Proc. Nat. Acad. Sci USA
Vol. 72, No. 8, pp. 3172–3175, August 1975
Cell Biology

Synthesis of vitellogenin in cultures of male and female frog liver regulated by estradiol treatment *in vitro*

(*in vitro* steroid action/*Xenopus* liver/yolk protein synthesis)

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Communicated by F. H. C. Crick, May 27, 1975

ABSTRACT **Using the frog *Xenopus laevis*, we show that the addition of physiological concentrations of estradiol to cultures of liver from untreated males rapidly induces the synthesis of large amounts of vitellogenin. Sustained synthesis of vitellogenin requires continuous exposure to estradiol. A nonestrogenic steroid, dexamethasone, does not induce vitellogenin synthesis but does induce increased synthesis of a different protein in liver cultures.**

The major proteins of egg yolk are derived from a precursor protein, vitellogenin (1), which is synthesized in the liver of mature females of many egg-laying animals, secreted into the bloodstream, and incorporated by growing oocytes (2). *In vivo*, estradiol regulates vitellogenin synthesis in females, and injection of the hormone induces vitellogenin synthesis in male liver, which normally does not produce this protein (3, 4). The molecular mechanism of steroid hormone action within a target tissue is believed to involve the binding of the hormone to a specific cytoplasmic receptor, the molecular modification of the hormone-receptor complex, and the binding of this complex to an acceptor site in the nucleochromatin (5). In keeping with the model, proteins that bind estradiol tightly have been isolated from both the cytoplasm and nucleochromatin of amphibian and bird liver (6–8). Thus, the synthesis of vitellogenin by liver and its regulation by estrogens provides an attractive eukaryotic cell system in which to study the steps and mechanisms of gene control.

Although induction of vitellogenin synthesis in male liver *in vivo* is well documented (9), induction has not so far been demonstrated *in vitro*. This communication shows that under proper conditions administration of estradiol to cultures of male liver is sufficient to induce vitellogenin synthesis. Nonestrogenic steroids do not induce vitellogenin synthesis, but dexamethasone treatment of liver *in vitro* induces the synthesis of a different protein. Culture conditions were established that permit high levels of protein synthesis and radioactive labeling, and sensitive methods of sodium dodecyl sulfate ($NaDodSO_4$)/gel electrophoresis and fluorography were used to analyze radioactive proteins.

MATERIALS AND METHODS

Culture Media and Preparation of Liver Cultures. The complete culture medium was a 50% dilution of Eagle's minimal essential medium with Earle's salts (Gibco Bio-Cult, Glasgow, Scotland) and contained a final concentration of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 2 mM glutamine, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. A similar medium contained no methionine. Livers (0.5–2.5 g) were taken from small, sexually mature frogs (13–45 g) under sterile conditions into ice-cold complete culture medium, chopped with scissors, and rinsed thoroughly. About 60 mg of tissue (3–5 pieces) were placed in each well of a Linbro FB-16-24-TC culture dish and covered with 1 ml of complete culture medium. Cultures were incubated at 25° in air on a rocking table (4 cycles/min). The medium was changed daily. Estradiol-17β, progesterone, testosterone, and dexamethasone (all from Sigma) were added to 1-ml cultures in 5 μl of propylene glycol; control cultures received propylene glycol only.

Labeling of Liver Proteins with [^{35}S]Methionine. The liver pieces were rinsed twice in 1–2 ml of culture medium lacking methionine at 25° and were then incubated for 6 hr at 25° in 0.5 or 1 ml of medium containing 20 μCi/ml of [^{35}S]methionine (Amersham) at a suitable specific activity (see *Results*). After the cultures had been centrifuged at 2000 × *g* for 5 min at 4°, the medium was removed and frozen at −20°. The tissue was washed three times with 100 mM NH_4HCO_3, 5 mM $NaHSO_3$, 25 mM Tris·HCl; pH 7.0, and frozen. It was homogenized at 0° in 0.5 ml of the same buffer containing 0.1% 2-mercaptoethanol and 150 μg/ml of phenylmethylsulfonylfluoride, in a microchamber Sorvall Omnimixer at top speed for 1 min. After aliquots had been taken to measure incorporation of isotope, the homogenate was centrifuged at 15,000 × *g* for 20 min, at 4°, and the supernatant was analyzed by $NaDodSO_4$/gel electrophoresis. This centrifugation removed particulate material without altering the pattern of radioactive bands seen in whole homogenates.

$NaDodSO_4$/Gel Electrophoresis. Ninety microliters of each culture medium were boiled with 20 μl of 10% $NaDodSO_4$, 5% 2-mercaptoethanol, 50% glycerol, 0.005% bromophenol blue, and analyzed by electrophoresis on 7.2% acrylamide slab gels as described (10). One hundred microliters of each homogenate supernatant were lyophilized, boiled in 100 μl of 2% $NaDodSO_4$, 1% 2-mercaptoethanol, 0.001% bromophenol blue, 2 mM Na_2EDTA; pH 6.8, and electrophoresed on 9% acrylamide slab gels. After free radioactivity had been cut away at the dye front, gels were fixed for 1 hr in 45% methanol/7% acetic acid, and radioactive bands were located by fluorography (11) using hypersensitized film (12).

Preparation of Nonradioactive Vitellogenin. High blood levels of vitellogenin were induced in fully grown female

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; complete culture medium, 50% dilution of Eagle's minimal essential medium with Earle's salts.

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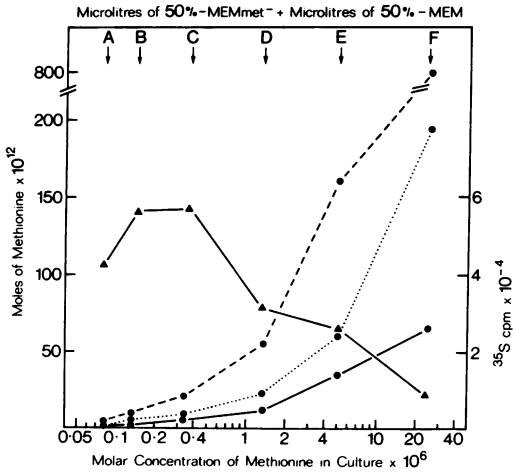

FIG. 1. Each culture of female liver contained 1 ml of medium prepared by mixing the volumes of culture medium lacking methionine (50%—MEMmet⁻) and complete culture medium (50%—MEM) indicated at the top of the figure (A, 1000 + 0; B, 999 + 1; C, 995 + 5; D, 975 + 25; E, 900 + 100; and F, 500 + 500), and was incubated with 20 μCi of [^{35}S]methionine (specific activity 250 Ci/mmol, Amersham) for 6 hr at 25°. Methionine uptake into tissue was determined from duplicate 20-μl aliquots of whole tissue homogenates dried on a GF/A filter (Whatman). Incorporation into protein was determined from duplicate aliquots, 100 μl for media and 20 μl for whole homogenates, precipitated with 10% trichloroacetic acid/5 mM methionine, incubated 15 min at 90°, collected and washed on filters, dried, and counted at 80% efficiency. Results expressed as moles of methionine ($\times 10^{12}$): (●- - -●) total uptake into whole tissue homogenates; (●···●) acid-insoluble incorporation into whole tissue proteins; (●—●) acid-insoluble incorporation into secreted proteins; (▲—▲) ^{35}S cpm ($\times 10^{-4}$) recovered as acid-insoluble secreted protein in 100-μl aliquots of media.

frogs by injecting 1 mg of estradiol-17β in 0.1 ml of propylene glycol into the dorsal lymph sac 6–18 days before killing. Vitellogenin was precipitated from the serum using dimethylformamide (13), dissolved in ice-cold 0.2 M NaCl, reprecipitated with dimethylformamide, and finally dissolved in 0.2 M NaCl and stored at −20°.

RESULTS

Choice of [^{35}S]methionine specific activity

When protein synthesis is monitored by incorporation of a radioactive amino acid, the concentration of nonradioactive molecules must be high enough to allow protein synthesis to proceed without diluting the isotopic precursor so much that incorporation of radioactivity becomes inconveniently low. Fig. 1 shows the effect of varying the methionine concentration on the uptake of methionine from the medium and its incorporation into protein synthesized by female liver during a 6-hr period. Uptake into the tissue and incorporation into both exported and nonexported proteins increase as the methionine concentration in the medium rises. On a linear scale, the level of acid-soluble methionine within the tissue increases in direct proportion to the methionine concentration in the medium. The number of acid-insoluble counts in the medium is limited in culture medium lacking methionine due to low rates of protein synthesis, and is low in methionine-rich medium due to dilution of the isotope. The results suggest that a mixture of 9 volumes of culture medium lacking methionine and 1 volume of complete culture medium (9 + 1 medium) composes a suitable medium for active protein synthesis and experimentally convenient levels of isotope incorporation.

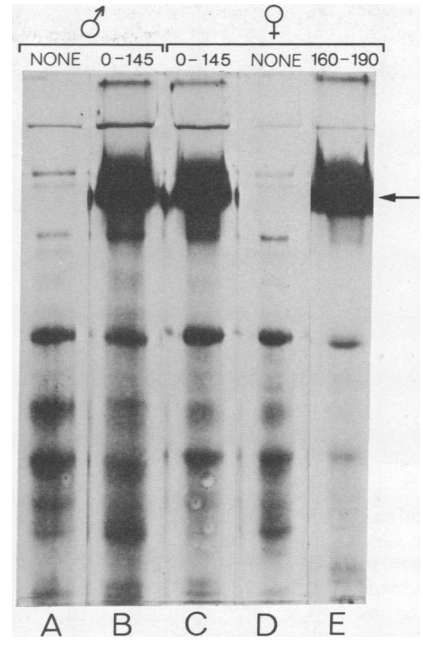

FIG. 2. Radioactive protein bands in samples of media analyzed on 7.2% acrylamide/NaDodSO$_4$ gel. Cultures A-D of male and female liver were maintained for 145 hr in complete culture medium without estradiol or with estradiol at 500 ng/ml (1.8×10^{-6} M). Culture E was maintained for 160 hr in complete culture medium without estradiol and then for 30 hr in complete culture medium with added hormone. Cultures were labeled in 9 + 1 Medium with or without added hormone as appropriate, using [^{35}S]methionine as described (Fig. 1). The *arrow* on right indicates the position of the vitellogenin band. (A and D) No estradiol; (B and C) estradiol present from 0–145 hr of culture; (E) estradiol present from 160–190 hr of culture.

Effect of plasma and serum

Experiments similar to those in Fig. 1 showed that neither 5% female *Xenopus* plasma nor 5% male human serum had any effect on the rate of protein synthesis in liver cultures. For this reason, and to avoid contamination with steroids present in plasma and serum, neither plasma nor serum was used in subsequent experiments. The level of protein synthesis was the same in cultures immediately after preparation and in cultures maintained in complete culture medium for at least 14 days provided the medium was changed daily. These results show that a simple medium is sufficient to establish and maintain short-term *Xenopus* liver cultures.

Induction of vitellogenin synthesis by estradiol-17β *in vitro*

Vitellogenin is secreted from liver cells soon after its synthesis (9, 14). We observe that tissue homogenates of labeled cultures contain little or no radioactive vitellogenin, even when large amounts are found in the medium. The fact that virtually all vitellogenin is exported greatly simplifies the detection and analysis of the protein.

Fig. 2 presents the pattern of radioactive proteins found in the media from cultures of male or female liver in the presence or absence of estradiol-17β. The results are typical of the effect of continuous hormone treatment *in vitro* for a period of several days. Male liver does not normally synthesize vitellogenin (track A), but is induced to do so by treat-

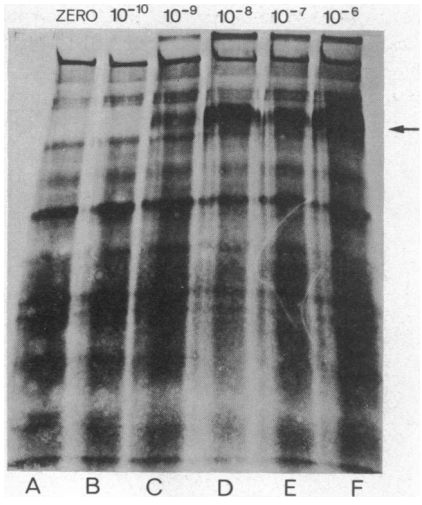

FIG. 3. Radioactive protein bands in samples of media analyzed on 7.2% acrylamide/NaDodSO$_4$ gel. Cultures of male liver were maintained for 8 days in culture medium without estradiol (A) or with added estradiol-17β (B–F) at concentrations of 0.05 ng/ml to 500 ng/ml (1.84×10^{-10} M to 1.84×10^{-6} M) in 10-fold steps. Cultures were radioactively labeled as in Fig. 2. The *arrow* on right indicates the position of the vitellogenin band. Molar concentration range of estradiol is shown at the top.

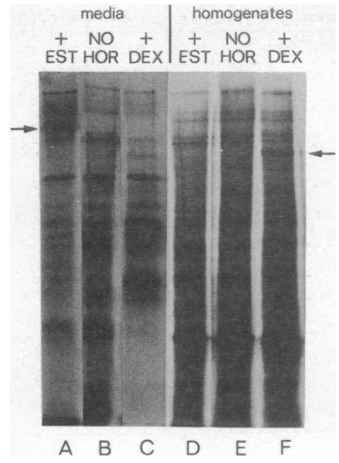

FIG. 4. Radioactive protein bands in samples of media and homogenate supernatants on 9% acrylamide/NaDodSO$_4$ gel. Cultures of male liver were maintained for 4 days in complete culture medium without added steroids. Incubation then continued for 10 days in complete culture medium without hormones (B and E), or with 2×10^{-7} M estradiol-17β (A and D), or with 2×10^{-7} M dexamethasone (C and F). Cultures were radioactively labeled as in Fig. 2. The *lefthand arrow* indicates the position of vitellogenin (molecular weight 200,000). A high level of synthesis of a different, predominantly nonsecreted, protein (approximate molecular weight 170,000) is induced by dexamethasone treatment *in vitro*, *arrow on right*.

ment with estradiol *in vitro* (track B). Other exported proteins characteristic of normal male liver continue to be synthesized and secreted in the presence of estradiol. Newly established cultures of female liver synthesize vitellogenin (2), and female liver cultured for several days in the presence of estradiol continues to synthesize large amounts of vitellogenin (track C) in addition to the other secreted proteins that exist in male liver cultures. In the absence of estradiol, vitellogenin synthesis in female liver cultures gradually declines, and by the sixth day without estradiol female liver no longer synthesizes vitellogenin (track D), although synthesis of the other secreted proteins continues. If estradiol is added to these non-vitellogenic female cultures, synthesis of vitellogenin resumes within 30 hr (track E). Subsequent experiments showed that pieces of male liver cultured without estradiol for a week are also induced to synthesize vitellogenin after hormone is added. As judged from the patterns of radioactive bands on NaDodSO$_4$ gels, vitellogenin is the only protein in either media or homogenates whose synthesis is induced by estradiol.

Approximately 10% (v/w) of freshly excised liver is due to trapped serum (2). Our culture media contain no serum or plasma. Thus, after 1 week in culture, with daily changes of the medium, all residual serum will probably have been washed away. The fact that both male and female liver, after 7 days in culture, can still respond to estradiol strongly suggests that no other factors are needed to induce synthesis of vitellogenin. We conclude that vitellogenin synthesis can be induced in male liver by treatment with estradiol *in vitro*, that sustained vitellogenin synthesis in both normal female and induced male liver requires the continued presence of estradiol, and that the hormone plus liver cells constitute a complete system for induction and control of vitellogenin synthesis.

Estradiol dose response and liver response to other hormones

Fig. 3 shows the response of male liver cultured for 8 days in the presence of different concentrations of estradiol-17β. There is no vitellogenin synthesis in the absence of estradiol (track A). Estradiol at 1.84×10^{-10} M does not induce vitellogenin synthesis (track B), but at a concentration of 1.84×10^{-9} M (track C) and at higher levels (tracks D–F), vitellogenin synthesis is induced. The lowest effective concentration of estradiol, about 2 nM, is similar to normal estrogen levels in the plasma of female vertebrates (15–17) and close to the minimum effective concentration of steroids known to induce protein synthesis in other cell systems (18, 19). These facts strongly suggest that estradiol-17β, rather than some minor contaminant, is the true inducing agent.

Estradiol-17β was also compared with progesterone, testosterone, and dexamethasone to see if other steroids would induce vitellogenin synthesis. Cultures of male liver were maintained for 4 days in complete culture medium to wash away endogenous hormones. The four steroids were then added to separate cultures at four concentrations, from 2×10^{-10} M to 2×10^{-7} M, in 10-fold steps. Incubation continued for 10 days, and every 3 days cultures were radioactively labeled and samples of media and homogenates were analyzed. The only cultures that made vitellogenin were those containing 2×10^{-9} M to 2×10^{-7} M estradiol-17β. No vitellogenin was found in either media or homogenates of cultures incubated with the other steroids. The *in vitro* specificity of the liver response agrees with estrogen dependence of the *in vivo* response (20), emphasizing the normal function of this *in vitro* system.

Although nonestrogenic steroids do not induce the synthesis of vitellogenin, do they induce increased synthesis of any other protein? Fig. 4 compares the radioactive proteins in the media and homogenates of control cultures and cultures treated *in vitro* for 10 days with either estradiol or dexamethasone. As in previous experiments, vitellogenin is present in the medium of the estradiol-treated culture only. Synthesis of a different protein is induced by dexamethasone. This second protein has a lower molecular weight than vitellogenin and is found predominantly in the tissue ho-

mogenate. Increased synthesis of this protein is induced by dexamethasone within hours at concentrations of 2×10^{-9} M or higher and not by the other steroids tested. The identity of the protein remains to be determined.

Identity of the protein induced by estradiol

The protein synthesized by liver cultures in response to estradiol is identified as vitellogenin for the following reasons. (*i*) It is an exported protein synthesized in female liver but not normally in male liver. (*ii*) Synthesis in either case depends only on the addition of estradiol at physiological concentrations. (*iii*) On $NaDodSO_4$ gels it has a molecular weight of 200,000 and migrates with vitellogenin extracted from female serum. (*iv*) It can be precipitated with dimethylformamide. (*v*) It was found to be the only protein exported from induced male cultures that incorporates [^{32}P]phosphate, a property consistent with the fact that vitellogenin is heavily phosphorylated (13, 20–22), and is the only phosphoprotein secreted from vitellogenic liver (2). (*vi*) Radioactive media occasionally contained a labeled protein running with lipovitellin extracted (23) from yolk, presumably the result of limited proteolysis at the unusually sensitive site that is cleaved during the release of lipovitellin from vitellogenin *in vivo* (1).

DISCUSSION

The main object of the work described here was to see if the induction of vitellogenin synthesis in male liver by estradiol, which has hitherto only been described *in vivo*, can also be obtained *in vitro*. We find that under simple, defined culture conditions, vitellogenin synthesis in isolated male liver is induced by physiological concentrations of estradiol, no other added factors being necessary. We also find that a continuous supply of estradiol is necessary and sufficient to sustain vitellogenin synthesis in isolated female liver, which strongly suggests that the production of vitellogenin in the female frog is dependent upon the continuous release of estrogens from the ovary (15). Uninduced male liver synthesizes no vitellogenin, and female liver maintained in the absence of estradiol gradually ceases to do so. Non-vitellogenic liver from either sex synthesizes vitellogenin upon addition of estradiol. The response is very fast, vitellogenin being clearly detectable within 6 hr and probably within 2 hr. These findings imply that the organization of the quiescent vitellogenin gene is the same in the two sexes, despite the fact that males normally never make vitellogenin at any time in their lives. The short time between the addition of estradiol and the first appearance of vitellogenin suggests that activation of the vitellogenin gene may depend only upon the availability of estradiol, and probably does not require cell division.

We have also found that this frog liver system synthesizes two different proteins in response to two different steroids. Estradiol-17β induces vitellogenin synthesis. Dexamethasone induces the synthesis of a different, as yet uncharacterized, protein. The existence of two distinct hormone responses from a single tissue should be helpful in analysis of the mechanism of gene activation in this eukaryotic system.

We warmly thank Ruth Longthorne for her excellent and cheerful technical assistance. We are also grateful to Drs. R. M. Benbow, F. H. C. Crick, J. B. Gurdon, R. A. Laskey, N. T. Williams, and R. Zigmond for their interest and encouragement in the preparation of this manuscript. The helpful suggestions of Dr. M. Balls as to the culture conditions for frog liver were much appreciated. This work was supported by the Helen Hay Whitney Foundation of New York City (L.J.W.) and the Medical Research Council of Great Britain (J.K.).

1. Bergink, E. W. & Wallace, R. A. (1974) *J. Biol. Chem.* **249,** 2897–2903.
2. Wallace, R. A. & Jared, D. W. (1969) *Dev. Biol.* **19,** 498–526.
3. Wallace, R. A. (1967) *J. Cell Biol.* **35,** 137A.
4. Clemens, M. J., Lofthouse, R. & Tata, J. R. (1975) *J. Biol. Chem.* **250,** 2213–2218.
5. O'Malley, B. W. & Means, A. R. (1974) *Science* **183,** 610–620.
6. Ozon, R. & Bellé, R. (1973) *Biochim. Biophys. Acta* **297,** 155–163.
7. Lebeau, M. C., Massol, N. & Baulieu, E. E. (1974) *FEBS Lett.* **43,** 107–111.
8. Gschwendt, M. & Kittstein, W. (1974) *Biochim. Biophys. Acta* **361,** 84–96.
9. Clemens, M. J. (1974) *Progr. Biophys. Mol. Biol.* **28,** 69–107.
10. Knowland, J. (1974) *Genetics* **78,** 383–394.
11. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46,** 83–88.
12. Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.*, in press.
13. Ansari, A. Q., Dolphin, P. J., Lazier, C. B., Munday, K. A. & Akhtar, M. (1971) *Biochem. J.* **122,** 107–113.
14. Schirm, J., Gruber, M. & Ab, G. (1973) *FEBS Lett.* **30,** 167–169.
15. Gallien, L. & Chalumeau-Le Foulgoc, M-T. (1960) *C. R. H. Acad. Sci.* **251,** 460–462.
16. Marot, J. & Ozon, R. (1973) *Comp. Biochem. Physiol.* **44B,** 1103–1111.
17. Mikhail, G., Wu, C. H., Ferin, M. & Vande Wiele, R. L. (1970) in *Karolinska Symposia on Research Methods in Reproductive Endocrinology,* 2nd Symposium March 23–25, 1970, pp. 347–358.
18. Baxter, J. D. & Tomkins, G. M. (1971) *Proc. Nat. Acad. Sci., USA* **68,** 932–937.
19. Sibley, C. H. & Tomkins, G. M. (1974) *Cell* **2,** 213–220.
20. Redshaw, M. R., Follet, B. K. & Nicholls, T. J. (1969) *J. Endocrinol.* **43,** 47–53.
21. Follet, B. K. & Redshaw, M. R. (1968) *J. Endocrinol.* **40,** 439–456.
22. Wallace, R. A. (1970) *Biochim. Biophys. Acta* **215,** 176–183.
23. Wallace, R. A. (1965) *Anal Biochem.* **11,** 297–311.