Coordination of ribosome content and polyome formation during estradiol stimulation of vitellogenin synthesis in immature male chick livers

(estradiol induction/yolk protein/ribosome synthesis/biochemical coordination)

ROBERT E. BAST, SANFORD A. GARFIELD*, LEE GEHRKE, AND JOSEPH ILAN

Department of Anatomy and Developmental Biology Center, Case Western Reserve University, Cleveland, Ohio, 44106; and * Department of Anatomy, School of Medicine, University of Virginia, Charlottesville, Virginia 22901

Communicated by E. Margolish, March 21, 1977

ABSTRACT To elucidate the mechanisms by which protein synthesis is affected by estradiol, we characterized cockerel liver polysomal profiles during hormone induction and withdrawal. We describe a method for isolating intact polyosomes which results in preparations that are stable even after storage in solution at 10° for 16 hr. In addition, our procedure eliminates the necessity for starving animals prior to experiments. Recovery of radioactive polyosomes indicated that yield is about 90% and that our polysomal preparations appear to represent polyosomal distribution in vivo. Using this approach we show that estradiol injection stimulates ribosome content 6-fold and that formation of polyosomes is coincident with the induction of vitellogenin synthesis. We also demonstrate that the size and number of polyosomes increase and decrease in a coordinated fashion with the rate of vitellogenin synthesis. The kinetics of ribosome synthesis and the fact that at least 80% of the newly synthesized ribosomes are directly recruited into polyosomes indicate that ribosomes might be limiting the rate of protein synthesis during the stimulatory phase of the hormone cycle. Here we describe the dynamics of polyome formation and dissociation in cockerel liver during hormonal induction and withdrawal. For these studies we used a method for isolating intact polyosomes which resulted in preparations that produced unchanged profiles even after storage at 10° in solution for 16 hr (Bast and Ilan, unpublished data). The results of our experiments also show that estradiol stimulates ribosome content 6-fold and that formation of polyosomes is coincident with the induction of vitellogenin synthesis. The size and number of polyosomes also increase and decrease with the rate of vitellogenin synthesis in a coordinated fashion. Furthermore, it seems that ribosomes might be limiting the rate of protein synthesis during the stimulatory phase of the hormone cycle.

A preliminary account of these findings was recently published (14).

MATERIALS AND METHODS

Animals. Newly hatched White Leghorn cockerels obtained from local hatcheries were kept in a 37° brooder and fed ad libitum. Birds weighing 100 ± 10 g, about 2 weeks old, were induced by subcutaneously injecting 2.5 mg of 17β-estradiol dissolved in 1,2-propanediol per 100 g of body weight. Control birds received solvent only. Isotopes were also injected subcutaneously.

Isolation of Polyosomes. Various times after hormone injection, birds were lightly anesthetized with ether and exsanguinated by cardiac puncture. After the gallbladder was removed, the liver was quickly excised and placed in a chilled, weighed beaker containing buffer A (50 mM Tris-HCl, pH 7.8 at 4°/25 mM KCl/5 mM MgCl2/250 mM sucrose/1 mM dithiothreitol/50 µM of cycloheximide per ml/100 µg of sodium heparin per ml). All following steps were performed at 2-4°.

The liver was rinsed twice with fresh cold buffer A, diced, and homogenized in 3 volumes of buffer B (100 mM Mes [2-(N-morpholino)ethanesulfonic acid], pH 6.0 at 4°/300 mM KCl/20 mM MgCl2/2 mM dithiothreitol/1 mg of sodium heparin per ml/5 µg of cycloheximide per ml) using a Dounce homogenizer and five strokes of the loose-fitting pestle. One-ninth volume of 10% Triton X-100 was added; the sample was mixed with three more strokes of the pestle and left to sit for 5 min. The homogenate was centrifuged at 480 × g (max) for 5 min to sediment nuclei and large pieces of cell debris. Centrifugation was continued for an additional 10 min at 12,000 × g (max) to pellet mitochondria and lysosomes. The supernatant was carefully poured off and combined with ½ volume of 1 M MgCl2. The pH was adjusted to 5.5 with 1 M acetic acid. After 1 hr at 4° the pH 5.5 solution was layered over several ml of buffer C (100 mM Tris [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], pH 7.1 at 20°/25 mM KCl/1 M su-

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.
FIG. 1. Time course of vitellogenin accumulation in the serum of cockerels. Birds were injected with 17β-estradiol at time 0 and killed at various times for determination of protein-bound, alkaline-labile phosphorus. Blood was collected by cardiac puncture. Vitellogenin concentration was calculated from phosphorus concentration by assuming that vitellogenin is 8% phosphorus by weight.

crose/2 mM dithiothreitol/500 μg of sodium heparin per ml/5 μg of cycloheximide per ml) and centrifuged at 29,600 × g (max) for 10 min. The polysomes, which formed a loose translucent gel, and usually a residual amount of buffer C were gently resuspended with a Teflon pestle in twice the liver-weight volume of buffer E (100 mM Tes, pH 7.5 at 4°/300 mM KCl/2 mM dithiothreitol/500 μg of sodium heparin per ml/5 μg of cycloheximide per ml). The gel of polysomes was fully dissolved by buffer E within 10 min, after which the polysome solution was clarified by centrifugation at 29,600 × g (max) for 5 min. We found that if buffers C and E contained Mg2+, then the polysomes were extremely difficult to resolubilize. It appears that in this procedure the polysomes carry a residual amount of Mg2+, which is sufficient to retain their integrity when they are precipitated with 0.1 M MgCl2. Polysomes were analyzed by layering 5–15 A260 units over a 12-ml linear 20–50% sucrose gradient. The sucrose was dissolved in buffer D (100 mM Tris-HCl, pH 7.6 at 4°/25 mM KCl/10 mM MgCl2/2 mM dithiothreitol/500 μg of sodium heparin per ml/5 μg of cycloheximide per ml/20 or 50% sucrose). Gradients were centrifuged in a Beckman Instruments SW41 rotor at 4° for 90 min at 40,000 rpm. The gradients were analyzed using a Gilford model 2400-2 recording spectrophotometer and density gradient scanner.

Chemical Determinations. Protein-bound, alkaline-labile serum phosphorus was determined by the technique of Martin and Doty (15) as modified by Wallace and Jared (16).

RESULTS

Time course of protein-bound, alkaline-labile phosphorus in serum of estradiol-induced cockerels

We felt it important to measure the rate of synthesis and the ultimate serum concentration of vitellogenin in our system, as these parameters may vary with species and age (5). Vitellogenin is a glycolipophosphoprotein which contains virtually all the protein-bound, alkaline-labile phosphorus present in the sera of estradiol-treated roosters (17, 18). Therefore, the level of phosphorus in the serum (see Materials and Methods) is an accurate representation of the vitellogenin concentration in the serum (19, 20). Fig. 1 shows a typical experiment determining the time course of the appearance of protein-bound, alkaline-labile phosphorus in the serum of cockerels at various times after hormone injection. As indicated on the left axis, the serum of a normal cockerel contains only trace amounts of alkaline-labile phosphorus. Within 1 day, detectable amounts of alkaline-labile phosphorus appear in the serum protein. Its concentration increases in a linear manner, reaching a peak on day 4 after injection. Thereafter, the concentration declines, till by day 7 the amount of alkaline-labile phosphorus present in serum protein is quite close to that in a control bird. Since the phosphorus content of vitellogenin is 3% by weight (7), the amount of vitellogenin in the serum can be calculated. This is shown on the right axis. The maximum vitellogenin concentration, i.e., day 4, is about 1.2 mg/ml of serum.

Bergink et al. (4) determined that the half-life of 32P-labeled vitellogenin in the serum of estrogen-treated roosters is 8.3 ± 0.4 hr. By assuming that a 100-g chick has 4 ml of serum (21), and knowing the vitellogenin concentration (Fig. 1), the rate of vitellogenin secretion by the liver can be calculated. At the peak of the induction, day 4, a 5 to 6-g cockerel liver is secreting 10.3 mg of vitellogenin per day into the serum.

Since the de novo synthesis of prodigious amounts of such a large protein [vitellogenin is a dimer with a monomer molecular weight of 240,000 (7)] might be accomplished by drastic changes in the protein-synthesizing apparatus of the liver, we decided to investigate changes in ribosome synthesis and distribution.

Determining the quality of isolated polysomes

The validity of this report ultimately depends on isolating polysomes representative of those in the intact cell; therefore, we decided to demonstrate the effectiveness of our isolation procedure. This topic will be dealt with exhaustively in a future communication (Bast and Ilan, unpublished data). Uninduced birds and birds induced 3 days previously were injected intra-peritoneally with [3,4,5-3H]leucine 45 min before they were killed. Polysomes were isolated and analyzed. Fig. 2A shows that no peak of radioactivity coincides with the monosome A260 peak in uninduced livers, indicating that no degradation occurred. On the contrary, significant radioactivity is apparent in the heavy polysome region, indicating the presence of large polysomes in quantities too low to be detected by their A260. As a control, an aliquot of the sample was treated with RNase, causing all the radioactivity to move from the polysomal region into the monosomal region.

Fig. 2B shows the pattern of labeled leucine incorporated in vivo into nascent polypeptides by cockerel livers 3 days after hormone injection. These livers are more active in protein synthesis, as is evident from the increased amount of A260 and radioactivity associated with the polysomal region of the gradient. Once again RNase treatment causes the 260 nm absorbing material and the radioactivity to migrate as monosomes. Therefore, the profiles appear to represent the in vivo distribution of ribosomes in polysomal structures.

Effect of estradiol on liver polysomes

Fig. 3 shows a typical series of liver polysomal profiles generated during the full course of estrogen stimulation. Polysomes could not be detected by their absorbance at 260 nm in preparations from uninduced liver. However, by 0.75 day after injection, peaks appeared in the polysomal region of the profile with greater absorbance in the smaller polysome region than in the larger polysome region. By day 4 the absorbance profile gradually shifted to one in which larger polysomes predominated and the amount of absorbance in the monosome and subunits region was relatively much decreased. From day 5 through day 7 the polysome profiles gradually returned to that of an uninduced liver, with the larger polysomes dissociating first. Increased absorbance in the polysomal region between days 0 and 4 was accompanied by an apparent decrease in the relative proportion of absorbance in the monosomal region. This may be due to recruitment of existing monosomes into poly-
Fig. 2. Distribution of labeled leucine in the nascent polypeptides on polysomes from (A) uninduced and (B) induced liver. Livers were labeled in vivo by injecting [3H]leucine 45 min before the birds were killed. (Right) An aliquot of each polysome preparation was treated with 5 μg of pancreatic ribonuclease per ml for 15 min at 4° as a control to show that radioactivity distribution was attached to polysomes.

1.0 0.6 0.2 0.4
300 200 100 50
Fraction number

However, the major source of increased absorbance in the polyosomal region must be attributed to the entry of newly synthesized ribosomes into polysomes. This is evident from the data presented in Fig. 4. The changes in total ribosome content per g of liver during various stages of estradiol induction are shown. The ribosome concentration in liver at 0.75 day and 1 day appears to be lower than in livers from uninduced birds. This is most likely due to edema, a response common to many estrogen-sensitive organs (22). By day 3 ribosomal concentration is greater than that found in uninduced liver. At the time of peak rate of vitellogenin synthesis, i.e., day 4, there is a 3-fold increase in the amount of ribosomes per g of liver over the value in uninduced control liver. However, on day 4 the liver weight of an induced animal is twice that of an uninduced control. This increase in weight is mainly due to water uptake. Therefore, the total ribosomal content in induced liver is 6 times that of uninduced liver when the increase in liver weight is accounted for. After day 4 the ribosome concentration gradually decreases, until by day 7 it has returned to almost the level in uninduced liver.

Fig. 4 also shows the proportion of ribosomes organized as polysomes. During estradiol stimulation and the consequent increasing rate of vitellogenin synthesis, the percent of ribosomes organized as polysomes increased from 42% in control liver to 84% in a maximally induced 4-day liver. The percent total ribosomes in polysomes jumped from 42 to 73% in just 0.75 day, in contrast to gradual increases in amount of ribosomes per g of liver and rate of vitellogenin synthesis. From day 2–4 the percent ribosomes in polysomes remained quite constant, varying between 77 and 85%. Thereafter the organization of
FIG. 3. Alterations of polysomal profiles during induction. Cockerels were injected with 17β-estradiol on day 0 and killed at 1, 2, 3, 4, 5, 6, and 7 days. The polysomal profiles were cut out and weighed. For estimation, the slope of the leading edge of the monosomal peak was extended to the base line. Subunits and monosomes were then cut from the profile and the weight of the polysomal region was determined.

ribosomes as polysomes declined precipitously until on day 7 the level returned to that found in control liver, i.e., 38%.

DISCUSSION

In order to explore the mechanisms by which vitellogenin synthesis is regulated by estradiol, we decided to characterize liver polysomes during hormone induction and withdrawal. First, the rate of vitellogenin accumulation at various times during the hormone cycle was determined. Phosphorus was detected in the serum at 1 day after injection. From day 1 to day 4 the phosphorus concentration increased in a linear manner. After day 4 the concentration declined till by day 7 there was once again almost no phosphorus detectable in the serum.

The data represented in Fig. 2 verify that conditions used for polysome, monosome, and subunit isolation are qualitative and should allow us to study the relationship between these components of the protein-synthesizing machinery during estradiol induction of vitellogenin synthesis. The absence of a radioactive peak in the monosomal region of sucrose gradients is an indication that polysomes are not extensively degraded during isolation and analysis. Yet, the treatment of aliquots with RNase and the consequent shift of radioactivity and absorbance to the monosomal region identify these properties as due to polysomal material.

We would like to stress that bird livers usually contain large amounts of ribonuclease and glycogen, and that it is common practice to starve animals to deplete glycogen reserves. Starvation brings about vast changes in polysomal profiles. Therefore, our preparations never involve starving the animal. The use of low pH and high salt concentration in the postmitochondrial supernatant dissociates RNase, glycogen, and pigment granules from polysomes. Therefore, when Mg²⁺ is added, the polysomes, ribosomes, and ribosomal subunits are chemically precipitated and separated from the above contaminants by centrifugation through a sucrose layer.

Polysome profiles were studied during the entire hormone induction cycle. Monosomes and disomes comprise the bulk of various times thereafter. Polysomes were isolated and analyzed as described in Materials and Methods. A parallel group of uninduced birds of the same age was killed during the experiment. Polysomal profiles from the livers of these birds were always similar to those of induced birds on days 0 and 7.
Table 1. Amount of ribosomal protein and vitellogenin synthesis at various times during induction

<table>
<thead>
<tr>
<th>Time interval, day</th>
<th>Protein synthesized, mg/liver Vitellogenin</th>
<th>Ribosomallevation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>3.75</td>
<td>2.9</td>
</tr>
<tr>
<td>2-3</td>
<td>7.15</td>
<td>6.8</td>
</tr>
<tr>
<td>3-4</td>
<td>10.1</td>
<td>24.3</td>
</tr>
</tbody>
</table>

* Rate of synthesis = (dx/dt) + Kd x; x = concentration; t = time; Kd = degradation coefficient = ln 2/t1/2 for ribosomes is assumed to be 2 days; for vitellogenin, 8.4 hr.

† For calculation of ribosomal protein synthesis assume 10 A290 units = 1 mg of ribosomes per ml and 50% of ribosome is protein.

The ribonucleoprotein particles present in the unstimulated and 7-day livers. Between these time points striking changes occur in the ribosome distribution and concentration (see Fig. 3). After an initial drop, due to the rapid uptake of water, the ribosome concentration begins to increase by day 2, with the maximum concentration occurring on day 4 after injection. Despite this 2.5 to 3-fold increase of ribosome concentration, the percentage of ribosomes in polysomes remains the same, i.e., 73–85% from day 0.75 until vitellogenin synthesis begins to decline after day 4. From day 4 to day 7 both ribosome concentration and their percentage in polysomes decrease, till on day 7 both values have returned to those of unstimulated livers. The curves shown in Figs. 1 and 4 are strikingly parallel and indicate that a coordinated regulation of all cellular functions is involved in the response to estradiol stimulation.

If one of the major products of this increased protein synthesis is vitellogenin, it should result in the appearance of a substantial amount of large polysomes in the profile. They would be necessary to direct the synthesis of the large vitellogenin polypeptide. The polysomal profiles we obtained show that this is not the case. This may be due to the fact that during induction ribosomes are limiting the rate of protein synthesis. Moreover, it may be that vitellogenin is not the major product of the hormone-induced increase in protein synthesis. As an example we have calculated the rates of vitellogenin and ribosomal protein synthesis during the stimulatory phase of the induction (Table 1). The calculations show that except for the earliest part of the stimulation more ribosomal proteins than vitellogenin are synthesized and at the peak of the induction period twice as much ribosomal protein is synthesized. The synthesis of ribosomal proteins is not the only example of coordinate regulation. Elongation factors (23) and other soluble factors (24) involved in protein synthesis demonstrate enhanced activity after hormone treatment. It seems that this response involves not only the stimulation of secreted proteins [i.e., vitellogenin and low density lipoprotein (25)], but also drastic increases in the functional protein-synthesizing machinery. Thus, when attempting to define the regulatory step in the synthetic pathway of a protein, one must be certain that an alteration in activity or quantity is the specific regulatory action and not a nonspecific response to increased demand for overall protein synthesis brought about by a coordinated regulatory mechanism.

This work was supported in part by U.S. Public Health Service Grant HD 06727 and a grant from the Population Council. R.E.B. is a recipient of a postdoctoral fellowship award from the Population Council. S.G. was a recipient of a Child Health and Human Development Postdoctoral Fellowship (I FO 2HD 54435). L.G. is supported by U.S. Public Health Service Training Grant 5-T01-EM00265.