Rates of Induction of Specific Translatable Messenger RNAs for Ovalbumin and Avidin by Steroid Hormones

(estrogen and progesterone action/mRNA/oviduct/differentiation/protein synthesis)

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ABSTRACT In the chick oviduct, injections of estrogen and progesterone induce synthesis of the specific proteins ovalbumin and avidin, respectively. We have studied the rate of induction of the specific messenger RNA molecules for these proteins after a single injection of estrogen or progesterone. The mRNAs were extracted, partially purified, and quantified *in vitro* in a heterologous protein-synthesizing system. Single injections of estrogen in chicks previously withdrawn from all steroid hormones for 2 weeks led to rapid increases in ovalbumin mRNA within 3 hr, which coincided with increases in the rate of ovalbumin synthesis. Maximal ovalbumin mRNA activity occurred by 18-20 hr. The half-life of the mRNA was estimated to be 8-10 hr and corresponded to the half-life for cessation of intracellular ovalbumin synthesis after a single injection of an estrogen. Similarly, after an injection of progesterone into chicks first treated with estrogen, appearance of avidin mRNA preceded demonstrable accumulation of this specific protein in oviduct cells. The mRNA for avidin was first apparent at 6 hr, and reached maximal concentrations between 18 and 24 hr after injection. These data confirm that both estrogen and progesterone act on oviduct to induce rapid accumulation of specific mRNAs before and coincident with the appearance of the cell-specific induced proteins. The overall results of these experiments are compatible with the hypothesis that the production of mRNA is a rate-limiting step in the steroid hormone-mediated induction of protein synthesis.

In the chick oviduct, both estrogen and progesterone selectively induce the synthesis of the specific proteins, ovalbumin and avidin, respectively (1-5). Studies in our laboratory have shown that both of these sex hormones induce changes in the pattern of synthesis of nuclear RNA before the appearance of specific proteins, in a manner that suggested differential gene transcription (3, 6, 7). Recently, methods have been devised to extract the mRNAs for both ovalbumin and avidin from oviduct (8, 24, 10, 12). These messengers can be translated with fidelity in a heterologous protein-synthesizing system derived from rabbit reticulocytes. Finally, we have demonstrated that the presence of these specific translatable mRNAs is absolutely dependent upon prior hormone administration (8-11).

In this communication, we examine the rate of induction of the specific messenger RNA molecules for ovalbumin or avidin after a single injection of estrogen or progesterone, respectively. The rate of estrogen induction of extractable ovalbumin mRNA, measured by the reticulocyte cell-free system, correlates extremely well with the increase in mRNA activity in

oviduct tissue as measured by the rate of incorporation of [³H]lysine into ovalbumin. Likewise, the appearance of avidin mRNA activity precedes avidin accumulation after injection of progesterone. The rates of induction of mRNA by these two sex hormones are compared, and the implication of these studies is discussed.

MATERIALS AND METHODS

Animals. 7-Day-old female Rhode Island Red chicks received daily subcutaneous injections of 5 mg of diethylstilbestrol (DES) in sesame oil for 2 weeks. They were then withdrawn for 2 weeks before again receiving a single subcutaneous injection of 1 mg of DES. In experiments using progesterone, the steroid was administered as a single injection to chicks previously treated with DES (5 mg/day) for 12 days. Chicks were killed by cervical dislocation at various times after this injection, as described in the text and figure legends.

In Vitro Labeling Conditions. The magnum portions of the oviducts were removed, and cut into 5- to 10-mg pieces. The tissue was then transferred to a 25-ml Erlenmeyer flask and incubated in 2 ml of Medium 199 without amino acids, but containing 1.2 mg/ml of NaHCO₃ and 5 μ Ci of L-[³H]lysine (19 Ci/mmol) (Amersham-Searle Corp.). Incubation was at 37° in the presence of 95% O₂-5% CO₂, and flasks were shaken continuously. After 2 hr of incubation, the tissues were removed from the medium, weighed, and a 5% (w/v) homogenate was prepared in 10 mM sodium phosphate-15 mM sodium chloride (pH 7.5). The homogenate was then centrifuged at 105,000 $\times g$ for 60 min at 4°. The supernatant fluid was used for determination of radioactive ovalbumin and Cl₃CCOOH-precipitable radioactivity.

Isolation of Partially Purified Oviduct mRNA. Total oviduct nucleic acid was prepared from chicks at various times after hormone administration (8, 10). The nucleic acid preparations were treated with DNase (electrophoretically pure, Worthington Biochem. Corp.) at a concentration of 2 μ g/ml in 2 mM Mg acetate-10 mM Tris-acetate (pH 7.0) for 60 min at 4°. The mixture was then again extracted with phenol and precipitated from ethanol. The DNase-treated RNA was then filtered on nitrocellulose membrane filters (Millipore) and eluted off the filters (12, 13). The oviducts from about 30 chicks were grouped together and used for each RNA preparation.

Abbreviation: DES, diethylstilbestrol.

Specific Protein Synthesis in the Rabbit Reticulocyte Lysate System. The assay contained in a final volume of 0.5 ml: rabbit reticulocyte lysate (1:4, v/v), 0.2 ml; ATP (pH 7.0), 0.2 mM; phosphoenol pyruvate (pH 7.0), 7.5 mM; pyruvate kinase, 0.3 IU; $MgCl_2$, 2.0 mM; Tris·HCl (pH 7.4 at 23°), 20 mM; KCl, 100 mM; 20 μ M each of 19 amino acids (minus valine); L-[^{14}C]valine, 10 μ M (572 dpm/pmol); and 5–10 μ g of the filtered nucleic acid. Incubation was for 60 min at 37°. 25- μ l Aliquots were removed from each tube, and precipitated in Cl_3CCOOH and counted by the standard procedures (14, 15); 400- μ l Aliquots were used to measure the radioactive ovalbumin or avidin synthesized by immunoprecipitation methods (8, 11).

RESULTS

Ovalbumin synthesis in oviduct tissue *in vitro*

The rate of *in vivo* synthesis of ovalbumin in oviduct after a single subcutaneous injection of 1 mg of DES to withdrawn chicks is shown in Fig. 1. It can be seen that 2 weeks after estrogen withdrawal, a basal rate of synthesis of ovalbumin exists but it is very low. When the experiments were repeated with liver slices, there were no counts in the immunoprecipitate, indicating that the small basal rate of synthesis of ovalbumin in chicks withdrawn from estrogen for 2 weeks is real and not an artifact of the assay system. Nevertheless, within 3 hr after a single injection of DES, there is a definite increase in the rate of ovalbumin synthesis. The rate reaches a peak at 18–20 hr, and returns to baseline by 36 hr. The insert in Fig. 1 shows the rate of synthesis of total oviduct protein in the same tissue. Here, the rate peaks slightly earlier at 12 hr. Moreover, there is a preferential synthesis of ovalbumin over other proteins since the rate of the former increases about 10-fold, whereas that for total protein only increases about 4-fold.

FIG. 1. Rate of L-[3H]lysine incorporation into ovalbumin and total protein after a single injection of DES. Withdrawn chicks were given a single subcutaneous injection of 1 mg of DES in sesame oil at zero time. At indicated times after the injection, the magnum portion of the oviduct was incubated in culture medium in the presence of L-[3H]lysine as described in *Methods*. The tissue was homogenized, and centrifuged as described in *Methods*. Aliquots of the supernatant fraction were assayed for ovalbumin by antiserum against ovalbumin (8). *Insert*: aliquots of the supernatant fluid were precipitated in 10% trichloroacetic acid, dissolved in NCS, and counted in spectrofluor-toluene. Each point represents the mean of at least three separate experimental determinations.

The increase in the rate of incorporation of L-[3H]lysine after DES administration could result either from an absolute increase in the rate of synthesis of ovalbumin or from a decrease in the L-lysine pool size within the protein-synthesizing compartment. Table 1 contains the results of experiments to confirm the exact mechanism of this increase. It can be seen from Table 1A that there is no significant change in the total pool of lysine in the supernatant fraction of a 5% homogenate, as determined by amino-acid analysis. This still cannot exclude subtle changes in pool size. Table 1B excludes this possibility since the increase in rate of L-[3H]lysine incorporation (ratio of the peak over the basal value) does not vary with different doses of L-[3H]lysine introduced into the system as would occur if permeability and compartmentalization were a factor.

Estrogen effects on ovalbumin messenger RNA levels

Previous studies in our laboratory showed that the activity of mRNA in the oviduct is dependent upon the hormonal state of the animal (8, 9, 11). There was also a good correlation of ovalbumin mRNA activity with ovalbumin synthesis and accumulation after repeated injections of DES (9). In the present study, the early rates of induction of ovalbumin mRNA after a single injection of DES were examined by assay of the total extractable oviduct mRNA in the reticulocyte assay system. As can be seen from Fig. 2, significant amounts of ovalbumin mRNA activity were not detected at 0 hr. Messenger RNA activity was first demonstrable at 3 hr after a single injection of 1 mg of DES. This activity increases with time after the injection and peaks at about 18 hr. Thereafter it again declines to undetectable concentrations by 48 hr. An excellent correlation exists between mRNA activity, as directly quantified by the reticulocyte cell-free system (Fig. 2),

TABLE 1.

(A) Lysine concentration in chick oviduct homogenate	
	nmol of lysine per ml of supernatant
Basal (0 hr)	8.01
Estrogen-stimulated (18 hr)	6.37
(B) Incorporation of various amounts of L-[3H]lysine into oviduct protein	
	Cl_3CCOOH -precipitable cpm (5 μ Ci [3H]lysine)
	Cl_3CCOOH -precipitable cpm (1 μ Ci [3H]lysine)
Basal (0 hr)	6.5
Estrogen-stimulated (18 hr)	5.6

(A) The magnum portion of the oviduct was incubated in culture medium, homogenized in buffer (5% w/v), and centrifuged as described in *Methods*. Aliquots of the supernatant fraction were analyzed for lysine in a Beckman 121 amino-acid analyzer. Each number represents the mean of two separate experimental determinations.

(B) The rate of L-[3H]lysine incorporation into total protein was determined as described in *Methods*, with 5 μ Ci and 1 μ Ci L-[3H]lysine per ml of culture medium, respectively, at 0 hr and at 18 hr after administration of DES. Each number represents the mean ratio of two separate experimental determinations.

FIG. 2. Effect of estrogen on ovalbumin mRNA activity, as compared to the rate of ovalbumin synthesis in oviduct. Withdrawn chicks were given a single subcutaneous injection of 1 mg of DES in sesame oil at zero time. For each time point after the injection, 30 chicks were killed, and total RNA was extracted and filtered (10, 11); mRNA activity was assayed in the reticulocyte lysate system (8). Each reaction tube contained 10 μ g of exogenous mRNA and the components described in *Methods*, with [14 C]valine as a label. mRNA activity is represented by the bars whereas the rate of protein synthesis (L-[3 H]lysine incorporation), as described in legend to Fig. 1, is superimposed (dashed line) for comparison. The background radioactivity in the mRNA assays ranged from 200–250 cpm and has been subtracted from the values shown.

and mRNA activity, as reflected by the rate of incorporation of L-[3 H]lysine into ovalbumin (see Fig. 1). There is some biological variation in the response, but good correlation is always obtained when experiments are done on the same batch of chicks within about 2 months. There is also some variation in response according to the dose of DES (manuscript in preparation) in that larger doses of estrogenic compounds produce a more extended curve, since tissue concentrations of the hormone remain elevated longer. For example, it is possible to delay the decline in mRNA content until 20–24 hr with a 5-mg injection of DES. An estimate of the half-life for ovalbumin mRNA can be made from the slope of the falling off of mRNA activity shown in Figs. 1 and 2. 50% of the activity has disappeared by 8–10 hr after the peak activity at 18 hr, suggesting an 8- to 10-hr $T_{1/2}$ for ovalbumin mRNA. This apparent $T_{1/2}$, however, probably represents an overestimation since a small amount of continued synthesis of ovalbumin mRNA may well be occurring on the descending limb of the activity curve, as evidenced by a slightly steeper slope obtained with lower doses of DES. Recent experiments have suggested that these specific mRNAs may have complex decay curves (manuscript in preparation).

Progesterone effects on avidin messenger RNA content

Fig. 3 shows the results of experiments in which chicks treated with estrogen were given a single injection of progesterone at 0 time and concentrations of avidin mRNA and oviduct content of avidin protein were then quantified at various times over the next 240 hr. Neither avidin mRNA nor avidin itself can be detected in oviduct tissue before administration of progesterone. After injection of the steroid, mRNA

was first detected at 6 hr, while accumulation of avidin protein was noted at 6–12 hr. Intracellular accumulation of avidin continues until 36–48 hr, at which time avidin mRNA is less than 50% of the peak level at 18–24 hr. Avidin was again unmeasurable at 120 and 240 hr and, correspondingly, avidin mRNA activity was also undetectable.

Avidin represents less than 1% of the total protein in egg white. Thus, it might be expected that there would be quantitatively less avidin mRNA in oviduct than ovalbumin mRNA. In these experiments detectable mRNA concentrations produced only 30–200 cpm of radioactive avidin when translated in the reticulocyte lysate system. The amount of mRNA initially detected at 6 hr was of marginal statistical significance. Results were similar, however, in three separate experiments using groups of chicks treated in an identical fashion.

Recently, we have increased the efficiency of our translation system (see legend to Fig. 4). When samples obtained from chicks stimulated with estrogen for 6 or 18 hr were incubated in the lysate system for 3 hr at 25°, the curves shown in the left panel of Fig. 4 were obtained. Maximal amounts of ovalbumin are synthesized in the presence of 10 μ g of oviduct mRNA fraction whether estrogen was administered for 6 or 18 hr. Moreover, the shapes of the dose-response curves are similar, and under these conditions, no ovalbumin is detectable with mRNA from unstimulated chicks. These results show that the differences in mRNA activity shown in Fig. 2 are not due to differential rates of synthesis of ovalbumin from similar amounts of messenger, but probably represent increased intracellular concentrations of the mRNA for ovalbumin. The new assay conditions result in a 50% increase in the amount of ovalbumin translated from 10 μ g of the mRNA fraction (18 hr) isolated from ovi-

ducts of chicks treated with a single injection of estrogen (compare Figs. 2 and 4).

The dose-response curve for avidin synthesis from oviduct mRNA extracted 6 or 18 hr after a single injection of progesterone is shown in the right panel of Fig. 4. Again, the shape of the curve is the same whether the mRNA is derived from animals stimulated at 6 or 18 hr and maximal avidin synthesis is achieved at the same RNA concentration (18 μ g). Two major differences can be seen when we compare the ovalbumin and avidin data. First, maximal avidin synthesis requires 18 μ g of mRNA, whereas only 9–10 μ g is required for optimal ovalbumin production. Secondly, the new assay conditions result in a more than 2-fold increase in the amount of avidin recovered in the antibody-antigen complex.

With both assays shown in Fig. 4 the amount of specific protein synthesized with increasing concentration of mRNA reaches a plateau and in fact begins to decline somewhat. This type of curve could be caused by the presence of inhibitory substances present in the mRNA preparations. At any rate, in order to assure reproducibility in the induction of mRNA with time, one must use an amount of mRNA on the linear portion of the curves. Such was the case in the present experiments.

DISCUSSION

The hormonal induction of specific protein synthesis has been demonstrated in many different systems, involving a host of different steroid hormones (for review, see ref. 16). This process can be prevented by various inhibitors of protein synthesis, e.g., puromycin or cycloheximide (4, 17). This finding implies that *de novo* synthesis of specific proteins occurs under such conditions. By the use of immunoprecipitation assay

techniques with antibodies against the specific proteins, this has been shown to be the case in several systems (18–20). That this also applies to the estrogen induction of ovalbumin in the chick oviduct was demonstrated by studies done both in our laboratory (ref. 6, and present study) and by Palmiter *et al.* (21). In this study, we have also shown that the induction process is real and not a labeling artifact secondary to a change in the amino-acid pool upon hormone treatment.

Inhibitors of RNA synthesis at appropriate concentrations can block the induction of specific proteins by steroid hormones (4, 16). It has always been assumed from these experiments that RNA synthesis is required for the process. We have reported that in the chick oviduct estrogen induces the appearance of ovalbumin mRNA activity, which parallels *in vivo* ovalbumin accumulation (9), and withdrawal of estrogen treatment results in disappearance of ovalbumin mRNA activity (8). The present communication documents the appearance of ovalbumin mRNA activity within 3 hr after a single estrogen injection. The changes in mRNA activity correlate well with the changes in the rate of ovalbumin synthesis. The half-life of the mRNA is probably less than the 10 hr suggested in Fig. 2 (manuscript in preparation). This again corresponds to the $T_{1/2}$ for cessation of ovalbumin synthesis after a single injection of estrogen. This finding indicates that the intracellular accumulation of mRNA for ovalbumin is the rate-limiting factor in the estrogen induction of this protein in chick oviduct.

Similar conclusions were drawn from experiments performed to determine the mode of action of a second steroid hormone, progesterone. We have reported the *de novo* induction of synthesis of the specific oviduct protein avidin in response to progesterone administration (3, 4). This response is of con-

FIG. 3. Effect of progesterone on avidin mRNA activity and accumulation of avidin in oviduct. Chicks treated with estrogen (12 days) were given a single subcutaneous injection of 1 mg of progesterone in sesame oil at zero time. For each time point 30 chicks were killed. All but two oviducts were used for extraction and partial purification of avidin mRNA (11, 12) and the mRNA activity for avidin was assayed in the reticulocyte lysate system (8, 12). Avidin was measured in a 105,000 $\times$ *g* supernatant fluid prepared from the remaining two oviducts by the [14 C]biotin assay (4, 5). mRNA activity is shown by the bars, whereas tissue accumulation of avidin is represented by the solid lines. Data represent average values from three separate experiments.

FIG. 4. Synthesis of ovalbumin (left) or avidin (right) *in vitro* as a function of the amount of oviduct mRNA. Chicks were given a single injection of DES (1 mg) or progesterone (1 mg), as described above. 30 Animals were killed at 6 and 18 hr after hormone administration, and a mRNA fraction was prepared (8, 11, 12). Samples were incubated in the lysate system (8) for 3 hr at 25° and specific protein synthesis was quantitated by immunoprecipitation (8, 11). To each tube to be assayed for avidin synthesis was added 50 μ l of a 30% ammonium sulfate pellet prepared from oviduct cytosol, as described by Schrader and O'Malley (25). ▲—▲, 0 hr; ○—○, 6 hr; ●—●, 18 hr.

siderable interest because progesterone results in very little change in net cellular RNA or protein synthesis (3-5). This report (see Fig. 3) reveals that after administration of the steroid hormone, and before and coincident with detectable avidin synthesis, the specific mRNA for avidin can be shown to appear in the tissue. Avidin is a protein that accumulates and is stored within oviduct cells before eventual secretion into egg white. Unfortunately our assay is not sufficiently sensitive to measure the *in vivo* rate of avidin synthesis in oviduct after progesterone stimulation. The [^{14}C]biotin assay (4) only measures total accumulation of avidin in oviduct and, as expected, shows a temporal delay relative to both mRNA accumulation and mRNA translation. Therefore, we cannot make a direct comparison of the fluctuation of mRNA content with the rate of synthesis of this protein, as is possible for ovalbumin.

The data illustrated in Fig. 4 were obtained under optimal conditions for cell-free translation of ovalbumin or avidin mRNA. The total amount of avidin synthesized was 75% of the total amount of ovalbumin made. Even though maximal avidin synthesis required twice the amount of mRNA fractions required in maximal ovalbumin synthesis, it is surprising that the total values were so similar. Ovalbumin comprises about 60% of total egg-white protein, whereas avidin represents less than 1%. One would theorize that the mRNA for ovalbumin would be present in a much greater concentration than that for avidin, even after a single injection of each steroid. One reason for this apparent discrepancy might be that since the oviducts have been prestimulated with estrogen but subsequently withdrawn from all hormone for 14 days, the short-term response of this metabolically quiescent tissue to a single injection of estrogen was most probably submaximal. Indeed, the peak of intracellular ovalbumin mRNA activity was only a fraction (6-8%) of that attainable after continuous endogenous or exogenous estrogenic stimulation. Avidin mRNA activity, however, was measured after a single injection of progesterone in estrogen-stimulated chicks. The value obtained was 25% of that obtained in mature hen oviduct.

The previous series of indirect experiments were consistent with the fact that both estrogenic and progestational steroids act in the oviduct to alter gene transcription in a manner that leads to the production of mRNA for the specific proteins ovalbumin and avidin, respectively. Administration of these hormones have resulted in quantitative changes in rapidly labeled nuclear RNA and RNA polymerase (3, 22) and qualitative changes in the populations of newly synthesized nuclear RNA, as measured by nearest-neighbor dinucleotide analysis and DNA-RNA hybridization (3, 23). Our present methods now allow a direct assessment of mRNA activity, and the experiments reported in this study appear to confirm that both estrogen and progesterone act on oviduct to induce rapid accumulation of specific mRNAs. We cannot yet define the precise mechanism of this response in that accumulation of mRNA might result from an increased rate of synthesis or a decreased rate of degradation or a combination of these events. However, considering the magnitude of the response,

the rate of accumulation of mRNA in the tissue, the estimated intracellular turnover ($T_{1/2}$) of the mRNA, and the baseline level of mRNA before stimulation, it appears likely that the steroid hormone acts in part to increase the rate of synthesis of these specific mRNAs. The overall results are consistent with the hypothesis that generation of mRNA is the rate-limiting factor in steroid hormone-mediated induction of protein synthesis.

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