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*SEQUENCES OF RNA AND PROTEIN SYNTHESIS DURING EARLY ESTROGEN ACTION**

BY TERRELL H. HAMILTON

DEPARTMENT OF ZOOLOGY, THE UNIVERSITY OF TEXAS, AUSTIN

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That RNA synthesis is a major aspect of early estrogen action in the uterus of a previously ovariectomized rat is now well documented.[1-5] Following administration of a single dose of estradiol-17b to such experimentals, synthesis within the uterine cells of a variety of biological molecules is accelerated, and evidence exists that this increase is one of qualitative[3] as well as of quantitative[1] differences in comparison to the control system. By four hours, rises in levels of RNA, nucleotides, protein, and phospholipids can be discerned,[2, 6-8] and it is well known that this *in vivo* uterine-response system is a valuable one for unraveling of the biochemical basis of early estrogen action in particular, and of hormone action in general.

Parallels between the biochemical basis of hormone actions in vertebrate and invertebrate organisms are now apparent,[9, 10] and it is a major goal of contemporary endocrinology—which seeks to explain on a molecular basis the ability of hormones to influence both the *synthesis* and *activity* of other biological molecules—to unify cellular theories for hormonal mechanisms of representative organisms throughout the animal kingdom. The current direction of thinking in many laboratories is that ontogenetic as well as phylogenetic tissue specificities[11, 12] to particular hormones result from genetic programing of intracellular responses to these. Thus, DNA–RNA interactions are involved in hormone actions, and the suggestions of Jacob and Monod[13] and Monod *et al.*[14] concerning the relation of hormone action to protein synthesis assume a new relevance, posing models which should be capable of testing in the near future.

Below are reported isotopic and inhibitor experiments which indicate the following about estrogen-induced synthesis of RNA and proteins throughout the four-hour *in vivo* response of uterine cells to single injections of 10 μg of estradiol-17b at zero hour in ovariectomized rats: (1) that between 30 min and 1 hr a small rise in protein synthesis occurs, lasting until $2^1/_2$ hr, at which time a marked acceleration of protein synthesis occurs; (2) that RNA synthesis during these test periods is accelerated by the time of 1 hr, rising markedly to a peak at 3–4 hr; (3) that actinomycin D, by preventing DNA-dependent RNA synthesis, prevents the typical estrogen-induced, 4-hr rise in RNA and protein synthesis with the RNA, *but not the protein* synthesis being restricted to levels below those of controls; and (4) that the time-sequence studies (1, 2) and inhibitor studies (3) illustrate (*a*) the involvement

of DNA-dependent RNA synthesis in early estrogen action, and (*b*) an action of estrogen on protein synthesis which is not actinomycin D-sensitive.

Thus, evidence is presented to support the thesis that estrogen initially induces, in ways yet unknown, protein synthesis which initiates or underwrites RNA synthesis, thus setting the stage for the following more extensive protein synthesis so characteristic of uterine responses to estrogen.

Materials and Methods.—Ovariectomized Holtzman rats of uniform weight (180–190 gm) and age were used in all experiments. All hormone injections were intravenous with 10 μg being administered at zero hour. Inhibitors were injected intraperitoneally in buffered saline at 15 min before, and 2 hr after, zero hour (doses: 5 mg for puromycin and 500 μg for actinomycin D). Merck, Sharpe and Dohme (Rahway, New Jersey) kindly supplied generous amounts of the latter inhibitor. All isotope injections were intraperitoneal at zero hour, with individual experimentals receiving either 50 μc of glycine-2-C^{14} or 40 μc of uridine-2-C^{14}. In all experiments (Figs. 1 and 2), the preceding doses were also injected into experimentals at 2 hr.

Estimates of C^{14}-labeling of protein are based on the following extraction technique: uteri were homogenized in 0.01 *M* tris buffer (pH 7.2, 4°C), and, following a 15-min SDS-DNase-RNase incubation at room temperature, the supernatant was collected and subjected to TCA precipitation followed by lipid removal as previously described.[1] Final counting of radioactivity was with a Tri-Carb liquid scintillation counter.

C^{14}-labeled RNA from homogenized uterine tissue was extracted in 0.01 *M* buffer (pH 7.2) as follows: after a 10-min SDS-DNase-PVS incubation at room temperature, unruptured cells and cell membranes were removed by moderate centrifugation. The supernatant was then twice extracted with water-saturated phenol, with the resulting aqueous solution (minus last interphase) being increased by addition of a 2× volume of 100% ethanol. This solution was stored for 24 hr at −10°C for precipitation of nucleic acid. The precipitate was collected, dissolved in buffer with PVS, and reprecipitated by ethanol. Dissolved in buffer, the RNA was then hydrolyzed by RNase (45 min, 25°C), and the solution made 5% PCA. After centrifugation, the supernatant specific activity was recorded.

Results.—(a) *Time-sequence studies on estrogen-induced synthesis of uterine proteins:* Figure 1 shows the rise in C^{14}-labeling of protein in uteri following injection of estradiol-17b and glycine-2-C^{14} at zero hour. At intervals of time indicated in the figure, experimentals were autopsied and the uterine proteins isolated for recording of specific activity as described above (see *Materials and Methods*). For such a time-sequence study of hormonal acceleration of synthetic processes, it is necessary to have a control for each time period. The results of these experiments indicate that between 30 min and 1 hr, a small rise in protein synthesis occurs in the estrogen-stimulated uterus, which is about 20 per cent over that of controls. Ranges for controls and hormone-treated animals overlap at 1 hr (as well as for periods up to $2^1/_2$ hr), but the means are different from 1 hr to $2^1/_2$ hr, with the experimental groups always having larger mean values than those of control groups. This, along with the fact that at 30 min both groups have approximately the same level of C^{14} incorporation, indicates that a small "hump" rise in protein synthesis occurs between 30 min and 1 hr following estrogenic stimulation of the atrophied, previously estrogen-deficient uterus.

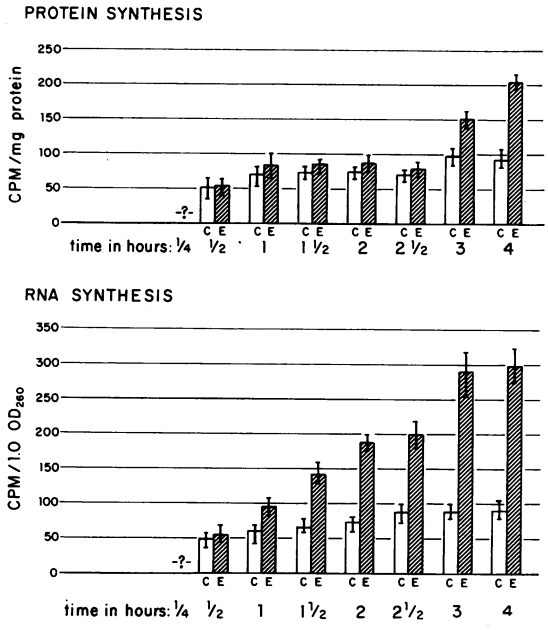

FIG. 1.—Time sequences for estrogen-induced acceleration of uterine protein (top half of fig.) and RNA synthesis (bottom half of fig.). Controls (*C*) for each experiment (*E*) are designated by open bars adjacent to bars giving experimental values. Each bar (ranges designated by brackets) represents a mean value for three experiments, with each of the latter representing values based on results from pooled uteri from three rats. Protein synthesis is indexed by glycine-2-C^{14} labeling, and RNA synthesis by uridine-2-C^{14} labeling (see *Materials and Methods*).

From 3 to 4 hr following administering of isotope and hormone, the specific activity of uterine protein rises markedly, indicating a rapid increase in rate of protein synthesis.

Two points are of further interest here. First, all attempts to recover isotope in proteins after 15 min failed in the sense that repeatable results could not be obtained. Values varied from background counts to values of 30 or 55 cpm/mg of protein. Second, evidence of delayed uptake by the uterus of the peritoneal-injected C^{14} amino acid in controls comes from the fact that at 3 and 4 hr control values rise appreciably over the control, plateau value held from 1 to $2^1/_2$ hr. The cause and meaning of this is unknown. For the present, it is interpreted as another example of experimental weakness of an *in vivo* system—a system where conditions for "flooding" with isotopic precursor may vary from one animal to another in ways not amenable to control by the investigator.

(b) *Time-sequence studies on estrogen-induced synthesis of uterine RNA:* Compared to the preceding findings (top half of Fig. 1), this experiment (bottom half of Fig. 1) indicates a rapid, rather linear rise in uridine-2-C^{14} labeling of uterine RNA. Again, in ways described in the preceding paragraph, difficulties were encountered in recording consistent labeling patterns at 15 min; but by 30 min, and continually to 3 hr, rises in the specific activity of uterine RNA can be correlated with time from zero hour.

In the experiment cited, increased labeling of uterine RNA in hormone-treated experimentals ceased by 3 hr, the 4-hr value being approximately the same. Pre-

liminary experiments, using younger, lighter-weight rats (along with the same doses of hormone and isotopes as used in the present one) had demonstrated a labeling rise in uterine RNA at 4 hr over that at 3 hr. This is cited merely to demonstrate the difficulty in reproducing results in *in vivo* experiments such as these—difficulties presumably attributable in this case to the problem of isotope saturation of the RNA precursor pool and of tissue or cellular uptake of the precursor. It will be noted (bottom half of Fig. 1) that control values demonstrate rises in isotopic incorporations into uterine protein from $2^1/_2$ to 4 hr following intraperitoneal injections of 20 μc of uridine-2-C^{14}. Nevertheless, mean values for RNA labeling in estrogen-stimulated experimentals show a significant rise in specific activity per optical density$_{260}$ unit from $2^1/_2$ to 4 hr—a rise continuing the trend for such from 1 to $2^1/_2$ hr.

Together, the two experiments summarized in Figure 1 indicate that RNA synthesis in the estrogen-stimulated uterus of the ovariectomized rat precedes that of protein synthesis. A further aspect of this is that, by one hour following injection of estrogen, a small "hump" rise in protein synthesis occurs concomitantly with the onset of the more extensive initial rise in RNA synthesis.

One possible conclusion from this is that estrogen initially activates, induces, or initiates a class of protein synthesis which (by underwriting the RNA synthesis which then occurs?) makes possible the later, more extensive burst of protein synthesis so characteristic of "late" estrogen action in the uterus. To study further such a possibility, inhibitors of RNA and protein synthesis were used, and these experiments are described below.

(c) *Influence of actinomycin D and puromycin on uterine RNA and protein synthesis in control and estrogen-stimulated experimentals:* Previous workers[1, 6] have demonstrated that puromycin, an inhibitor of protein synthesis, suppresses the rise in amino acid-C^{14}-labeling of uterine protein which normally occurs by four hours following injections of estrogenic steroid hormones in ovariectomized rats. The recent use, by various workers, of actinomycin D to inhibit DNA-dependent RNA synthesis raises the obvious question of the ability of this inhibitor to prevent the acceleration of RNA synthesis which normally follows estrogenic stimulation of uteri in previously estrogen-deficient rats.

Briefly stated, experiments testing the point (Fig. 2) indicate that combined

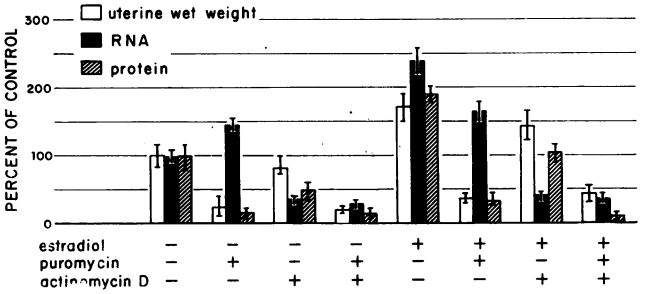

FIG. 2.—Influences of puromycin and/or actinomycin D on control and estrogen-stimulated uteri of ovariectomized rats. RNA synthesis is indexed by uridine-2-C^{14} labeling, and protein synthesis by glycine-2-C^{14} labeling (see *Materials and Methods*). Control values (- - -) are as follows: uterine wet weight, 67.6 mg per uterus; RNA synthesis, 426cp m/1.0 OD$_{260}$; and protein synthesis, 298 cpm/mg. Experimental groupings are as described in Fig. 1. The doses of isotope given at zero hour were repeated at 2 hr, and experiments were terminated at 4 hr.

injections of estradiol-17b and actinomycin D result in suppression of RNA synthesis to levels below those of controls as well as of estrogen-treated animals, and in restriction of protein synthesis to levels near those of controls. These conclusions are for uterine responses at four hours following injections of isotope, hormone, and inhibitor.

For the experiment where only actinomycin D is administered to experimentals (again see Fig. 2), RNA synthesis is restricted to levels below those of controls, and the same is noticed for protein synthesis. This finding, in light of the finding noted in the preceding paragraph, suggests that estrogen is capable of inducing some uterine protein synthesis simultaneously with inhibition of RNA synthesis by actinomycin D.

The preceding indicates, indirectly, a role of protein synthesis in early estrogen action which is independent of synthesis of DNA-dependent RNA. (Influences of early estrogen action on the activity of DNA-dependent RNA or on rate-limiting mechanisms for protein synthesis by nuclear and/or cytoplasmic polysomes are, of course, alternate hypotheses incapable of testing by the experiments cited in the present paper.) Thus, puromycin and actinomycin D may be tested together as well as in combination with estrogen, and these two experiments indicate that the two inhibitors suppress both RNA and protein synthesis to levels below those of respective control values, and that the two inhibitors with estrogen exert essentially the same inhibitory influence on RNA and protein synthesis in uterine cells (viz., to levels below those of controls).

These findings, then, suggest at the minimum that while actinomycin D can prevent estrogen-induced synthesis of uterine RNA and protein synthesis, only RNA synthesis is suppressed to levels below control ones. This observation raises the interesting possibility of an early influence of estrogen on protein synthesis in the uterus which does not involve the synthesis of DNA-dependent RNA.

(d) *Influence of actinomycin D on uterine wet weight in control and estrogen-treated experimentals:* Summarizing information in Figure 2, the influences of actinomycin D on wet weight of the uterus correspond qualitatively to changes noticed for protein synthesis (see (c) of *Results*). This may indicate that increased fluid imbibition of previously atrophied, but estrogen-stimulated, uteri reflects synthesis of proteins.[2, 6]

Discussion.—While there are a variety of conclusions which might be drawn from the data presented above, the following seem of particular interest in view of some recent findings by others.[2–5]

(1) *Relative times for onsets of uterine RNA and protein synthesis following estrogenic stimulation:* Noteboom and Gorski,[3] working with immature, nonovariectomized rats (body weights of approximately 50 gm), have reported evidence for increased labeling of uterine RNA by two hours following estrogenic stimulation, but not for increased labeling of uterine proteins. The findings reported here differ from theirs in the latter aspect. About the difference, two possibilities are suggested: (1) their doses of isotope (1–4 μc) may have been insufficient for saturation of amino acid pools available to uterine cells for synthesis of proteins; and (2) their experimental animals, not being ovariectomized, may have had in circulation a low level of endogenous estrogen maintaining uterine protein synthesis at time of injection of isotopes and hormone (5 μg of estradiol-17b). The possibility has yet to be

ruled out that time periods for estrogenic stimulation of RNA and protein synthesis in estrogen-free cells are different from those necessary for increases in such synthetic processes when cells are already functioning at a certain, low level of estrogen-maintained activity.

(2) *The early influences of estrogens on RNA and protein synthesis:* The following amplifies comments made above (section (c) of *Results*) and relates these to the work of Ui and Mueller.[2] They report, for example, that the influence of actinomycin D on hormone-free animals or on hormone-treated experimentals suppresses protein synthesis to about the same level (i.e., just beneath control levels). The difference between this finding and that of the present report (hormone-treated individuals having approximately control-level synthesis of proteins in the presence of actinomycin D inhibition of RNA synthesis, see Fig. 2) is attributed to the different experimental approaches to the problem of securing labeled proteins. However, these workers note that for their system protein synthesis is not seriously impaired by actinomycin D, and that uterine wet weights increase somewhat when estrogen and actinomycin D are coadministered. Thus they conclude from this that a "part of the information (i.e., RNA) for hormone response is already available to the protein synthetic machinery at the control uteri. ..."

Two lines of evidence support Ui and Mueller's conclusion. First, and this they note, Noteboom and Gorski[3] have presented evidence suggesting that RNA polymerase activity is increased in uterine nuclei 1 hr after estrogenic stimulation. They further report that the rise in such activity is inhibited by puromycin. Second, the results of the present report indicate (Fig. 2) that coadministration of puromycin and actinomycin D in conjunction with estrogen treatment suppresses both rises in uterine wet weights and in rates of protein synthesis—two responses which occur at higher levels when only actinomycin D and estrogen are given to experimental animals.

The question now arises about the means whereby estrogen leads to early protein synthesis in control uteri. Noteboom and Gorski[3] leave undecided the question of an estrogen-induced increase of activity or synthesis of RNA polymerase. The present findings, and those of Ui and Mueller,[2] suggest that a rise in synthesis of RNA polymerase is a likely possibility. However, it is clear that studies on the general topic have, to the present time, been mostly qualitative in the sense that molecules resulting from alteration of control rates of synthesis have not been identified, but have been measured in unspecific ways concerned with concentrations (total amounts and specific activity). Future progress in this area will now depend upon species characterization of uterine proteins, nucleic acids, nucleotides, and phospholipids whose formations and (presumably) activities change in ways still undescribed following estrogenic stimulation at zero hour.

Summary.—(1) Evidence is presented to demonstrate that, during the 4-hour uterine response to estrogen, over-all RNA synthesis is accelerated prior to acceleration of protein synthesis.

(2) Concomitant with the early rise in uterine RNA synthesis following estrogenic stimulation, a small "hump" rise in protein synthesis occurs by 1 hr. Such a pattern of estrogen-induced synthesis of uterine protein holds until $2^1/_2$–3 hr, at which time a rapid, more extensive phase of protein synthesis occurs.

(3) Actinomycin D suppresses estrogen-induced synthesis of RNA to levels below those of controls, but protein synthesis in this case is restricted to control values. Since actinomycin D in hormone-free controls limits protein synthesis to lower levels, and since combined estrogen-puromycin-actinomycin D treatments give maximum inhibition of RNA and protein synthesis, evidence exists for a component of uterine hormone response which is not sensitive to actinomycin D, but is sensitive to puromycin.

(4) The findings (1–3) hint that an early action of estrogen on the uterus involves induction or activation of protein synthesis which underwrites DNA-dependent RNA synthesis, and thereby controls the following more extensive phase of protein synthesis. The mechanism for this awaits elucidation.

The author continues to be indebted to F. L. Hisaw, H. S. Forrest, and R. H. Barth, Jr., for assistance in various ways.

The following abbreviations are used: RNA for ribonucleic acid; DNA for deoxyribonucleic acid; RNase for ribonuclease; DNase for deoxyribonuclease; TCA for trichloroacetic acid; PCA for perchloric acid; PVS for polyvinyl sulfate; SDS for sodium dodecyl sulfate; and cpm for counts per minute.

Concentrations of compounds used in this study for isolation of protein, RNA, and ribonucleotides are as follows: RNase, 20 μg per ml; Dnase, 20 μg per ml; PVS, 2%; SDS, 0.2%.

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*DEVELOPMENTAL HEMOGLOBIN ANOMALIES IN A CHROMOSOMAL TRIPLICATION: D_1 TRISOMY SYNDROME**

BY E. R. HUEHNS,† F. HECHT,‡ J. V. KEIL, AND A. G. MOTULSKY§

DEPARTMENTS OF MEDICINE AND GENETICS, UNIVERSITY OF WASHINGTON, SEATTLE

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Recent work on the structure and genetic control of human hemoglobin suggests that there are four loci determining the structure of each of the four different α-, β-, γ-, and δ-globin chains.[1] It has also been suggested that other genetic loci control their rates of synthesis.[2] Family studies indicate that the structural genes