PRIMARY INDUCTION OF VITELLOGENIN SYNTHESIS IN MONOLAYER CULTURES OF AMPHIBIAN HEPATOCYTES

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ABSTRACT

Direct induction of vitellogenin production in cultured male amphibian hepatocytes by estradiol-17β has been accomplished. Liver cells were isolated from adult male bullfrogs by collagenase perfusion and maintained as monolayers in serum-free medium containing insulin and estradiol. Vitellogenin production was measured by direct immunoprecipitation from radioactively labeled secreted protein with a specific antiserum against vitellogenin. Significant quantities of vitellogenin were detected in the exported protein on the second day of hormone treatment. Vitellogenin production increased with duration of culture in the presence of estradiol until by the eighth day ~90% of secreted protein was vitellogenin. This response is largely comparable to that obtainable in vivo.

Indirect immunofluorescence microscopy was used to identify cells synthesizing vitellogenin in response to estradiol. An increase in cytoplasmic fluorescence could be seen in cells throughout the cultures, with increasing time in the presence of estradiol. By the sixth day of treatment, the majority of cells showed significant fluorescence labeling. The results suggest that studies on the mechanisms underlying the primary activation of the vitellogenin gene may now be conducted under well defined conditions in a monolayer liver cell culture system.

KEY WORDS amphibian hepatocyte culture · estradiol · vitellogenin · induction · immunofluorescence microscopy

The steroid hormone regulation of egg-yolk protein synthesis in oviparous vertebrates is widely used for studying the control of gene expression in higher organisms (7, 12, 23). After injection of estrogen into these animals, the liver synthesizes and secretes the high molecular weight egg-yolk precursor protein, vitellogenin (11, 25), a protein which is normally made only in females during yolk deposition (15). The circulating vitellogenin is then selectively taken up by the ovary and cleaved to form the egg-yolk proteins lipovitellin and phosvitin (2, 6, 8).

At peak hormonal response, vitellogenin is the predominant protein synthesized by the liver (12, 18, 23). The commitment of such a large part of the liver's protein synthetic capacity to the production of a single protein along with the availability of large amounts of tissue for biochemical analysis makes this a generally attractive system for studies on the hormonal regulation of gene expression.

One of the major limitations of studies on the estrogen regulation of vitellogenin production has been the lack of a suitable estrogen-responsive liver cell culture system. The ability to directly induce vitellogenin synthesis in liver cultures would simplify, for both technical and systematic reasons, a variety of experiments found difficult to conduct in vivo. To this end we have developed methods for the high-yield isolation and mono-

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layer culture of amphibian hepatocytes (21, 22). These cell cultures, when maintained in a serum-free, chemically defined medium, retain many of the normal liver cell functions found in vivo. We have also found that estrogen dramatically increases the vitellogenin production of cells derived from female frogs (20). However, in view of the fact that females normally produce vitellogenin for egg-yolk production during their reproductive cycle, this response may not represent the first or primary activation of the gene.

Male animals which do not synthesize detectable amounts of vitellogenin will respond as dramatically as their female counterparts when given an injection of estrogen. As a result, most studies on the early events involved in the hormonal control of the vitellogenin gene have been conducted on males. The direct induction of vitellogenin synthesis in male liver cell cultures would provide a clearly defined system in which to carry out further studies of this sort. In this paper, we report the full primary induction of vitellogenin synthesis in cultures of amphibian liver cells obtained from male animals. We also show by indirect immunofluorescence microscopy that the vast majority of the cultured cells are capable of responding to estrogen by producing vitellogenin.

MATERIALS AND METHODS

Liver Cell Isolation and Culture

Hepatocytes were isolated from mature, male bullfrogs (*Rana catesbeiana*) by collagenase perfusion and maintained as monolayers on collagen-coated culture dishes in serum-free, isotonic Waymouth's MB 752/1 medium (Grand Island Biological Co. (Gibco), Grand Island, N. Y.) as described previously (21), except that bovine insulin (Ely Lilly & Co., Indianapolis, Ind.) was included in all culture media at a final concentration of 10 mU/ml to prevent any drop in protein secretion during the culture period (22). Between 5.0 and 10.0 $\times$ 10^8 cells with 95% viability are obtained under these conditions, and attachment efficiencies exceed 95%. Estradiol-17β in propylene glycol was added to the culture media at the designated concentrations before use. Control cultures received propylene glycol only. The final concentration of propylene glycol in medium never exceeded 0.1%.

Preparation of Antivitellogenin Antiserum

Partially purified vitellogenin was isolated from haparinized plasma obtained from chronically estrogen-treated animals according to the procedure of Ansari et al. (1). Samples were lyophilized and stored dry at −90°C until use.

Adult New Zealand white rabbits were injected subcutaneously with 2–3 mg of vitellogenin, prepared as described above, in Freund's complete adjuvant (Gibco) then injected again 3 wk later and weekly thereafter with 1–2 mg in 1.0 M NaCl. Serum was prepared from blood collected at weekly intervals. Pooled serum samples were then extensively absorbed to remove antibodies produced to any contaminants in the vitellogenin used for injection. This was accomplished by slowly adding male frog serum to the crude antivitellogenin serum, with stirring at room temperature until a visible precipitate formed. The mixture was stored at 4°C overnight and then clarified by brief centrifugation. The supernate was collected and the entire process was repeated until no further precipitation occurred. The immunoglobulin fraction was prepared from the final supernate by ammonium sulfate precipitation as outlined by Rhoads et al. (17), lyophilized, and stored at −90°C. Before use, the antibody preparation was dissolved in phosphate-buffered saline (PBS, 0.85% NaCl—0.01 M NaH_2PO_4, pH 7.4) at a concentration of 20.0 A_{280}/ml.

Quantitation of Vitellogenin Production by Direct Radioimmunoprecipitation

Vitellogenin production was measured by direct immunoprecipitation of ^{3}H-labeled secreted protein in culture medium by a modification of the procedure outlined by Rhoads et al. (17). Duplicate 200-μl aliquots of medium were precipitated with 100 μl of antivitellogenin antibody plus 25 μg of carrier vitellogenin under the conditions described. The final precipitate was washed twice with PBS containing 10 mM L-leucine and then dissolved in 0.5 ml of 2% sodium dodecyl sulfate (SDS). The sample was then mixed with 10 ml of Biofluor (New England Nuclear, Boston, Mass.) and radioactivity was measured with 38% efficiency. Background values for immunoprecipitates formed in the presence of labeling medium were typically below 75 cpm. Additional experiments showed >90% recovery of labeled vitellogenin with the use of this method.

Culture medium radioactivity in acid-precipitable material was determined by the filter-disk technique of Mans and Novelli (14). Filters were counted in toluene-based fluor with 40% efficiency.

Indirect Immunofluorescence Microscopy of Vitellogenin-Producing Cells

Hepatocytes were cultured on 35-mm dishes at a density of 4.5–5.0 $\times$ 10^5 cells/cm^2. On the indicated days, the plates to be examined were rinsed extensively with PBS, fixed in 1% formaldehyde, and extracted with 80% acetone by the method of Pastan et al. (16). One ml of antivitellogenin IgG, previously purified by ion exchange chromatography (13), was then added to the plates at a concentration of 0.1 A_{280}/ml and incubated for 10 min at 37°C. The dishes were next rinsed five times with PBS, after which 1 ml of fluorescein-labeled goat anti-rabbit IgG (Miles Yeda, Israel), diluted 1:20 with PBS, was added. The plates were then incubated for an additional 10 min. The dishes were subsequently rinsed again in PBS, covered with buffered glycerol, coverslipped, and viewed in a Zeiss Photomicroscope II equipped with epifluorescent illumination. Pictures were recorded on Tri-X film.

RESULTS

Characterization of Antivitellogenin Antibody

To measure vitellogenin production by the cultured hepatocytes, we developed a procedure in which vitellogenin is selectively precipitated from radioactively labeled total secreted protein by an

antiserum specific for vitellogenin. However, attempts to purify vitellogenin for antibody production by a variety of chromatographic techniques were unsuccessful. All preparations contained material of less than the subunit molecular weight of vitellogenin (≃200,000) as shown by SDS gel electrophoresis. We therefore injected rabbits for antibody production with partially purified vitellogenin prepared from the serum of estrogen-treated animals by dimethylformamide precipitation. The resulting antiserum was then extensively absorbed with male frog serum to remove antibody directed against the contaminants in the vitellogenin preparations used for injection. The absorbed antiserum was then analyzed for its specificity for vitellogenin.

Immunodiffusion and immunoelectrophoretic analysis of the absorbed antisera indicated that the antibody recognizes a single major component present in the serum of females and estrogen-treated males (results not shown). Evidence that the secreted protein precipitated by the antibody was indeed vitellogenin was provided by analyzing the migration of a radioactively labeled immunoprecipitate after electrophoresis under denaturing conditions in a polyacrylamide gel. Hepatocytes were isolated from an estrogen-stimulated frog, then maintained in primary culture for several hours in the presence of [^{3}H]leucine or [^{32}P]O_4. Vitellogenin in the radioactively labeled secreted protein present in the culture medium was then immunoprecipitated, and the precipitate was solubilized, electrophoresed in the presence of SDS, and the amount of radioactivity in individual slides of the gel was determined. The results of such an analysis are shown in Fig. 1. The majority of the immunoprecipitable tritium-labeled protein was found in two peaks near the top of the gel. The position of these peaks coincided with that of the two high molecular weight subunits of vitellogenin found on Coomassie blue-stained gels of partially purified vitellogenin (position indicated by arrows in Fig. 1). Others have noted such a doublet of vitellogenin subunits under similar electrophoresis conditions (4).

Since vitellogenin is a highly phosphorylated protein, vitellogenin secreted by hepatocytes maintained in the presence of [^{32}P]O_4 should label with this isotope. As shown in Fig. 1, the [^{32}P] present in an immunoprecipitate migrated to the same position as that of the tritium-labeled protein. However, a comparison of the [^{32}P] to [^{3}H] ratio in the two high molecular peaks of radioactivity

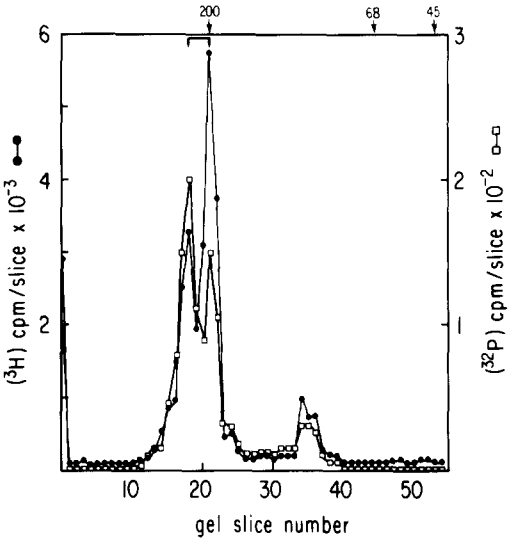

FIGURE 1 SDS polyacrylamide gel electrophoresis of ^{3}H and ^{32}P-labeled anti-vitellogenin immunoprecipitates. A female frog was injected with 1.5 mg estradiol, and 4 d later its hepatocytes were isolated and cultured on 100-mm dishes as described (22). The cells were maintained during the first 24 h of culture in medium containing 5.0 μCi/ml L-[3,4,5-^{3}H]leucine or 5.0 μCi/ml NaH$^{32}PO_4$. The medium was then collected and 0.4 ml was immunoprecipitated with antivitellogenin antiserum using 10 μg vitellogenin as carrier (see Materials and Methods). The immunoprecipitates were then electrophoresed on tubular 5.6% acrylamide gels as described by Fairbanks et al. (9). The gels were cut into 1-mm slices, dissolved in 60% $HClO_4$-30% H_2O_2 at 75°C in glass scintillation vials, and radioactivity was measured after addition of 10 ml Biofluor (New England Nuclear, Boston, Mass.). [^{3}H]leucine-labeled immunoprecipitate, ● (left ordinate). ^{32}P-labeled immunoprecipitate, □ (right ordinate). The vitellogenin doublet is indicated by arrows. The positions and molecular weights (× 1,000) of three electrophoretic markers are shown at top of figure. The markers used are myosin (200,000), bovine serum albumin (68,000), and ovalbumin (45,000).

shows a considerable difference between the two. This result suggests a different degree of phosphorylation of the polypeptides in each band. This may be the reason for the apparent molecular weight difference between the two subunits seen on SDS gels. Alternatively, there may be two distinct molecular weight species made with different amounts of phosphorylated and nonphosphorylated proteins present within each group.

In addition to the two high molecular weight bands of immunoprecipitable radioactivity just described, a small amount of material was present in

a band of lower molecular weight (Fig. 1). For two reasons, we believe this to be a proteolytic fragment of vitellogenin which remains immunoprecipitable. First, the polypeptides in this band are phosphorylated since some of the immunoprecipitable [^{32}P] was present in this region. Second, this material migrated in the position of a fragment of similar molecular weight seen on SDS gels of preparations of vitellogenin allowed to degrade in vitro (data not shown). In view of the results described above, we believe that the rabbit antibody we have prepared is specific for vitellogenin.

Induction of Vitellogenin Synthesis by Estradiol

The antivitellogenin antibody described in the first section was used to measure via radioimmunoprecipitation the production of vitellogenin by male hepatocytes maintained in the continuous presence or absence of estradiol. When cells were plated at or near confluence and kept in serum-free medium containing insulin and estradiol 17-β, the hepatocytes responded by synthesizing and secreting vitellogenin (Fig. 2). Initially (day 1), <1% of the acid-precipitable radioactivity in the medium was precipitated with antivitellogenin antibody. However, on the second day of culture in the presence of estradiol, significant quantities of vitellogenin were detected in the secreted protein. On succeeding days, vitellogenin production increased steadily until, by the eighth day of treatment, ~90% of the protein exported was vitellogenin. In parallel cultures maintained over the same time period in medium containing insulin and 0.1% propylene glycol (estradiol solvent), <1% of secreted protein was immunoprecipitable on each day analyzed. Overall, the duration of the lag and the magnitude of the response to estradiol in the cultured hepatocytes closely resemble those found in vivo (18, 23).

High concentrations of hormone were needed to elicit a significant response (Table I). Little or no effect of estradiol on vitellogenin production could be seen at concentrations below 10^{-7} M. The greatest response observed came at the highest concentration tested, 10^{-5} M.

Indirect Immunofluorescence Microscopy of Vitellogenin-Producing Cells

The ability to induce vitellogenin production in this culture system enabled us to examine, by indirect immunofluorescence microscopy, the proportion of cells which respond to estradiol. Cultures were first fixed and permeabilized, then incubated consecutively with the IgG fraction of the vitellogenin antibody and next, fluorescein-labeled goat anti-rabbit IgG. The fixation and staining method employed gave a low background

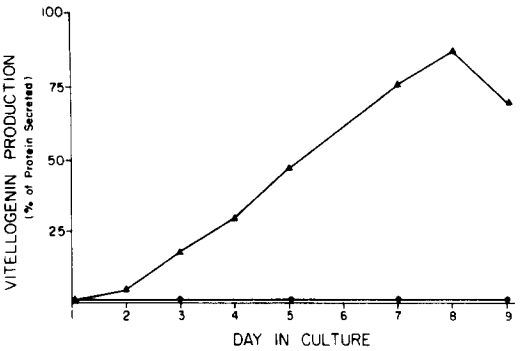

FIGURE 2 Estradiol induction of vitellogenin synthesis in monolayer cultures of amphibian hepatocytes. Liver cells obtained from male donors were maintained on 35-mm dishes at a cell density of 6.5×10^5 cells/cm^2. Hormone-treated cultures were maintained in the continuous presence of 10^{-5} M 17β-estradiol, whereas control cultures received hormone solvent only. On the indicated days, secreted protein was labeled by incubating individual plates for 12 h in 1 ml of culture medium having a leucine concentration 1/1,000 that of normal plus 5.0 μCi L-[3,4,5-^{3}H]leucine. At the end of the labeling period, the medium was collected and stored at −90°C until use. Medium from each plate was analyzed in duplicate for both immunoprecipitable vitellogenin and TCA-insoluble material as described in Materials and Methods. Each point is the mean of data obtained from triplicate plates in a typical experiment. The standard deviation of each point was within 10% of the mean. Cells maintained in the presence of estradiol, ▲. Control cultures receiving no estradiol, ●.

TABLE I

Dose Response of Vitellogenin Production to Estradiol

Hormone concentration	Vitellogenin as % secreted protein
M	
10^{-5}	47.7 ± 3.0
10^{-6}	13.5 ± 2.6
10^{-7}	4.4 ± 1.4
10^{-8}	1.5 ± 0.2
10^{-9}	0.95 ± 0.06

Cultures were maintained in the presence of the designated concentration of hormone and, at the end of 5 d, vitellogenin production was measured as described in Materials and Methods. Results are expressed as the mean ± SD of data from triplicate plates.

fluorescence in control cultures, i.e., cells maintained without estradiol (Fig. 3). Some light nuclear staining was seen in these cells, along with some widely scattered fluorescent spots associated with cell debris. Both of these sources of fluorescence were not due to a specific interaction with antivitellogenin antibody since a similar pattern and level of fluorescence was seen when antivitellogenin IgG was omitted.

Immunofluorescence microscopy of vitellogenin in cells cultured for 6 d in the presence of estradiol showed a dramatically increased level of fluorescence compared with that seen in controls (Fig. 4). During this time, the estrogen-treated cells developed highly granular areas of cytoplasm, perhaps due to an increase in secretory vesicles (compare Fig. 4*a* with Fig. 3*a*). Fluorescence labeling of vitellogenin within the cells was restricted primarily to this granular cytoplasm. An experiment was conducted to ensure that the fluorescence staining observed in the estradiol-treated cultures was due to labeling of vitellogenin in cells actually producing the protein. Control cultures were incubated with medium previously in contact for 24 h with cells actively producing vitellogenin, or with medium containing 50 μg/ml added vitellogenin. No significant change in fluorescence from that seen in the control cultures of Fig. 3 could be detected.

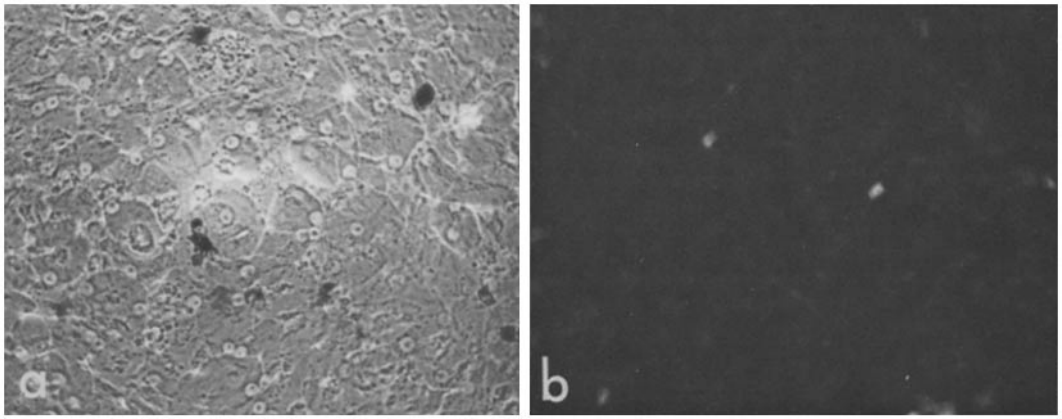

FIGURE 3 Phase-contrast and fluorescence micrographs of control hepatocyte cultures treated with antivitellogenin IgG. Cells were maintained for 6 d without estradiol, then processed for indirect immunofluorescence microscopy as described in Materials and Methods. *(a)* Phase contrast. × 150. *(b)* Fluorescence micrograph of same field as in *a*. Exposure time 20 s. × 150.

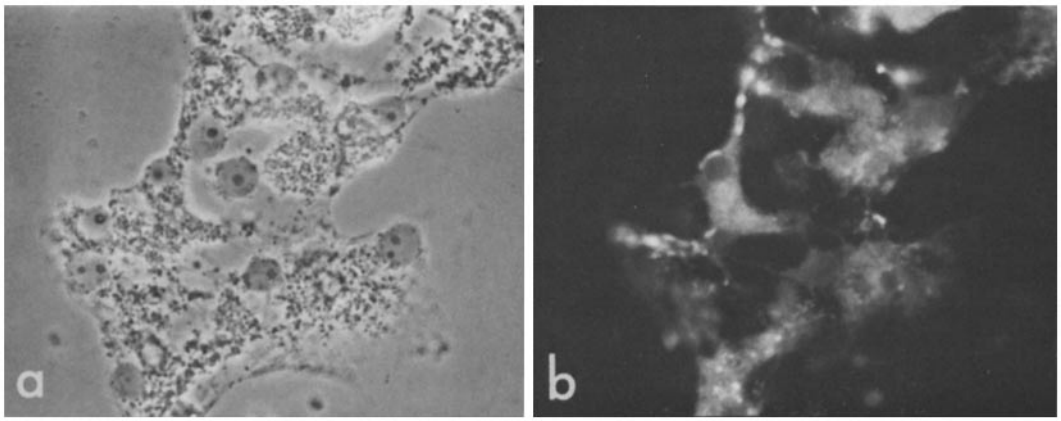

FIGURE 4 Phase-contrast and fluorescence micrographs of estradiol-treated cultures with antivitellogenin IgG. Cells were maintained for 6 d in the presence of 10^{-5} M estradiol, then processed for indirect immunofluorescence microscopy as described in Materials and Methods. *(a)* Phase contrast. × 590. *(b)* Fluorescence micrograph of same field as in *a*. Exposure time, 5 s. × 590.

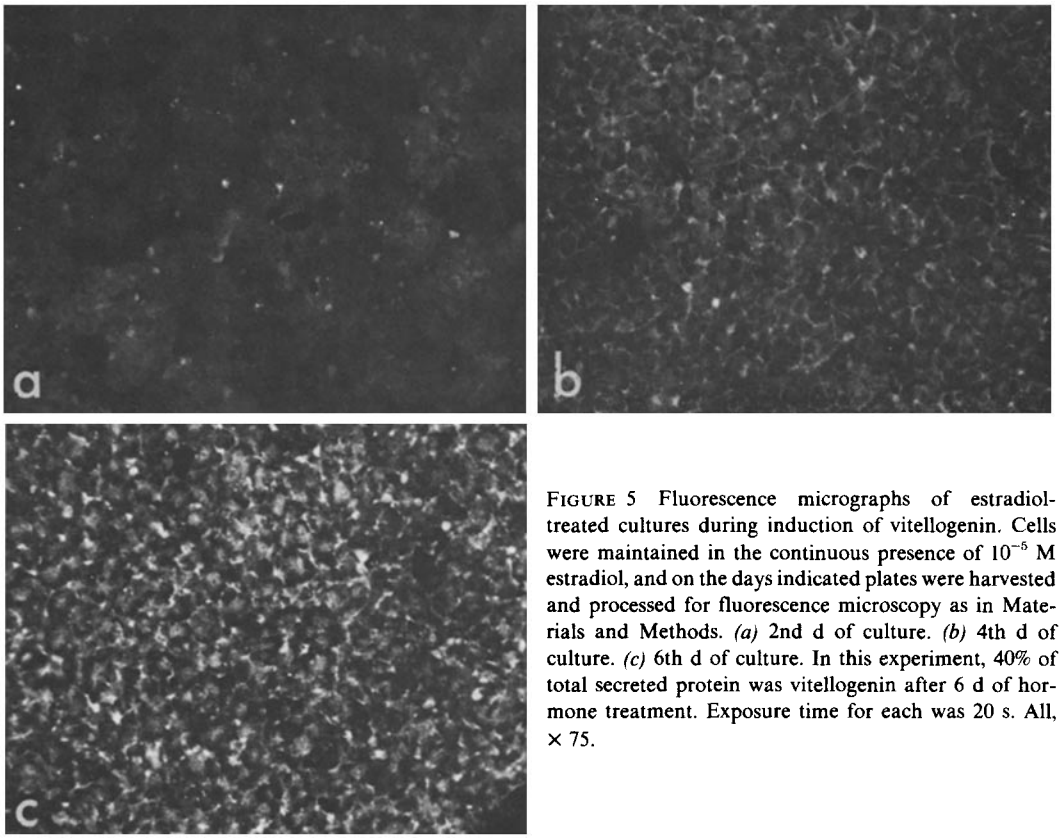

FIGURE 5 Fluorescence micrographs of estradiol-treated cultures during induction of vitellogenin. Cells were maintained in the continuous presence of 10^{-5} M estradiol, and on the days indicated plates were harvested and processed for fluorescence microscopy as in Materials and Methods. *(a)* 2nd d of culture. *(b)* 4th d of culture. *(c)* 6th d of culture. In this experiment, 40% of total secreted protein was vitellogenin after 6 d of hormone treatment. Exposure time for each was 20 s. All, × 75.

This result suggests that the extensive fluorescent labeling found in the estrogen-treated cultures is not due to the cellular adherence or uptake of vitellogenin produced by only a few of the cultured hepatocytes.

To estimate the proportion of cells which respond to estradiol, cells were cultured in the continuous presence of hormone and on various days were examined for the presence of vitellogenin by this indirect immunofluorescence procedure. The low-magnification micrographs in Fig. 5 show a progressive increase in cellular fluorescence from the second through the 6th d of culture. Scattered fluorescence was found on day 2 (Fig. 5 *a*), but a significant amount of fluorescence was seen by the 4th d of culture (Fig. 5 *b*), particularly in areas of high cell density. By the 6th d of treatment (Fig. 5 *c*), the entire culture showed bright fluorescence with virtually every cell showing some degree of labeling. Some variation in fluorescence intensity from cell to cell was noted which correlated with differences in the organization of the granular matrix within the cytoplasm described previously. Areas of high particle density were brightly fluorescent, while cells having an even distribution of such particles had a more subdued staining. In view of these results, it appears that the majority of cells placed into culture have the capacity to respond to estradiol by synthesizing vitellogenin. However, in view of the limited resolution of this technique, the possibility that a small population of cells is not responsive to estradiol cannot be excluded.

DISCUSSION

In this report, we have demonstrated that monolayer cultures of amphibian hepatocytes prepared from male frogs retain the ability to respond directly to estradiol by synthesizing and secreting vitellogenin. Employing a sensitive radioimmunoprecipitation assay to measure vitellogenin production, we have shown that the lag and the magnitude of the response found in culture are very similar to those described in vivo. In addition, it appears that the majority of cells placed in culture respond to estradiol, as demonstrated by

indirect immunofluorescence microscopy of vitellogenin within producing cells.

During the course of these studies, it became clear that the conditions chosen for maintaining amphibian hepatocytes in culture were of particular importance for obtaining a response to estradiol. In general, conditions that maintain hepatocyte functions in culture permit a greater cellular response to estradiol. We have found that the addition of insulin to the culture medium helped the cells to maintain a higher overall rate of protein synthesis during the culture period (22), and that a high cell density tends to extend hepatocyte longevity. Both conditions are necessary for maximizing vitellogenin production. In contrast, various animal sera, as well as thyroid hormone and the synthetic glucocorticoid dexamethasone, did not enhance overall cell viability or vitellogenin induction by estradiol.

We have found that high concentrations of estradiol are necessary for inducing high levels of vitellogenin production. This was not surprising in view of the fact that large quantities of hormone (mg/kg body weight) must be injected to obtain a maximum response from the intact animal. In fact, serum levels after a single injection can reach close to 10^{-6} M (5). One plausible reason for the requirement of such high hormone levels both in culture and in vivo is that estradiol is catabolized rapidly by the liver, resulting in considerably lower effective intracellular concentrations of hormone. Alternatively, the hormone may not be transported efficiently into the cells of the liver, resulting in a lower effective intracellular concentration of estrogen.

Recently, it has been shown that under appropriate conditions cubed liver preparations from the amphibian *Xenopus laevis* will respond directly to estrogen (10, 24). However, the magnitude of the response does not approach that found in vivo (10), perhaps due to progressive tissue necrosis under these culture conditions (3, 19). The liver cell culture system we described offers a number of important advantages for studies on the hormonal regulation of vitellogenin production. Since the hepatocytes can be prepared in both high yield and purity (21), many identical samples of cells can be obtained from a single animal. Also, the liver cells maintain viability in culture and respond directly to estrogen under well defined conditions in serum-free medium. In general, the culture system provides for easy manipulation of a wide range of experimental variables.

In summary, we have shown that primary monolayer cultures of amphibian hepatocytes respond directly to estradiol by synthesizing vitellogenin. The availability of such a clearly defined system will certainly simplify further studies on the hormonal control of vitellogenin gene expression.

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