RNA Transcription and Uterine Growth: Differential Effects of Estradiol, Estriol, and Nafoxidine on Chromatin RNA Initiation Sites†

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ABSTRACT: We have previously shown that the differential effects of estradiol (E_2), estriol (E_3), and Nafoxidine (N) on uterine growth and endogenous RNA polymerase activities are related to the retention of estrogen receptor–hormone complexes in uterine nuclei. These finding support the concept that estrogen exerts regulatory effects on target cells at the level of transcription. To examine these relationships further, we have utilized the rifampicin-challenge assay to investigate the effects of these hormones on the numbers of RNA chain-initiation sites specific for *Escherichia coli* RNA polymerase present on uterine chromatin. Treatment of immature rats with E_2 and E_3 resulted in rapid elevations in the numbers of RNA chains capable of being initiated between 1 and 4 h postinjection. By 6 h the number of RNA chains initiated on chromatin isolated from E_3-treated animals had returned to control levels. E_2 treatment resulted in sustained increases in the number of chromatin-associated RNA-initiation sites for *E. coli* RNA polymerase, which reached a maximum level at 12 h postinjection and gradually declined to control levels 48 h after treatment. Nafoxidine treatment, while only slightly increasing the number of RNA chains transcribed by exogenous RNA polymerase 6 h after injection, caused large increases 24 h after hormone administration. This increase was sustained up to 72 h postinjection. These differential effects of E_2, E_3, and N on RNA synthesis employing chromatin templates and exogenous RNA polymerase correlate with previous reports from our laboratory, demonstrating that a secondary rise in RNA polymerase II and sustained elevation in polymerase I activity induced by E_2 and N, but not E_3, are prerequisite for true uterine growth. The failure of E_3 to increase the number of RNA chains initiated beyond 4 h is consistent with the endogenous nuclear RNA polymerase data and suggests these changes in RNA polymerase activity are necessary for E_2 and N stimulation of RNA synthesis on uterine chromatin by *E. coli* RNA polymerase. These data confirm and extend our previous findings that long-term nuclear retention of receptors is a requirement for estrogen regulation of transcriptional events associated with true uterine growth.

A large body of evidence has accumulated over the last decade which suggests that estrogens modify target cell growth and differentiation, at least in part, by altering RNA transcriptional events (Hamilton, 1968; O'Malley and Means, 1974; Katzenellenbogen and Gorski, 1975; Yamamoto and Alberts, 1976). In either the immature or ovariectomized rat uterus, estradiol administration results in increased synthesis and accumulation of RNA, which reflect increases in endogenous nuclear RNA polymerase activities (Glasser et al., 1972; Hardin et al., 1976). These changes in RNA polymerase activities, at least for the first 6 h following hormone administration, do not appear to be due to an increase in the number of polymerase molecules (Cournvalin et al., 1976; Weil et al., 1977). Rather, these changes may reflect either alterations in chromatin template activity or increases in RNA polymerase activities by some means other than an increase in the number of enzyme molecules.

Estrogen-induced changes in chromatin template capacity as measured by the ability of chromatin DNA to act as a template for excess exogenous bacterial RNA polymerase were one of the first demonstrations that estrogen exerts an effect on uterine biochemistry at the level of transcription (Hamilton, 1968). Such studies demonstrated that estrogen administration to immature or ovariectomized rats resulted in early increases in uterine chromatin template activity. However, due to the complexity of the transcription process, these studies provided little information as to which components of the chromatin were altered or which steps in transcription were affected by the hormone.

Procedures have been recently adapted from procaryotic systems for measuring the initiation of RNA chains by bacterial RNA polymerase on eucaryotic chromatin templates (Cedar and Felsenfeld, 1973; M. Tsai et al., 1975). Both of these techniques utilize conditions under which each site available for initiation of RNA synthesis by RNA polymerase is capable of synthesizing only one RNA chain. Once this chain is completed, the enzyme cannot reinitiate synthesis. The latter technique takes advantage of the ability of the antibiotic rifampicin to specifically inhibit bacterial RNA polymerase not bound in a highly stable preinitiation binary complex (Chamberlin, 1974). When a mixture of chromatin and bacterial RNA polymerase is challenged with a mixture of ribonucleoside triphosphates and rifampicin, only those RNA polymerase molecules bound in a highly stable binary complex between DNA and the enzyme are capable of synthesizing RNA chains. This technique allows the measurement of the number of RNA chains initiated by the exogenous enzyme on a given quantity of chromatin DNA (M. Tsai et al., 1975). The sites measured by this assay procedure may not be identical to those used to initiate RNA synthesis on the chromatin template by the endogenous RNA polymerases in vivo. Rather, these measurements represent an indication of the activity of the chromatin template and do not necessarily represent the specific promoters expressed in vivo following hormone administration (M. Tsai et al., 1975). However, this assay has been used to demonstrate that, in the case of the chick oviduct, estrogen treatment results in an increase in the number of chromatin RNA initiation sites transcribed in vitro by *E. coli* RNA polymerase (Schwartz et al., 1975). In later studies it was shown there was a temporal correlation between the number of chromatin-associated estrogen receptor–hormone complexes and the number of initiation sites capable of being transcribed by RNA polymerase (S. Tsai et al., 1975; Kalimi et al., 1976).

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These findings agree well with the proposal that steroid hormones induce tissue-specific responses by initially interacting in the target cell with specific cytoplasmic molecules termed receptors. This interaction results in the transfer of the receptor–hormone complex to the cell's nucleus where it binds to chromatin. At least some of these complexes appear to interact with specific chromatin acceptor sites. This latter interaction is thought to result in altered patterns of transcription which ultimately results in the alteration of biochemical events required for the expression of specific hormonal response patterns (Clark et al., 1973a; O'Malley and Means, 1974; Gorski and Gannon, 1976; Yamamoto and Alberts, 1976).

In previous studies from this laboratory, we have shown that treatment of the immature rat with various estrogenic compounds results in altered uterine growth patterns. The nature of these altered growth patterns is dependent upon the nuclear retention of the estrogen receptor–hormone complexes (Anderson et al., 1972, 1973, 1975; Clark et al., 1973b, 1977). Estrogenic compounds such as estradiol or Nafoxidine that cause long-term nuclear retention of the receptor–hormone complex result in true uterine growth, as measured by increased uterine dry weight and DNA content 24 h after hormone treatment. On the other hand, no significant uterine growth is observed following single injections of short-acting estrogens, such as estriol or dimethylstilbestrol, which are retained in the nucleus for short periods of time (Anderson et al., 1972, 1975; Clark et al., 1976b, 1977; Lan and Katezenellenbogen, 1976; Capony and Rochefort, 1977; Ruh and Baudendistel, 1977). Additionally, we have demonstrated that nuclear retention of the receptor–hormone complex in uterine nuclei is temporally correlated with stimulation of endogenous nuclear RNA polymerase activities (Hardin et al., 1976).

In this paper, we show that long-acting estrogens like estradiol and Nafoxidine increase and sustain at elevated levels the number of *E. coli* RNA polymerase initiation sites measured in vitro which are present on rat uterine chromatin. Estriol, a short-acting estrogen, fails to stimulate significant increases in these chromatin-associated RNA polymerase initiation sites for exogenous polymerase.

Materials and Methods

Animals. Immature (21–23 day old) female rats of the Sprague-Dawley strain were obtained from the Texas Inbred Mouse Co., Houston, Texas. Animals were kept in a controlled environment of 72 °F with constant relative humidity and a light–dark cycle of 12 h each with the light cycle starting at 7 a.m. Food and water were provided ad lib. Estradiol and estriol were dissolved in 0.9% (w/v) NaCl which contained 1% (v/v) ethanol, while Nafoxidine was solubilized in distilled water which contained 1% (v/v) ethanol. Estradiol (1.0 μg), estriol (1.0 μg), Nafoxidine (50 μg), or 0.25 mL of vehicle was administered as a single subcutaneous injection at various times prior to sacrifice. Animals were sacrificed by cervical dislocation, and uteri were rapidly removed, stripped of adhering fat, and placed in ice-cold 0.9% (w/v) NaCl prior to chromatin isolation.

Isolation of Chromatin. All steps of the isolation procedure were performed at 0–4 °C. A modified version of the procedure of Spelsberg et al. (1971) was used for chromatin isolation. Uteri were drained, weighed, and minced finely with iris scissors. Ten to twelve volumes of 0.5 M sucrose–TKM was added to the minced tissue. The tissue was homogenized by 30-s pulses with a Polytron PT-10 (Brinkman) at a rheostat setting of 4.2 until no tissue clumps were visible. The homogenate was filtered through two layers of organza (100 mesh) into a glass homogenizer equipped with a tight-fitting Teflon pestle. The filtrate was further homogenized by six to ten strokes of the motor-driven pestle. Nuclei were collected by centrifugation at 10 000*g* for 10 min in a Beckman JS-13 rotor. Nuclear pellets were resuspended in 1.8 M sucrose–TKM and centrifuged at 13 000*g* for 45 min in a Beckman JS-13 rotor. The resultant pellet was resuspended in 0.5 M sucrose–TKM + 0.2% (v/v) Triton X-100. The pellet was collected by centrifugation at 10 000*g* for 10 min in a Beckman JS-13 rotor. The final nuclear pellet was washed three times by resuspension in 80 mM NaCl, 20 mM Na_2EDTA[1] (pH 6.3) followed by centrifugation at 2000*g* for 10 min. The washed nuclear pellet was resuspended in 50 mL of 0.01 × SSC and held on ice until >95% of nuclei had lysed as judged by phase-contrast microscopy. The chromatin was collected by centrifugation at 10 000*g* for 10 min and resuspended in 0.01 × SSC to a final DNA concentration of 0.3–0.5 mg/mL. The chromatin preparations were stored at 0–4 °C and were used within 5–7 days of isolation.

DNA Isolation. DNA was isolated from chromatin by the Marmur procedure (Marmur, 1961) with Proteinase K (25 μg/mL) being substituted for Pronase.

E. coli RNA Polymerase Isolation. E. coli RNA polymerase was isolated from frozen, 0.75 log phase *E. coli* K-12 cells by the Polymin P procedure of Burgess and Jendrisak (1975). Enzyme preparations were stored at −20 °C in storage buffer [0.01 M Tris-HCl (pH 8.0), 0.1 M Na_2EDTA, 0.1 M DTT, 0.1 M NaCl, 50% glycerol (v/v)] at a concentration of 10 mg/mL. Immediately prior to assay, enzyme preparations were diluted to appropriate concentrations with BSA (1 mg/mL) and were used immediately in transcription assays.

Determination of Rifampicin-Resistant E. coli RNA Polymerase Initiation Sites. RNA synthesis in the presence of rifampicin and heparin using uterine chromation as a template was performed by a modification of the procedure of M. Tsai et al. (1975). Reactions were run in 6 × 50 mm disposable culture tubes in a final volume of 150 μL. Varying concentrations of the enzyme (0–40 μg) were first incubated at 37 °C for 30 min with 1.5 μg of chromatin DNA or 0.5 μg of purified rat DNA suspended in 95 μL of preincubation buffer containing 12.5 μg of BSA, 10 μmol of Tris-HCl (pH 7.9), 1.25 μmol of $MnCl_2$, 2.5 μmol of β-mercaptoethanol, and 6.25 μmol of $(NH_4)_2SO_4$. At the end of the preincubation period, RNA synthesis was initiated by the addition of 30 μL of a mixture containing 5 μCi of [^{3}H]UTP (12–16 Ci/mmol), 18.75 nmol each of ATP, CTP, GTP, and UTP, 100 μg of heparin, 5 μg of rifampicin, and 0.1 μmol of potassium phosphate (pH 8.0). The reaction mixtures were incubated for an additional 15 min at 37 °C. Reactions were terminated by removing 50-μL aliquots from each reaction which were spotted on 2.5-cm DE 81 filters. These were washed and counted as described by Roeder (1974). Under these assay conditions, 1 pmol of [^{3}H]UMP incorporated represents 90 cpm.

Isolation and Sizing of in Vitro Synthesized RNA. RNA synthesized as described above was adjusted to 0.5% $NaDodSO_4$ and incubated in the presence of 50 μg of proteinase K at 37 °C for 30 min. An equal volume of redistilled phenol saturated with 0.01 M disodium acetate (pH 5.0), 0.01 M Na_2EDTA, and 0.5% $NaDodSO_4$ was added and the mixture shaken at room temperature for 15 min. The mixture was

[1] Abbreviations used: TKM buffer, 10 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 2.5 mM KCl; EDTA, ethylenediaminetetraacetic acid; SSC, 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0); DTT, dithiothreitol; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; TE buffer, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA.

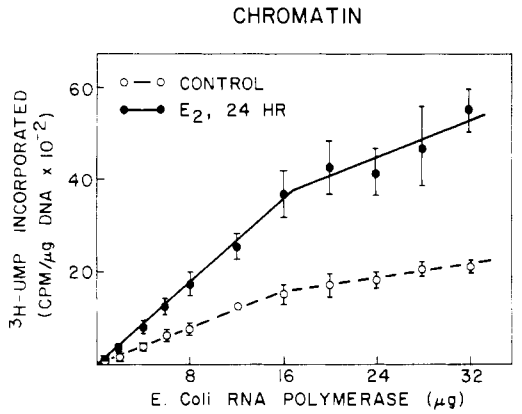

FIGURE 1: Titration of rat uterine chromatin with increasing concentrations of *E. coli* RNA polymerase. Animals were injected with either saline or estradiol as described in the text. At 24 h after injection, animals were sacrificed, chromatin was isolated, and RNA synthesis was measured using chromatin DNA as a template. Reactions were run in the presence of rifampicin and heparin: E_2 (●); control (○).

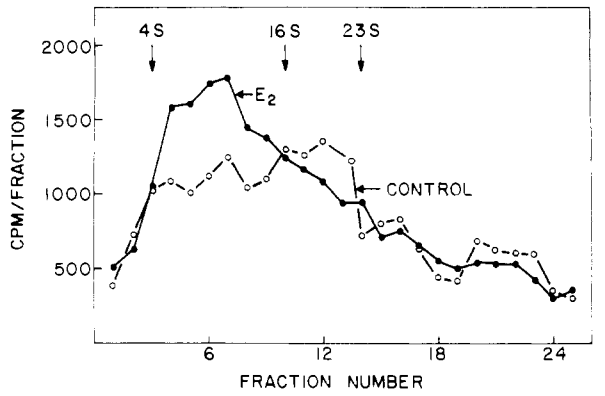

FIGURE 2: Sucrose gradient analysis of RNA synthesized on chromatin. Uterine chromatin was isolated from either animals injected with E_2 or with saline 24 h prior to sacrifice. Five micrograms of chromatin DNA was preincubated with 100 μg of *E. coli* RNA polymerase for 15 min at 37 °C. Nucleoside triphosphates, rifampicin, and heparin were added, and RNA synthesis was allowed to continue for 15 min. RNA was isolated and sized on 5–20% sucrose gradients as described under Materials and Methods. The chain length of RNA in each fraction was calculated. This value was then used to determine the number average chain length of the RNA. The number average chain length for RNA synthesized using E_2 chromatin as template was 390 nucleotides, control chromatin was 420 nucleotides: E_2 (●); control (○).

centrifuged at 6000*g* for 15 min; the upper phase was removed and reextracted with equal volumes of phenol and of chloroform–isoamyl alcohol (24:1, v/v). After extraction as above, the phases were separated by centrifugation. The upper phase was removed and 50 μg of purified tRNA was added as carrier. The RNA was precipitated by the addition of 2 volumes of absolute ethanol followed by storage at −20 °C for 18 h.

The precipitated RNA was collected by centrifugation and resuspended in 100 μL of distilled H_2O, and the number average chain length of the synthesized RNA was determined on 5–20% linear sucrose gradients as described by M. Tsai et al. (1975) using tRNA and rRNA isolated from *E. coli* as markers.

DNA Determination. DNA was estimated by the diphenylamine method of Burton (1956).

Chemicals. Unlabeled nucleoside triphosphates were obtained from P-L Biochemicals and [5-^{3}H]UTP was from Schwarz/Mann. Estradiol-17β, estriol, heparin, and rifampicin were from Sigma. Nafoxidine was a generous gift of the Upjohn Co. Frozen *E. coli* K-12 cells were obtained from Grain Processing, while Polymin P was a gift of BASF, Rhein, Germany. Proteinase K was from E. M. Labs, Elmsford, N.Y. All other chemicals were of the highest available grade.

Results

Figure 1 illustrates typical titration curves of rat uterine chromatin incubated with increasing amounts of *E. coli* RNA polymerase in the presence of rifampicin and heparin. The latter two components were included to inhibit RNA chain reinitiation and RNase activity, respectively (M. Tsai et al., 1975). Several points are evident from this type of curve. The amount of RNA being synthesized increased linearly with increasing enzyme concentrations until a transition point was reached. Previous studies (M. Tsai et al., 1975) have shown that the coordinates of this transition point can be used to calculate the number of high-affinity binding sites for RNA polymerase as well as the number of RNA chains initiated by *E. coli* RNA polymerase in vitro.

The data presented in Figure 1 also demonstrated that treatment of immature rats with E_2 24 h prior to preparation of chromatin significantly increased the number of rifampicin-resistant RNA chains initiated. Chromatin isolated from both control or E_2-treated animals exhibited similar patterns of saturation with increasing concentrations of RNA polymerase; however, E_2-treated chromatin allowed the initiation of over twice as many RNA chains, as reflected by the increased incorporation of [^{3}H]UMP into RNA.

The increased incorporation of [^{3}H]UMP into RNA when E_2-treated chromatin was used as a template for RNA synthesis as compared to levels of incorporation employing control chromatin as a template may have resulted from either increasing the numbers of RNA chains initiated or an increased length of the RNA synthesized. This results from the conditions employed in the assay, in which only RNA polymerase molecules bound to chromatin in a stable preinitiation binary complex are able to synthesize RNA. To obtain estimates of the size of RNA synthesized on rat uterine chromatin using exogenous RNA polymerase in the presence of rifampicin and heparin, the RNA synthesized under these conditions was isolated by phenol extraction and sized on 5–20% sucrose density gradients as described under Materials and Methods. A typical gradient is shown in Figure 2.

The data presented in Table I indicate that the RNA synthesized using either control or E_2-treated chromatin as a template was approximately the same size with respect to the number average chain length when assayed by sucrose density gradients. The number average chain length of the RNA synthesized in this study ranged from 390 to 470 nucleotides (Table I). This variation in size was not sufficient to account for any treatment effects with respect to the number of initiation sites for *E. coli* RNA polymerase that we observed in the rifampicin assay. These findings indicated that the estrogen-induced increase in [^{3}H]UMP incorporation into RNA using the uterine chromatin templates was the result of increased numbers of RNA chain-initiation sites for *E. coli* RNA polymerase in vitro rather than an increased length of the RNA synthesized.

As can be seen in the experiment illustrated in Figure 2, there appears to be a possible shift in distribution of chain lengths to a smaller size in the RNA synthesized on chromatin isolated from animals treated with estradiol. This possible shift is not reflected in the calculated number average chain length. Since this shift was not seen in all gradients, it may possibly be due to incomplete disaggregation of the sample prior to

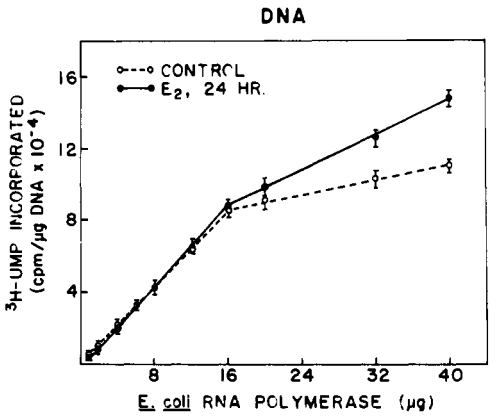

FIGURE 3: Titration of uterine DNA with increasing concentrations of *E. coli* RNA polymerase. DNA was isolated from the chromatin preparations used in Figure 1. Reactions were conducted as described in Figure 1 and in the text: E_2 (●); control (○).

TABLE I: Size of RNA Product Synthesized and Number of Sites Available for Initiation of RNA Synthesis in Vitro on Various Templates.[a]

template	size of RNA prod (nucleot)	initiat. sites/pg of DNA
control chromatin	415	42 000
E_3 chromatin, 4 h	440	63 000
E_2 chromatin, 4 h	395	54 600
E_2 chromatin, 12 h	410	105 000
E_2 chromatin, 24 h	390	88 250
N chromatin, 12 h	445	64 260
N chromatin, 24 h	475	82 740
control DNA	310	
E_2 DNA, 24 h	335	

[a] RNA was synthesized and isolated as described under Materials and Methods employing the templates indicated above. The isolated RNA was sized on linear 5–20% sucrose gradients as described using *E. coli* tRNA and rRNAs as molecular weight markers. The number average chain length in nucleotides was determined, and this value along with the quantity of [^{3}H]UMP incorporated at the transition point of titration curves were used to calculate the numbers of initiation sites per picogram of DNA template using the procedures described in M. Tsai et al. (1975).

layering on the gradient. The other possibility is the RNA being synthesized when using chromatin isolated from hormone-treated animals is being degraded due to ribonucleases bound to the chromatin. However, since the RNA being measured is synthesized in the presence of large amounts of heparin, ribonuclease activity is minimal. It appears that the overall effect of estrogen treatment is to increase the number of sites present on uterine chromatin capable of supporting initiation of RNA chains by *E. coli* RNA polymerase and not an increase in RNA chain elongation followed by preferential chain scission by endogenous ribonucleases.

One of the points investigated in this study was the role of chromatin structure in steroid-induced RNA synthesis in target tissues. One approach to this problem is illustrated in Figure 3. The DNA present in chromatin used as templates for the synthesis of RNA in Figure 1 was extracted as described under Materials and Methods. When these DNAs were used in titration analysis in the presence of rifampicin and heparin, both E_2 and control DNAs had nearly the same transition point. This was in sharp contrast to the data obtained when chromatin was used as a template (Figure 1). It should also be noted, as expected, that the purified DNAs are much more efficient templates for RNA synthesis than is chromatin.

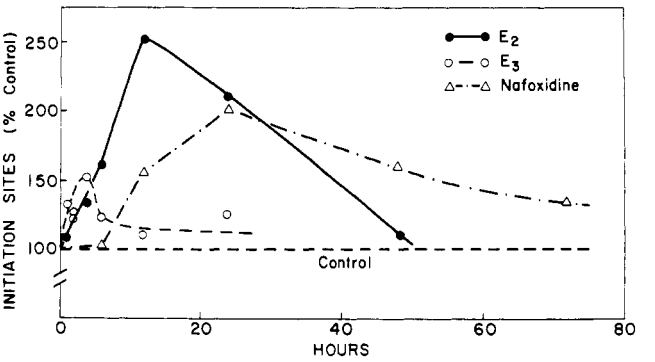

FIGURE 4: Time course of chromatin sites capable of initiating RNA synthesis following hormone injections. Animals were injected with either saline, estradiol, estriol, or Nafoxidine and sacrificed at indicated times. Uteri were removed and chromatin was isolated and used in titration assays as described in the text. Control levels represents 42 000 initiation sites/pg of DNA: E_2 (●); E_3 (○); N (△).

At higher concentrations of RNA polymerase, the two curves diverge significantly. This portion of the chromatin titration curve represents regions of weak interactions of the enzyme with the template. The differences in this region of the titration curves probably represent nonspecific effects due to damage to the template during the DNA isolation procedure. These data indicate chromatin structure is important for the expression of steroid hormone effects on RNA synthesis.

Several points should be made concerning the data presented in Figures 1 and 3. The transition points for the titration curves in both cases occur at about 16 μg of RNA polymerase; however, in the case of chromatin this represents 1.5 μg of chromatin DNA as template, while for purified DNA 0.5 μg of DNA was used. The data plotted on the ordinate have been normalized to a per microgram DNA basis for comparison purposes. When 1 μg of chromatin DNA or purified DNA was assayed, the transition points occurred at approximately 11 and 32 μg of DNA, respectively.

Previous work from this and other laboratories has shown that the nuclear retention of receptor–estrogen complexes is important in the estrogenic stimulation of such events as uterine growth and endogenous nuclear RNA polymerase activities (Anderson et al., 1972, 1973, 1975; Clark et al., 1973b; Hardin et al., 1976; Katzenellenbogen and Ferguson, 1975; Lan and Katzenellenbogen, 1976; Ruh and Baudendistel, 1977; Capony and Rochefort, 1977). This work has been facilitated by the availability of different classes of estrogenic compounds. We have classified these compounds as either short- or long-acting estrogens (Clark et al., 1977). E_3 represents a short-acting estrogen which is retained in the nucleus for short periods (1–4 h). E_2 is a physiological (long-acting) estrogen which is retained in target-cell nuclei for intermediate periods of time (6–24 h), while N is a long-acting estrogen with a very long nuclear retention time (>24–48 h).

The data presented in Figure 4 summarize the effects of estrogens on immature rat uterine chromatin RNA synthesis initiation sites for *E. coli* RNA polymerase following a single injection of hormone. Chromatin was isolated at each indicated time point, and the number of initiation sites was determined as described under Materials and Methods and Figure 1. The most important observation made from these data was that RNA initiation sites for *E. coli* RNA polymerase present on uterine chromatin increased and declined temporally with the accumulation and depletion patterns of receptor–estrogen complexes (Anderson et al., 1972, 1973, 1975; Clark et al., 1973a,b, 1977). Both estradiol and estriol induced early (1–4 h) increases in the numbers of chromatin sites capable of

forming binary complexes with RNA polymerase but only estradiol resulted in prolonged stimulation of this parameter for a period of 12 h. However, by 48 h the level of sites capable of initiating RNA synthesis had returned to control levels in estradiol-treated animals. This corresponds to the depletion of receptor–estrogen complexes from the nuclei of these animals.

Nafoxidine treatment which results in long-term nuclear retention of receptor–hormone complexes also produced prolonged increases in the number of uterine chromatin sites available for RNA synthesis in vitro. The number of sites resulting from this treatment peaked at 24 h after treatment and remained elevated 72 h following hormone injection. These results indicate that increased numbers of chromatin sites for initiation of RNA chain synthesis are dependent on the presence of nuclear estrogen–receptor hormone complexes.

Discussion

For a number of years it has been known that treatment of immature rats with estrogen results in increases in uterine RNA synthesis. This effect is thought to be at least partially at the level of chromatin transcription (Hamilton, 1968). This increased RNA synthesis may result from the interaction of the estrogen receptor–hormone complex with specific chromatin acceptor sites (Clark et al., 1973a; O'Malley and Means, 1974; Gorski and Gannon, 1976; Yamamoto and Alberts, 1975). Earlier studies which measured chromatin template activity using saturating amounts of exogenous RNA polymerase were difficult to interpret due to the complex nature of the transcription reactions (Chamberlin, 1974; M. Tsai et al., 1975). The present study has utilized techniques which only measure RNA synthesis which begins at synthesis sites specific for the initiation of RNA by exogenous RNA polymerase on uterine chromatin.

The data presented in Figure 1 indicate that when rat uterine chromatin is titrated with increasing quantities of *E. coli* RNA polymerase a definite transition point is reached in the titration curve. It has been previously demonstrated that when chromatin is preincubated in the presence of RNA polymerase and the reaction is subsequently challenged with rifampicin and nucleoside triphosphates, only the RNA polymerase molecules bound to chromatin in stable preinitiation complexes are capable of initiating RNA synthesis (M. Tsai et al., 1975). Therefore, our initial studies indicate that treatment of the immature rat with estradiol results in a marked increase in the numbers of RNA chain initiated by *E. coli* RNA polymerase using uterine chromatin templates. These results are in agreement with previous reports on changes in chromatin template activity induced by estrogen treatment and support the concept that estrogens modulate, in some unknown fashion, uterine chromatin transcription.

The absolute number of chromatin sites available for the initiation of RNA synthesis by exogenous polymerase and the RNA polymerase concentration at which a transition point is reached in the titration assay are much higher in this system than in the chick oviduct (compare Figures 1 and 4 with M. Tsai et al., 1975). The chick oviduct represents a tissue composed of a few cell types, while the estrogen-stimulated rat uterus represents a complex mixture of cell types, which appear to respond differently to estrogen treatment. This complex cellular response probably results in the large number of sites present on uterine chromatin capable of supporting RNA synthesis.

It has been reported that one of the effects of estrogen on RNA metabolism is to increase the rate of RNA chain elongation by endogenous RNA polymerase (Barry and Gorski, 1971). Results presented in Table I and Figure 2 indicate that the number average chain lengths of RNA synthesized under our conditions are the same for E_2-treated and control chromatins but that chromatin isolated from estrogen-treated animals synthesizes twofold more RNA at 12 h postinjection. These findings suggest that estrogen treatment results in an increase in the number of specific sites on chromatin available for the initiation of RNA synthesis rather than an alteration in the length of the RNA synthesized. The length of the RNA synthesized should not be affected by ribonuclease activities associated with the chromatin due to the presence of heparin during the synthetic phase of the reaction scheme.

Billing et al. (1969a,b) have presented data which indicate that changes observed in RNA synthetic activity in the rat uterus within the first few hours following estradiol administration are due to changes in nucleotide pool sizes. They observed that 5 h after hormone treatment there were marked increases in RNA synthesis, particularly, an increased synthesis of tRNA and rRNA. In a subsequent series of papers from the same laboratory, an early increase in very high-molecular-weight RNA synthesis independent of pool changes was noted (Knowler and Smellie, 1971, 1973). To circumvent the problem of precursor pool size changes, several groups have measured RNA polymerase activities in isolated nuclei under conditions of assay which maintain constant levels of nucleotides (Glasser et al., 1972; Borthwick and Smellie, 1975; Hardin et al., 1976). The results of these studies show estrogen administration in vivo causes distinct alterations in RNA polymerase activities independent of precursor pools.

The quantitative and qualitative changes occurring in rat uterine RNA synthesis following estrogen administration, particularly at early times following injection of the hormone, have been difficult to accurately measure. These difficulties are undoubtedly a result of a number of factors. Among these is the nature of the organ. The uterus is a complex organ made up of a number of cell types which appear to respond to estrogens in distinct manners. For instance, Nafoxidine preferentially stimulates growth and appearance of nuclear bodies in luminal epithelium cells (Clark et al., 1978). It seems entirely possible that estrogens may cause specific alterations in RNA synthesis in certain uterine cell types, and these changes might go undetected when the whole tissue is examined.

We have previously shown that a correlation exists between the patterns of endogenous nuclear RNA polymerase activities and the retention of estrogen–receptor complexes in the nuclei of the immature rat uterus (Hardin et al., 1976). All classes of estrogenic compounds that we have examined resulted in altered RNA polymerase activities. Only the estrogen–receptor complexes which were retained in nuclei for periods greater than 6 h stimulated a secondary rise in RNA polymerase II activity and a sustained elevation in RNA polymerase I activity required for true uterine growth. When we examined the effects of E_2, E_3, and N on the number of sites on uterine chromatin, available for initiation of RNA synthesis by exogenous RNA polymerase, a similar pattern was found as for endogenous RNA polymerase activity. Only E_2 and N resulted in large sustained increases in initiation sites for RNA synthesis, while E_3 treatment induced only an early but small transient increase in the parameter (Figure 4).

It is interesting to note that the increase in sites on chromatin for initiation of RNA synthesis by exogenous RNA polymerase from E_2- and N-treated animals paralleled the secondary rise in nuclear RNA polymerase II activity as well as the increases in polymerase I activity previously described for the immature rat uterus (Hardin et al., 1976). The failure of E_3 to induce the necessary elevations of RNA polymerase I and II activities

associated with true uterine growth has been attributed to the short-term nuclear residence of the E_3-receptor complex in the nucleus (Anderson et al., 1972, 1973, 1975; Clark et al., 1977). Taken together with the observations in the present study that E_3 failed to increase the numbers of sites for the initiation of RNA synthesis present on uterine chromatin beyond 4 h postinjection strongly suggests that the secondary increases in RNA polymerase II and sustained elevation in polymerase I activities are prerequisite for E_2 and N stimulation of RNA chain-initiation sites on chromatin. The periods after hormone treatment at which initiation sites for RNA synthesis are maximally increased by estrogens also coincide with the onset of DNA synthesis (Stormshak et al., 1976; J. Hardin, unpublished observations). All of these data taken together indicate that treatment of the immature rat with long-acting estrogens (E_2 and N) results in many complex changes at the level of the nuclear receptor–hormone complex, which are obligatory for estrogen-induced changes in RNA polymerase activity and subsequent increases in the chromatin template activity.

Regardless of the data presented in this paper, the question of whether and how estrogens effect uterine RNA synthesis remains complex. Frolik and Gorski (1977) were unable to detect any changes in in vitro labeling patterns of RNA from uteri treated in vivo with estradiol. It appears that more sensitive techniques will have to be used to ultimately resolve the controversy surrounding early estrogen effects or lack of effects on the uterine RNA synthesis. Several approaches would appear to be applicable to this problem. In particular, the use of RNA isolation techniques which effectively inhibit ribonuclease activity such as the guanidine technique (Deeley et al., 1977) should be attempted. The most sensitive assay to study estrogen-induced changes in RNA populations would then involve isolating total poly(A)-containing RNA and using this RNA as a template for reverse transcriptase to prepare a total cDNA. This cDNA could then be used to estimate the number of different poly(A) sequences in RNA preparations isolated from uteri of rats exposed to different estrogen treatment regimes by performing RNA excess hybridizations (Bishop et al., 1974; McKnight et al., 1975; Monohan et al., 1976). Only by using such sensitive techniques can the controversy surrounding estrogen effects on uterine RNA synthesis be resolved.

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Studies of the Effects of Ultraviolet Radiation on the Structural Integrities of Ribosomal RNA Components of the *Escherichia coli* 50S Ribosomal Subunit†

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ABSTRACT: The effects of 254-nm radiation on the structural integrities of free and 50S ribosome-bound 5S and 23S ribosomal ribonucleic acids (rRNA) have been elucidated. Irradiation of aqueous solutions of *Escherichia coli* 50S ribosomes with 253.7-nm radiation results in the formation of single-strand breaks in double-stranded regions of the 23S rRNA component, but not in rRNA chain scission, and destabilization of the secondary structure of the 23S rRNA toward denaturation. The minimum doses of 253.7-nm radiation required for the first detection of the two effects are 7×10^{19} quanta for the production of single-strand breaks in double-stranded regions of the 23S rRNA, and $\leq 2.3 \times 10^{19}$ quanta for destabilization of the 23S rRNA secondary structure. Free 23S rRNA is resistant toward photoinduced chain breakage at doses of 253.7-nm radiation up to at least 2.3×10^{20} and is much less sensitive toward destabilization of secondary structure than ribosome-bound 23S rRNA. In contrast to the photosensitivity of 50S ribosome-bound 23S rRNA toward chain breakage, 50S ribosome-bound 5S rRNA is resistant toward chain breakage at doses of 253.7-nm radiation up to at least 2.3×10^{20} quanta. Ribosome-bound 5S and 23S rRNA are also not photosensitive toward intermolecular 5S/23S rRNA cross-linkage.

Recent photochemical studies of *Escherichia coli* 30S ribosomes have indicated that exposure of aqueous buffered solutions of 30S ribosomes to 254-nm radiation results in the introduction of chain breaks into the 16S rRNA[1] component (Gorelic, 1976a,b). Two types of chain breaks were identified in these studies. One type of chain break, designated chain nicks, corresponds to single-strand breaks in base-paired regions of the 16S rRNA and is first introduced into the 16S rRNA at a dose of 254-nm radiation of 6×10^{19} quanta. The second type of chain break is designated chain scission and corresponds to single-strand breaks in single-stranded regions or double-strand breaks in double-stranded regions of the 16S rRNA. This second type of chain break is first introduced into the 16S rRNA at doses of 254-nm radiation of ca. 3×10^{20} quanta. In contrast to the results obtained with 30S ribosome-bound 16S rRNA, RNA chain breaks were not detected in 16S rRNA irradiated in the free state with doses of 254-nm radiation up to and including 3×10^{20} quanta.

The *E. coli* 50S ribosome, like the 30S ribosome, is a macromolecular complex constituted of RNA and protein molecules. It would therefore be reasonable to expect that irradiation of aqueous buffered solutions of 50S ribosomes with 254-nm radiation might result in changes in the structural integrities of the rRNA components of the 50S ribosome analogous to the changes that have been reported for the 16S rRNA component of UV-irradiated 30S ribosomes. There are reasons to believe, however, that the sensitivities of the rRNA components of *E. coli* 30S and 50S ribosomes toward UV-mediated changes in their structural integrities could differ in at least two respects. The recent results of neutron scattering studies of rRNA and protein distributions in the *E. coli* ribosomes suggest that there are substantially more RNA–RNA type interactions in the *E. coli* 50S ribisome than in the 30S ribosome (Moore et al., 1974; Stührmann et al., 1976). Although the molecular natures of the photophysical processes responsible for UV-mediated RNA chain breakage have not been identified, it is likely that either RNA–RNA or RNA–protein interactions are involved in these photoprocesses. Consequently, a *first* difference might be that the rRNA components of the *E. coli* 50S ribosome could be substantially more sensitive than the 16S rRNA of 30S ribosomes toward UV-mediated chain breakage if RNA–RNA type interactions are primarily responsible for chain breakage. The *E. coli* 50S ribosomes contain, in addition to their protein complement, two rRNA species, whereas there is only a single 16S rRNA species in the *E. coli* 30S ribosome. The results of studies of pyrimidine photochemistry in aqueous solutions have indicated that UV irradiation of appropriate mixtures of pyrimidines can

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[1] Abbreviations used: Me_2SO, dimethyl sulfoxide; tRNA and rRNA, transfer and ribosomal ribonucleic acids, respectively; poly (U), poly(uridylic acid); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet; CD, circular dichroism; ORD, optical rotatory dispersion; T_m, melting temperature.

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