Proc. Natl. Acad. Sci. USA
Vol. 74, No. 6, pp. 2384–2388, June 1977
Biochemistry

Regulation by estrogen of the vitellogenin gene

(estradiol-17β action/dose response/membrane-bound polysomes/cell-free translation/mRNA)

JAMES K. SKIPPER AND TERRELL H. HAMILTON

Department of Zoology, The University of Texas, Austin, Texas 78712

Communicated by Howard Bern, April 8, 1977

ABSTRACT **The vitellogenin gene is inactive in the liver of male *Xenopus laevis*, unless exogenous estrogen is administered. We have previously shown that conventional doses of estradiol-17β result in the appearance of new hepatic messenger RNAs, some of which are encoded for vitellogenin. We now report that much higher doses of the hormone (2 mg/frog per day for 4 days) are required to elicit maximal responses. The relative levels of membrane-bound polysomes and vitellogenin mRNA were determined as a function of time and dose of hormone. Translation of total polysomal RNA in a cell-free system derived from wheat germ was used to estimate the relative levels of vitellogenin messenger RNA. Faithful translation of this messenger RNA was indicated by two lines of evidence: labeled cell-free products were immunoprecipitated with antivitellogenin antibody, and the migration of the major labeled product in sodium dodecyl sulfate/acrylamide gels was identical to that of native vitellogenin. Our results establish conditions for maximal estrogen-induced responses in this system, and are compatible with the hypothesis that a major regulatory mechanism of steroid hormones in the control of protein synthesis is that of gene activation and regulation of messenger RNA levels.**

Investigations of a variety of experimental systems used to study the molecular mode of action of sex steroid hormones have led some investigators to conclude that a major mechanism for controlling protein synthesis is the hormone-dependent regulation of specific mRNAs (1–4). Definitive results relevant to this problem have been obtained from work with target tissues in which the steroid hormone evokes the synthesis of a specific protein in large quantities. The male African clawed frog (*Xenopus laevis*), lacking endogenous estrogen, does not synthesize vitellogenin. However, treatment of male frogs with exogenous hormone evokes a prodigious and prolonged synthesis of vitellogenin, the precursor of the yolk proteins. This response of the male liver offers a valuable experimental system for the study of hormonal activation of a specific gene.

These considerations have prompted us (4) and others (5–7) to exploit this system (for recent review, see ref. 8). We recently demonstrated that estrogen administered to the male *Xenopus* caused the appearance of new mRNA in the liver (4). This mRNA, microinjected into living *Xenopus* oocytes, directed the synthesis *de novo* of the yolk proteins. Our observation has been confirmed and extended by Berridge and Lane (9), who also demonstrated that the primary translational product of this estrogen-induced mRNA is vitellogenin. This precursor is processed within the oocyte into the individual yolk proteins, lipovitellin and phosvitin (10).

In general, the isolation and fractionation of polysomes have been useful intermediate steps in the purification of various species of mRNAs (11, 12); hence, a major aim of the work now reported was to determine the temporal influence of exogenous estrogen on the relative abundance of polysomes in hepatic tissue of male *Xenopus*. Because none of the previous reports dealing with dose responses *in vivo* demonstrated maximal vitellogenic responses (13–15), we have also determined the effects of large doses of estrogen on the relative levels of polysomes and vitellogenin mRNA. Finally, we present evidence that estrogen-induced mRNA obtained from the liver of male *Xenopus* is faithfully translated in a wheat germ system as assessed by specific immunoprecipitation coupled with electrophoresis of the translational products in sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels.

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Adult *Xenopus* males were obtained from the South African Snake Farm (Fish Hoek, Cape Province, South Africa), and maintained at 19° in running, charcoal-filtered tap water (4). Frogs were fed beef liver thrice weekly. For primary stimulation, frogs received 2 mg of estradiol-17β in 0.2 ml of propylene glycol. Some frogs received only one injection; others received several injections, administered as one per day for the indicated number of consecutive days. For secondary stimulation, frogs thus treated were given a single injection after the primary response had subsided, 30 or more days after the initial injection(s). All male frogs weighed 48–54 g.

Vitellogenin labeled *in vivo* was prepared by injecting male frogs with estradiol-17β and then 250 μC of a mixture of 15 ^{14}C-labeled amino acids (New England Nuclear). The hormone and isotope were given at 4 days and 6 hr, respectively, before the blood serum was collected. Vitellogenin was then isolated from serum by dimethylformamide precipitation (16), TEAE-cellulose chromatography (17), and, finally, gel filtration through "Ultragel" AcA-22 (LKB Instruments, Inc.). The final preparation showed no heterogeneity upon electrophoresis in 5% or 10% $NaDodSO_4$/polyacrylamide gels. The S-30 fraction of wheat germ was prepared as described by Roberts and Paterson (18), with the pre-incubation step omitted.

Methods for the preparation and analysis of hepatic polysomes from *Xenopus* are similar to those described by others (19, 20). The details of our methods will be published elsewhere.* Total RNA was extracted from purified polysomes as described by Kates (21), and washed three times in LiCl (22).

For cell-free protein synthesis, the reaction mixture contained in a final volume of 0.5 ml: 200 μl of wheat germ extract (S-30 fraction); *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)–KOH, pH 7.6, 20 mM; dithiothreitol, 2 mM; ATP, 1 mM; GTP, 20 μM; phosphocreatine, 8 mM; KCl, 125 mM; magnesium acetate, 2.5 mM; creatine kinase, 50 μg; each of the 20 amino acids except methionine, 20 μM; [^{35}S]methionine, 24 nM (168–300 Ci/mmol, New England Nuclear); and various amounts of RNA preparations as indicated in the relevant figures. Incubation conditions employed were 25° for 90 min. To determine total incorporation, duplicate 20-μl aliquots of the reaction mixture were processed by the filter-paper disk method of Mans and Novelli (23). Reactions were terminated by addition of pancreatic RNase before immunoassay. To measure vitellogenin synthesized in the wheat germ system, duplicate

* J. K. Skipper and T. H. Hamilton, unpublished data.

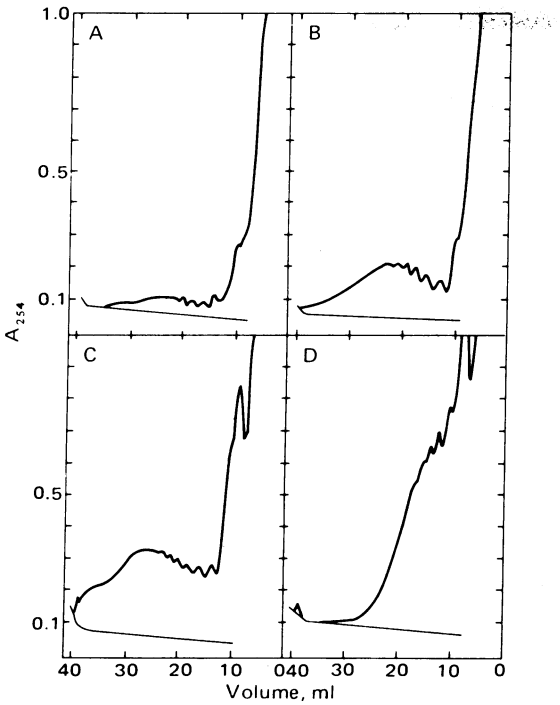

FIG. 1. Changes in the sedimentation profiles of hepatic polysomes of male *Xenopus* at various times after treatment with estradiol-17β. Crude subcellular fractions containing total polysomes were prepared from the pooled livers of two to four animals per group after the indicated treatments. Aliquots derived from 50 mg of tissue were layered on 7–47% linear sucrose gradients for analysis.* The direction of sedimentation is from right to left. Control animals (*A*) received no hormone. All experimental animals received four consecutive daily injections (2 mg/day), and thereafter at the indicated times their hepatic polysomes were obtained for analysis: (*B*) 4 days, (*C*) 8 days, and (*D*) 30 days.

200-μl aliquots of the reaction mixture were added to monospecific antivitellogenin antibody (24) for immunoprecipitation. This assay was performed by mixing: 200 μl of wheat germ reaction mixture; 40–45 μg of carrier vitellogenin in phosphate-buffered saline (PBS) (45 mM KH_2PO_4; 25.6 mM NaOH; 150 mM NaCl; 0.7 mM NaN_3) and 200 μl of antibody capable of precipitating 60 μg of vitellogenin. This mixture was incubated at room temperature for 30 min and then at 5° overnight. Immunoprecipitates were collected by centrifugation at 1200 × *g* for 20 min, washed once in 2 ml of PBS buffer (containing 1% Triton X-100, wt/vol, and 20 mM methionine), and washed twice in 2 ml of PBS. These conditions resulted in immunoprecipitates containing more than 93% of the labeled antigen. The precipitates were dissolved in 100 μl of a "$NaDodSO_4$ sample buffer" (25) by heating for 5 min in a boiling-water bath. Duplicate aliquots of this sample were counted in Aquasol scintillator (New England Nuclear), and 50-μl aliquots of the sample were layered on 0.1% $NaDodSO_4$/5% polyacrylamide gels for electrophoresis (25). After electrophoresis, the gels were sliced (1 mm), digested in 1 ml of NCS solubilizer (Amersham/Searle) for 24 hr at 50°, and assayed for radioactivity in 10 ml of scintillation fluid [6 g of 2,5-diphenyloxazole (PPO) and 75 mg of [1,4-bis[2(5-phenyloxazolyl)]benzene (POPOP) per liter of toluene] using a Beckman LS-250 scintillation counter. The counting efficiency for ^{35}S was 81% and for ^{14}C was 86%.

Triplicate aliquots of subcellular fractions were used for RNA determinations, as described by Fleck and Munro (26). Purified RNA was estimated from absorbance measurements by assuming that 1 mg of RNA dissolved in 1 ml yields an A_{260} of 20.

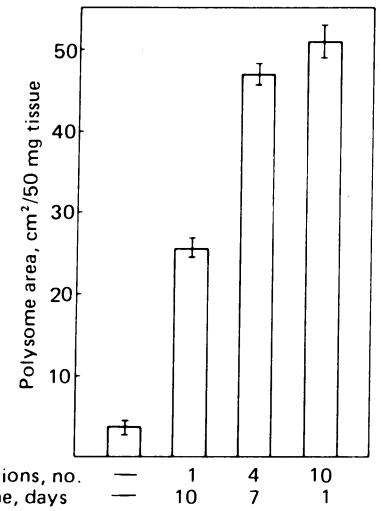

FIG. 2. Changes in the content of hepatic polysomes of male *Xenopus*, as a function of number of doses of estradiol-17β administered. Frogs received the indicated number of consecutive daily doses of hormone (2 mg/day), and after the indicated number of days following the last injection their total hepatic polysomes were analyzed as described in Fig. 1. The total number of days for hormonal response was 11 days for each group. The polysomal area of the gradients (dimers and larger aggregates) was measured by use of a planimeter.* Each bar designates the mean and SD of four experiments using four frogs per group.

For electron microscopy, hepatic samples were fixed for 12 hr at 4° in a solution containing 3% glutaraldehyde, 1.5% acrolein, 1% paraformaldehyde (all wt/vol), and 50 mM sodium cacodylate (pH 7.2). Samples were again fixed for 2 hr at 4° in 2% osmium tetroxide/25 mM sodium cacodylate (pH 7.2), and stained *en bloc* in 0.5% uranyl acetate for 24 hr at 4°. After dehydration, samples were embedded in an Epon/Araldite mixture (27) and stained in 0.5% uranyl acetate followed by lead citrate (28). Thin sections were photographed using a Hitachi HU-11E electron microscope operated at 50 kV.

RESULTS

Fig. 1 shows typical sucrose gradient profiles of total polysomes obtained from hepatic tissue of control and estrogen-treated male frogs at various times after treatment. Because all the polysome samples were obtained from equivalent amounts of tissue, the differences in area under the polysome region of the absorbancy profiles represent changes in polysome content per unit of tissue weight. Such data also provide qualitative information on the relative content of the various size classes of polysomes. Hepatic tissue of untreated frogs contained relatively few polysomes, whereas hormone treatment resulted in a substantial increase of the polysomes within 4 days after multiple injections of estrogen. At 8 days, when vitellogenin synthesis is near maximal, the level of polysomes had further increased, and a large size class of polysomes appeared in the lower third of the gradient. At 30 days, when vitellogenin synthesis has ceased, the level of polysomes had decreased concomitant with a marked disappearance of the larger size classes. We have obtained similar results when purified polysomes were analyzed from equal amounts of tissue taken from control and estrogen-treated frogs.*

To determine the dose(s) of estradiol-17β required to evoke maximum levels of hepatic polysomes, several groups of frogs were given different numbers of primary daily injections. Subcellular fractions containing total polysomes were then examined on sucrose gradients. Area measurements were made of the polysome regions and are shown in Fig. 2. A near maximal response occurred after four daily injections; 10 daily in-

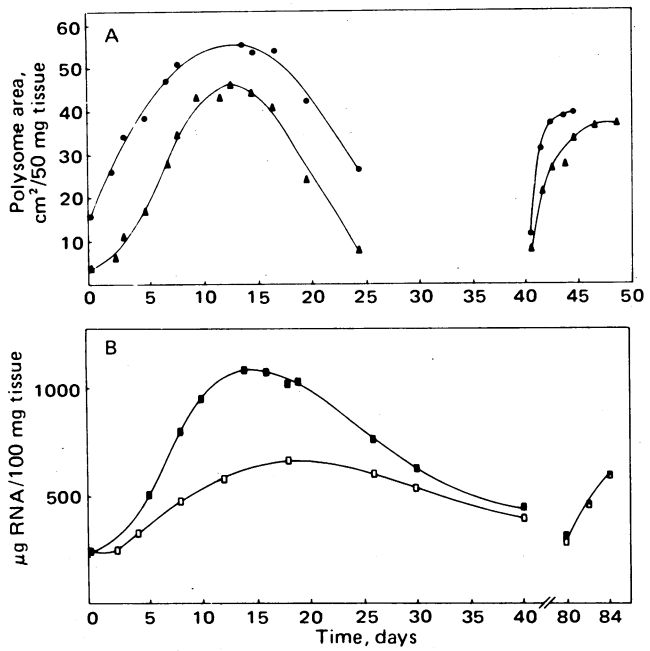

FIG. 3. Changes in the content of hepatic polysomes (*A*) and whole-tissue RNA (*B*) in male *Xenopus* as a function of time after primary and secondary treatment with estradiol-17β. For primary treatment, all frogs were given as indicated either one or four consecutive daily doses (2 mg per dose) of the hormone. For secondary treatment, at 40 (*A*) or 80 (*B*) days, only a single dose of the hormone was given. Analysis of polysomes and determination of whole-tissue RNA were as described under *Materials and Methods* and in Fig. 1. ●, total polysomes; ▲, membrane-bound polysomes; ■, frogs given seven primary injections; □, frogs given only one primary injection.

jections were necessary to elevate polysome levels to a maximum. No further increase in polysome levels was observed after 11 to 15 daily injections.

Fig. 3*A* charts the temporal changes in levels of total and membrane-bound polysomes after four daily injections of estrogen. Initially, free polysomes were present in larger amounts than membrane-bound polysomes. At 12–15 days, the membrane-bound polysomes had increased about 13-fold per unit of wet weight of tissue. This fraction constituted from 80 to 82% of the total polysomes. After 15 days, a steady decline in the level of membrane-bound polysomes was observed. At 25 days, this fraction was near its control level; the fraction of total polysomes, however, remained significantly above control. By 40 days, both fractions had returned to control levels. After administration of a secondary injection at 40 days, both fractions of polysomes increased more rapidly than observed during primary stimulation.

Fig. 3*B* depicts the effects of both one and four primary injections of estrogen on the accumulation of whole-tissue RNA as a function of time after treatment. Multiple injections resulted in a greater accumulation of RNA than a single injection. The peak response of RNA levels coincided with the maximum accumulation of polysomes observed in Fig. 3*A*. The decrease in RNA levels after 15 days, however, was less pronounced than that of polysomal content. Again, a secondary injection evoked a more rapid accumulation of RNA than was observed during primary stimulation.

Electron micrographs of hepatic parenchymal cells (Fig. 4) also illustrate the pronounced accumulation of estrogen-induced membrane-bound polysomes or rough endoplasmic reticulum. Another notable change in the ultrastructure of these cells was the appearance of numerous Golgi complexes, presumably engaged in the posttranslational modification and processing of vitellogenin.

For translation in a cell-free protein-synthesizing system, total polysomal RNA was extracted from purified hepatic polyribosomes obtained from control or estrogen-treated frogs. In preliminary work, we found that the total incorporation into acid-precipitable products was variable as well as reduced at standard concentrations of KCl (60–70 mM). When the final

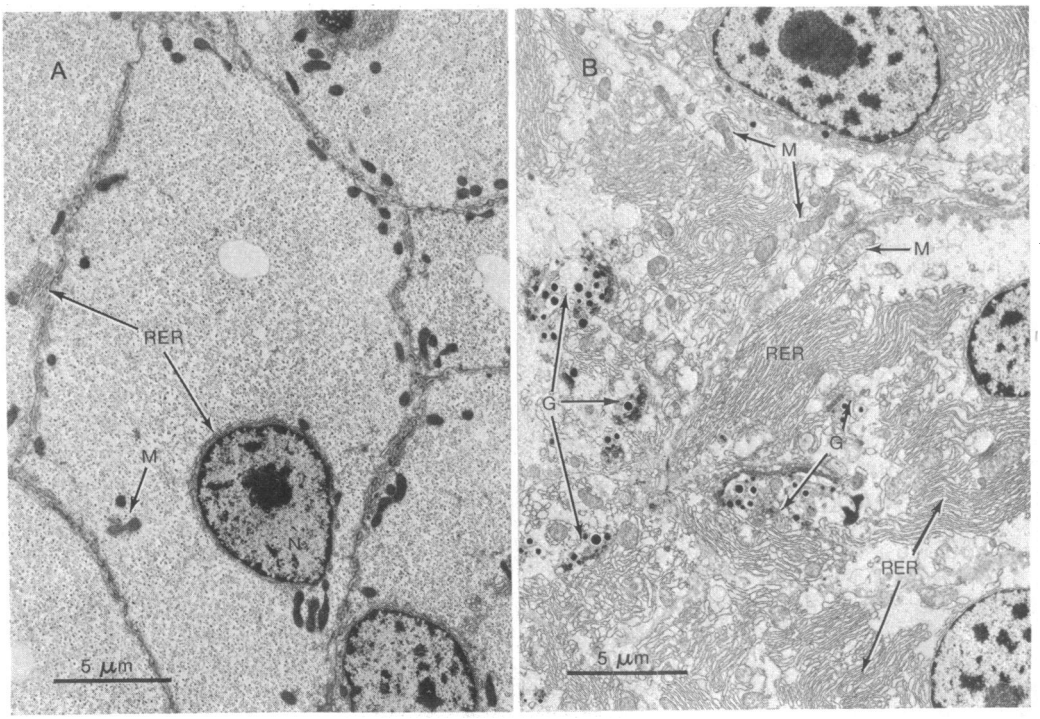

FIG. 4. Effect of estradiol-17β on the ultrastructure of hepatic cells of male *Xenopus*. Control frogs (*A*) were given four daily injections of propylene glycol, and experimentals (*B*) received four daily injections of the hormone (2 mg per dose). Eight days later the livers of both groups were taken for fixation and processing. Abbreviations: N, nucleus; M, mitochondrion; RER, rough endoplasmic reticulum; and G, Golgi complex.

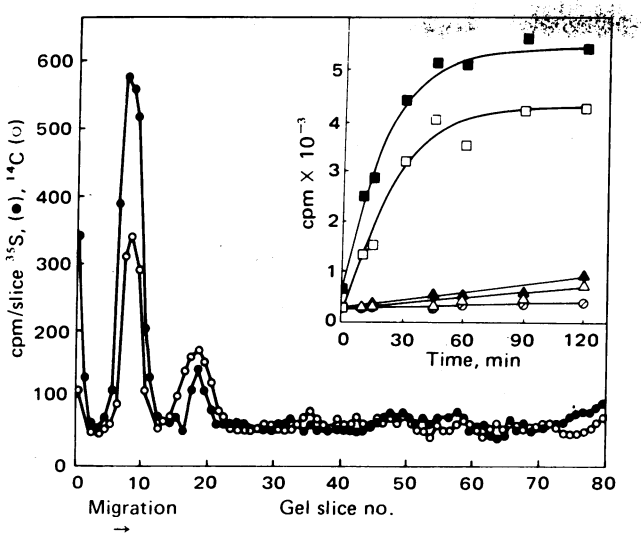

FIG. 5. Electrophoresis of labeled products in $NaDodSO_4$/polyacrylamide gels and the kinetics of incorporation (*Inset*) of [^{35}S]methionine in the wheat germ system. Preparations of polysomal RNA were obtained from *Xenopus* males 8 days after seven injections of estradiol-17β or propylene glycol, and were designated as induced or control RNA, respectively. Labeled immunoprecipitates obtained from the wheat germ system (^{35}S) and labeled vitellogenin of male serum (^{14}C) were subjected to electrophoresis in 0.1% $NaDodSO_4$/5% polyacrylamide gels. Count profiles were plotted without subtracting background counts. RNA (40 mg) was added to the wheat germ system, and incorporation was measured at the times indicated thereafter. For other details, see *Materials and Methods*. ■, total incorporation after addition of induced RNA; □, immunoprecipitable incorporation after addition of induced RNA; ▲, total incorporation with no RNA added; △, immunoprecipitable incorporation with control RNA; and ⊘, total incorporation with induced RNA and 5 μg of RNase.

concentration of KCl was increased to 125 mM, the levels of incorporation of [^{35}S]methionine into polypeptide products were increased 30–40%. The *inset* of Fig. 5 shows the kinetics of incorporation into both acid-insoluble material and immunoprecipitable products. Incorporation was rapid for the first 30 min and slowed thereafter to 90 min. From 90 to 120 min no further incorporation was observed. Incorporation was dependent on exogenous RNA, and completely sensitive to RNase. A very low level of endogenous background incorporation was evident in the wheat germ system. No significant incorporation of [^{35}S]methionine into immunoprecipitable products was observed upon addition of RNA from hepatic polysomes obtained from untreated frogs; however, 70–77% of the total incorporation was immunoprecipitable by specific antivitellogenin antibody upon addition of polysomal RNA isolated from polysomes taken from frogs at the peak response following multiple estrogen injections.

To further identify the labeled, immunoprecipitable products of the cell-free system, the antigen–antibody pellets obtained after synthesis *in vitro* and from native vitellogenin labeled *in vivo* were subjected to electrophoresis in 0.1% $NaDodSO_4$/5% acrylamide gels. Fig. 5 shows that the major labeled peptide contained in the precipitates from the cell-free system migrated to the same position as did native vitellogenin precipitated from the serum of estrogen-treated males. Both precipitates contained a minor component that migrated in front of the major peptide product. Because native vitellogenin has only one component (10), we think that the minor component of both precipitates probably resulted from protease contamination in the antibody preparation. Other minor, labeled peaks can be seen throughout the gels. Determinations of the molecular weight for vitellogenin in the gel system routinely gave values ranging from 185,000 to 210,000.

Table 1 shows the activity of various RNA preparations in the wheat germ system. Total polysomal RNA obtained from the livers of either rats or control frogs did not direct incorporation of [^{35}S]methionine into immunoprecipitable vitellogenin in the cell-free system. In marked contrast, polysomal RNA obtained from estrogen-treated frogs directed pronounced incorporation into vitellogenin. The content of vitelloginin mRNA (per unit of RNA) appears to be approximately equal in RNA preparation from frogs given either one or four daily injections. However, because four daily injections resulted in more polysomal RNA per unit of tissue weight (Fig. 3), the level of vitellogenin mRNA is clearly higher on the basis of weight of tissue in animals given four daily injections.

DISCUSSION

Hormonal stimulation of protein synthesis in many tissues is characterized by an acceleration of ribosome formation, resulting in a net increase in the cytoplasmic concentration of ribosomes (29, 30). Also, an increased and concomitant proliferation of endoplasmic reticulum occurs in tissues that synthesize large quantities of hormone-induced protein destined for export (31, 32). Considerable evidence indicates that secretory proteins are synthesized on membrane-bound polysomes (33, 34). Our data demonstrate a massive and prolonged proliferation of membrane-bound polysomes or rough endoplasmic reticulum in male *Xenopus* liver, as a function of time and dose of hormone. These changes paralleled the known temporal changes in the rate of synthesis and secretion of vi-

Table 1. Analysis of translation of polysomal RNA in the wheat germ system

Source of hepatic RNA	Estrogen treatment: Daily injections	Estrogen treatment: Days after last injection	Incorporation into protein, cpm/50 μl: Total	Incorporation into protein, cpm/50 μl: Immunoprecipitated	Vitellogenin, %
—	—	—	150	0	0
Male rat	—	—	3875	155	4.0
Male *Xenopus*	—	—	4322	138	3.2
	1	4	5431	2335	43.0
	1	11	6702	4892	73.0
	4	1	6250	2812	45.0
	4	8	5915	4554	77.0
	4	12	6109	5009	82.0

All frogs received 2 mg/day of estradiol-17β for the indicated number of consecutive days, and on the designated day after the last of these injections total hepatic polysomal RNA was isolated as already described. Forty milligrams of induced or control RNA (see Fig. 5) was added to the cell-free system derived from wheat germ. The radioactivity values are averages of triplicate determinations of total incorporation and of incorporation precipitated by monospecific antivitellogenin antibody.

tellogenin (5, 13). The polysomes prepared and analyzed in our work were obtained from all the hepatic cell types. Of these, only the parenchymal cells are responsive to estrogen (15), constituting about 50% of the hepatic cell population; consequently, the hormone-induced polysomes of parenchymal cells were contaminated by polysomes from nonresponsive cells. Our measurements of changes in the levels of total and membrane-bound polysomes (Fig. 3A) are, therefore, minimal estimates of the changes in parenchymal cells.

An early report (13) indicated that single doses of estradiol-17β larger than 1 mg resulted in sporadic deaths and a high incidence of edematous frogs. We found these problems only if doses of 2 mg of the hormone were given for 10 or more consecutive days. These treatments, however, resulted in only 10–15% deaths. Because four injections of 2 mg/day increased levels of polysomes to near maximal values (Fig. 2) without killing the frogs, we employed this treatment in most of the experiments now reported. The requirement of chronic hormone treatment to achieve maximal vitellogenic responses in this system is probably related to the frog's high capacity to catabolize estrogen (J. K. Skipper, K. D. Lanclos, and T. H. Hamilton, unpublished data). The determination of the time and dose of hormone treatment required to elicit maximal responses (Fig. 2) is of practical significance to investigations aimed at the purification of vitellogenin mRNA or other hepatic regulatory molecules evoked by estrogen.

The data obtained by using the wheat germ system demonstrate the faithful translation of vitellogenin mRNA assessed by two criteria: immunoprecipitation of labeled peptide products by specific antibody and the comigration of the labeled immunoprecipitable products with native vitellogenin in $NaDodSO_4$/acrylamide gels (Fig. 5). Other laboratories have recently succeeded in translating vitellogenin mRNA in cell-free systems derived from reticulocytes (5, 9). An unusually large percentage of the total labeled peptide formed in the wheat germ system was incorporated into immunoprecipitable products. Because different mRNA species have different K^+ optima for translation in wheat germ systems (35), the various mRNA species present in the RNA preparations we tested are probably not translated at equivalent rates. We also recognize that the content of methionine in vitellogenin may be higher than that in the average liver protein. If so, a disproportionate labeling of vitellogenin with [^{35}S]methionine would have occurred; consequently, the percentage of label we observe in immunoprecipitable products is probably not equivalent to the percentage of vitellogenin mRNA present in a particular RNA sample. The vitellogenin mRNA content (per unit of RNA) appears to be approximately equal in RNA preparations from frogs given either one or four daily injections. However, because four daily injections resulted in more polysomal RNA per unit of tissue weight (Fig. 3), the level of vitellogenin mRNA is clearly higher on the basis of weight of tissue in animals given four daily injections.

In conclusion, our data indicate that estrogenic stimulation of male *Xenopus* liver increases the level of vitellogenin mRNA in concert with changes in the levels of total hepatic RNA, rough endoplasmic reticulum, and membrane-bound polysomes (compare Figs. 3, 4, and Table 1). Also, G. U. Ryffel and R. Weber (personal communication), using hybridization with cDNA for a more direct measure of vitellogenin mRNA, have obtained comparable estimates of the relative proportion of the mRNA in male *Xenopus* liver when hormone treatments comparable to ours were given.

We thank Hazel Clinton, Mary Schwarzbeck, and Jon Siiteri for excellent technical assistance. Stephen Farmer and J. R. Tata of the National Institute of Medical Research, London, kindly provided antivitellogenin antibody. Ruben Mitchell of the Cell Research Institute, University of Texas, prepared the electron micrographs. This work was supported by grants from the National Institute of Child Health and Human Development (HD-03803-07 and -08). J.K.S. was a recipient of a postdoctoral fellowship from the National Institute of Arthritis and Metabolic Disease (AM-05103).

The costs of publication of this article were defrayed in part by the payment of page charges from funds made available to support the research which is the subject of the article. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

1. O'Malley, B. W. & Means, A. R. (1974) *Science* **183**, 610–613.
2. Palmiter, R. D. & Smith, L. T. (1973) *Mol. Biol. Rep.* **1**, 129–134.
3. Schimke, R. T., Rhoads, R. E., Palacious, R. & Sullivan, D. (1973) *Karolinska Symp. Res. Methods Reprod. Endocrinol.* **6**, 357–375.
4. Lanclos, K. D. & Hamilton, T. H. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3934–3938.
5. Shapiro, D. T., Baker, H. J. & Stitt, D. T. (1976) *J. Biol. Chem.* **251**, 3105–3110.
6. Wahli, W., Wyler, T., Weber, R. & Ryffel, G. U. (1976) *Eur. J. Biochem.* **66**, 457–465.
7. Witliff, J. L. & Kenney, F. T. (1972) *Biochim. Biophys. Acta* **269**, 485–492.
8. Tata, J. R. (1973) *Cell* **9**, 1–14.
9. Berridge, M. V. & Lane, C. D. (1976) *Cell* **8**, 283–297.
10. Bergink, E. W. & Wallace, R. A. (1974) *J. Biol. Chem.* **249**, 2897–2903.
11. Heywood, S. M., Dowben, R. M. & Rich, A. (1967) *Proc. Natl. Acad. Sci. USA* **57**, 1002–1009.
12. Sarkar, S. & Makherjee, S. P. (1973) *Prep. Biochem.* **3**, 583–604.
13. Wallace, R. A. & Jared, D. W. (1968) *Can. J. Biochem.* **46**, 953–959.
14. Follett, B. K. & Redshaw, M. R. (1968) *J. Endocrinol.* **40**, 439–456.
15. Redshaw, M. R., Follett, B. K. & Nicholls, T. J. (1969) *J. Endocrinol.* **43**, 47–53.
16. Ansari, A. Q., Dolphin, P. J., Lazier, C. B., Munday, K. A. & Akhtar, M. (1971) *Biochem. J.* **122**, 107–113.
17. Wallace, R. A. (1965) *Anal. Biochem.* **11**, 297–311.
18. Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2330–2334.
19. Venkatesan, N. & Steele, W. J. (1972) *Biochim. Biophys. Acta* **287**, 526–537.
20. Blobel, G. & Potter, V. R. (1967) *J. Mol. Biol.* **28**, 539–542.
21. Kates, J. (1973) in *Methods in Cell Biology*, ed. Prescott, D. M. (Academic Press, New York), Vol. 7, p. 531.
22. Palmiter, R. D. (1974) *Biochemistry* **13**, 3606–3615.
23. Mans, R. J. & Novelli, G. D. (1961) *Arch. Biochem. Biophys.* **94**, 48–53.
24. Berridge, M. V., Farmer, S. R., Green, C. D., Henshaw, E. C. & Tata, J. R. (1976) *Eur. J. Biochem.* **62**, 161–171.
25. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412.
26. Fleck, A. & Munro, H. N. (1962) *Biochim. Biophys. Acta* **55**, 571–583.
27. Mollenhauer, H. H. (1964) *Stain Technol.* **39**, 111–114.
28. Reynolds, E. S. (1963) *J. Cell Biol.* **17**, 208–212.
29. Moore, R. J. & Hamilton, T. H. (1964) *Proc. Natl. Acad. Sci. USA* **52**, 439–446.
30. Tata, J. R. & Widnell, C. C. (1966) *Biochem. J.* **98**, 604–620.
31. Tata, J. R. (1970) *Biochem. J.* **116**, 617–360.
32. Palmiter, R. D., Christensen, A. K. & Schimke, R. T. (1970) *J. Biol. Chem.* **245**, 883–845.
33. Siekevitz, P. & Palade, G. E. (1960) *J. Biophysic. Biochem. Cytol.* **7**, 619–630.
34. Ganoza, M. C. & Williams, C. A. (1969) *Proc. Natl. Acad. Sci. USA* **63**, 1370–1376.
35. Efstratiadis, A. & Kafatos, F. C. (1976) *Eukaryotes at the Subcellular Level*, ed. Last, J. (Marcel Dekker, New York), pp. 1–124.