*EARLY ESTROGEN ACTION: NUCLEAR SYNTHESIS AND ACCUMULATION OF PROTEIN CORRELATED WITH ENHANCEMENT OF TWO DNA-DEPENDENT RNA POLYMERASE ACTIVITIES**

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During the early action of estrogen in the uterus of the ovariectomized rat, nuclear RNA synthesis *in vivo* is stimulated prior to the sequential stimulations of DNA-dependent RNA polymerase assayed in isolated nuclei incubated in the absence or the presence of ammonium sulfate.[1] The stimulation by the hormone of nuclear RNA synthesis *in vivo* also precedes the stimulation of nuclear protein synthesis *in vivo*, which in turn precedes the enhancement of cytoplasmic protein synthesis.[2] These sequences of stimulation of RNA and protein synthesis in the nucleus have also been described for the early action of thyroid hormone in the liver.[3]

We now report data which demonstrate for early estrogen action that the increases in synthesis and concentration of nuclear protein *in vivo* parallel, respectively, the stimulations of the Mg^{2+}-activated and Mn^{2+}–$(NH_4)_2SO_4$-activated RNA polymerase reactions in whole nuclei assayed *in vitro*. The hormone-induced stimulation of nuclear protein synthesis precedes the increase in nuclear content of protein, and the stimulation of RNA polymerase in nuclei assayed in the absence of ammonium sulfate precedes the stimulation of the polymerase assayed in the presence of the salt at high concentration. A small but significant increase in the nuclear concentration of RNA as well as of protein was observed between 12 and 72 hours of estrogen action, coinciding with a dramatic increase at that time in concentration of cytoplasmic ribonucleoprotein (RNP) particles. We conclude that the synthesis and accumulation in the uterine nucleus of RNA and protein induced by the hormone represents an amplification of the protein-synthesizing machinery of the nucleus, necessary for an underwriting of the accelerated synthesis and transport to the cytoplasm of ribosomes or ribosomal components. The process of nuclear synthesis and transport to the cytoplasm of RNP particles is relatively rapid, and after the initial stimulation of nuclear RNA synthesis, the rate of this process appears to be inversely correlated with the concentration of the particles in the cytoplasm.

Materials and Methods.—Nearly all the materials as well as the experimental procedures employed have been described previously.[1, 2, 4, 5] By the procedure of Widnell, Hamilton, and Tata,[5] nuclei were isolated from uteri of 180-gm Sprague-Dawley rats either intact or ovariectomized for at least 3 weeks. This procedure provides enzymically active nuclei with little damage and cytoplasmic contamination. Whole nuclei thus isolated were washed twice in 0.32 *M* sucrose containing 3 mM Mg^{2+} by centrifugation at 700 gm for 10 min. The nuclei were then resuspended in 0.25 *M* sucrose containing 1 mM Mg^{2+} for assay of RNA polymerase or in ice-cold H_2O for determination of DNA,[6] RNA,[7] or protein.[8] In several experiments, isolated nuclei were washed in the presence of 5% triton X-100 (ref. 9), prior to chemical determination of DNA, RNA, and protein. All nuclear preparations were checked by phase microscopy for absence of cytoplasmic

(myofibril) contamination, and 20–30% of the total DNA was recovered in the final preparation. The assay of the Mg^{2+}-activated, and Mn^{2+}-$(NH_4)_2SO_4$-activated RNA polymerase reactions in uterine nuclei *in vitro* was exactly as described by Hamilton, Widnell, and Tata.[1b] Both RNA polymerase reactions were dependent upon the presence of all four nucleoside triphosphates, and were inhibited by addition to the incubation medium of DNase or actinomycin D.

Assay of rate of synthesis of nuclear protein rapidly labeled *in vivo* was as described by Means and Hamilton.[2] Rats were pulse-labeled by intraperitoneal (i.p.) administration of 100 μc of 1-H^3-methionine 10 min prior to killing and removal of uteri for isolation of nuclei as noted above and described elsewhere.[1, 5] The results were quantitated as counts per min of the labeled precursor incorporated per mg of nuclear protein. The dose of tritiated methionine used and the time of pulse-labeling were sufficient for saturation of the uterine precursor pool.[10] The cytoplasmic concentration of uterine polyribosomes was determined by isolation of the RNP particles from the supernatant fraction remaining after sedimentation of nuclei from the tissue homogenate.[4] The results were quantitated as the amount in mg of protein of RNP preparation isolated per mg of DNA in the tissue homogenate. Ovariectomized animals received 10 μg i.p. of estradiol-17β at time zero, and again at intervals of 24 hr thereafter or at 36 hr in experiments concerned with long-term effects of the hormone.

Results.—In Table 1 are listed the effects of either serial administrations of 10 μg of estradiol-17β or diestrus on the activities of the two[11] DNA-dependent RNA polymerase reactions assayed *in vitro* and on nuclear protein synthesis *in vivo* in uterine nuclei from ovariectomized rats. The Mg^{2+}-activated polymerase reaction was stimulated 14 per cent and 140 per cent at 1 hour and 12 hours, respectively, after administration of the hormone to ovariectomized rats. This

TABLE 1. *Effect of diestrus or administration of estradiol-17β on synthesis of nuclear RNA in vitro or nuclear protein in vivo in the uterus of the intact or ovariectomized rat.*

Time of killing after initial administration of hormone at time zero (hr)	RNA Polymerase (cpm/mg DNA) Mg^{2+}-activated reaction	MN^{2+}-$(NH_4)_2SO_4$-activated reaction	Specific activity of nuclear protein (cpm/mg protein)
Ovariectomized animals			
0 (control)	2104	3744	340
0.3	2056	3670	—
0.5	—	—	228
1	2840	3820	—
2	—	—	406
4	4328	3838	1180
8	—	—	1630
12	5100	4220	—
24	5288	5594	—
48*	5326	5824	492
72*	5428	5268	358
Intact animals			
0 (diestrus)	5172	6820	302
0 (estrus)	6690	6738	—

Estradiol-17β (10 μg dissolved in 0.1 ml 1, 2-propanediol) was administered to ovariectomized rats at time zero and at later times in the experiments designated. For assay of the two RNA polymerase reactions, animals were killed at the time indicated, and the uteri removed for isolation of nuclei and incorporation of C^{14}-ATP into RNA *in vitro* as previously described. For assay of nuclear protein synthesis *in vivo*, each animal was administered 100 μC 1-H^3-methionine (50 mc/mmole; from New England Nuclear Corp., Boston) 10 min prior to killing and removal of the uteri for isolation of nuclei and determination of specific activity of the total protein therein. From four to eight ovariectomized or intact rats were used in each control or experimental group.

* Hormone administered again at intervals of 24 hr (RNA polymerase), or at 36 hr (nuclear protein).

observation is in good agreement with previous findings.[1, 12] This elevated activity of RNA polymerase (approx. 140–150%) was maintained to 72 hours by serial administrations of the hormone, approaching the activity observed for the polymerase reaction in uterine nuclei isolated from intact animals in diestrus. The activity of the Mn^{2+}–$(NH_4)_2SO_4$-activated RNA polymerase reaction in uterine nuclei assayed *in vitro* was not stimulated during the interval of 12 hours following administration of the hormone.[1] From 12 to 72 hours, however, this polymerase reaction was stimulated 40–60 per cent.

The specific activity of nuclear protein rapidly labeled *in vivo* in the uterus of the ovariectomized rat exhibited an initial decrease of 50 per cent at 30 minutes during the course of estrogen action (Table 1). This was followed by a 400 per cent rise at 8 hours, and then a decline to a value at 72 hours near that of the untreated control or diestrous rat. The initial depression and subsequent stimulation of the specific activity of nuclear protein *in vivo* during early estrogen action from 30 minutes to 8 hours has been described previously.[2, 13]

In Table 2 are given our data for nuclear ratios of RNA/DNA and protein/DNA and for concentration of cytoplasmic RNP in the estrogen-stimulated or diestrous uterus of the overiectomized or intact rat. The ratio of nuclear RNA/DNA showed a slight increase of 10–20 per cent between 12 and 72 hours of estro-

TABLE 2. *Effect of diestrus or administration of estradiol-17β on the RNA/DNA and protein/DNA ratios of uterine nuclei and on uterine concentration of DNA and cytoplasmic RNP in the intact and ovariectomized rat.*

Time after initial administration of hormone at time zero (hr)	Nuclear Ratio* RNA/DNA	Nuclear Ratio* Protein/DNA	DNA/ uterus (mg)	Cytoplasmic RNP (mg protein/ mg DNA)	Wet weight/ uterus (mg)
Ovariectomized animals					
0 (control)	0.23 ± 0.03	2.20 ± 0.14	0.98	45	68 ± 5
0.5	0.24 ± 0.04	2.05 ± 0.18	0.91	48	—
1	—	—	0.85	47	—
2	0.28 ± 0.04	2.32 ± 0.15	0.96	51	87 ± 6
4	0.27 ± 0.04	2.30 ± 0.06	0.92	63	102 ± 10
12	0.29 ± 0.05	2.30 ± 0.15	0.97	96	117 ± 9
24†	0.32 ± 0.05	3.36 ± 0.11	1.10	223	130 ± 8
36	—	—	1.22	—	135 ± 9
48†	0.32 ± 0.06	3.64 ± 0.09	1.20	405	140 ± 11
72†	0.33 ± 0.06	3.78 ± 0.13	1.24	413	210 ± 19
Intact animals					
0 (diestrus)	0.32 ± 0.05	3.54 ± 0.20	1.26	420	213 ± 16
0 (estrus)	0.34 ± 0.04	3.28 ± 0.16	1.49	—	—

Ovariectomized (control) animals were administered 10 μg estradiol-17β at time zero and at later times in the experiments designated. Animals were killed at the times indicated for removal and weighing of uteri and isolation of nuclei. Each nuclear preparation was washed twice prior to determinations of RNA/DNA and protein/DNA ratios. Polyribosomal preparation was isolated from the supernatant fraction remaining after sedimentation of nuclei. Aliquots of the tissue homogenate were taken for DNA determination. Each control or experimental group consisted of eight animals. With the exception of the data on uterine DNA and cytoplasmic concentration of RNP particles, each value given is the average of triplicate experiments with the ranges indicated. The other experimental details were as described elsewhere and under *Materials and Methods*.

* Average value for triplicate experiments with their range above and below (±) the average designated.

† Hormone administered again at intervals of 24 hr (nuclear ratios and DNA), or at 36 hr (RNP particles and uterine weight).

gen action in the ovariectomized rat, approaching the value of 0.32–0.34 observed for diestrous or estrous uteri of intact animals. The ratio of uterine nuclear protein/DNA demonstrated a 40–60 per cent increase between 12 and 24 hours of estrogen action. Thereafter to 72 hours and in diestrous or estrous uteri of intact animals, the ratio of nuclear protein/DNA was essentially constant, being 3.3–3.8. Preliminary experiments have shown that most of the RNA and protein of the extrachromosomal nuclear fraction of either estrogen-deficient or estrogen-stimulated uteri is recovered in RNP particles of a ribosomal subunit nature when distributed on a sucrose density gradient.

In accordance with a previous finding,[4] the concentration of cytoplasmic RNP particles in the uterus of the overiectomized rat increased eightfold during 72 hours of estrogen action (Table 2). The wet weight of the uterus increased about 200 per cent during this period of hormone action. Values for DNA and wet weight per uterus at 72 hours of estrogen action in the ovariectomized animal were close to their respective values obtained for diestrous uteri.

TABLE 3. *Effect of diestrus or administration of estradiol-17β on the RNA/DNA and protein/DNA ratios of preparations of uterine nuclei isolated from the intact or ovariectomized rat and washed with detergent.*

Time after administration of hormone at time zero (hr)	Nuclear Ratio*		Time after administration of hormone at time zero (hr)	Nuclear Ratio*	
	RNA/DNA	Protein/DNA		RNA/DNA	Protein/DNA
0 (control)	0.18, 0.20	2.24, 2.03	24	0.28, 0.33	3.09, 3.27
12	0.21, 0.25	2.18, 2.34	0 (diestrus)	0.30, 0.36	3.33, 3.15

The conditions of this experiment were as described in Table 2, with the exception that the isolated nuclear preparations were washed in the presence of 5% triton X-100 (w/v) as described under *Materials and Methods.*

* Values for duplicate experiments are cited.

Finally, the data of Table 3 show that the trends for the effects of estrogen on nuclear RNA/DNA and protein/DNA ratios described above are also exhibited when the nuclei are washed in the presence of 5 per cent triton X-100 (see ref. 10 and *Materials and Methods*). It has been reported[10] that the detergent at the concentration cited causes the removal of the outer nuclear membrane from liver nuclei, so we applied the procedure to uterine nuclei in order to minimize the problem of cytoplasmic ribosome contamination of the nuclear preparations obtained.

Discussion.—An analysis of the temporal nature of the uterine biochemical parameters here described for estrogen action makes clearer the sequences of synthesis of nuclear RNA and protein in relation to their turnover to the cytoplasm in ribosomes[14, 15] or to their accumulation within the nucleus. It is useful in this context to consider the experimental values reported for estrogen treatment in Tables 1 and 2 as percentages of the values observed for untreated (control) ovariectomized rats.

Figure 1 thus summarizes our findings for the effects of estrogen on six such parameters in the ovariectomized rat or in the intact rat in diestrus. The early effects (*A*) of exogenous estrogen in stimulating nuclear RNA synthesis *in vivo*

from 2 to 20 minutes and in depressing nuclear and other subcellular protein synthesis at 30 minutes have been discussed in previous papers.[2, 13] Recently, Church and McCarthy[16] have also observed an extremely early effect of estrogen on nuclear RNA synthesis *in vivo* in the uterus. They find by RNA-DNA hybridization experiments that within minutes after administration of

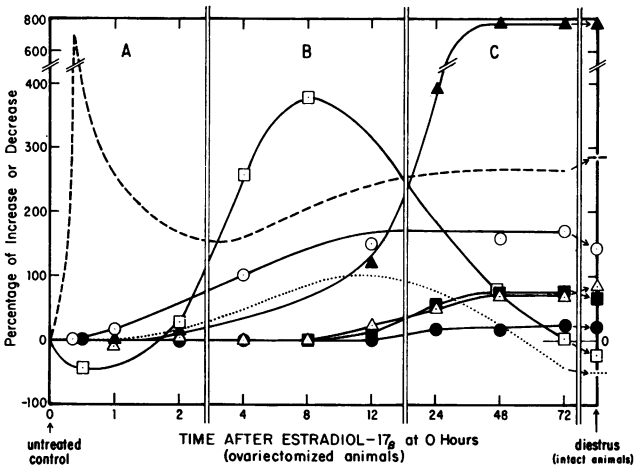

FIG. 1. Time course for the effects of administration to the ovariectomized rat of estradiol-17β on six of the uterine biochemical parameters listed in Tables 1 and 2. The data from the tables are expressed as percentage of increase or decrease in the values over or under the values observed for the untreated control animal. The symbols on the right ordinate indicate the biochemical parameters of the uterus of the intact animal in diestrus similarly expressed as percentages of the values for the untreated control.

O—O, Activity of the Mg^{2+}–activated RNA polymerase reaction; △—△, activity of the Mn^{2+}–$(NH_4)_2SO_4$-activated RNA polymerase reaction; □—□, specific activity of nuclear protein rapidly labeled *in vivo* with 1-H^3-methionine; ●—●, ratio of nuclear RNA/DNA; ■—■, ratio of nuclear protein/DNA (percentage values not shown prior to 8 hr, see Table 2); ▲—▲, concentration of cytoplasmic RNP; — specific activity of nuclear RNA rapidly labeled *in vivo* with H^3-uridine; ...., 1-C^{14}-leucine incorporation activity *in vitro* of cytoplasmic polyribosomal preparation. The last two parameters are taken from refs. 1 and 4.

estrogen to the ovariectomized rabbit there is an induced synthesis of nuclear RNA which is qualitatively as well as quantitatively different from that of the untreated control.

Why the rate of nuclear RNA synthesis *in vivo* during the early course of estrogen action should first accelerate (Fig. 1*A*), then decline, and then rise and plateau from 12 to 72 hours (Fig. 1*B*, *C*) is unknown. The trend may reflect an early enhancement of synthesis of chromosomal RNA[17] which then declines after certain genetic loci are completely activated. We have recently discovered that the RNA/DNA ratio of uterine but not liver chromatin increases significantly during the first 15 minutes following estrogen treatment *in vivo*. From 15 minutes to 12 hours, the initially increased ratio of RNA/DNA for the uterine chromatin remains constant. Thereafter, from 12 to 72 hours, both the RNA/DNA and the protein/DNA ratios of the chromatin increase.[18] Alterna-

tively, the increase in nuclear RNA synthesis *in vivo* may result in an early drain on the uterine ATP pool,[19] which we have surmised[2, 13] is the cause of the decreased protein synthesis in the organ during the first hour.

Two other correlations between the curves for uterine biochemical parameters during estrogen action shown in Figure 1 warrant particular attention:

First, there is a close association from two to eight hours between nuclear protein synthesis *in vivo* and the activity of RNA polymerase assayed in the presence of Mg^{2+} and the absence of ammonium sulfate (Fig. 1*B*). Although the identity of the proteins synthesized by the nucleus at eight hours remains to be determined, the marked rise thereafter in concentration of cytoplasmic RNP particles (Fig. 1*C*) suggests that ribosomal protein is one type synthesized. The product *in vitro* of the Mg^{2+}-activated RNA polymerase reaction is a ribosomal type of RNA both for hepatic[11] and uterine[1] nuclei. Tata[20] has also observed a correlation between nuclear protein synthesis *in vivo* and Mg^{2+}-activated RNA polymerase *in vitro* soon after hormone-induced growth of rat liver. Whether the effect of estrogen on RNA polymerase activity in uterine nuclei, occurring after a preceding stimulation of nuclear RNA synthesis *in vivo*, is dependent upon a preceding synthesis of nuclear protein is uncertain. The findings, however, of Summers, Noteboom, and Mueller[21] indicate that in nuclei of HeLa cells a continuous synthesis of protein is necessary for preservation of RNA-polymerase activity assayed at low ionic strength. A comparable conclusion can be drawn from previous observations[1, 12] that pretreatment of ovariectomized or immature rats with cycloheximide or puromycin abolishes the estrogen-stimulated activity of this RNA polymerase reaction in uterine nuclei.

Second, the 40–60 per cent rise from 12 to 48 hours in the nuclear protein/DNA ratio parallels nicely the 40–60 per cent enhancement of the Mn^{2+}–$(NH_4)_2SO_4$-activated RNA polymerase reaction during the same interval of time (Fig. 1*C*). In this instance the activity of RNA polymerase, assayed *in vitro* at high ionic strength, is very closely associated with an increase in nuclear content of protein, but not with nuclear protein synthesis *in vivo*. The rise in salt-activated RNA-polymerase activity observed 12–24 hours after hormone administration may be an example[21, 22] of the synthesis *de novo* of the polymerase enzyme. The product *in vitro* of the RNA-polymerase reaction occurring in the presence of 0.4 *M* ammonium sulfate is a more DNA-like type of RNA.[1, 11]

Our findings suggest that, in addition to accelerating the synthesis and transport to the cytoplasm of ribosomes, estrogen may effect an elevation of the protein-synthesizing capacities of subnuclear fractions by increasing their content of RNP. Indeed, an amplification of the RNA-(messenger and chromosomal RNA as well as ribosomal RNA) and protein-(RNA polymerase as well as ribosomal protein) synthesizing capacities of the uterine nucleus may well be necessary for the underwriting of the increased synthesis of ribosomes or ribosomal components which turn over to and accumulate in the cytoplasm as polyribosomes.[4] McCarthy, Parsons, Carter, and Laszlo[23] have shown that RNP particles isolated from subfractions of the rat-liver nucleus synthesize protein by essentially the same mechanism as those found in the cytoplasm. Since recent investigations have shown by electron microscopy and autoradiography that Mg^{2+}-activated RNA

polymerase resides in the nucleolus, whereas Mn^{2+}–$(NH_4)_2SO_4$-activated RNA polymerase is restricted almost entirely to the extranucleolar chromatin in the rat-liver nucleus,[24, 25] the increase in the nuclear content of RNP in the estrogen-stimulated uterus is expected to occur both in the nucleolar and the extranucleolar fractions.

In conclusion, our findings, along with those of Trachewsky and Segal,[26] point to early and sequential effects of estrogen on nuclear metabolism of RNA and protein in the uterus of the ovariectomized rat. An extremely early effect[27] of the hormone, occurring probably in the nucleolus and by derepression or activation of a portion of the genome controlling synthesis of a few specific molecules of RNA, appears in some way to result in an increased nuclear synthesis of RNA and protein, which in turn are transported as RNP particles to the cytoplasm. Furthermore, a modest increase in the RNP content of the nucleus may be necessary to underwrite the accelerated synthesis of the RNP destined for the cytoplasm. The involvement of chromosomal RNA in this sequence of hormonal responses remains to be clarified. The roles of acidic and basic proteins also remain to be determined. Both the amino acid-incorporation activity *in vitro* of cytoplasmic RNP particles and the specific activity of nuclear protein *in vivo* decrease from 8 to 12 hours to 72 hours as the cytoplasm becomes saturated with the particles (see ref. 4 and Fig. 1). This observation suggests that an unknown control mechanism, perhaps at the site of the nuclear membrane, whereby the nuclear rate of synthesis and transport to the cytoplasm of ribosomes or ribosomal components is inversely correlated with their concentration in the cytoplasm. At the minimum, our findings indicate that the turnover to the cytoplasm of nuclear ribosomes or ribosomal components must be a relatively rapid process. Only in this way can a dramatic rise in cytoplasmic RNP occurring simultaneously with a relatively minor increase in nuclear RNP be reconciled with the evidence for a nuclear origin of RNA and of ribosomes or ribosomal components.[28–33]

Conclusions.—From the data reported, the following major conclusions are drawn for the early action of estrogen in the uterus of the ovariectomized rat: (1) that a slight and delayed increase in the nuclear content of RNA and protein reflects an amplification of the protein-synthesizing machinery of the nucleus necessary for underwriting the accelerated synthesis and transporting to the cytoplasm of ribosomes or ribosomal components; (2) that the process of nuclear synthesis of ribosomal RNA and protein and their turnover to the cytoplasm in ribosomes or ribosomal components is a relatively rapid one; and (3) that by an unknown control mechanism, in operation after the initial stimulation of nuclear RNA synthesis, the rate of nuclear synthesis and transport to the cytoplasm of ribosomes or ribosomal components is inversely correlated with their concentration in the cytoplasm.

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