Translation of hormone-induced messenger RNA in amphibian oocytes: I: Induction by estrogen of messenger RNA encoded for vitellogenic protein in the liver of the male African clawed toad (*Xenopus laevis*)

[estrogen action/polyribosomes/poly(A)-rich RNA/microinjection/lipovitellin/phosvitin]

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ABSTRACT Induction of the synthesis of the vitellogenic proteins, lipovitellin and phosvitin, in the liver of the male African clawed toad (*Xenopus laevis*) was investigated as a function of time after treatment with estradiol-17 β [1,3,5(10)-estratriene-3,17 β -diol]. The appearance of mRNAs encoded for lipovitellin and phosvitin in the cytoplasmic fraction of the liver was assayed by microinjections of hepatic mRNA preparations [either polyribosomes or poly(A)-rich RNA] into oocytes obtained from mature female toads. Oocytes were then incubated in the presence of radioactive amino acid(s) at 19° for periods of time varying from 4 to 18 hr after microinjection. The results show that at 2 hr after hormone treatment more mRNA was present in the cytoplasm, and that from 2 to 72 hr after treatment the level of induced mRNA increased almost linearly to 110% above the control values. Experiments employing specific lipovitellin antiserum indicated no radioactive lipovitellin among the proteins synthesized in oocytes microinjected with hepatic mRNAs isolated from 3 to 9 hr after hormone treatment. However, a marked synthesis of immunoprecipitable, radioactive lipovitellin and an enhanced incorporation of [³H]serine occurred in the oocytes microinjected with hepatic mRNA preparations obtained from toads treated with hormone for 12 or more hr. The identities of the proteins encoded by the mRNAs induced early in estrogen action (2-9 hr) in the male amphibian liver are unknown. It is surmised that some of these proteins may function in the regulation of the subsequent synthesis of the vitellogenic proteins.

Gurdon and his coworkers (1-3) have developed a sensitive technique for the assay of messenger RNA (mRNA). The assay involves microinjections in nanogram amounts of polyribosomes or of other preparations containing mRNA into *Xenopus* oocytes for efficient and long-term translation. For the study of mRNA and its translation in living cells, the amphibian oocyte system offers several advantages. First, translation of exogenous mRNAs in the oocyte has been shown with minimal estimates to be 100-1000 times more efficient than in the reticulocyte cell-free system (1). Second, it seems obvious that translation of mRNA in a living cell is subject to less artifactual conditions. Third, there is good evidence that the oocyte lacks specificity with respect to the type of mRNA capable of being translated (4).

Because of these unique properties, we have recently exploited the amphibian oocyte system for the study of estrogen-induced mRNAs encoded for the vitellogenic proteins in the liver of the male *Xenopus*. In the male toad untreated with the hormone the genes for the vitellogenic proteins, phosvitin and lipovitellin, are repressed. Several laboratories (5, 6) have previously shown that after treatment *in vivo* for 9 or more hr with estrogen these yolk proteins appear in the liver and are exported to the plasma. We now show that estrogen-induced mRNA preparations, obtained from male

toad livers later than 9 hr after treatment and microinjected into the oocyte, are translated for the synthesis *de novo* of lipovitellin and phosvitin. In obtaining these results, we microinjected either 300 or 900 ng of polyribosomal RNA in the form of polyribosomes, or 9 ng of poly(A)-rich RNA. Our investigation has also revealed an earlier induction of mRNAs at 2-9 hr after hormone treatment. The identities and function of the proteins synthesized by these mRNAs remain unknown. Our observations are consistent with those for several other model systems for estrogen action, indicating that an early stimulation of mRNA as well as ribosomal RNA is a characteristic event in the subcellular action of the hormone (7, 8).

The amphibian oocyte, capable of translating exogenous mRNAs with a high efficiency, promises to be a valuable system for the study of specific messengers and proteins in the early action of steroid and other hormones. Presently, most investigations of estrogen-induced mRNAs are contingent on a preceding knowledge of the translation product (e.g., ovalbumin, phosvitin, lipovitellin). To date, these proteins have proven to be secretory ones exported from the target organs responsive to hormonal stimulation. We emphasize that exploitation of the amphibian oocyte system, whereby the translations of the hormone-induced mRNAs are compared to those of the control and endogenous mRNAs, permits unknown as well as known proteins induced by hormone action to be displayed by column chromatography or gel electrophoresis. In other words, translation of exogenous mRNAs in the amphibian oocyte permits qualitative as well as a quantitative analysis of the effect of specific hormones on the populations of mRNAs in their characteristic target organs.

MATERIALS AND METHODS

Mature male toads (*Xenopus laevis*) weighing 50-60 g were supplied by Charles Fletcher of Glen Burnie, Md. Toads, kept at 19° in well water and fed a diet of dried beef heart and liver, were maintained on a cycle of 12 hr of light and dark. All animals received in the dorsal lymph sac either 1 mg of estradiol-17 β [1,3,5(10)-estratriene-3,17 β -diol] in 0.2 ml of propylene glycol or the carrier alone.

Male toads were killed by decapitation and the hepatic polyribosomes were isolated as described by Staehelin *et al.* (9) from a 10% homogenate prepared in 0.25 M sucrose, 50 mM Tris-HCl, pH 7.4, 100 mM KCl and 10 mM MgCl₂ containing 100 μ g/ml of heparin and 10% rat liver RNase inhibitor fraction (10).

FIG. 1. Kinetics of incorporation of $[^3\text{H}]$ serine in *Xenopus* oocytes, following microinjections of hepatic polyribosomes obtained from estrogen-treated or control male toads. Polyribosomes were isolated from the livers of male toads at 72 hr after treatment with hormone or propylene glycol only. Preparations of suspended polyribosomes containing 300 ng of RNA in 50–70 nl of the injection buffer were microinjected into oocytes. Oocytes were then incubated in Barth's medium containing $[^3\text{H}]$ serine as described under the *Materials and Methods*. ●, Oocytes microinjected with polyribosomes from hormone-treated toads; ○, oocytes microinjected with polyribosomes from control toads; Δ, oocytes microinjected with the injection buffer only.

Cytoplasmic RNA was isolated from a 10% hepatic homogenate in 0.14 M sucrose, 0.25 M KCl, 10 mM MgCl_2 , 10 mM Tris, pH 8.2, and 1% Triton X-100. The homogenate was centrifuged at $17,000 \times g$ for 10 min and the supernatant fraction was removed. This fraction was made 0.5% with respect to sodium dodecyl sulfate, and was extracted with an equal volume of phenol and chloroform (11). The phenol phase was then extracted with $\frac{1}{2}$ volume of 10 mM Tris, pH 9.0, and 1 mM MgCl_2 (12). The two aqueous phases were combined and deproteinized by extractions with phenol and chloroform. RNA was finally precipitated from the combined aqueous phases by addition of 2 volumes of 95% ethanol and 2% potassium acetate.

Poly(A)-rich RNA was isolated from cytoplasmic RNA exactly as described by Schutz *et al.* (13). The cellulose column method for the isolation of the poly(A)-rich RNA fraction was equivalent to the oligo(dT)-cellulose column method. Both columns bound poly(A)-rich RNA in high salt buffers, and eluted poly(A)-containing RNA when H_2O was added. Identical elution patterns were obtained with both columns. Preparations of polyribosomes or poly(A)-rich RNA were suspended in the injection buffer (15 mM Tris, pH 7.8, 80 mM NaCl, 1.0 mM KCl) to a final concentration of 5 mg/ml or 50 $\mu\text{g}/\text{ml}$, respectively. These preparations containing mRNA from estrogen-treated and control toads were microinjected into *Xenopus* oocytes essentially as described by Gurdon *et al.* (1, 2). After suspension of the mRNA preparations in the injection buffer, 50–70 nl was microinjected into each oocyte by use of a micropipette constructed as described by Smith and Ecker (14). Oocytes (25 to 35 per group) were incubated in 5 ml of Barth's medium [as modified by Gurdon (15)] containing 10 μCi of $[^3\text{H}]$ serine per ml (1.2 Ci/mmol) and/or 50 μCi of ^{14}C -labeled amino-acid mixture (New England Nuclear Corp.) for 18 hr at 19° , or for shorter periods of time as indicated. In some experiments, the proteins of incubated oocytes were analyzed by

FIG. 2. Incorporation of $[^3\text{H}]$ serine in *Xenopus* oocytes microinjected with hepatic polyribosomes isolated at the indicated times after administration of estrogen to male toads. All oocytes were microinjected with 50–70 nl of polyribosomes (300 ng of RNA) as described in Fig. 1. The results given here and in Fig. 7 are expressed as the percent increase in the incorporation of radioactive precursor in oocytes microinjected with polyribosomes isolated from hormone-treated toads at indicated times over the incorporation observed for oocytes microinjected with control polyribosomes isolated at the same indicated times.

TEAE-cellulose chromatography as previously reported by Wallace (16). Elution of the yolk protein was continuously monitored at 280 nm. Protein was determined by the method of Lowry *et al.* (17).

Immunoprecipitation of radioactive lipovitellin with an excess of specific lipovitellin antiserum was performed as described elsewhere (5). The counting efficiency for ^3H in Aquasol (New England Nuclear Corp.) was 40%, whereas the counting efficiency for ^{14}C was 62%. When the two isotopes were counted together, their respective counting efficiencies were 21% and 46%.

RESULTS

At various times after treatment of male toads with estradiol- 17β , hepatic polyribosomes were isolated and adjusted to a concentration of 5 mg of RNA per ml. Immediately thereafter, the suspended polyribosomes were microinjected (50–70 nl containing 300 ng of RNA) into oocytes. Fig. 1 demonstrates that the incorporation of $[^3\text{H}]$ serine into acid-insoluble protein by polyribosomes isolated from toads treated for 72 hr with estrogen was essentially linear throughout an incubation period of 18 hr. Fig. 1 also shows that oocytes microinjected with polyribosomes isolated from hormone-treated toads incorporated $[^3\text{H}]$ serine at an increased rate, compared to oocytes microinjected either with polyribosomes isolated from control animals or with the same volume of the injection buffer only.

The temporal stimulation of $[^3\text{H}]$ serine incorporation in oocytes, microinjected with hepatic polyribosomes isolated at various times after hormone treatment, is shown in Fig. 2. A near linear increase in the incorporation of $[^3\text{H}]$ serine by oocytes microinjected with polyribosomes isolated from the hormone-treated toads is evident. Enhanced incorporation increasing to approximately 100–110% above the control values at 72 hr after treatment was observed in four replicate experiments.

Wallace (18) and others have reported that the serine component of phosvitin represents about 55% of the total amino-acid composition. The sizable increase in the incorporation of $[^3\text{H}]$ serine into acid-insoluble protein in the microinjected oocyte (Fig. 2) strongly suggested the synthesis

FIG. 3. Synthesis by *Xenopus* oocytes of vitellogenic protein as a result of microinjection of hepatic polyribosomes obtained from estrogen-treated male toads. Oocytes (40 per group) were injected with 50–70 nl of polyribosomes (300 ng of RNA) isolated from the livers of male toads at 72 hr following treatment with either (A) propylene glycol or (B) estradiol-17 β . Oocyte proteins were chromatographed in TEAE-cellulose (15) and fractions of 10 ml each were collected. ●—●, Incorporation of [^{3} H]serine; —, absorbance at 280 nm.

de novo of phosvitin mediated by the exogenous mRNA. To examine this possibility, we used TEAE-cellulose chromatography to identify the radioactive proteins synthesized in the oocytes. *Xenopus* phosvitin and lipovitellin were added as carriers to the supernatant fraction, and co-chromatographed on the TEAE-cellulose column. The chromatograms (Fig. 3) are representative of five replicate experiments, and show that the carrier proteins, lipovitellin and phosvitin, eluted in fractions 35 (pH 9.5) and 85 (pH 8.6), respectively. The identification of the two proteins according to their separation in TEAE-cellulose is in excellent agreement with the chromatograms reported by Wallace (16). The TEAE-cellulose chromatogram of Fig. 3A shows the radioactivity profile obtained from oocytes microinjected with polyribosomes isolated from control toads. One

FIG. 4. Cellulose chromatogram of cytoplasmic poly(A)-rich RNA. Cytoplasmic RNA was dissolved in a minimal volume of elution buffer (10 mM Tris, pH 7.6, 500 mM KCl, 0.2 mM MgCl $_2$), and layered on a cellulose column (Sigma cell type 38) previously equilibrated with the same buffer. The column was washed with a high salt buffer until the absorbance at 260 nm had dropped to background, and the poly(A)-rich RNA was finally eluted with H $_2$ O. The poly(A)-rich RNA was consistently determined to be 1.0–1.15% of the total cytoplasmic RNA.

FIG. 5. Specific activity of hepatic poly(A)-rich RNA, as a function of time after estrogen administration in the male toad. Male toads treated with hormone at the indicated times received during the final hour of hormone treatment 1 mCi of carrier-free ^{32} P, and the poly(A)-rich RNA was isolated as described under *Materials and Methods*. The hatched and open bars represent the percent of increase in the specific activity above control values for total cytoplasmic RNA and poly(A)-rich RNA, respectively. ●, Specific activity of poly(A)-rich RNA; ○, specific activity of cytoplasmic RNA.

small peak of radioactivity was found to elute in fraction 20. No appreciable radioactivity was observed, however, in either the lipovitellin or phosvitin region of the chromatogram. In contrast, Fig. 3B shows the radioactivity profiles obtained from oocytes microinjected with hepatic polyribosomes obtained from male toads at 72 hr after treatment with estrogen. Two major peaks of radioactivity are evident: one peak eluting at the position of lipovitellin, and the other at that of phosvitin. In addition to these two major peaks of radioactivity, unidentified peaks eluting in fractions 45, 55, and 65 were also detected.

To study further estrogen-induced synthesis of mRNA in the liver of the male toad, we isolated poly(A)-rich RNA from the cytoplasmic RNA. Fig. 4 shows a cellulose chromatogram of cytoplasmic RNA isolated from *Xenopus* liver. The fractions eluting between tubes 60–68 were designated poly(A)-rich RNA (13). The total recovery of the RNA loaded onto the column was 92%. Since the preceding experiments indicated that estrogen treatment caused an enrichment of the mRNA population of polyribosomes, an increase in the specific activity of the poly(A)-rich RNA fraction of cytoplasmic RNA was also expected. The changes in the specific activities of both the poly(A)-rich RNA and cytoplasmic RNA labeled with ^{32} P at various times after estrogen treatment are shown in Fig. 5. A rapid increase in the specific activity of the poly(A)-rich RNA occurring between 6 and 9 hr after hormone treatment is evident. Between 9 and 24 hr of hormone treatment, the specific activity of this RNA increased approximately 13-fold above the control value observed at 24 hr. In contrast, the specific activity of total cytoplasmic RNA was increased about 180% at 24 hr after hormone treatment. No change in the acid-soluble radioactivity was observed. From these data, it is evident that the hormone caused an increased incorporation of ^{32} P into cytoplasmic RNA, with the enhanced specific activity occurring to a much greater extent in the poly(A)-rich RNA.

FIG. 6. Immunoprecipitation of lipovitellin isolated from *Xenopus* oocytes microinjected with hepatic polyribosomes, obtained at the indicated times after estrogen treatment. For immunoprecipitation, oocytes were incubated in the presence of 50 $\mu\text{Ci}/\text{ml}$ of medium of a ^{14}C -labeled amino-acid mixture. For determination of the ratio of ^3H to ^{14}C , oocytes were incubated in medium containing 10 $\mu\text{Ci}/\text{ml}$ of ^3H serine and 50 $\mu\text{Ci}/\text{ml}$ of ^{14}C -labeled amino-acid mixture. Immunoprecipitations were performed under conditions of antibody excess. ●, Immunoprecipitable lipovitellin radioactivity; O, ratio of the specific activity of ^3H serine and ^{14}C -labeled amino acids.

In experiments employing specific antiserum for lipovitellin, we found no immunoprecipitable, radioactive lipovitellin in oocytes microinjected with hepatic polyribosomes isolated at 9 or fewer hr of hormone treatment (Fig. 6). However, a marked synthesis of immunoprecipitable, radioactive lipovitellin occurred in the oocytes microinjected with polyribosomes isolated at 12 hr after treatment. Unlike lipovitellin, amphibian phosvitin is not antigenic, and thus a radioimmunoassay for phosvitin was not possible. We examined the appearance of phosvitin in these microinjected oocytes by double labeling with ^3H serine and ^{14}C amino acids. Fig. 6 also shows that the ratio of ^3H to ^{14}C was essentially invariant in oocytes microinjected with polyribosomes isolated at 1, 3, and 6 hr after hormone treatment, although total incorporation was increased at 3 and 6 hr (compare Fig. 2). At 9 and 12 hr after hormone treatment, however, the ratio of ^3H to ^{14}C was significantly enhanced in oocytes microinjected with the isolated polyribosomes. We consider this good evidence for phosvitin synthesis. The differences between the lag periods observed for phosvitin and lipovitellin synthesis, on the other hand, are interpreted as being more apparent than real, and as being a consequence of the different sensitivities of the two assays employed.

To determine if the poly(A)-rich RNA isolated from hepatic cytoplasmic RNA was enriched with mRNA as a result of hormone treatment, 9 ng of poly(A)-rich RNA in 50 nl of injection buffer was microinjected into oocytes. These oocytes were incubated as described for oocytes microinjected with polyribosomes (see Fig. 2). Fig. 7 shows that incorporation of ^3H serine was increased 135% above controls at 24 hr after treatment. We also repeated the microinjection experiments described in Fig. 2 under conditions designed to compare the mRNA activity of polyribosomes with that of the poly(A)-rich RNA. Here, we assumed that 1% of the total polyribosomal RNA is mRNA and that 100% of the

FIG. 7. Incorporation of ^3H serine in *Xenopus* oocytes microinjected with hepatic poly(A)-rich RNA or polyribosomes, isolated at various times after administration of estrogen to male toads. Oocytes were microinjected with 50 nl containing either 9 ng of poly(A)-rich RNA or 900 μg of polyribosomes. ●, Oocytes microinjected with poly(A)-rich RNA; ○, oocytes microinjected with polyribosomes.

poly(A)-rich RNA is mRNA. In all of our experiments, hepatic poly(A)-rich RNA was consistently 1.0–1.15% of the total cytoplasmic RNA (see Fig. 4). On this basis, we adjusted the concentration of the polyribosomes so that 50 nl of the injection buffer contained 900 ng of polyribosomal RNA. Thus, Fig. 7 also shows that when both types of mRNA preparations are adjusted to an equivalent content of mRNA and subsequently microinjected into oocytes obtained from the same female toad, then essentially the same degree of ^3H serine incorporation is observed. These experiments show that both types of mRNA preparations are translated with the same efficiency in the oocyte system when compared on a basis of mRNA content rather than total RNA microinjected.

DISCUSSION

We have taken advantage of the fact that the *Xenopus* oocyte, although rich in lipovitellin and phosvitin, does not synthesize these yolk proteins. These proteins are formed as vitellogenin in the liver, secreted into the blood, and then sequestered by developing oocytes (19, 20). After microinjections of mRNA preparations obtained from the livers of estrogen-treated male toads, oocytes incubated in the presence of ^3H serine actively synthesize lipovitellin and phosvitin. The amounts of exogenous mRNA (3–9 ng) microinjected into the oocytes in our experiments are far below saturation levels (K. D. Lanclos and T. H. Hamilton, unpublished work). Thus the increase in the incorporation of ^3H serine by oocytes, described in Figs. 2, 3, 6, and 7 as a result of microinjections of preparations containing mRNA, indicates the appearance of new as well as more mRNAs in the cytoplasm of the male liver as a consequence of hormone treatment.

The marked rise in the specific activity of hepatic poly(A)-rich RNA between 6 and 9 hr after hormone treatment (Fig. 5) is in good agreement with the induction of lipovitellin *in vivo* described by Wittliff and Kenney (5, 6). Their observation that treatment *in vivo* with actinomycin D during the first 6 hr of estrogen action abolishes induction of lipovitellin

synthesis is also consistent with the observations and conclusions we now report.

Of particular interest in our investigation of the action of estrogen in the male *Xenopus* liver is the lag period of 6–9 hr following a single injection of hormone before the onset of synthesis of yolk protein (Fig. 6). This lag may represent the time required for an obligatory synthesis of certain regulatory proteins. There is now good evidence for the induction of specific cytoplasmic and nonhistone chromosomal proteins in the rat uterus at very early times following estrogen administration (21–24). It has been suggested, but not proven, that these proteins are involved in gene activation. Also, there is now evidence that the male *Xenopus* liver contains receptor proteins for estrogen (25), and that these are present in very small quantities (J. K. Skipper, K. D. Lanclos, and T. H. Hamilton, unpublished work). Gorski *et al.* (26) have shown that during the first 6 hr of estrogen action in the rat uterus there is an induced synthesis of the estrogen receptor. Hence, a preceding synthesis of receptor molecules for estrogen might also account for the protracted response of the toad liver to the hormonal stimulus.

In conclusion, our laboratory is particularly interested in the synthesis of regulatory or other proteins that precede the vitellogenic response in the amphibian liver and the estrogenic response in the mammalian uterus. Because regulatory proteins generally occur and function at extremely low concentrations within the cell, the amount of mRNA encoded for such proteins would probably be extremely small in relation to the total cellular concentration of mRNA. The oocyte system should be extremely advantageous in experiments of this nature because of its high sensitivity as a result of the repeated translations of the microinjected RNA. Commonly used cell-free systems lack this capacity for repetition of translation. We believe that the amphibian oocyte provides an excellent translation system ("the egg as a test tube") for assaying minute quantities of specific mRNA molecules that appear in the nucleus as well as the cytoplasm at early times following hormone treatment. An elucidation of the nature and amounts of the regulatory and other proteins appearing early in the action of estrogen in the liver of the male amphibian should provide valuable information concerning the molecular mode of action of the hormone with respect to specific gene activation.

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