Sequential Changes in the Protein Synthetic Activity of Male Xenopus laevis Liver Following Induction of Egg-yolk Proteins by Estradiol-17β

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SUMMARY

Administration of a single injection of estradiol-17β to adult male Xenopus laevis induces the synthesis and secretion by the liver of the egg-yolk protein complex vitellogenin. Explants taken from livers of hormone-treated animals and maintained in organ culture continue to produce this protein at a rate which is dependent on the length of time between estrogen injection of the donor and preparation of the explants. Organ culture, therefore, can be used to quantitate the vitellogenic response and to study its time course in detail.

An increase in the rate of incorporation of radioactive amino acids into secreted protein is first detectable in explants taken from animals 12 hours after estradiol injection. The response becomes increasingly pronounced as the period after hormone treatment of the donors is prolonged, and it is maximal in explants taken from frogs 12 days after estrogen administration. At this time production of vitellogenin can account for as much as 90% of total protein synthesis in the liver. Labeling of intracellular protein with [14C]serine is stimulated 2-fold and incorporation into secreted protein increased 36-fold under conditions where the rate or extent of uptake of radioactive precursor into the acid-soluble pool does not change. After a lag period of 2 to 4 hours the secretion of vitellogenin in culture continues at a constant rate for up to 3 days. At least part of the stability of yolk protein synthesis in culture is attributable to long lived messenger RNA species.

The major component of vitellogenin labeled either in vivo or in culture has a molecular weight of approximately 180,000 as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Material labeled with [3H]serine gives a similar distribution of radioactivity on gel electrophoresis as that labeled with [35S]methionine, with no peak corresponding to the molecular weight of phosvitin. It is suggested that phosvitin is not a primary product of translation but is synthesized as part of the large subunit of vitellogenin.

Hormonal induction of specific protein synthesis provides a means of examining mechanisms involved in the regulation of gene expression in differentiated cells of higher organisms. An appropriate system for such studies is the estrogen-dependent production of egg-yolk proteins by the liver in the frog Xenopus laevis (1–3). The female of this species secretes yolk proteins into the bloodstream in the form of a large complex, vitellogenin (4, 5), which is subsequently taken up by the ovaries for conversion into the components of the yolk platelets, phosvitin and lipovitellin (6).

Male frogs do not normally synthesize vitellogenin but can be induced to do so following a single injection of estradiol-17β (1, 3, 7). As a result of such hormone treatment, not only is there induction of the egg-yolk proteins, but also an increase in the total protein synthetic activity of the liver (3, 8, 9). However, the quantitative nature of these changes has not been clearly established. In particular, the fraction of total hepatic protein synthesis which is directed toward yolk protein production has not been reported. We have attempted to answer this question by measuring rates of amino acid incorporation into secreted and nonsecreted protein in cultured explants of liver taken from male animals at various times after a single injection of estradiol-17β.

The complex nature of vitellogenin in Xenopus has been demonstrated by several groups (4, 5, 7). It has a molecular weight of approximately 450,000, contains 12% lipid, 1.4% phosphorus, and 0.7% carbohydrate by weight, and binds calcium, iron, and the bile pigment biliverdin. The amino acid composition is characterized by an unusually high serine content (11.5%), due to the serine-rich phosvitin component (5), but evidence concerning the number of polypeptides comprising the protein moiety of vitellogenin has only appeared very recently (10). We have examined the distribution of radioactivity in newly synthesized and secreted polypeptides in an attempt to determine whether phosvitin and lipovitellin are secreted as discrete components of the vitellogenin complex. Such information would enable us to gain some insight into the degree of complexity of the biosynthetic response of the liver to estrogens in egg-laying animals.

Preliminary results of part of this study have been reported (11, 12).

EXPERIMENTAL PROCEDURE

Materials—[14C]Serine (50 to 60 Ci per mol), [3H]serine (6.3 X 10⁶ Ci per mol), and [35S]methionine (73 X 10⁶ Ci per mol) were obtained from the Radiochemical Centre, Amersham, England. Actinomycin D was from Koch-Light, Colnbrook, England. Estradiol-17β was from Koch-Light, Colnbrook, England. Actinomycin D was...
was supplied by Sigma Chemical Co., and α-amanitin was a generous gift from Prof. Th. Wieland.

Animals—Adult male *Xenopus laevis* were supplied by the South African Snake Farm, Fish Hoek, Cape Province, South Africa. They were maintained in tap water at 20° and fed twice weekly on chopped beef heart. Estradiol-17β (1 mg per animal) was injected via the dorsal lymph sac in 0.1 ml of propylene-1,2-diol. Control animals were injected with solvent alone.

**Incubation of Liver Explants in Culture**—The medium used for incubation of liver explants was a modified Wolf and Quimby medium (13) in which NaHCO₃ was replaced by 10 mM Hepes1 buffer, pH 7.5. Antibiotics included in the medium were: penicillin G (Glaxo, 0.12 mg per ml), streptomycin sulfate (Glaxo, 0.27 mg per ml), and ampicillin (Beecham Research Laboratories, 25 μg per ml). Livers were removed rapidly from pithed frogs and washed in this medium in the cold. All subsequent operations were performed at room temperature under sterile conditions. Pieces of tissue measuring approximately 1 × 1 × 0.5 mm were prepared, washed, and then transferred onto squares of nylon bolting cloth in 30-mm diameter sterile Petri dishes. Each dish contained six individual explants (6 to 12 mg wet weight of liver) in 1 ml of medium with 0.5% Eagle's MEM serum. Half of the total explants were removed from their dishes, rinsed in 0.9% NaCl, blotted, and weighed. All of the tissue from each dish then was homogenized in 1 ml of 0.9% NaCl and protein precipitated by addition of an equal volume of 10% (w/v) trichloroacetic acid containing unlabeled amino acid corresponding to the radioactive one used (1 mg per ml). For estimation of incorporation into nonsecreted protein, the precipitates were washed twice more with 5% (w/v) trichloroacetic acid, dissolved in 1 ml of 0.3 M NaOH, incubated for 1 hour at 37°, and reprecipitated with 10% trichloroacetic acid. After a further wash in 5% trichloroacetic acid, the precipitates were dissolved in 1 ml of 0.3 M NaOH and 0.1 ml aliquots were assayed for radioactivity in 10 ml of Triton X-100-toluene scintillation fluid with 0.9 ml of water added. The counting efficiency in the Beckman LS 2000 machine used was 91% for 1C.

For estimation of incorporation of labeled amino acids into secreted protein, 0.1-ml aliquots of culture medium were directly pipetted onto Whatman No. 3MM filter discs (2.4 cm diameter) which then were processed according to the procedure of Mans and Novelli (14). Radioactivity in duplicate filters for each culture period measured at 70% efficiency for 1C in toluene-based scintillation fluid (15).

Incorporation of radioactivity into both nonsecreted and secreted protein is expressed as counts per min or disintegrations per min per mg wet weight of tissue incubated, thus allowing direct comparisons between rates of tissue and secretory protein synthesis. The values for duplicate cultures do not usually vary by more than 10% either side of the mean when the results are calculated in this way.

**Ascorbic Acid Uptake into Acid-soluble Pool**—Explants were removed from culture, blotted well without washing, and weighed. Total tissue from each dish was then homogenized in 1 ml of 5% trichloroacetic acid and the precipitates washed by centrifugation and resuspension in a further 1 ml of trichloroacetic acid. The washings were combined and 0.25-ml aliquots were assayed for radioactivity in triplicate in 10 ml of the Triton scintillator with 0.75 ml of water added to each sample. The counting efficiency was 91% for 1C. Ascorbic acid uptake by duplicate cultures is expressed as disintegrations per min per mg wet weight of tissue.

**Purification of Authentic Vitellogenin**—Vitellogenin was purified from pooled sera of male *Xenopus* which had been injected 12 days previously with 1 mg of estradiol-17β, using the dimethylformamide precipitation procedure at pH 7 to 7.5 as described by Asnari et al. (4). Radioactive vitellogenin, labeled by injection of 20 μCi of [³⁵S]methionine into each animal 24 hours before death, had a maximum specific activity of 9.8 X 10⁴ dpm per μg. For estimation of radioactivity each slot of the gel slab was cut away using a slab gel apparatus modified from the commercially available F-C apparatus (A. H. Thomas, Philadelphia, Pa.). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out using a slab gel apparatus as described below.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis**—Liver explants were cultured for 24 hours in the presence of either [³⁵S]methionine (5 or 7.6 μCi per ml) or [³⁵S]serine (5 μCi per ml). Aliquots of the medium were then taken for analysis of labeled proteins and processed in one of two ways: (a) 20-μl aliquots were utilized directly by adding 2-mercaptoethanol, sodium dodecyl sulfate, glycerol, and bromophenol blue and were boiled as described below; (b) 200-μl aliquots were treated with 10% trichloroacetic acid to precipitate protein. The precipitates were washed three times with 5% trichloroacetic acid, twice with ethanol, and once with ether and resuspended in 30 μl of water. The suspensions were then prepared for electrophoresis as described below.

Electrophoresis was carried out using a slab gel apparatus modified from the commercially available F-C apparatus (A. H. Thomas, Philadelphia, Pa.). Sodium dodecyl sulfate polyacrylamide gels were prepared essentially as described by Maizel (16). Gels contained 0.1% sodium dodecyl sulfate and were discontinuous with a 3.6% acrylamide spacer (pH 6.7) and 7.5% acrylamide resolving gel (pH 8.3). The electrode buffer consisted of Tris-HCl (0.6%), glycine (0.8%), and sodium dodecyl sulfate (0.1%), pH 8.3. Electrophoresis was carried out for 18 hours at 20 mA. Eight samples could be run on one gel slab using this procedure.

For estimation of radioactivity each slot of the gel slab was cut transversely using an automatic slicer designed by Dr. R. Brimacombe at this Institute, giving approximately 40 3-mm fractions per slot. Each slice was digested in 0.5 ml of 100-volume hydrogen peroxide for 16 hours at 50° and 10 ml of Aquasol then were added. After standing overnight at 4° (to reduce chemiluminescence), radioactivity in the gel fractions was measured in a Beckman LS 200 scintillation counter. Background counts were subtracted from the data shown. Gels were calibrated with a series of stand and polypeptides ranging in size from the λ2 subunit of reovirus (140,000 daltons) to trypsinogen (24,000 daltons).

**RESULTS AND DISCUSSION**

**Effects of Estradiol Treatment in Vivo** on Synthesis of Secreted and Nonsecreted Protein by Cultured Tissue—I n order to measure the effects of estradiol on the synthesis of secreted and tissue protein by *Xenopus* liver as a function of time after administration of the hormone in vivo we have cultured explants for various periods of time and have monitored the incorporation of a radioactive amino acid into acid-insoluble material in the culture medium and the liver. Fig. 1 shows the rates of labeling of secreted, nonsecreted, and total protein in culture using tissue from animals killed at different times after hormone injection. Previous work has shown that a dose of 1 mg of estradiol per adult male frog is necessary to obtain prolonged induction of yolk protein synthesis (1). At this dose of the hormone a progressively greater rate of secretory protein labeling per unit weight of liver is observed up to 12 days after injection, but by 18 days the effect is declining (Fig. 1). Much less dramatic effects on the initial rates of labeling of nonsecreted proteins occur after a single hormone injection. At 6 days after estradiol administration, when the rate of tissue protein labeling is increased by a factor of approximately 2 and the rate of secretory protein labeling is stimulated more than 30-fold, no changes in the rate or extent of uptake of radioactive

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1 The abbreviation used is: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
FIG. 1. Time courses of labeling of secreted, nonsecreted, and total protein by *Xenopus* liver explants in culture at various times after estradiol administration *in vivo*. Explants were cultured in medium containing [14C]serine (0.5 μCi per ml) for various times. Incorporation into secreted and nonsecreted protein was assayed as described under "Experimental Procedure." Data are shown for the first 12 hours of incubation, but incorporation continued linearly for a further 12 hours. A, control liver. B to E, liver from animals injected with estradiol for 3, 6, 12, or 18 days before death, respectively. X—X, total protein; O—O, nonsecreted protein; ●—●, secreted protein. Insets (Δ—Δ) show kinetics of uptake of radioactive serine into the acid-soluble fraction of tissue from control (A) and 6-day estradiol-treated (C) *Xenopus* during the first 2 hours of incubation.

Fig. 2. Secreted and tissue protein synthesis in culture at early times after estrogen treatment *in vivo*. Explants were taken from livers of animals treated with estradiol for various times *in vivo* and cultured for 24 hours in the presence of [14C]serine (0.5 μCi per ml) as described under "Experimental Procedure." O—O, nonsecreted protein; ●—●, secreted protein.

precursor are apparent (Fig. 1, A and C). The vitellogenic response of *Xenopus* liver is clearly established by 12 to 18 hours after giving the hormone (Fig. 2) and the rate of amino acid incorporation into secreted protein increases steadily thereafter (Figs. 1 and 2). In contrast, the doubling of incorporation into nonsecreted protein is observed within 12 hours but does not increase subsequently. However, the latter observation does not enable us to distinguish between a true increase in synthesis of nonsecreted liver protein and an effect on labeling due to changes in precursor specific activity. We have not been able to detect any effect of estradiol given less than 12 hours before removal of the liver into organ culture on labeling of either secreted or tissue protein. This finding agrees with the results of Wittliff and Kenney (3) and Zelson and Wittliff (17) who used an immunological method to detect the earliest synthesis of the lipovitellin component of vitellogenin *in vivo*. In estrogen treated rooster liver the comparable time for first appearance of yolk proteins in the plasma after hormone treatment is 4 hours (18). Since actinomycin D given with the hormone blocks appearance of the yolk proteins in both roosters (19) and *Xenopus* (3), it is probable that the latent period represents at least the time required for synthesis and processing of RNA species required for expression of the steroid-induced response.

Simultaneous measurements of rates of labeling of secreted and nonsecreted protein by *Xenopus* liver in culture have enabled us to calculate the proportion of newly synthesized protein which is destined for export, assuming that the rate of secretion of protein during the period of 6 to 24 hours in culture is equal to its rate of synthesis. The rate of secretory protein synthesis accounts for as much as 78% of the rate of total protein synthesis in the liver within 3 days of estradiol treatment and rises to a maximum of 96% at 12 days (Fig. 1). Although we have not taken into account the possibility of some turnover of newly synthesized intracellular protein during the labeling period, this is unlikely to influence the results to any great extent.

Our results showing the secretion of large amounts of protein by organ cultures of livers of male *Xenopus* treated *in vivo* with estradiol-17β confirm and extend previous reports. Wallace and co-workers (1, 6) also have shown that cultured pieces of liver from hormone-treated frogs secrete a distinct phosphoprotein complex into the medium. Earlier, these workers had demonstrated (1) that a 1-mg dose of estradiol *in vivo* elicits an increasing rate of synthesis and secretion of vitellogenin into the plasma.
in vivo up to 12 days after treatment and that thereafter the rate declines. Our analysis of the rate and proportion of protein synthesis which is represented by secretory products in culture at various times after estrogen administration (Fig. 1) is therefore in agreement with this finding.

It should be noted that there are marked differences between the time courses of labeling of secreted and tissue proteins in the hormone-treated explants (Fig. 1, B to E). Incorporation of radioactivity into intracellular protein increases linearly soon after the addition of the labeled amino acid, but there is a pronounced delay in appearance of label in secreted protein. During the first 3 to 4 hours of incubation, very little radioactive material is secreted. At the end of this period, the rate of appearance of labeled protein in the medium increases markedly and this enhanced rate is then maintained for the remainder of the 24 hour period of culture. There is a fall in the rate of tissue protein labeling after 4 hours (Fig. 1, C and D), suggesting a precursor-product relationship between newly synthesized intracellular material and proteins subsequently appearing in the medium. This effect also has been noted in estrogen-treated Xenopus liver by others (2, 20). There is relatively little difference in the rates of labeling of nonsecreted proteins among any of the cultures from estrogen-treated animals after the 6-hour time point. The duration of the lag period in appearance in the medium of labeled proteins is independent of the time after hormone treatment in vivo (Fig. 1) or of the temperature of incubation of the cultures, at least up to 25°. The lag time most probably reflects a processing time during which addition of carbohydrate and lipid moieties to the newly synthesized polypeptides is occurring. Phosphate incorporation into vitellogenin, on the other hand, appears to be more closely associated in time with synthesis of the polypeptide chains in Xenopus liver (20), although Schirm et al. (21) have reported a 20-min time lag between synthesis and phosphorylation of the yolk proteins in the estrogen-treated rooster liver system.

Stability of Protein Synthesis in Culture—When explants were tested for their ability to respond to estradiol in vitro, we failed to observe any effect of the hormone upon its addition to the culture medium. No response of control male Xenopus liver to estradiol occurred even in the presence of female serum, pituitary extract, growth hormone, or insulin, either alone or in various combinations. However, once the vitellogenic response has been initiated in vivo, secretory protein synthesis constitutes a constant proportion of the total protein synthesis for up to 3 days in culture (Fig. 3), in the presence of absence of estradiol in the medium. At least part of the stability of secreted protein production in vitro (and presumably in vivo) can be attributed to the stability of messenger RNA required for synthesis of the complex. When explants were incubated in a sufficient concentration of actinomycin D to block [H]uridine incorporation into RNA by more than 80% within 12 hours, synthesis and secretion of protein continued at a high rate for up to 48 hours, declining thereafter (Fig. 4). The same result was obtained using α-amanitin, although the extent of inhibition of RNA labeling by this compound was only 40 to 50%, presumably reflecting loss of activity of the nucleoplasmic RNA polymerase II in the liver (22). Nonsecreted protein labeling was also quite resistant to the effects of actinomycin and amanitin, suggesting that the metabolic stability of many cellular mRNA species in Xenopus liver is high. We have made no attempt to calculate the minimum half-life for secretory protein mRNA since the decline of protein synthesis in the absence of transcription may not be due to loss of mRNA (23).

![Fig. 3. Maintenance of secretory protein synthesis by hormone-induced explants in culture. Explants were taken from livers of frogs injected with estradiol 24, 48, and 72 hours previously. Replicate cultures were set up and incubated for up to 3 days as described under "Experimental Procedure." [3H]Serine (0.5 μCi per ml) was added for a 24-hour period on the first, second, or third day of culture and incorporation of radioactivity into secreted and nonsecreted protein was determined at the end of this period. Secretory protein synthesis is expressed as a fraction of total protein synthesis in order to correct for variations in precursor specific activity in the different explants and at different times in culture. The actual values for radioactivity in protein were within the range shown in Fig. 2. X—X, 24 hours after estrogen treatment; O—O, 48 hours after estrogen treatment; ●—●, 72 hours after estrogen treatment.

![Fig. 4. Stability of protein synthesis in culture in the presence of inhibitors of RNA synthesis. Explants were taken from a 3-day estrogen-treated Xenopus and cultured for up to 4 days in the continuous presence or absence of actinomycin D (5 μg per ml) or α-amanitin (1 μg per ml) as described under "Experimental Procedure." [3H]Serine was added in fresh medium with or without the same inhibitors for a 24-hour period on each of the days of the experiment and incorporation of radioactivity into secreted protein was determined at the end of this period. X—X, no inhibitors present; O—O, actinomycin D added; ●—●, α-amanitin added.

It is possible to interpret the evidence for a progressively increasing rate of secreted protein synthesis following a single hormone injection as reflecting accumulation of stable mRNA for those proteins within the liver cells. As Kafatos (24) has pointed out, synthesis of a relatively stable mRNA at a constant rate is the most reasonable mechanism by which a cell may synthesize increasing amounts of a specific protein product in response to

* Unpublished observations.
from culture media. Gels were prepared, sliced, and counted as described under "Experimental Procedure." A, 2 male Xenopus injected 12 days previously with 1 mg of estradiol were each given 50 μCi of [35S]methionine via the dorsal lymph sac. Blood was collected 24 hours later and vitellogenin was prepared by dimethylformamide precipitation from the serum. Labeled vitellogenin, 200 pg, was loaded on to the gel. B, 50 μl of culture medium from a 24-hour incubation of liver explants taken 13 days after estrogen injection and cultured in [35S]methionine (7.6 μC per ml). C, as in B but liver explants taken from control male Xenopus. The arrows indicate the positions of marker polypeptides used to calibrate the gels. λ1, μ1, μ0, ν1, and ν2 refer to the reovirus markers and "trypp." to trypsinogen. Note the change of scale of the ordinate between Gels B and C.

Environmental signals, including hormones. Bergink et al. (18) have produced data suggesting the synthesis of long-lived mRNA(s) for yolk proteins at a constant rate during the response of the rooster liver to estrogen administration in vivo and it may be that such a mechanism operates during the 12 days after administration of a 1-mg dose of estradiol to male Xenopus.

Characterization of Estrogen-induced Protein Secreted into Culture Medium—The ability of liver explants from estrogen-treated male Xenopus to synthesize yolk components in vitro has been studied by several workers (1, 2, 6). The nature of the secretory products has been established by TEAE-cellulose chromatography (1, 6) and by specific dimethylformamide precipitation followed by gel electrophoresis (2). Neither of these methods, however, has provided any information on the number of polypeptide components in the secreted vitellogenin complex. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis we have analyzed the polypeptide size distributions in labeled proteins secreted by explants from control and estrogen-treated animals and compared them with the pattern obtained with vitellogenin synthesized in vivo (Fig. 5).

The major component of authentic vitellogenin (Fig. 5A) has a molecular weight of approximately 180,000, but radioactivity also is found in regions of the gel corresponding to smaller poly- peptides. A large polypeptide with an electrophoretic mobility identical to the large subunit of authentic vitellogenin is heavily labeled by hormone-induced explants in culture (Fig. 5B) whereas control male Xenopus liver cultures secrete no polypeptides of molecular weight higher than 70,000 (Fig. 5C). Secreted polypeptides labeled by incubation of hormone-induced explants with either [35S]methionine (Fig. 5B) or [3H]serine (Fig. 6) give very similar distributions of radioactivity on sodium dodecyl sulfate gels, suggesting that no one component has a particularly high serine to methionine ratio. It is to be noted especially that a serine-rich peak is not found in the region of the gel where phosphovitin (which has a molecular weight of about 40,000) might be expected to run (Fig. 6). After this work was completed, Bergink and Wallace (10) reported molecular weight determinations on the subunit of vitellogenin. They obtained values in the range of 195,000 to 210,000 (by sodium dodecyl sulfate gel electrophoresis) and 185,000 to 195,000 (by agarose gel filtration) for the polypeptide size, provided that no degradation occurred during the isolation of the protein. These figures are close to our own estimates.

Conclusions

We have shown that, at the time of maximum response to injection of estradiol, as much as 90% of the total protein synthetic activity of male Xenopus liver is devoted to secretory products. Of these, virtually the whole appears to be the 180,000-dalton component of vitellogenin (Fig. 5B). Thus a consequence of estrogen treatment is to render the amphibian liver, which normally synthesizes many diverse proteins, as specialized toward the production of one product as is the mammalian reticulocyte for globin (26) or the chicken oviduct for the egg-white proteins (28). Hepatic tissue from estrogen-treated frogs, therefore, may constitute an important potential source of an mRNA coding for a single secretory polypeptide.

The absence of [3H]serine incorporation into polypeptides in the region of the gel corresponding to a molecular weight of 40,000 suggests that phosphovitin (which contains 52% of the serine residues of each vitellogenin molecule) must be synthesized and secreted as part of a larger polypeptide and converted to the form found in yolk platelets only during or after uptake by the ovary. A similar conclusion has been arrived at independently by Bergink.
and Wallace (10). Since phosvitin is not a primary translation product it may be concluded that "phosvitin mRNA" as such does not exist.

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