also allowed considerable progress to be made in the analysis of the kinetics of synthesis and utilization of vitellogenin mRNA, particularly in amphibia and birds (Berridge *et al.*, 1976; Baker & Shapiro, 1977; Ryffel *et al.*, 1977; Gordon *et al.*,

1978). Vitellogenesis in fish has not been studied as extensively as in the other vertebrates mentioned above. Although the induction by oestrogen of vitellogenin has been demonstrated in several species of fish (Plack *et al.*, 1971; Campbell & Idler, 1976; Wallace, 1978), little is known about vitellogenin genes or their transcripts in these vertebrates. Roach & Davies (1980) have, however, reported that in the catfish *Ameiurus nebulosus* both the vitellogenin and its corresponding mRNA are significantly smaller than those reported in all other vertebrates. It raised the possibility that fish vitellogenin genes and their products may somehow not fall into the general vertebrate pattern. Recently, we have observed that circulating vitellogenin in the rainbow trout (*Salmo gairdneri*) is a dimer of M_r 500 000, comprising two subunits of equal size (Y. Valotaire, unpublished work). The possibility of major differences between catfish and trout vitellogenins prompted us to analyse vitellogenin mRNA from control and oestrogen-stimulated male trout liver.

Materials and methods

Animals

Male trout (*Salmo gairdneri*), 150–350 g, were injected on three consecutive days with 50 µg of oestradiol-17β/100 g body wt. and killed 7 days after the last injection. The animals were monitored before

Abbreviations used: poly(A)⁺ RNA, polyadenylated RNA; poly(A)[−] RNA, RNA not retained on oligo-(dT)-cellulose; cDNA, complementary DNA; R_{ot} , product of RNA concentration and the time of incubation (expressed as mol of ribonucleotides · litre^{−1} · s); $R_{ot\frac{1}{2}}$, R_{ot} value that is needed to allow hybridization to proceed halfway to completion; 20× SSC, 3 M-NaCl/0.3 M-trisodium citrate; SDS, sodium dodecyl sulphate.

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death for the appearance of vitellogenin in the serum by rocket immunoelectrophoresis with rabbit anti-(trout vitellogenin) antiserum.

Materials

[³H]dCTP (sp. radioactivity 21 Ci/mmol), [α -³²P]dCTP (sp. radioactivity 3000 Ci/mmol) and [³⁵S]methionine (sp. radioactivity 700 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Deoxyribonucleoside triphosphates, actinomycin D, S₁-nuclease and creatine kinase (EC 2.7.3.2) were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. Proteinase K and (dT)₁₂₋₁₈ were purchased from Boehringer-Mannheim, Lewes, Sussex, U.K. Micrococcal nuclease was obtained from P-L Biochemicals, Milwaukee, WI, U.S.A., and oligo(dT)-cellulose was from Collaborative Research, Cambridge, U.K. Avian-myeloblastosis-virus reverse transcriptase was a gift from Dr. J. W. Beard (Life Sciences Inc., St. Petersburg, FL, U.S.A.). All other chemicals were of analytical grade and were obtained from Sigma (London) or BDH Chemical Co., Poole, Dorset, U.K.

Methods

Total RNA from 20–30 g of liver was extracted by the LiCl/urea procedure (Auffray & Rougeon, 1979) with minor modifications (Tenniswood & Simpson, 1982). Poly(A)⁺ RNA was prepared by oligo(dT)-cellulose chromatography as described by Aviv & Leder (1972), with average yields of 10–30 µg of poly(A)⁺ RNA/mg of total RNA (40–50% higher from livers of treated than of control animals).

DNA complementary to poly(A)⁺ RNA from both control and oestrogen-treated male trout liver was synthesized by the method of Williams & Penman (1975), with the minor modifications described by Smith *et al.* (1979). Either [³H]dCTP (100 µCi) or [α -³²P]dCTP (20 µCi) was included in the reaction to obtain cDNA labelled with either radioisotope.

RNA and cDNA preparations were electrophoresed in 1.2%-agarose slab gels under denaturing conditions (McMaster & Carmichael, 1977), with *Xenopus* rRNA, *Escherichia coli* rRNA and wheat-germ tRNA as size markers. Electrophoresis was performed at room temperature at 100 V for 4 h. Gels were stained with ethidium (1 µg/ml) and photographed under 306 nm light by using an orange filter and Ilford FP4 film.

cDNA-RNA-excess hybridization reactions were carried out as described by Deeley *et al.* (1977). Poly(A)⁺ RNA was used at a concentration of 0.1 mg/ml or 1 µg/ml in 10³–10⁴ excess relative to the amount of [³H]cDNA (500 d.p.m./pg) used.

Incubations were carried out at 69°C for various times up to 80 h, and the extent of hybridization was determined by digestion with S₁-nuclease (Milcarek *et al.*, 1974; James & Tata, 1980).

Total, poly(A)⁺ RNA and poly(A)[−] RNA isolated from both control and oestradiol-stimulated male trout liver were translated in a micrococcal-nuclease-treated reticulocyte-lysate cell-free translation system (Pelham & Jackson, 1976). After 90 min at 26°C the samples were either directly processed for SDS/10%-polyacrylamide-gel electrophoresis (Laemmli, 1970) or immunoprecipitated with specific anti-(trout vitellogenin) antibodies. After electrophoresis, the gels were autoradiographed or fluorographed as described by Bonner & Laskey (1974).

Results and discussion

Extraction of RNA

RNA was prepared from both control and oestrogen-treated male trout, and was then fractionated into poly(A)⁺ RNA and poly(A)[−] RNA by two passages through oligo(dT)-cellulose. When the integrity and relative abundance of RNA were examined by staining with ethidium bromide after agarose-gel electrophoresis under denaturing conditions, as shown in Fig. 1, poly(A)⁺ RNA from oestrogen-stimulated male livers displayed a high-*M*_r RNA (31 S; 7200 nucleotides), which was not present in control liver preparations, although an RNA species is faintly visible as a 32 S (7600 nucleotides) species. The poly(A)⁺ RNA preparations from both groups of fish do show several other abundant RNA species in common, in particular one component migrating to the 22 S position (2700 nucleotides), which is most likely albumin mRNA, although it is slightly larger than the 2300 nucleotides reported for *Xenopus* albumin mRNA (Schoenberg, 1981). The size of vitellogenin mRNA in oestrogen-treated male trout is markedly larger than that reported for catfish (Roach & Davies, 1980). On the other hand, it is similar to that found for vitellogenin mRNA from a variety of other species, including *Xenopus* (Tata & Smith, 1979), chicken (Deeley *et al.*, 1977; Gordon *et al.*, 1978) and locust (Applebaum *et al.*, 1981). Also, in common with vitellogenins from other vertebrates, its synthesis is under oestrogen control.

Cell-free translation of total, poly(A)⁺ RNA and poly(A)[−] RNA from control and oestrogen-treated male trout liver

When poly(A)⁺ RNA from oestrogen-stimulated trout liver was translated in a nuclease-treated reticulocyte-lysate cell-free system, the incorporation of [³⁵S]methionine into acid-insoluble material was linear with time up to 90 min, with a 40-fold

Fig. 1. Agarose-gel electrophoresis of glyoxal/dimethyl sulphoxide-denatured male trout liver RNA

RNA was denatured with 1 M-glyoxal in 50% (v/v) dimethyl sulphoxide before electrophoresis on 1.2%-agarose gels (McMaster & Carmichael, 1977). Track 1, 2 μ g of poly(A)⁺ RNA isolated from the livers of oestrogen-treated male trout; track 2, 10 μ g of poly(A)⁺ RNA isolated from the livers of control male trout; track 3, 2 μ g of poly(A)⁻ RNA isolated from livers of oestrogen-treated male trout; track 4, 10 μ g of poly(A)⁻ RNA isolated from control livers. Arrows indicate the positions of the presumptive vitellogenin (Vg) and albumin (Alb) mRNA.

stimulation over background incorporation. Total RNA and poly(A)⁻ RNA were translated significantly less efficiently. Electrophoretic analysis on SDS/10%-polyacrylamide gels (Fig. 2) showed that the overall pattern of translation products obtained with poly(A)⁺, poly(A)⁻ or total RNA from oestrogen-stimulated male liver (tracks 1–4) or control male liver (tracks 5–8) as templates are similar, although the control male RNA was translated approx. 15% less efficiently than the corresponding RNA from stimulated livers. Among the protein bands found to be common to RNA from both oestrogen-stimulated and control trout liver RNA preparations is one corresponding to albumin (M_r 70000). Among the translation products found only with oestrogen-stimulated liver poly(A)⁺ RNA is a band corresponding to a protein of M_r 200000. This protein corresponds in size to trout vitellogenin, and its authenticity was confirmed by immunoprecipitation with a well-characterized rabbit anti-(trout vitellogenin) antibody (Fig. 3). The antibody only immunoprecipitated the ³⁵S-labelled vitellogenin, and some proteolytic degradation products, from the translation of oestrogen-stimulated poly(A)⁺ RNA (tracks 3 and 4), but not from

Fig. 2. SDS/10%-polyacrylamide-gel electrophoresis of [³⁵S]methionine-labelled translation products of control and oestrogen-stimulated male trout liver RNA in a messenger-dependent rabbit reticulocyte cell-free translation system

Samples of RNA (2–45 μ g) were incubated in duplicate in 25 μ l of micrococcal-nuclease-treated rabbit reticulocyte lysate and analysed by gel electrophoresis as described in the text. Lanes 1–4, RNA preparations from livers of oestrogen-stimulated male trout: lane 1, total RNA (45 μ g); lane 2, poly(A)⁻ RNA (40 μ g); lanes 3 and 4, poly(A)⁺ RNA (4 and 2 μ g respectively). Lanes 5–8, RNA preparations from the livers of control male trout: lane 5, total RNA (45 μ g); lane 6, poly(A)⁻ RNA (40 μ g); lanes 7 and 8, poly(A)⁺ RNA (4 and 2 μ g respectively). The position of authentic vitellogenin (Vg) and albumin (Alb) are indicated by arrows.

control poly(A)⁺ RNA (tracks 7 and 8). Even with overexposure of the autoradiograms (results not shown), no vitellogenin-like bands could be seen in any of the tracks corresponding to control RNA samples, although a vitellogenin band becomes visible in the immunoprecipitates from total and poly(A)⁻ RNA from oestrogen-stimulated livers (tracks 1 and 2).

RNA-excess hybridization analysis

The induction by oestrogen of a species of RNA not normally present in male hepatocytes was further investigated by RNA-excess hybridization in liquid by using [³H]cDNA to poly(A)⁺ RNA from oestrogen-stimulated male trout. Analysis of the kinetics of hybridization by S₁-nuclease digestion (Bishop *et al.*, 1974) revealed several differences in the abundance and complexity of poly(A)⁺ RNA from control and oestrogen-stimulated male livers (Fig. 4). A maximum value of 80% S₁-nuclease resistance was obtained with both RNA preparations.

Fig. 3. SDS/10%-polyacrylamide-gel electrophoresis of translation products after immunoprecipitation with anti-(trout vitellogenin)

Duplicate samples (25 μ l) of the translation products shown in Fig. 2 were immunoprecipitated with 5 μ l of rabbit anti-vitellogenin and 200 μ l of goat anti-(rabbit γ -globulin) as second antibody. The immunoprecipitates were dissolved in 100 μ l of 8 M-urea/SDS sample buffer, and 25 μ l samples were run on 10%-polyacrylamide gels. Lanes 1–8 represent the immunoprecipitated samples of the translation products shown in Fig. 2. The arrow represents the position of authentic vitellogenin (Vg) from trout plasma.

Preparations from oestrogen-stimulated animals comprise at least two abundance classes of mRNA: a major class representing 47% of the poly(A)⁺ RNA, which hybridizes with $R_0 t_{1/2}$ 3.8×10^{-2} mol · litre⁻¹ · s, and a second, less abundant, class, with $R_0 t_{1/2}$ 7.0 mol · litre⁻¹ · s, making up the remaining 33% of the poly(A)⁺ RNA that was hybridized. Control male liver poly(A)⁺ RNA, on the other hand, displays a major abundance class with $R_0 t_{1/2}$ 1×10^{-1} mol · litre⁻¹ · s, representing 38% of the poly(A)⁺ RNA, and a less abundant RNA component, which hybridized with $R_0 t_{1/2}$ 7 mol · litre⁻¹ · s. Northern hybridization (Alwine *et al.*, 1977) with [³²P]cDNA to RNA from oestrogen-stimulated-male-liver RNA suggests that the 9% increase in the abundant mRNA after oestrogen stimulation is due to the induction of the 31S mRNA. Hybridization to a cloned *Xenopus* vitello-

Fig. 4. Kinetics of hybridization of poly(A)⁺ RNA isolated from the livers of oestrogen-stimulated (O, □) and control male trout (●, ■)

RNA-excess hybridization was performed as outlined in the text. [³H]cDNA (500 c.p.m./pg), prepared by using poly(A)⁺ RNA from oestrogen-stimulated male trout, was hybridized to RNA from control males (●, 1 μ g/ml; ■, 0.1 mg/ml) or to RNA from oestrogen-stimulated males (O, 1 μ g/ml; □, 0.1 mg/ml). The percentage hybridization was determined by S₁-nuclease digestion.

genin probe, at low stringency, confirmed that this mRNA codes for vitellogenin (results not shown). There are probably several less-abundant RNA species induced by oestrogen in vertebrate liver, including those coding for the vitamin-binding proteins (Murthy & Adiga, 1978; Muniyappa & Adiga, 1980), which are induced at approx. 1% of the extent of vitellogenin in male chickens after oestrogen stimulation.

To conclude, the response of male trout liver to oestrogen is very similar to that known for most egg-laying vertebrates (Tata & Smith, 1979). The hormone induces *de novo* large amounts of mRNA for vitellogenin, and accelerates the transcription of other genes, while not affecting the amounts of other mRNA species. Trout vitellogenin mRNA and protein are very similar in size to those found in most vertebrate and invertebrate species (Clemens, 1974; Tata & Smith, 1979; Applebaum *et al.*, 1981; Chinzei *et al.*, 1982; James *et al.*, 1982). In this respect, catfish vitellogenin and its mRNA (Roach & Davies, 1980) do not fit into the vertebrate pattern. The synthesis of vitellogenin cDNA that we have described here for the analysis of messenger abundance in male trout liver will enable us to clone the cDNA in bacterial plasmids. This, in turn, will lead to an understanding of the organization of vitellogenin genes in trout and their regulation by oestrogen.

We are grateful to Mr. John Steven for technical assistance. M. T. holds a Medical Research Council of Canada Fellowship. Y. V. was the recipient of a FEBS Travel Award.

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