COMPARATIVE EFFECTS OF ESTRADIOL-17β AND ESTRIOL ON UTERINE RNA POLYMERASES I, II, AND III IN VIVO

by Robert A. Webster* and Terrell H. Hamilton

Department of Zoology
The University of Texas
Austin, Texas 78712

Received February 17,1976

Summary: The early and later effects of estradiol-17β and estriol on the RNA polymerase activities of uterine nuclei obtained from ovariectomized rats were compared. At 4 hr of hormone action both estradiol-17β and estriol stimulated the activity of polymerase I, but not the activities of polymerases II and III. At 24 hr, however, the effect of estriol had disappeared, whereas estradiol-17β stimulated all three polymerase activities. These results indicate that estrogen-induced growth of the uterus occurs in two phases, initiation and maintenance. Estriol initiates uterine growth, but does not maintain the process. Estradiol-17β, in contrast, does both. The differences in the effects of the two estrogens may reside in their different binding affinities.

Increased RNA synthesis has been shown to be an early and important event in the action of estrogen in inducing growth of the uterus (1). The stimulation by estrogen of uterine growth has been correlated with an increase in RNA polymerase activity in isolated nuclei and an increase in the number of cytoplasmic ribosomes (2). Moreover, the time course of stimulation is different with respect to the different types of RNA polymerases (2). Several reports have indicated that the early hormonal effects of estradiol-17β and estriol in the uterus are similar (3,4), whereas the later effects are not (3,5,6). The purpose of the investigation now reported was to compare the effects of these two estrogens in vivo on the activities of the RNA polymerases I, II, and III in isolated uterine nuclei at early (4 hr) and later (24 hr) periods of hormone action.

Materials and Methods

Female Sprague-Dawley rats were used not less than three weeks after ovariectomy. Estradiol-17β and estriol (20 μg) were injected intraperitoneally

* Present address: Department of Molecular Medicine, Mayo Clinic, Rochester, Minnesota 55901.

in 0.5 ml 50% (v/v) propylene glycol, 0.9% NaCl. Control animals received only the carrier solution. Animals were treated in groups of 6 and, at the indicated time, killed by cervical dislocation. Their uteri were quickly excised and pooled in ice-cold 0.25 M sucrose, 1 mM $MgCl_2$. Each uterus was cleaned of fat and mesentery and then slit lengthwise.

Uterine nuclei were isolated essentially as described by Widnell et al. (7) with modifications. The finely minced tissue was homogenized in 10 volumes of 0.5 M sucrose, 5 mM $MgCl_2$ with the Polytron PT-20 (Brinkmann Instruments) for 30 sec at 60v and then 120 sec at 40v. The homogenate was filtered through polyester cloth, diluted to 0.45 M sucrose with H_2O, underlaid with the 0.5 M sucrose, 5 mM $MgCl_2$, and centrifuged 10 min at 1000 x g (avg.). The pellet was washed once in 0.5 M sucrose, 1 mM $MgCl_2$. The crude nuclei were purified by sedimentation through 2.1 M sucrose, 1 mM $MgCl_2$ for 60 min at 40,000 x g (avg.). The purified nuclei were washed once in 0.5 M sucrose, 1 mM $MgCl_2$, and then suspended in 0.25 M sucrose, 1 mM $MgCl_2$. Small aliquots were quick-frozen in a dry ice-ethanol bath (-70°C) and stored at -30°C. Freezing, storage, and thawing did not affect the nuclear RNA polymerase activities. Nuclear DNA was determined using calf thymus DNA as a standard (8,9,10).

Liver nuclei were isolated by essentially the same procedure. Approximately 7 gm of liver from one rat was homogenized in 3 volumes 0.5 M sucrose, 5 mM $MgCl_2$ in a teflon-glass homogenizer with 30 strokes of the motor-driven pestle operated at 60v. The remainder of the procedure was the same as that for uterine nuclei.

The RNA polymerase activities of the isolated nuclei were determined under the Mg-low salt and Mn-high salt assay conditions essentially as described by Widnell and Tata (11). α-Amanitin, however, was added to some reaction mixtures to allow for the assay of RNA polymerases I, II, and III after the terminology of Roeder and Rutter (12). α-Amanitin inhibits only polymerase II at low concentrations in vitro (13).

Polymerase I activity was determined in a Mg-low salt reaction mixture containing α-amanitin. A final volume of 0.5 ml contained: 50 μmoles Tris-HCl buffer (pH 8.5 at 25°C), 2.5 μmoles $MgCl_2$, 10 μmoles monothioglycerol, 0.1 μmole EDTA·Na_2, 0.1 μg α-amanitin, 0.3 μmole each ATP, GTP, and UTP, .003 μmole [^{3}H]CTP (6 μCi), and 0.1 ml nuclear suspension (uterine nuclei, 35-85 μg DNA; liver nuclei, 75-100 μg DNA). The reaction mixture was incubated at 30°C for 15 min. The reaction was stopped by the addition of 0.5 ml cold 0.1 M NaPPi, pH 7 with HCl, containing .025 μmole CTP and 50 μg RNA.

The combined activity of polymerases II + III was determined in a Mn-high salt reaction mixture that contained, in 0.5 ml: 50 μmoles Tris-HCl buffer (pH 7.5 at 25°C), 2.0 μmoles $MnCl_2$, 200 μmoles $(NH_4)_2SO_4$, 10 μmoles monothioglycerol, 0.1 μmole EDTA·Na_2, 0.3 μmole each ATP, GTP, and UTP, .003 μmole [^{3}H]CTP, and 0.1 ml nuclear suspension. The reaction mixture minus the CTP was "preincubated" at 30°C for 15 min, after which time the CTP in .05 ml was added, and the incubation continued for another 45 min. The reaction was stopped as for polymerase I.

The activity of polymerase III was determined with α-amanitin (0.1 μg) in the Mn-high salt reaction mixture described above. Polymerase II activity represented the difference between the polymerase III activity and the polymerase activity in duplicate. Reaction mixtures to be used as assay "blanks" had the [^{3}H]CTP added after the reaction was stopped.

The [^{3}H]RNA was precipitated by adding 1.0 ml cold 20% trichloroacetic acid (TCA) to the stopped reaction mixtures. The precipitates were collected with vacuum on membrane filters (25 mm dia., 0.8μ pore) and washed with 15 ml 15% TCA, 0.1 M NaPPi; 5 ml 10% TCA; 5 ml 70% ethanol, 10% K-acetate; and 5 ml 70% ethanol. The precipitates were solubilized from the dried filters by incubating in 0.8 ml 0.2 N NaOH for 60 min at room temperature. These extracts were neutralized with 0.2 ml 5% acetic acid and mixed with 10 ml of a toluene scintillation solution (5 gm PPO, 0.1 gm POPOP, 110 ml Bio-Solv

BBS-3 solubilizer per liter). The radioactivity was determined in a Beckman scintillation spectrometer. The ^{3}H counting efficiency was always about 37% as determined by the internal standard method. The average cpm (100-200) for the "blank" reaction mixtures were subtracted from those of each polymerase assay mixture.

Table 1. Effect of α-amanitin on the RNA polymerase activities of liver nuclei from intact rats.

medium	μg α-amanitin (per mg DNA)	polymerase activity[a]	% inhibition
Mg-low salt	0	353	0
	0.01	324	8.1
	0.05	309	12.5
	0.1	304	13.8
	0.5	287	18.8
	1.0	281	20.4
	5.0	276	21.9
Mn-high salt	0	614	0
	0.01	237	61.4
	0.05	96	84.4
	0.1	77	87.5
	0.5	70	88.6
	1.0	85	86.2
	5.0	69	88.8

[a] pmoles of CMP incorporated per mg DNA

Results

The data of Table 1 demonstrate the effect of increasing amounts of α-amanitin *in vitro* on the RNA polymerase activities of liver nuclei assayed in the Mg-low salt and Mn-high salt reaction mixtures described by Widnell and Tata (11). Of the three major forms of eucaryotic RNA polymerase (I, II, and III) that have been characterized thus far (12,14), only polymerase II was inhibited at low concentrations of α-amanitin *in vitro* (13). This property and the comparative ionic strength and divalent cation optima (12) provide the methodological basis for the assay of polymerases I, II, and III in whole nuclei (15). As shown in Table 1, the inhibition of nuclear polymerase II activity reached a constant level in both the Mg-low salt and Mn-high salt reaction mixtures at a ratio of 0.1 μg of α-amanitin per 100 μg of DNA, the maximum amount of DNA as nuclei per reaction. Thus, the nuclear polymer-

ase activities determined with 0.1 μg α-amanitin in the Mg-low salt and Mn-high salt reaction mixtures represent those of polymerases I and III, respectively. The activity in the Mn-high salt reaction mixture without α-amanitin is that of polymerases II + III. The activity of polymerase II was determined by subtracting the polymerase III activity from the combined activity of polymerases II + III.

Table 2 shows the effect on these polymerase activities in uterine nuclei of estradiol-17β *in vivo* in ovariectomized rats. At 4 hr after hormone treatment there was no change in the polymerase II activity. At 24 hr, however, there was a 60% increase in the activity of this polymerase. This confirms the results reported by Hamilton *et al.* (2) for the estrogenic

Table 2. Effect of estradiol-17β *in vivo* on the RNA polymerase activities of uterine nuclei from ovariectomized rats.

medium	polymerase activity	hours after estradiol-17β incorporation[a] 0	4	24	% inhibition by α-amanitin 0	4	24
Mg-low salt	I	37	79	164	—	—	—
Mg-low salt + α-amanitin (0.1 μg)	I	30	71	160	19	10	2
Mn-high salt	II+III	187	206	308	—	—	—
Mn-high salt + α-amanitin (0.1 μg)	III	14	22	30	93	89	90
————	II[b]	173	184	278	—	—	—

[a] pmoles of CMP incorporated per mg DNA
[b] obtained by subtracting polymerase III activity from polymerase II + III activity

stimulation of the Mn-high salt polymerase activity. The observed increase of 140% in polymerase I activity at 4 hr of estrogen action (Table 2) likewise confirms their results for the activity of the Mg-low salt polymerase (2). While these investigators observed no further increase in the latter activity up to 24 hr after hormone administration, our present results indicate an increase of about 400% in the activity of polymerase I at 24 hr after hormone treatment (Table 2).

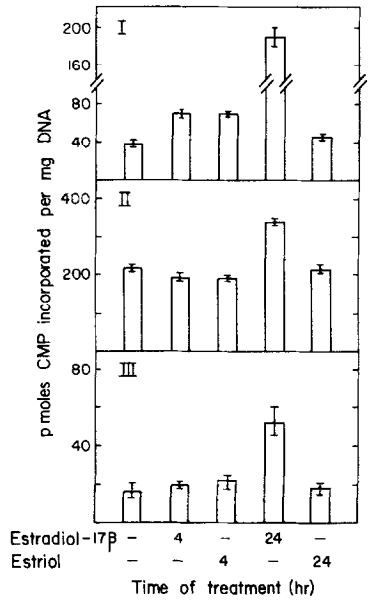

Fig. 1. Comparative effects of estradiol-17β and estriol in vivo on the activities of RNA polymerases I, II, and III in nuclei isolated from uteri of ovariectomized rats. Each bar denotes the range of variation for three replicate experiments.

The comparative effects of estriol and estradiol-17β in vivo on the uterine polymerase activities are shown in Fig. 1. At 4 hr after hormone treatment, the two estrogens were equally effective in stimulating polymerase I activity by about 80%. At 24 hr, however, only estradiol-17β evoked a significant increase in polymerase I activity of about 400%. At 4 hr, neither estrogen stimulated polymerase II activity, although both increased polymerase III activity slightly. At 24 hr, however, estradiol-17β stimulated polymerase II activity 60% and polymerase III activity 220%, while estriol caused no stimulation of either polymerase activity. Thus, both estriol and estradiol-17β are capable of inducing the short-term increase (4 hr) in polymerase I activity, but only estradiol-17β can effect the long-term increases (24 hr) in all three polymerase activities.

Discussion

Hisaw (3) demonstrated that both estriol and estradiol-17β can initiate uterine hypertrophy at 4-6 hr after administration of hormone, but that only estradiol-17β can maintain the growth response measured at 24 hr after treat-

ment. This is certainly related to the findings of Jensen et al. (16) demonstrating that the uterus initially takes up both estriol and estradiol-17β, but does not retain estriol as well as estradiol-17β. These results have been confirmed and extended by Anderson et al. (5,6). The difference in the retention by the uterus of the two estrogens may be related to their different binding affinities for cytoplasmic receptors. The respective dissociation constants for the two estrogen-receptor complexes are 2×10^{-9} M for estriol and 7×10^{-10} M for estradiol-17β (17).

Our results suggest that the estrogenic stimulation of uterine hypertrophy (2,3) has two phases: initiation and maintenance. Estriol can initiate uterine growth, but cannot maintain the process. Estradiol-17β, on the other hand, can do both. The activity of RNA polymerase I is stimulated during the initiation (4 hr) of uterine growth by both estrogens. Enhancement of the activities of polymerases II and III and additional increase in the polymerase I activity are observed only if the stimulation of the uterus is maintained to 24 hr of treatment with estradiol-17β, but not with estriol. Baquer and McLean (18) have shown that the activity of uterine ribose-5-phosphate isomerase was increased during initiation of growth at 2 hr of estrogen action. The activity of this enzyme increased again when growth was maintained for 16 hr, whereas the activities of many other uterine enzymes increased only when growth was maintained for 16 hr. Also DeAngelo and Gorski (19) and Cohen and Hamilton (20,21) have described specific proteins whose induction is restricted to the early (initiation) phase of estrogen action.

Considered together, the observations discussed and cited above indicate that the phenomenon of biphasic stimulations of multiple RNA polymerase activities correlated with biphasic inductions of synthesis of specific proteins or sets of proteins is a major feature of the action of estrogen(s) in stimulating growth and development of the uterus. The relevance of this correlation to the precise molecular mechanisms occurring in early and later estrogen action remains to be elucidated.

References

1. O'Malley, B. W., and Means, A. R., Science, 183, 610-620 (1974).
2. Hamilton, T. H., Widnell, C. C., and Tata, J. R., J. Biol. Chem., 243, 408-417 (1968).
3. Hisaw, Jr., F. L., Endocrinol., 64, 276-289 (1959).
4. Hamilton, T. H., Proc. Nat. Acad. Sci., U.S.A., 49, 373-379 (1963).
5. Anderson, J. N., Clark, J. H., and Peck, E., Biochem. Biophys. Res. Comm., 48, 1460-1468 (1972).
6. Anderson, J. N., Peck, E. J., and Clark, J. H., Endocrinol., 96, 160-167 (1975).
7. Widnell, C. C., Hamilton, T. H., and Tata, J. R., J. Cell Biol., 32, 766-770 (1967).
8. Burton, K., Biochem. J., 62, 315-323 (1956).
9. Schneider, W. C., J. Biol. Chem., 161, 293-303 (1945).
10. Munro, H. N., and Fleck, A., Methods of Biochemical Analysis, Vol. 14 (ed., D. Glick), pp. 113-176, Interscience Publishers, New York (1966).
11. Widnell, C. C., and Tata, J. R., Biochim. Biophys. Acta, 123, 478-492 (1966).
12. Roeder, R. G., and Rutter, W. J., Nature, 224, 234-237 (1969).
13. Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G., and Rutter, W. J., Science, 120, 447-449 (1970).
14. Chambon, P., Ann. Rev. Biochem., 44, 613-638 (1975).
15. Blatti, S. P., Ingles, C. J., Lindell, T. J., Morris, P. W., Weaver, R. F., Weinberg, F., and Rutter, W. J., Cold Spring Harbor Symp. Quant. Biol., 3, 649-657 (1970).
16. Jensen, E. V., Jacobson, H. I., Flesher, J. W., Saha, N. N., Gupta, G. N., Smith, S., Colucci, V., Shiplacoff, D., Neumann, H. G., DeSombre, E. R., and Jungblut, P. W., Steroid Dynamics (ed., G. Pincus, T. Nakao, and J. Tait), pp. 133-156, Academic Press, New York (1966).
17. Gorski, J., Toft, D., Shyamala, G., Smith, D., and Notides, A., Rec. Prog. Hormone Res., 24, 45-72 (1968).
18. Baquer, N. Z., and McLean, P., Biochem. Biophys. Res. Comm., 48, 729-734 (1972).
19. DeAngelo, A. B., and Gorski, J., Proc. Nat. Acad. Sci., 66, 693-698 (1970).
20. Cohen, M. E., and Hamilton, T. H., Biochem. Biophys. Res. Comm., 64, 633-639 (1975).
21. Cohen, M. E., and Hamilton, T. H., Proc. Nat. Acad. Sci., 72, 4346-4350 (1975).