Effect of Estrogen on Gene Expression in the Chick Oviduct

Comparative Aspects of RNA Chain Initiation in Chromatin Using Homologous Versus Escherichia Coli RNA Polymerase*

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Hen oviduct RNA polymerase II and Escherichia coli RNA polymerase holoenzyme and core enzyme were used to study the initiation of RNA synthesis on chromatin. In either the presence or absence of estrogenic stimulation, changes in the level of oviduct chromatin initiation sites as measured in the presence of either homologous or heterologous polymerases followed a similar pattern. Comparison of the initiation sites utilized by these enzymes on chick oviduct chromatin indicated that these enzymes compete with each other for the same initiation regions. In contrast to chromatin, however, the majority of the initiation sites on DNA which are utilized by the oviduct RNA polymerase II are different from those utilized by E. coli holoenzyme. These results suggest that chromatin proteins are involved in the selection of initiation sites on chromatin for RNA polymerases. The in vitro transcripts of these RNA polymerases on stimulated chick oviduct chromatin were analyzed by hybridization to a cDNA probe transcribed from ovalbumin mRNA. The relative concentration of ovalbumin sequences transcribed by these three polymerases was 4:1.5:1 for oviduct RNA polymerase II, E. coli core enzyme, and holoenzyme respectively. Therefore, the efficiency of transcribing a specific gene appears to depend on the interaction between RNA polymerase and chromosomal elements in the initiation region.

The steroid hormone-mediated induction of specific protein synthesis in target tissue has been well documented (1-8). In recent years, it has been demonstrated that specific messenger RNAs are induced prior to accumulation of specific proteins in the course of estrogen-medicated growth and differentiation of the chick oviduct (9, 10, 11). In addition, estrogenic hormones also induce dramatic changes in endogenous RNA polymerase activity (12, 13), nuclear RNA synthesis (14, 15), and chromatin composition and function (16) in oviduct cells. These results taken together indicate an action of steroid hormones at the transcriptional level. We have recently reported a method which enables us to measure the formation of an initiation complex for RNA synthesis between RNA polymerase and chromatin (17). Using this method, we have measured the number of initiation sites available to RNA polymerase on chick oviduct chromatin isolated from different developmental stages. Following administration of estrogen, an increase in the number of chromatin initiation sites occurred which correlated well with the increased growth and differentiation of chick oviduct oviduct during estrogen stimulation (18, 19). These results suggested that estrogen-mediated differentiation of the chick oviduct might involve regulation of the number of chromatin DNA sequences available for transcription by RNA polymerase. However, it should be noted that E. coli RNA polymerase was used exclusively for these studies. Although chromatin can be transcribed by bacterial RNA polymerase in vitro yielding a product that bears some similarity to RNA synthesized in vivo (20-22), serious questions have been raised concerning the biological validity of the use of bacterial enzyme for such studies. In order to assess the validity of using bacterial RNA polymerase, we have compared the initiation sites utilized by bacterial RNA polymerase with those utilized by homologous RNA polymerase and re-examined the effect of hormone on the number of available initiation sites in isolated chromatin.

Experimental Procedure

Materials

Nucleoside triphosphates were obtained from P-L Biochemicals. [3H]UTP (15 Ci/mmol) was purchased from Schwartz/Mann Corp. Rifampicin was obtained from Calbiochem, Inc. and heparin was obtained from Sigma Chemical Co. Escherichia coli paste (K 12) was purchased from Grain Processing Co. RNase free DNase was purchased from Worthington Co. RNase H was a generous gift of Dr. E. Chargaff of Columbia University, purified according to the procedure of Stavrianopoulos and Chargaff (26). This preparation of RNase II was found to digest more than 97% of the A moiety of the poly(rA)-poly(dT) duplex under the reaction conditions used. Frozen hen oviducts were purchased from Pel Freeze Inc. All other chemicals were reagent grade and were purchased from J T Baker Co., Fisher, or Mallinckrodt.

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Preparation of DNA and Chromatin from Chick Oviduct-Chick DNA was isolated from chick oviduct as reported previously (27).

Chick oviduct chromatin was prepared according to the method of Tsai et al. (17), except that 10% of the oviduct of chick was homogenized in 14 days with diethylstilbestrol or had been treated 14 days with diethylstilbestrol and then withdrawn from all hormone for an additional 12 days. Isolated chromatin was analyzed for DNA, histone, and acidic protein as described previously (17), and stored at approximatel y 1 mg/ml of DNA in 2 mM Tris-HCl pH 7.9 and 0.1 mM EDTA.

Preparation of E. coli RNA Polymerase—E. coli RNA polymerase holoenzyme was isolated from 1% log phase E. coli K-12 paste by a modified method of Burgess (28) through the DEAE-cellulose column. This material was further purified by DNA-cellulose chromatography as described by Bautz and Dunn (29) and then Bio-Gel A-5m in 1 M KCl as described by Burgess (28). The final enzyme preparation was precipitated with 60% saturated (NH₄)₂SO₄, and stored at -20°C in storage buffer (0.1 M Tris-HCl, pH 7.9; 0.01 M MgCl₂, 0.1 mM EDTA; 0.5 mM dithiothreitol; and 50% glycerol) at a concentration of 10 mg/ml.

E. coli RNA polymerase core enzyme was prepared by phosphocellulose chromatography according to the procedure of Burgess and Travers (30).

Assay of Oviduct RNA Polymerase II—Oviduct RNA polymerase was assayed in 0.25 ml volume containing 12.5 ml of Tris-HCl, pH 7.9; 0.05 μmol of (NH₄)₂SO₄, 0.5 μmol of 2-mercaptoethanol, 10 μmol of each of ATP, GTP, CTP, and UTP (57 pmol/μmol unless otherwise noted); and 20 μg of calf thymus DNA. RNA synthesis was carried out for 10 min at 37°C. Reactions were terminated by addition of cold 5% trichloroacetic acid containing 0.1 M sodium pyrophosphate, collected on glass fiber filters (Reeve Angel 504 AH), and counted in toluene based scintillation fluid (Toluene-Spectrafluor 1000-242 (v/v)) in a Beckman LS-233 scintillation spectrometer.

One unit of RNA polymerase is defined as the amount of enzyme needed to incorporate 1 nmol of UMP into trichloroacetic acid-precipitable material in 10 min at 37°C.

Preparation of Hen Oviduct RNA Polymerase II—Oviduct RNA polymerase II (B) was purified from frozen hen oviduct by a modified method of Keding et al. (31, 32). Frozen hen oviduct (200 g) was homogenized in a Waring Blender at high speed in 600 ml of Buffer A (50 mM Tris-HCl, pH 7.9; 0.1 mM EDTA; 0.5 mM dithiothreitol; and 5% glycerol) for 5 min. The homogenate was filtered through four layers of cheesecloth and diluted with Buffer A to 1.8 liters. Saturated (NH₄)₂SO₄ (405 ml) was added and the sample was homogenized at the high setting for 1 min. The resulting homogenate was again passed through four layers of cheesecloth, and solid (NH₄)₂SO₄ was added (0.13 g/100 ml of homogenate). After standing for 30 min, the precipitate was collected by centrifugation at 25,000 × g for 20 min. The pellet was homogenized in 600 ml of Buffer B (same as Buffer A except that glycerol concentration was 30%). Nucleic acid in the homogenate was precipitated by adding 5.5 ml of 1% protamine sulfate, pH 5.5/100 ml of solution. After stirring at 0°C for 20 min, the suspension was centrifuged for 1 hour at 31,000 rpm in a type 35 rotor. The supernatant solution ("protamine sulfate fraction") was stored at -80°C.

Protamine sulfate fractions corresponding to 600 g of hen oviduct were diluted with Buffer B until the salt concentration was lower than 0.08 M (NH₄)₂SO₄. Washed DEAE-Sephadex A-25 (405 ml) was added and the mixture was gently stirred for 45 min. The mixture was then filtered in a Buchner funnel, washed with 1 liter of Buffer B containing 0.08 M (NH₄)₂SO₄ and then transferred to a column (5 cm diameter). The column was further washed with Buffer B containing 0.08 M (NH₄)₂SO₄ until the A₂₆₀ was less than 0.1. Enzyme activity was then eluted from the column with 0.5 M (NH₄)₂SO₄ in Buffer B, precipitated with 1.5 volumes of saturated (NH₄)₂SO₄ solution, and stored at -80°C ("DEAE-fraction").

DEAE-fractions corresponding to 3.4 kg of oviduct were combined and diluted with Buffer B until the salt concentration was less than 0.08 M. The solution was loaded on a phosphocellulose (Whatman P320) column (4.5 × 24 cm) which was pre-equilibrated with Buffer B containing 0.06 M (NH₄)₂SO₄. After washing with Buffer B (0.08 M (NH₄)₂SO₄), RNA polymerase was eluted with 0.25 M (NH₄)₂SO₄ in Buffer B. The combined RNA polymerase fraction was diluted with an equal volume of Buffer B and loaded onto a DEAE-Sephadex A-25 column (4.5 × 24 cm). After washing with Buffer B containing 0.1 M (NH₄)₂SO₄, elution was carried out with a 1-liter linear gradient from 0.1 to 0.6 M (NH₄)₂SO₄ in Buffer B. Enzyme activity was assayed according to the method described earlier and precipitated with 1.5 volumes of saturated (NH₄)₂SO₄. The resulting pellet was dissolved in Buffer C (same as Buffer A except that glycerol concentration was 50%) and stored at -80°C at a concentration of 130 units/ml.

Conditions for RNA Synthesis without Reinitiation—For the comparison of E. coli RNA polymerase holoenzyme and core enzyme, RNA polymerase was preincubated with 5 μg of chromatin in 200 μl of a buffer containing 62.5 mM Tris-HCl, pH 7.6; 1.25 mM MgCl₂; 62.5 mM (NH₄)₂SO₄; and 2.5 mM 2-mercaptoethanol. After 40 min, RNA synthesis was initiated by the addition of 50 μl of solution containing 37.6 nM each of ATP, GTP, CTP, and UTP (116 rpm/ml) and 10 μg of rifampicin, and 200 μg of heparin, and carried out for 1.5 min at 37°C. In the comparison of oviduct RNA polymerase II and E. coli RNA polymerase, RNA polymerase was preincubated with 1.5 μg of DNA or 5 μg of chromatin at 37°C for 15 min in 200 μl of preincubation buffer as described above. RNA synthesis was initiated by the addition of 50 μl of solution containing 150 nM each of ATP, GTP, and CTP, 160 nM (NH₄)₂SO₄, 300 μg of oviduct chromatin or DNA; and either 800 μg of E. coli RNA polymerase holoenzyme, 1600 μg of core enzyme, or 400 units of oviduct RNA polymerase II. The reaction mixtures were incubated for 2 hours at 37°C.

The chromatin was then removed by centrifugation at 10,000 × g for 20 min. The supernatant solution was adjusted to 40 μg/ml of RNA-free DNase and incubated for 30 min at room temperature. This solution was brought to 0.5% in sodium dodecyl sulfate and 20 μg/ml in Proteinase K (EM Laboratories) and incubated for 15 min at 37°C. RNA was extracted with an equal volume of phenol/chloroform (1:1) three times or until no more interphase was present, and then precipitated with the addition of 2 volumes of absolute ethanol at -20°C overnight. The ethanol precipitate was dissolved in 0.5 ml of H₂O and passed through a Sephadex G-50 column equilibrated with 0.1 mM NaCl; 0.01 mM sodium acetate, pH 5.0; and 1 mM EDTA. The fractions which eluted in the void volume were precipitated with 2 volumes of ethanol. The RNA precipitate was collected by centrifugation and dissolved in a small amount of H₂O.

cDNA Hybridization—In vitro RNAs were hybridized to cDNA synthesized from ovalbumin mRNA according to the method described by Harris et al. (25). cDNA used in these experiments had an average chain length of 900 nucleotides.

RNA Product Analysis—RNA products synthesized by E. coli or oviduct RNA polymerases were analyzed with regard to their sensitivity to RNase A and RNase H. Conditions for these RNase treatments were as follows: 0.4-ml reaction mixtures containing 1 × SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0); 25 mM MgCl₂; 0.05 mM Tris-HCl, pH 8.0; 0.5 mg/ml of bovine serum albumin; and either 10 μg of RNase A (preheated at 100°C for 5 min to inactivate any contaminating DNase) or 2.5 μg of RNase H. Reactions were incubated at 37°C for 30 min. The amount of RNA degraded was measured by comparing the trichloroacetic acid-precipitable material in reactions to controls containing no RNase.

Results

Effects of Hormone on Level of Chromatin Initiation Sites—The number of initiation sites on chromatin can be measured by the rifampicin challenge technique described by Tsai et al. (17). Basically, this technique involves the preincubation of RNA polymerase with template to allow formation of stable preinitiation complexes. RNA synthesis is then initiated by the simultaneous addition of rifampicin and nucleoside triphosphates. In these conditions, RNA polymerase present in the preinitiation complex form will initiate the synthesis of an
RNA chain; however, no secondary initiation events from a single site will occur. As the amount of RNA polymerase added to a fixed amount of DNA is increased, a transition point is observed in the curve which corresponds to the amount of RNA polymerase required to saturate the available binding sites on chromatin. The coordinates of this transition point are indicative of the number of sites available for initiation of transcription (17). As reported earlier, the initiation sites on chromatin isolated from chick oviduct changed drastically during hormone stimulation and withdrawal (18, 19).

One major question raised in these studies was whether the number of initiation sites for other RNA polymerase, especially homologous RNA polymerase, also followed the same pattern of changes. To answer this question, enzyme saturation curves were carried out to measure the number of initiation sites available to *Escherichia coli* RNA polymerase core enzyme and hens oviduct RNA polymerase II on chromatin isolated from hormone-stimulated and withdrawn chicks. *E. coli* core enzyme, purified from holoenzyme by phosphocellulose chromatography to remove the σ subunit, behaves quite differently from holoenzyme in selecting initiation sites on bacteriophage DNA. Holoenzyme initiates RNA synthesis from the correct promoter sites and synthesizes RNA which closely resembles in vivo RNA (33). However, core enzyme initiates RNA synthesis nonspecifically, especially from nicked or terminal regions, and synthesizes RNA chains in a random fashion. When core enzyme was used, the enzyme saturation curve on chromatin showed two phases (Fig. 1) similar to the saturation curves of *E. coli* holoenzyme. With stimulated chromatin (Panel A), *E. coli* core polymerase incorporated 44 pmol of UMP at the transition point. This level decreased to 16 pmol on chromatin isolated from chick oviduct after hormone withdrawal for 12 days (Panel B). A similar 3-fold decrease of RNA synthesis was previously observed when initiation sites for *E. coli* holoenzyme were measured (18, 19).

For studies with oviduct RNA polymerase II, the rifamycin derivative, AF/013, was used instead of rifampicin, since rifampicin does not inhibit animal RNA polymerases. AF/013 inhibits initiation of RNA synthesis prior to the formation of the first phosphodiester bond (34, 35). This compound has been previously used to inhibit the reinitiation of RNA synthesis in order to allow a comparison of *E. coli* and calf thymus RNA polymerases in selection of initiation sites on calf thymus or SV40 DNA (36, 37). The enzyme saturation curve with hen oviduct RNA polymerase II on hormone-stimulated and withdrawn chromatin also had two phases (Fig. 2). With stimulated chromatin (Panel A) oviduct RNA polymerase II incorporated 100 pmol of UMP at the transition point. This level decreased to 24 pmol on withdrawn chromatin. A 3- to 4-fold decrease in RNA synthesis was also observed with *E. coli* holoenzyme when AF/013 was used (Panels C and D). At the present time it is not possible to assess the absolute number of initiation sites for either oviduct RNA polymerase II or core enzyme. Such an analysis would involve more detailed kinetic studies as well as size determination of the chromatin transcripts. However, because these parameters have not varied between stimulated and withdrawn chromatins in previous transcription studies, the ratio of RNA synthesis at the transition point on two chromatins will be equal to the ratio of initiation sites available. Therefore, regardless of the polymerase used, the relative change in the level of initiation sites during hormone stimulation and withdrawal followed a similar pattern.

![Fig. 1. Enzyme saturation curves on chick oviduct chromatin isolated from oviduct tissues of stimulated and withdrawn chicks by *Escherichia coli* RNA polymerase core enzyme. *E. coli* RNA polymerase core enzyme (0 to 25 μg) was preincubated with 5 μg of chromatin in 0.2 ml of preincubation mixture as described under "Methods" at 37°C. At the end of 40 min, 50 μl of nucleotides and rifampicin mixture were added and further incubated at 37°C for 15 min. RNA synthesized was precipitated with 5% trichloroacetic acid and counted in toluene base scintillation fluid. A, chromatin isolated from 14 days diethylstilbestrol-stimulated chicks (14DES). B, chromatin isolated from 12 days withdrawn chick after 14 days diethylstilbestrol (14DES + 12W).](http://www.jbc.org/)

**Competition of Enzyme for Initiation Sites on Chick Oviduct DNA and Chromatin**—It was of interest to determine whether oviduct RNA polymerase II and bacterial RNA polymerases utilize the same or different sites on chick DNA and chromatin. This problem can be approached by mixing increasing amounts of any two polymerases in the rifampicin challenge assay. In this type of competition assay, a preinitiation complex is formed between RNA polymerase and DNA at the initiation site prior to the onset of RNA synthesis. Therefore, if both enzymes utilize the same sites for initiation, competition should occur between the enzymes for available initiation sites under conditions where RNA polymerase is in excess. In these conditions the level of RNA synthesis for the mixture of two enzymes should be equal to the average value for the level of RNA synthesis for either enzyme alone. On the other hand, if both enzymes utilize different sites for initiation, competition for the formation of preinitiation complex should occur. In this case the level of RNA synthesis for the mixture should be equal to the sum of the levels of RNA synthesis of either enzyme alone. The theoretical activity of RNA synthesis for the two extreme cases of complete competition or no competition can be calculated according to the following equations. If both enzymes use different sites:

\[
V_{\text{same}} = V_{\text{E}} + V_{\text{C}}
\]
Initiation Sites on Chromatin for Eukaryotic RNA Polymerase

FIG. 2. Enzyme saturation curves on 14 days diethylstilbestrol-stimulated and withdrawn chromatin by hen oviduct RNA polymerase II and Escherichia coli RNA polymerase holoenzyme. Hen oviduct RNA polymerase II (0 to 50 μl with a concentration of 130 units/ml) and E. coli RNA polymerase holoenzyme (0 to 50 μl with a concentration of 400 μg/ml) were preincubated with 5 μg of chromatin at 37°C for 15 min. RNA synthesis was started with 150 nmol each of ATP, CTP, and GTP, and 15 nmol of UTP (575 cpm/pmol), and 50 μg of AF/013. At the end of 15 min, RNA synthesized was measured according to the method described under “Methods.” A, oviduct RNA polymerase II, 14 days diethylstilbestrol-stimulated (14DES) chromatin; B, oviduct RNA polymerase II, withdrawn chromatin (14DES + 12W); C, E. coli RNA polymerase holoenzyme, 14 days diethylstilbestrol-stimulated chromatin; D, E. coli RNA polymerase holoenzyme, withdrawn chromatin.

If both enzymes use the same sites:

$V_{X+Y} = \frac{V_{X}}{c} + \frac{V_{Y}}{c'}$  \hspace{1cm} (2)

where $V_{X+Y}$ is the enzyme activity when RNA polymerases $X$ and $Y$ are mixed together at concentrations $c$ and $c'$ respectively, $V_{X}$ and $V_{X'}$ are the enzyme activities of RNA polymerase $X$ at concentrations $c$ and $c'$, and $V_{Y}$ and $V_{Y'}$ are the enzyme activities of RNA polymerase $Y$ at concentrations of $c'$ and $2c'$.

The initiation sites utilized by oviduct RNA polymerase II and E. coli holoenzyme on purified chick DNA were first examined. Enzyme saturation curves of 1.5 μg of DNA for either polymerase alone or a mixture of the two enzymes are shown in Fig. 3. The stock concentrations of enzyme used in this experiment were 130 units/ml for oviduct enzyme and 400 μg/ml for E. coli enzyme. For oviduct RNA polymerase alone, the incorporation of [PH]UMP reached a saturation level of approximately 43 pmol (Curve A), while with E. coli RNA polymerase alone, a plateau level corresponding to 600 pmol was obtained (Curve B). This substantial difference in the saturation levels of UMP incorporation indicates that hen oviduct RNA polymerase II is only capable of utilizing a subclass of the initiation sites on DNA which E. coli RNA polymerase may utilize. This is consistent with the characteristics of RNA polymerase II from higher organisms, which are known to be relatively inefficient at transcribing intact native DNA templates. Based on the enzyme saturation curves of either polymerase alone and Equations 1 and 2, theoretical curves were calculated representing the expected levels of RNA synthesis if both polymerases use different sites (Curve C) or the same sites (Curve D) on DNA. The experimental points for the mixture of the two enzymes (solid circles) followed the theoretical Curve C quite closely. These data suggested that E. coli RNA polymerase and hen oviduct RNA polymerase II utilize different initiation sites on chick DNA. It should be noted that at the highest enzyme concentrations used, the experimental points for the mixture of polymerases became intermediate between Curves C and D. If enzyme concentrations were increased further, the competition between the enzymes became even more prominent (data not shown). These results are explicable because at very high enzyme to DNA ratios RNA polymerase will stack up one by one along the DNA strands and may thus sterically hinder the transcription process.

The initiation sites utilized by E. coli RNA polymerase holoenzyme, core enzyme and oviduct RNA polymerase II on chick oviduct chromatin were next examined. Chromatin proteins undoubtedly play a major role in the regulation of gene expression. It is thus possible that proteins in the chromatin might be involved in the selection of initiation sites for RNA polymerase. If this is true, it would be expected that these different RNA polymerases would utilize the same sites for initiation in chromatin, despite having unique specificities on DNA. The results of a competition experiment between E. coli holoenzyme and core enzyme on oviduct chromatin from...
These experiments were performed under conditions in which utilization, in general, the same initiation sites in chromatin. These results suggest that chromatin proteins may play a major role in determining the absolute number of initiation sites. Therefore, with or without a factor E. coli RNA polymerase utilized the same initiation regions on chromatin.

The results of an analogous competition experiment utilizing E. coli holoenzyme and oviduct RNA polymerase II are shown in Fig. 5. Again, the experimental points for the mixture of the two polymerases followed very closely the curve predicted assuming complete competition between the two enzymes for initiation sites. Thus, both homologous and bacterial RNA polymerases utilized the same initiation regions on chromatin for initiation. These results suggest that chromatin proteins may play a major role in determining the absolute number of sites in DNA available to RNA polymerase for the initiation of RNA synthesis.

Analysis of in Vitro Transcription Products—Competition experiments demonstrated that different RNA polymerases utilize, in general, the same initiation sites in chromatin. These experiments were performed under conditions in which each initiation site is allowed to initiate only a single chain. It is possible, however, that under conditions in which reinitiation of RNA synthesis can occur, different RNA polymerases might vary in the relative efficiencies with which they transcribe different regions of the chromatin. To test this possibility RNA was transcribed from estrogen-stimulated chick oviduct chromatin in the absence of any initiation inhibitor. This RNA was then analyzed for the presence of sequences homologous to a specific portion of the genome (ovalbumin gene) by hybridization to a complementary DNA (cDNA) probe transcribed from pure ovalbumin mRNA by avian myeloblastosis virus reverse transcriptase (9, 25). The concentration of mRNA specific sequences present in these transcripts can be estimated by comparing the initial slope of the titration curves to that observed for hybridization of cDNA to the purified mRNA from which it was transcribed. As shown in Fig. 6, oviduct polymerase II transcribed more ovalbumin mRNA sequences (0.04% of total RNA) from oviduct chromatin than E. coli RNA polymerase core enzyme, which in turn transcribed more ovalbumin sequences than E. coli holoenzyme. The relative quantity of ovalbumin sequences in the transcripts of oviduct polymerase II, E. coli core enzyme, and E. coli holoenzyme calculated from the initial slopes of the hybridization curves was 3.8:1.5:1, respectively. Thus, oviduct RNA polymerase II transcribes ovalbumin mRNA sequences more efficiently than either of the E. coli RNA polymerases.

A control experiment was carried out to show that the

![Fig. 4. RNA initiation sites utilized by Escherichia coli RNA polymerase holoenzyme and core enzyme on chick oviduct chromatin.](http://www.jbc.org/)

![Fig. 5. RNA initiation sites utilized by Escherichia coli holoenzyme and hen oviduct RNA polymerase II on chick oviduct chromatin. Hen oviduct RNA polymerase II (C, Curve A), E. coli holoenzyme (Δ, Curve B), or a mixture of the two enzymes in equal volumes (○) was incubated at 37° with 5.0 μg of chick oviduct chromatin (14 days diethylstilbestrol-stimulated) in 0.2 ml of preincubation buffer for 40 min. RNA synthesis was initiated by the addition of 0.05 ml of ribonucleoside triphosphate mixture containing 0.2 mg/ml of rifampicin and 4.0 mg/ml of heparin, and carried out for 1.5 min at 37°. Theoretical curves were constructed from the experimental Curves A and B assuming either no competition (Curve C) or complete competition (Curve D) between the two polymerases for RNA initiation sites.)
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Reaction contained 1.5 ng of cDNA and was carried out for 96 hours in mRNA by the procedure of Harris et al. (9). Each hybridization hybridized to complementary DNA transcribed from ovalbumin extracted essentially by the procedure of Harris et al. (25). RNA was centrifuged at 12,000 x g for 20 min to remove chromatin. RNA was chromatin with either 800 pg of holoenzyme (0) or 200 units of oviduct chromatin (m) and in which 300 pg of chick DNA was used in place of which no exogenous RNA polymerase or α-amanitin was added to the transcribed by added exogenous RNA polymerase and not due to ovitum cDNA were detected in this control sample. There-

oviuduct RNA polymerase II (A). Controls were all run in 2.5 ml in holoenzyme (O), 1600 pg of core enzyme (©), or 400 units of hen ovitum RNA polymerase II (Δ). Controls were all run in 2.5 ml in which no exogenous RNA polymerase or α-amanitin was added to the chromatin (©) and in which 300 μg of chick DNA was used in place of chromatin with either 800 μg of holoenzyme (O) or 200 units of ovitum enzyme (Δ). Reactions were incubated for 2 hours at 37° and then centrifuged at 12,000 x g for 20 min to remove chromatin. RNA was extracted essentially by the procedure of Harris et al. (25). RNA was hybridized to complementary DNA transcribed from ovalbumin mRNA by the procedure of Harris et al. (9). Each hybridization reaction contained 1.5 ng of cDNA and was carried out for 96 hours in 0.05 ml of 0.01 M Tris-HCl, pH 7.9, 1 mM MnCl₂, 2 mM 2-mercapto-ethanol; 50 mM (NH₄)₂SO₄; 4 μg/ml of α-amanitin (for E. coli enzymes); 300 μg of chick ovitum chromatin (14 days diethylstilbestrol-stimulated) and either 800 μg of E. coli RNA polymerase holoenzyme (©), 1600 μg of core enzyme (©), or 400 units of hen ovitum RNA polymerase II (Δ). Controls were all run in 2.5 ml in which no exogenous RNA polymerase or α-amanitin was added to the chromatin (©) and in which 300 μg of chick DNA was used in place of chromatin with either 800 μg of holoenzyme (O) or 200 units of ovitum enzyme (Δ). Reactions were incubated for 2 hours at 37° and then centrifuged at 12,000 x g for 20 min to remove chromatin. RNA was extracted essentially by the procedure of Harris et al. (25). RNA was hybridized to complementary DNA transcribed from ovalbumin mRNA by the procedure of Harris et al. (9). Each hybridization reaction contained 1.5 ng of cDNA and was carried out for 96 hours in 0.05 ml of 0.01 M Tris-HCl, pH 7.0/0.6 M NaCl at 68°. The amount of cDNA resistant to S₁ nuclease was determined as previously described (9).

ovalbumin mRNA sequences in the RNA transcript were not due to endogenous contaminating sequences present in the chromatin preparation. The chromatin was incubated under the normal conditions of RNA synthesis without any exogenous enzyme. After RNA synthesis, carrier yeast RNA equivalent to the amount of RNA normally synthesized was added, and the sample was then processed identically with the sample synthesized by RNA polymerase. Few sequences homologous to ovalbumin cDNA were detected in this control sample. Therefore, the hybridization we observed was due to new sequences transcribed by added exogenous RNA polymerase and not due to contaminating transcripts, may be partially due to the presence of contaminating endogenous ovalbumin sequences present in the ovitum enzyme preparation. If present, this contamination could only cause a maximum 60% increase in ovalbumin mRNA sequences in the chromatin transcripts. Experimentally we observe a 380% increase in ovalbumin mRNA sequences in chromatin transcripts prepared using the ovitum enzyme. Therefore, the increase cannot be accounted for by contributions from contaminating ovalbumin mRNA sequences in the preparation of ovitum RNA polymerase II.

It has been reported that RNA transcribed by eukaryotic RNA polymerase II from chromatin is largely in an RNA-DNA hybrid form (38, 39). To test this possibility RNA synthesized from m ovitum chromatin by E. coli or ovitum RNA polymerase was treated with either pancreatic RNase A, which will digest single-stranded RNA, or E. coli RNase H, which will degrade RNA in an RNA-DNA hybrid form (36). The results are shown in Table I. The majority of ovitum chromatin transcripts were sensitive to pancreatic ribonuclease A, 90% for E. coli RNA polymerase transcripts and 82% for ovitum RNA polymerase. However, the transcripts were only slightly sensitive to RNase H. These results suggested that the majority of the transcripts synthesized by bacterial enzyme or homologous enzyme existed in a single-stranded configuration, rather than in a hybrid form. This view was further supported by the treatment of chromatin transcripts with both RNase A and RNase H. Only a small amount of the RNA, 1% for E. coli enzyme transcripts and 8% for ovitum enzyme transcripts, was further digested by hybridase compared to RNase A treatment alone. Similar results were also observed when different enzyme and chromatin concentrations were used in the synthesis of RNA, or when the chromatin was briefly sonicated prior to RNA synthesis (data not shown). Pretreating chromatin transcripts at 100° for 10 min prior to RNase A treatment increased the amount of RNA which was degraded to a base level of 94 to 95%. Thus, very little of the RNA synthesized from ovitum chromatin under these experimental conditions appeared to be present as an RNA-DNA hybrid. The discrepancy between the results obtained by Howk et al. (38) and Jacquet et al. (39) and the results reported here may be due to a different degree of reinitiation of RNA synthesis. It should be noted that these nuclease digestions were carried out at 25 mM MgCl₂, 0.15 M NaCl, and 15 mM sodium citrate. In such conditions, secondary structure may exist in the RNA which could account for the resistance of 9 to 10% of the transcripts to both RNase A and RNase H digestion.

Discussion

Following estrogen administration to the unstimulated chick (primary stimulation) the level of initiation sites for bacterial RNA polymerase on chromatin isolated from ovitum tissue
ments were performed utilizing the rifamycin challenge assay. Based on this technique, E. coli RNA polymerase holoenzyme changes in the amount of chromatin sequences available for transcription. This prompted us to re-examine the chromatin than on withdrawn chromatin. Thus, the changes in saturation curves was 3- to 4-fold higher on stimulated measured at the transition point of the respective enzyme using any of these three enzymes, the level of RNA synthesis chromatin isolated from stimulated and withdrawn chicks. oviduct RNA polymerase II behave similarly in transcribing hormone induction of chromatin initiation sites using RNA may act primarily at the level of transcription to increase the specific induced protein, ovalbumin, suggesting that hormone receptor and intracellular messenger RNA for a specific previous studies in the literature. Butterworth et al. (40) have reported that rat liver RNA polymerase II and RNA polymerase from Micrococcus luteus bind to and transcribe from different sites on rat liver chromatin. The methods utilized for this study also involved mixing experiments between the two enzymes; however, the experiments were performed in the absence of any inhibitor of reinitiation. Thus, it is possible that during the course of the 10-min incubation a given initiation site was utilized sequentially and repeatedly by the two different RNA polymerases without any significant competition occurring in terms of total RNA synthesis. Recently, Cedar (41) has reported that calf thymus chromatin can support 30 times fewer specific sites for the homologous RNA polymerase than for the bacterial enzyme. The method utilized by Cedar involved the preincubation of enzyme and template with three of the four ribonucleotides, followed by simultaneous addition of the fourth ribonucleotide and a high concentration of ammonium sulfate to inhibit any further initiation. The explanation for the discrepancy between the results of Cedar and the results reported here are presently unclear. One possibility is that the presence of a high concentration of ammonium sulfate removed a portion of the calf thymus RNA polymerase II bound to chromatin. The effects of ammonium sulfate on transcription by eukaryotic RNA polymerases have not been wel well characterized, but we have noted that the animal

### Table I

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<tr>
<th>Treatment</th>
<th>E. coli enzyme</th>
<th>Oviduct enzyme</th>
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<tbody>
<tr>
<td>Control</td>
<td>28,084</td>
<td>12,172</td>
</tr>
<tr>
<td>RNase A</td>
<td>2,905</td>
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<td>9,1,344</td>
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<td>Heated at 100°, 10 min, than RNase A</td>
<td>1,308</td>
<td>5</td>
</tr>
</tbody>
</table>

Based on the specifics of the results, it is evident that the levels of RNA synthesis observed for the three RNA polymerases tested were not remarkably different on chromatin. The differences which were observed in the levels of RNA synthesis could be accounted for by slightly different efficiencies of the enzymes in escaping rifamycin (or rifampicin) inhibition or small variations in the size of the RNA product. Little competition should occur by the technique during the actual process of RNA synthesis even if overlapping sequences are transcribed, since only one round of transcription from any site can occur and ampic time is allowed for this to take place. It should be noted, however, that if two polymerases initiate at different sites within a limited region (50 to 80 nucleotides) of chromatin, they would appear to compete with each other by this technique. Thus, we interpret our result to show that different RNA polymerases utilize the same initiation regions on chromatin in contradiction with some previous studies in the literature. Butterworth et al. (40) have reported that rat liver RNA polymerase II and RNA polymerase from Micrococcus luteus bind to and transcribe from different sites on rat liver chromatin. The methods utilized for this study also involved mixing experiments between the two enzymes; however, the experiments were performed in the absence of any inhibitor of reinitiation. Thus, it is possible that during the course of the 10-min incubation a given initiation site was utilized sequentially and repeatedly by the two different RNA polymerases without any significant competition occurring in terms of total RNA synthesis. Recently, Cedar (41) has reported that calf thymus chromatin can support 30 times fewer specific sites for the homologous RNA polymerase than for the bacterial enzyme. The method utilized by Cedar involved the preincubation of enzyme and template with three of the four ribonucleotides, followed by simultaneous addition of the fourth ribonucleotide and a high concentration of ammonium sulfate to inhibit any further initiation. The explanation for the discrepancy between the results of Cedar and the results reported here are presently unclear. One possibility is that the presence of a high concentration of ammonium sulfate removed a portion of the calf thymus RNA polymerase II bound to chromatin. The effects of ammonium sulfate on transcription by eukaryotic RNA polymerases have not been well characterized, but we have noted that the animal

In order to compare the initiation sites utilized by different RNA polymerases on DNA and chromatin, competition experiments were performed utilizing the rifamycin challenge assay. Based on this technique, E. coli RNA polymerase holoenzyme was found to initiate at different sites on protein-free chick DNA than the homologous hen oviduct RNA polymerase II. This result is consistent with the work of Meilhoc and Chambron (36), who have shown using a similar technique that calf thymus RNA polymerase I (A), II (B), and E. coli holoenzyme utilized different sites on calf thymus DNA. In both cases, the E. coli RNA polymerase was found to be far more efficient at the formation of preinitiation complexes on DNA than the homologous enzymes. This could be due to the fact that the purified animal RNA polymerases have been separated from a soluble component, analogous to the bacterial σ factor, which facilitates formation of stable binary complexes on native DNA. Alternatively, certain components of chromatin could be involved in the formation of preinitiation complexes with eukaryotic RNA polymerases, so that these enzymes do not possess the ability to efficiently transcribe deproteinized native DNA.

Analogous competition experiments utilizing chick oviduct chromatin demonstrated that both homologous RNA polymerase II and E. coli holoenzyme initiated RNA synthesis at the same sites. This was also true when E. coli holoenzyme was compared to E. coli core enzyme. Furthermore, the levels of RNA synthesis observed for the three RNA polymerases tested were not remarkably different on chromatin. The differences which were observed in the levels of RNA synthesis could be accounted for by slightly different efficiencies of the enzymes in escaping rifamycin (or rifampicin) inhibition or small variations in the size of the RNA product. Little competition should occur by the technique during the actual process of RNA synthesis even if overlapping sequences are transcribed, since only one round of transcription from any site can occur and ampic time is allowed for this to take place. It should be noted, however, that if two polymerases initiate at different sites within a limited region (50 to 80 nucleotides) of chromatin, they would appear to compete with each other by this technique. Thus, we interpret our result to show that different RNA polymerases utilize the same initiation regions on chromatin in contradiction with some previous studies in the literature. Butterworth et al. (40) have reported that rat liver RNA polymerase II and RNA polymerase from Micrococcus luteus bind to and transcribe from different sites on rat liver chromatin. The methods utilized for this study also involved mixing experiments between the two enzymes; however, the experiments were performed in the absence of any inhibitor of reinitiation. Thus, it is possible that during the course of the 10-min incubation a given initiation site was utilized sequentially and repeatedly by the two different RNA polymerases without any significant competition occurring in terms of total RNA synthesis. Recently, Cedar (41) has reported that calf thymus chromatin can support 30 times fewer specific sites for the homologous RNA polymerase than for the bacterial enzyme. The method utilized by Cedar involved the preincubation of enzyme and template with three of the four ribonucleotides, followed by simultaneous addition of the fourth ribonucleotide and a high concentration of ammonium sulfate to inhibit any further initiation. The explanation for the discrepancy between the results of Cedar and the results reported here are presently unclear. One possibility is that the presence of a high concentration of ammonium sulfate removed a portion of the calf thymus RNA polymerase II bound to chromatin. The effects of ammonium sulfate on transcription by eukaryotic RNA polymerases have not been well characterized, but we have noted that the animal
polymerases are more easily removed from chromatin by heparin than is the E. coli enzyme. However, this is only speculation at the present time and further characterization of the two methods utilized will be necessary to fully understand the differences observed.

Even though different RNA polymerases transcribe chromatin from the same sites, they do not necessarily have to utilize any specific site with the same efficiency. To examine this question, we have tested the RNA transcribed from chromatin in the absence of any initiation inhibitor by E. coli and hen oviduct RNA polymerases for the presence of sequences homologous to ovalbumin mRNA. Hen oviduct RNA polymerase II was found to transcribe 2 to 3 times more sequences homologous to ovalbumin cDNA than either of the E. coli enzymes. Similar results were also obtained by Steggles et al. (22) who found that eukaryotic RNA polymerase II was more efficient than bacterial RNA polymerase in transcribing globin mRNA sequences from bone marrow chromatin. One interpretation of these results is that homologous RNA polymerase is more efficient than bacterial enzyme at utilizing the initiation site for the ovalbumin gene. Other interpretations, however, are possible. For instance, the homologous enzyme might synthesize a longer portion of the ovalbumin gene than the bacterial enzyme within the limits of the ovalbumin sequences present in the cDNA. Final evaluation of the explanation for the higher percentage of ovalbumin sequences in the transcripts of homologous enzyme than bacterial enzyme awaits further experimentation.

The formation of a preinitiation complex between E. coli RNA polymerase and native chick DNA which could initiate RNA synthesis in the rifampicin challenge assay was shown to occur through a highly temperature-dependent step (17, 42). This step was thought to involve the local opening of the DNA strands in the initiation region. On chromatin, however, the formation of stable preinitiation complexes was only slightly dependent on the temperature of formation. This low temperature dependency was similar in magnitude to that observed on denatured DNA, where no opening of the DNA strands occurs prior to RNA chain initiation. This led us to postulate that the initiation sites on chromatin might be held by chromatin proteins in a conformation which facilitates opening of the DNA strands during formation of the stable preinitiation complex. If this is indeed the case, such chromatin protein would be expected to be major determinants of the initiation sites utilized in chromatin. This hypothesis is thus consistent with our findings that different RNA polymerases utilize the same sites in chromatin. In this regard it is interesting to note that when superhelical SV40 form I DNA, which is known to contain a specific region in which the DNA strands are destabilized, is used as template, the majority of initiation sites utilized by calf thymus RNA polymerase I(A) or II(B) were the same (37). It should be emphasized, however, that at the present time we do not have any evidence to indicate that the initiation sites used in vitro correspond to the identical sites used for RNA chain initiation in vivo, especially in light of the results of Honjo and Reeder (43).

Our current working hypothesis for the transcription process in chromatin is that certain chromatin proteins interact with the DNA to form a region utilized for RNA chain initiation. In such regions, the formation of preinitiation complexes between enzyme and DNA is greatly favored due to destabilization of the DNA strands. This destabilization allows RNA polymerases from different origins to recognize the same sites for initiation. However, the efficiency with which specific genes in the chromatin are transcribed by RNA polymerase can vary according to the affinity of the interaction between RNA polymerase and the DNA in the initiation region. This hypothesis by no means rules out the possibility that soluble protein factors may be involved in the selective transcription of specific genes. It does, however, emphasize the importance of utilizing chromatin to study gene regulation in eukaryotic organisms.

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