Effect of Estrogen on Gene Expression in the Chick Oviduct

STUDIES ON THE INITIATION OF RNA SYNTHESIS ON CHROMATIN IN VITRO*

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Studies on the effect of the temperature requirement for RNA chain initiation by Escherichia coli RNA polymerase on DNA, chromatin, and reconstituted chromatin were carried out in order to better understand the nature of the initiation process. Varying the temperature or ionic strength during preincubations had little effect on the formation of stable preinitiation complexes (RS complexes) between RNA polymerase and chromatin. This observation was in marked contrast to similar studies performed on native DNA and indicates that initiation sites for RNA synthesis on chromatin are different from those on double-stranded DNA and resemble more closely initiation of RNA synthesis on single-stranded DNA. These results suggest that the local unwinding of the initiation region which is required for RNA chain initiation on native DNA may not be a prerequisite for RNA initiation on chromatin. The results of studies on reconstituted chromatin devoid of different classes of chromatin proteins demonstrate that both histone and nonhistone fractions are essential in maintaining the characteristics inherent to initiation of RNA synthesis on chromatin. Removal of either moderately lysine-rich histone or arginine-rich histone fractions led to the complete loss of the characteristic “chromatin type” initiation pattern for RNA synthesis, whereas, removing lysine-rich (F₁) histone had no effect.

Single strand specific and double strand specific antibodies to DNA and a single strand specific nuclease were used as probes to examine the structure of chromatin sites for initiation of RNA synthesis. Their effects on RNA synthesis suggest that initiation sites on chromatin are not located in freely accessible single-stranded or double-stranded regions of DNA.

Isolated chromatin has frequently been used as a template for exogenously added RNA polymerase to study the control of in vitro transcription. Such studies on the transcription process are important to our ability to understand the regulatory mechanisms inherent to gene expression in eucaryotes. The most thorough studies on the functional interaction of RNA polymerase with its template were carried out by Chamberlin and co-workers using Escherichia coli RNA polymerase and bacteriophage Τ7 DNA (1-8). Using this system, several steps prior to formation of the first phosphodiester bond in the RNA chain have been elucidated. RNA polymerase first binds rapidly and reversibly to random sites in the DNA forming nonspecific complexes. Through a series of association and dissociation reactions, RNA polymerase eventually locates at a promoter region and forms a specific preinitiation complex (I complex). This complex then undergoes a transition to form a stable preinitiation complex (RS complex) which can rapidly initiate RNA synthesis in the presence of nucleoside triphosphates. Initiation of RNA synthesis by RNA polymerase is very sensitive to rifampicin inhibition when the enzyme is present in either the free form or in a nonspecific enzyme-DNA complex (4, 6, 7). However, when RNA polymerase is bound at specific initiation sites in an RS complex form, the enzyme is 2 orders of magnitude less sensitive to rifampicin attack. Therefore, the formation of RS complexes can be measured in the presence of a concentration of rifampicin which is sufficient to inhibit the free enzyme and nonspecific enzyme DNA complexes but allows stable preinitiation complexes (RS complexes) to initiate a single RNA chain (5, 11). Using this method we have been able to measure changes in the number of initiation sites for E. coli RNA polymerase on chick oviduct chromatin during hormone-induced differentiation (12-14). The changes in the number of initiation sites correlate very well with the appearance of hormone-receptor complexes in nuclei and the subsequent accumulation of specific messenger RNAs and tissue growth and differentiation.

Useful information concerning the initiation of RNA synthesis can be derived by studying the effect of temperature on the formation of RS complexes (15). The transition from I complex to RS complex is a temperature-dependent process presumably involving a local unwinding of DNA strands at the promoter sequence (14, 16-18). Therefore, when RNA polymerase is incubated with DNA at temperatures below 10°C, there are no stable preinitiation complexes formed as measured by their
ability to rapidly initiate RNA chains. When incubation temperatures are raised above 10° a sharp cooperative increase in the formation of RS complexes is observed. Thus, the formation of the RS complex on DNA is highly temperature-dependent. In contrast, the temperature dependence for formation of stable preinitiation complexes on a chromatin template is much less, and in fact resembles that of denatured DNA (21). These results suggest that in vitro initiation sites on chromatin might be single-stranded in nature (12). This hypothesis is consistent with the results of Howk et al. (19) and Groner et al. (20) which demonstrated that the initial in vitro transcriptional product on chromatin using RNA polymerase II was a RNA-DNA hybrid. Crick has also proposed that in eukaryotic cells RNA chain initiation may occur at single-stranded regions of DNA (21). Levy and Simpson, however, found Crick’s hypothesis unacceptable after measuring the amount of single-stranded regions in isolated chromatin (22). These apparent discrepancies in the literature prompted us to study in detail the characteristics of RNA chain initiation on native chromatin and reconstituted chromatin complexes. We have examined the effects of temperature, antibodies, and a single strand specific nuclease on RNA chain initiation in order to better understand the structure of the chromatin sites for initiation of RNA synthesis.

**EXPERIMENTAL PROCEDURE**

**Materials**—Nucleoside triphosphates were obtained from P-L Biochemicals, [H]UTP (15 Ci/mmole) and ultrapure urea were the products of Schwarz/Mann Corp. Rifampicin and calf thymus DNA were purchased from Calbiochem, Inc. Heparin was obtained from Sigma Chemical Corp. and 1/4 log Escherichia coli pasteur (K-12) was purchased from Grain Processing Co. All other chemicals were reagent grade and were obtained from Fisher or J. T. Baker Corp.

**RNA Polymerase Isolation**—E. coli RNA polymerase containing the α subunit was isolated from 1/4 log E. coli K-12 by a modification of the method of Burgess (23) through the DEAE-cellulose column step. This material was further purified by DNA-cellulose chromatography as described by Bautz and Dunn (24) and then by Bio-Gel A-1.5 m chromatography under conditions of high salt as described by Burgess (23). The final enzyme preparation was stored at −20° in storage buffer (0.01 M Tris/HCl pH 7.9, 0.01 M MgCl₂, 0.1 M dithiothreitol, 0.1 mM EDTA, and 50% glycerol) at a concentration greater than 10 mg/ml. The enzyme obtained in this manner was greater than 98% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Preparation and Chemical Analysis of Chromatin**—Oviducts were removed from chicks which had received daily injections of 2.5 mg of diethylstilbestrol for 14 days, rinsed in cold saline (0.9% NaCl solution) and stored at −100° until needed. Chromatin was isolated according to the methods of Spelsberg and Hnilica (30). Sigma Chemical Corp. and K-12 Log Escherichia coli paste Houston, Texas. Conditions for RNA Synthesis—The conditions for RNA synthesis are a modification of the method of Tsai et al. (12). Briefly, E. coli RNA polymerase, diluted with 1 mg/ml of bovine serum albumin to specified concentration, was preincubated with DNA (1.5 µg) for 15 min or chromatin (5 µg) for 40 min at indicated temperatures in 200 µl of preincubation buffer containing 12.5 µmoles of Tris/HCl, pH 7.9, 0.5 µM of MnCl₂, 12.5 µM of (NH₄)₂SO₄, pH 7.9, and 0.5 µM of 2-mercaptoethanol. At the end of the preincubation period, RNA synthesis was initiated by the addition of 50 µl of a solution containing 37.5 nmoles of each of ATP, GTP, CTP, and [H]UTP (115 cpm/µmol) and 10 µg of rifampicin and allowed to continue at 37° for an additional 15 min. The reactions were terminated by the addition of cold 0.1 M trichloroacetic acid containing 0.01 M sodium pyrophosphate. The resultant RNA precipitates were collected on glass filters (Reeve Angel 934 AH) and counted in toluene based scintillation fluid (Toluene/ Spectrafluor. 1000/242 (v/v) in a Beckman LS-234 scintillation spectrometer. The size of several RNA transcripts has also been measured. Although there are some small variations, the variations cannot be accounted for by the differences observed in this paper.

**Preparation of Histone-Non-histone DNA Complexes**—The procedure for the fractionation of non-histone proteins from DNA has been previously reported (26). Briefly, the histone fraction and the non-histone (acidic protein) fractions AP₁, AP₂ are dissociated from DNA by incubation for 10 min at 60°C (12). The AP₁ fraction which is insoluble in 10 mM Tris/HCl, pH 6.8 (29) is 125S, 5566 Chromatin Sites for RNA Synthesis, and 5 mM urea. The soluble AP₂ fraction and the DNA associated with AP₁ and AP₂ are sedimented by centrifugation at 110,000 × g for 24 h. The histones are separated from AP₁ by dialysis against water and then exchanged with 0.1 M H₂SO₄. The AP₂ fraction which is insoluble in 10 mM Tris/HCl, pH 7.5, can be removed from DNA-AP₁ complex by sedimentation in a clinical centrifuge. The AP₂ is next removed from the DNA-AP₁ complex using 2 M NaCl, 5 mM urea, and 10 mM Tris/HCl buffer at pH 8.5 followed by subsequent sedimentation of the DNA-AP₁ complex. In this procedure, the relative distributions of total acidic protein into the various subfractions are as follows: AP₁; 15%, AP₂; 40%; AP₃; 35%; AP₄; 10%. F₁, F₂, and F₃ histone fractions were separated from F₄, and F₅ histone fractions according to the method of Van De Wiel and Van Holst (29). Various fractions of chromatin histone or non-histone proteins were then reconstituted with DNA or the DNA-AP₁ complex by gradient dialysis from 2.0 M NaCl to 0 M NaCl in the presence of divalent cation (1 mM MgCl₂), sodium bisulfite (2 mM) and 5 mM urea. The complexes were collected by centrifugation at 110,000 × g for 16 h and dialyzed against 2 mM Tris/HCl, pH 7.5 and 0.1 mM EDTA. It should be noted that (F₁ + F₂ + F₃) fraction was contaminated with 10 to 15% of F₄.

**Electrophoresis of Histone fractions in Sodium Dodecyl Sulfate-Acrylamide Gels**—Histones were isolated from different salt-washed chromatin according to the methods of Spelsberg and Hnilica (30). Sodium dodecyl sulfate gel electrophoresis at pH 10.0 was carried out according to the method of Panyim and Chalkley (31). Incubations of DNA and Chromatin with Specific Antibodies to Single Strand and Double Strand DNA—Single strand specific antibody to DNA, prepared by using a methylated bovine serum albumin λDNA complex as the antigen (32), was a gift from Dr. Lawrence Levine of Brandeis University, Mass. It was previously shown by Seaman et al. (32) that antibodies raised in this manner exhibit a high specificity for single strand rather than double strand DNA. The antibody to double-stranded DNA was provided by Dr. B. D. Stollar of Tufts University, Mass. It was obtained from a patient with systemic lupus erythematosus (33). Stollar has shown that 1 to 2% of the DNA in liver chromatin was accessible to the double strand specific antibody to DNA (33). Fifty microliters of various dilutions of the antibody were incubated with 50 µl of chromatin (5 µg) or DNA (1.5 µg) in 2 mM Tris/HCl, pH 7.5 and 0.1 mM EDTA at room temperature for 1 h. After this incubation, the initiation of RNA synthesis was assayed as described above.

**Effect of Mung Bean Nuclease I on Initiation of RNA Synthesis**—Mung bean nuclease I (a single strand specific DNase) was provided by Dr. M. Laskowski, Sr. of Roswell Park Memorial Institute, N. Y. This enzyme degrades single strand DNA at a rate 30,000 times greater than double strand DNA (34). Chromatin (5 µg) or DNA (1.5 µg) was incubated with 0.02 M sodium acetate (pH 5.3) at 37° in the presence or absence of 0 to 0.2 unit of nuclease (final volume 0.14 ml) for 30 min. Samples were then cooled to 0° and neutralized with 10 µl of 0.05 M NaOH. The nuclease-treated DNA or chromatin was then used to assay for the initiation of RNA synthesis according to the method described above. In control reactions which were designed to eliminate the physical effect of mung bean nuclease I on the RNA synthetic process itself, an equivalent amount of mung bean nuclease I was added to the samples after neutralization with NaOH and assayed for RNA initiation.

**RESULTS**

**Effect of Temperature on Formation of Stable Preinitiation Complex (RS Complex)**—Escherichia coli RNA polymerase was first preincubated with DNA or chromatin at various temperatures to allow for the formation of RS complexes. RNA synthesis was subsequently initiated by the addition of nucleotide triphosphates and rifampicin and carried out for 15 min at 37°. Under these conditions only enzyme in the RS complex form will be able to efficiently initiate an RNA chain; all other
enzyme and enzyme-DNA complexes will be inhibited (16-18). The transition curves shown in Fig. 1 depict the effect of temperature on formation of the stable preinitiation complex (RS complex) between RNA polymerase and either DNA or chromatin. The formation of the RS complex between native chick DNA and polymerase was highly temperature-dependent and revealed a 16/1 ratio at 37° versus 0°. The ratio of RS complex formed at 37° to 0° depends on the enzyme/DNA ratio as well as the integrity of the double-stranded DNA structure. At lower enzyme/DNA ratios or in the presence of nicked or single-stranded regions in the DNA, a lower temperature dependency was observed (12); this is due to the preferential binding of enzyme to nicked regions of DNA and the low temperature dependency for initiation of RNA synthesis in these regions. The transition temperature (midpoint of transition curve) is about 20° for this DNA. When chromat or denatured DNA was used as a template, the temperature dependency for initiation of RNA synthesis was very low. The RS complex formed at 37° on chromatin was only 2- to 3-fold higher than that observed at 0° and was now independent of the ratio of enzyme to template used. The endogenous RNA polymerase activity does not affect the results shown since only 100 to 300 cpm of UMP was incorporated in the absence of E. coli RNA polymerase. Since initiation of RNA synthesis on denatured DNA does not require DNA strand separation (I to RS complex transition), the temperature dependency for formation of the preinitiation complex on this DNA cannot occur at this step. Therefore, the small temperature dependency must occur at a different step leading to the formation of preinitiation complex such as binding of the enzyme to DNA. On chromatin, therefore, the same interpretation may also be valid for interpreting the minimal effect of temperature on the initiation process for RNA synthesis.

The temperature dependency for initiation of RNA synthesis was also examined using hen oviduct and calf thymus RNA polymerases (Table I). In these experiments rifampicin AF/013 was used in place of rifampicin. This inhibitor inhibits RNA synthesis prior to initiation (35). As shown in this table, there was very little temperature dependency observed when chromatin or heat-denatured DNA was used as a template. In contrast, when double-stranded DNA was used as template the level of RNA synthesis measured by the rifampicin-nucleotide triphosphate challenge technique was highly dependent on the preincubation temperature. Therefore, for both bacterial and homologous RNA polymerases, the temperature dependency for the formation of stable preinitiation complexes on chromatin was very low.

The temperature dependency for initiation of RNA synthesis has been interpreted as reflecting the energy required to open up or partially unwind the DNA strands at the promotor sequence (16, 17). The amount of energy required to unwind the double-stranded DNA is dependent on both the intrinsic \( T_m \) of the promotor region and the conditions of the ionic environment. Therefore, we would predict that at higher ionic concentrations, the transition temperature would increase. As compared to conditions of lower salt, the temperature transition curve at 0.0625 m (NH₄)₂SO₄ shifted to a higher temperature, on the average of 8 to 10° (Fig. 2A). This result supports the hypothesis that formation of an RS complex between DNA and RNA polymerase involves a temperature-dependent unwinding of the promotor sequences. When denatured DNA was used as a template (Panel B) the formation of RS complex once again was not highly dependent on the temperature and the transition observed was not a sigmoidal curve. The effect of salt on the initiation of RNA synthesis from denatured DNA was similar at all temperatures examined. At higher salt concentrations the level of RNA synthesis was somewhat reduced. Since initiation of RNA chains on denatured DNA

**TABLE I**

| Template           | Oviduct RNA polymerase | Calf thymus RNA polymerase |
|--------------------|------------------------|-----------------------------|
|                    | 37°        | 0°         | 37°/0°     | 37°        | 0°         | 37°/0°     |
| Chromatin          | 2,501     | 1,040     | 2.5       | 8,2986     | 3,143     | 2.9       |
| Denatured DNA      | 169,217   | 55,302    | 3.1       | 161,000    | 158,000   | 1.0       |
| Native DNA         | 10,743    | 1,653     | 6.5       | 13,362     | 1,624     | 8.2       |

**Fig. 1.** Dependence of RNA synthesis on temperature. *Escherichia coli* RNA polymerase (6.7 μg of enzyme protein with the DNAs and 8 μg with chromatin) was preincubated with chick oviduct native DNA (1.5 μg), denatured DNA (1.5 μg, heat-denatured at 100° for 10 min), or chromatin (5 μg) at different temperatures as described under "Experimental Procedures." At the end of the 40-min preincubation, 50 μl of nucleoside triphosphates and the rifampicin mixture were added and further incubated at 37° for 15 min. RNA synthesized was precipitated with 5% trichloroacetic acid and counted in toluene base scintillation fluid; • native chick DNA; ○ denatured chick DNA; and × chromatin.
does not require strand unwinding, this effect of salt on the formation of initiation complexes is most likely due to reduced binding of RNA polymerase to DNA which is known to decrease with increasing salt concentration. As for chromatin (Panel C), the formation of a RS complex was not highly dependent on temperature and was not significantly affected by salt even at a concentration as high as 0.125 M (NH₄)₂SO₄. The reduced dependence on temperature for formation of RS complexes suggests that the formation of initiation complexes on chromatin is quite different from that observed with double-stranded DNA, but resembles the results obtained when using single-stranded DNA. This led us to speculate that the initiation sites on chromatin may be single-stranded in nature.

Temperature Requirements for Initiation of RNA Synthesis on Reconstituted Chromatin Protein-DNA Complexes—As shown in our earlier report (12), several features of the initiation of RNA synthesis on DNA and on chromatin are distinct. First, 50 to 70% of the RNA polymerase bound to native chick DNA could initiate RNA synthesis in the rifampicin-nucleotide challenge assay; in contrast, only 3 to 5% of the bound polymerase could initiate RNA synthesis in the case of chick oviduct chromatin. Second, the temperature dependency for formation of stable preinitiation complexes on chromatin was significantly reduced as compared to DNA. Third, a distinct class of initiation sites was observed on DNA at low enzyme/DNA ratios, presumably from initiation at nick or gap regions; this class of sites was not detected on chromatin.

All of these differences must be a result of the presence of the chromatin proteins which are bound to the DNA. This prompted us to investigate whether any specific subfraction of the chromatin proteins was responsible for maintaining the characteristics inherent to initiation of RNA synthesis on chromatin. To investigate this possibility, various combinations of histone and non-histone fractions were reconstituted to DNA according to the procedure described under “Experimental Procedures.” The reconstituted protein-DNA complexes were preincubated with increasing amounts of RNA polymerase at either 0° or 37° followed by incubation with nucleoside triphosphates and rifampicin. Complexes of either the total non-histone fraction with DNA or the total histone fraction with DNA displayed a pattern of RNA synthesis which closely resembled that of protein-free DNA (Fig. 3). The formation of the stable preinitiation complex on these protein-DNA complexes retains a high temperature dependency although the level of RNA synthesis was reduced to 35% on DNA-histone complex. This reduction in RNA synthesis may be caused by histone proteins masking the initiation sites or physically

![Fig. 2](image-url) Effect of salt on the transition temperature for initiation of RNA synthesis. RNA polymerase was preincubated at different temperatures with DNAs or chromatin as described in Fig. 1, except different salt concentrations were added during the preincubation with (A) native chick DNA, (B) denatured chick DNA, and (C) chick oviduct chromatin. The preincubation conditions were: ---O, no (NH₄)₂SO₄; O--O, 0.0625 M (NH₄)₂SO₄; or x--x, 0.125 M (NH₄)₂SO₄.

![Fig. 3](image-url) Temperature-dependent RNA synthesis on different protein-DNA complexes. *Escherichia coli* RNA polymerase (0 to 15 μg) was preincubated with DNA (1.5 μg) (A), or non-histone-DNA complex (5 μg) (B), or histone-DNA complex (5 μg) (C), at 37° (●—●) or 0° (○—○) as described in Fig. 1. Histone-DNA and non-histone-DNA complexes were prepared as described under “Experimental Procedure.”
blocking the elongation process (36). These data suggest that even at "physiological" protein/DNA ratios, total histone or total non-histone protein fractions alone cannot convert the "DNA-like" initiation process to a "chromatin-like" initiation process. It thus appears that both histone and non-histone chromatin proteins are required to reconstruct the characteristics of the RNA initiation process observed with native chromatin.

Further studies were carried out to determine which subclasses of non-histone or histone proteins are required to produce the low temperature dependency which is characteristic of RNA chain initiation on chromatin. Various complexes devoid of a particular class of histone or non-histone protein were prepared and tested in the RNA initiation assay. As shown in Fig. 4, reconstitution of the total histone plus non-histone proteins (Panel B) could in fact result in a pattern of RNA chain initiation which was indistinguishable from native chromatin (Panel A). Protein-DNA complexes devoid of AP, and AP, (Panel C) or AP, and AP, (Panel D) showed an increase in total RNA synthesis, an increase in the temperature dependency for formation of preinitiation complexes, and the appearance of initiation from nicked DNA sites as observed at low enzyme/DNA ratios. All of these characteristics are typical of the "DNA-type" pattern of RNA chain initiation. The appearance of "DNA-type" initiation properties was positively dependent on the amount of the non-histone chromatin proteins which were removed and was not dependent on the particular class of protein as prepared by this fractionation procedure. This point is further substantiated by the results presented in Table II in which all protein-DNA complexes contained similar histone/DNA ratios, but different non-histone/DNA ratios. The non-histone content in these complexes varied directly with the "chromatin type" pattern of RNA chain initiation. It should be noted that using this fractionation procedure, it is not possible to obtain distinct fractions without any common proteins. Although there are some cross-contaminations between different fractions, there are no apparent correlations between the disappearance of particular non-histone fractions and changes in the characteristics of RNA chain initiation. In contrast, the quantity of total non-histone protein removed correlates very well with the changes in the characteristics of RNA chain initiation.

The results of transcription of reconstituted chromatin complexes devoid of certain classes of histone are shown in Fig. 5. The chromatin devoid of F, was prepared from chromatin exposed to 1.0 M NaCl in 50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂ for 30 min at 0°. By this treatment F₁ histone was completely removed while the majority of the other histones (>90%) remained bound to the DNA (Fig. 6). RNA synthesis as measured by the rifampicin-nucleotide challenge assay increased 2-fold after removal of F₁ histone. However, the temperature dependency for formation of RS complex

![Fig. 4. Temperature dependence on chromatin and different reconstituted protein-DNA complexes. RNA polymerase (0 to 12 µg) was preincubated with 5 µg of chromatin or reconstituted protein-DNA complexes at 0° (○-○) and 37° (●-●) as described in Fig. 1 except specific activity of UMP used was one-half that in Fig. 1. A, chick oviduct chromatin; B, reconstituted chromatin; C, reconstituted histone-A₂₆₄-DNA complex; D, reconstituted histone-A₂₄₄-DNA complex.](image)

![Fig. 5. Temperature dependence on chromatin devoid of different histone fractions. Escherichia coli RNA polymerase (0 to 20 µg) was preincubated with 5 µg of different protein-DNA complexes at 37° (●-●) or 0° (○-○) as described in Fig. 1. Different protein-DNA complexes were prepared according to the method described under "Experimental Procedure." A, reconstituted chromatin without F₁; B, reconstituted chromatin without F₁, F₁₉₂, and F₁₉₂; C, reconstituted chromatin without F₁₉₂ and F₁₉₂.](image)
remained similar to that observed with native chromatin. On the other hand, when $F_1$, $F_{2a}$, and $F_2b$ (lysine-rich and moderately lysine-rich histones) or $F_{2c}$, and $F_3$ (arginine-rich histones) were removed from the reconstitution mixture, the initiation of RNA synthesis on the reconstituted chromatin reverted to a "DNA-type" pattern. A dramatic increase in RNA synthesis and temperature dependency for formation of initiation complexes was noted. Therefore, both arginine-rich histone and moderately lysine-rich histone play a role in maintaining the characteristics of the "chromatin-type" pattern for initiation of RNA synthesis.

Effect of Single Strand Specific and Double Strand Specific Antibodies to DNA and Single Strand Specific Nuclease on Initiation of RNA Synthesis—This series of experiments was designed to examine whether blocking or eliminating single strand or double strand regions of chromatin DNA would reduce the number of newly initiated RNA chains. As can be seen in Fig. 7 the amount of RNA synthesis in the presence of rifampicin was not affected by the pretreatment (23° for 1 h) of chromatin with various antibody specific for single strand DNA. We also did not observe significant inhibition using various other pretreatment conditions such as: 0° for 3 h; 4° for 24 h; 37° for 1 h; minus 0.0625 M (NH₄)₂SO₄; or with up to 15% glycerol added to the incubation buffer (data not shown). In control experiments, however, this antibody at a 1/100 dilution or less inhibited RNA synthesis up to 80% when single-stranded DNA was used as template. These dilutions did not inhibit RNA synthesis when double-stranded DNA was used as a template. The small amounts of inhibition observed at very high concentrations of antibody could be due to nuclease contaminants in the antibody preparation. These results demonstrated that this antibody possessed the specificity for

![Sodium dodecyl sulfate gel electrophoresis of histone fraction isolated from control and salt-washed chromatin. Salt-washed chromatin was prepared as described under "Experimental Procedure."](image1)

![Bar graph](image2)

**Fig. 6.** Sodium dodecyl sulfate gel electrophoresis of histone fraction isolated from control and salt-washed chromatin. Salt-washed chromatin was prepared as described under "Experimental Procedure." Sodium dodecyl sulfate gel electrophoresis of histone fractions was carried out as described by Panyim and Chalkley (31). A, control chromatin; B, salt-washed chromatin.

**Fig. 7.** Effect of antibody to single strand DNA on the level of RNA synthesis. Antibody (1/10 dilution to 1/10,000 dilution) was incubated with chick oviduct DNAs (1.5 µg) or chromatin (5 µg) at 23° for 1 h. 8 µg of RNA polymerase and reaction mixture were added and further preincubated at 37° for 15 min as described under “Experimental Procedure.” Levels of RNA synthesis in the control samples were native DNA, 80,000 cpm; denatured DNA, 12,000 cpm; PM DNA, 111,000 cpm; and chromatin, 5000 cpm as expressed in UMP incorporated.
reacting only with single strand DNA and for inhibiting the initiation of RNA synthesis. Since the lack of inhibition on chromatin might be partially explained by the superhelical structure of chromatin DNA (37), we examined the effect of this antibody on PM, Form 1 DNA (superhelix DNA) (Fig. 7). RNA initiation on PM, DNA was only inhibited at high antibody concentration. Therefore, the lack of antibody inhibition on chromatin can be due to either superhelical or double-stranded properties of the chromatin DNA.

The antibody to double strand DNA obtained from a patient with systemic lupus erythematosus was also used to determine whether the double strand regions of chromatin DNA which can interact with this antibody are involved in the initiation of RNA synthesis. Stollar has shown that this antibody can react with 1 to 2% of the DNA in the liver chromatin (33). As shown in Fig. 8, the initiation of RNA synthesis on chromatin was only slightly inhibited by the antibody to double strand DNA. The control experiments showed that initiation of RNA synthesis on double strand but not single strand DNA was inhibited. When superhelical PM, DNA was used as template, antibody to double strand DNA also inhibited RNA synthesis in a similar fashion to that observed with native double strand DNA (data not shown). Taken together, these results indicate that initiation regions for RNA synthesis on chromatin are either covered by chromatin proteins or in a structure which is unavailable for reactions with antibodies.

Mung bean nuclease I can digest both single strand DNA and superhelical DNA containing a negative superhelix density (−80) above 0.05 (38). Nevertheless, pretreatment with concentrations up to 0.2 unit of Mung bean nuclease I had no effect on the initiation of RNA synthesis on chromatin (Fig. 9). In contrast, 0.01 unit of Mung bean nuclease was sufficient to inhibit more than 80% of RNA synthesis when single strand DNA was used as template (Fig. 10). This enzyme had absolutely no effect on RNA synthesis when native double strand DNA was used as a template.

These data suggest that chromatin initiation sites for RNA synthesis are not located in freely accessible single-stranded regions of DNA. However, this does not preclude the possibility that initiation occurs at single-stranded regions of DNA which have been covered by chromatin proteins or in regions which are too small to allow interactions with antibodies or nuclease molecules. One might question whether this result could be due to the condition of nuclease treatment. At pH 5.5, the structure of chromatin initiation sites could change from a single strand to double strand configuration. This reasoning seems unlikely since we have also observed that Neurospora crassa single strand specific nuclease, active at pH 7.0 (39), also has no effect on the level of RNA synthesis (data not shown).

**DISCUSSION**

We have previously shown that initiation of RNA synthesis on DNA differed from that on chromatin in several respects (12). The most distinct difference was the low temperature dependency for formation of the stable preinitiation complex (RS complex) on chromatin which was thought to be due to the single strand nature of the initiation sites on chromatin DNA (12). On single strand DNA, a temperature-dependent opening of DNA strands in the initiation region (transformation from I complex to RS complex) was not required for RNA chain initiation. However, the initiation of RNA synthesis on this denatured DNA was still slightly dependent on temperature. This low temperature dependence might arise from the effect of temperature on the affinity between RNA polymerase and DNA. Likewise, the small effect of temperature on the initiation of RNA synthesis on chromatin may also result from its effect on the dissociation and reassociation of enzyme from the template rather than transformation of I to RS complex.

When double strand DNA was used as a template, increasing the concentration of salt increased the transition temperature for formation of the RS complex (Fig. 2A). The effect of salt on
Chromatin Sites for RNA Synthesis

**FIG. 9.** Effect of a mung bean nuclease I on the level of RNA synthesis. Mung bean nuclease I (0 to 0.2 unit) was incubated with 5 µg of chromatin in the condition described under “Experimental Procedures.” After 30 min, the samples were neutralized with NaOH until pH 7.0 and the subsequent reactions were carried out as described in Fig. 7. In the control experiment (set B) mung bean nuclease I was added after the chromatin samples were neutralized with NaOH. In the control experiment 6500 cpm of UMP was incorporated.

**FIG. 10.** Effect of mung bean nuclease I on the level of RNA synthesis. Mung bean nuclease I (0 to 0.05 unit) was incubated with 1.5 µg of DNA or 5 µg of chromatin at 37°C for 30 min and the subsequent reaction was carried out as described in Fig. 9. 100% activity stands for 6500 cpm of UMP incorporated for chromatin, 72,000 cpm for native DNA, and 10,000 cpm for denatured DNA. A--A, denatured chick DNA; O--O, native chick DNA; •--•, chick oviduct chromatin.

The lack of a temperature-dependent and sigmoidal transition curve for initiation of RNA synthesis on chromatin can also be interpreted as the result of an extremely heterogeneous population of initiation sites which have slightly different transition temperatures. If this were the case, the temperature-dependent transition curve for formation of the available population of total initiation complexes would not be a sigmoidal transition curve but a series of small individual sigmoidal transitions. However, in this instance we would expect that increasing the concentration of salt should shift each individual curve similarly to DNA and thus the whole transition curve would be shifted to a higher temperature. Since salt had no effect on the temperature-dependent transition curve for chromatin, the lack of a sigmoidal transition appears not to be due to a heterogeneous population of initiation regions.

First, increasing the concentration of salt could decrease the affinity of enzyme binding to DNA. Thus, the energy released from this enzyme-DNA interaction which is required to open up the initiation sequence should decrease when the salt concentration is increased. Second, the salt could stabilize the DNA duplex structure. In this case, the energy required to open the initiation sequence would increase with the increasing salt concentration. In either case, the transition temperature would be higher at higher salt conditions due to the requirement for unwinding the initiation sequence prior to the initiation of RNA synthesis. On denatured DNA, however, the requirement for opening up the DNA strand is no longer a prerequisite. In this case, the salt will primarily affect the affinity with which the enzyme binds to DNA. Therefore, the number of RNA polymerase molecules bound to DNA will decrease with increasing concentration of salt (Fig. 2B). As for chromatin, the level of RNA synthesis was not significantly affected by salt. This suggests that the ionic interaction between RNA polymerase and chromatin DNA or strand separation prior to the initiation of RNA synthesis is not an important factor in determining the level of RNA synthesis. In this regard, it is interesting to note that recently in this laboratory (40), we have measured the effect of salt on the rate of formation of the stable preinitiation complex on DNA and chromatin. We found that salt had little effect on the rate of formation of preinitiation complexes on chromatin although it markedly affected the rate of formation of preinitiation complex on DNA. This difference was attributed to a nonspecific nonionic interaction of RNA polymerase with chromatin protein(s). This interaction is most probably the rate-limiting step for the formation of RS complex.

The transition curve could occur at either of two levels (17). The transition curve would be higher at higher salt conditions due to the requirement for unwinding the initiation sequence prior to the initiation of RNA synthesis. On denatured DNA, however, the requirement for opening up the DNA strand is no longer a prerequisite. In this case, the salt will primarily affect the affinity with which the enzyme binds to DNA. Therefore, the number of RNA polymerase molecules bound to DNA will decrease with increasing concentration of salt (Fig. 2B). As for chromatin, the level of RNA synthesis was not significantly affected by salt. This suggests that the ionic interaction between RNA polymerase and chromatin DNA or strand separation prior to the initiation of RNA synthesis is not an important factor in determining the level of RNA synthesis. In this regard, it is interesting to note that recently in this laboratory (40), we have measured the effect of salt on the rate of formation of the stable preinitiation complex on DNA and chromatin. We found that salt had little effect on the rate of formation of preinitiation complexes on chromatin although it markedly affected the rate of formation of preinitiation complex on DNA. This difference was attributed to a nonspecific nonionic interaction of RNA polymerase with chromatin protein(s). This interaction is most probably the rate-limiting step for the formation of RS complex.
The apparent differences between initiation of RNA synthesis on DNA and chromatin must be due to the presence of chromatin proteins. After removing either histone or non-histone proteins, the initiation of RNA synthesis resembles that seen on native DNA. These results indicate that both histone or non-histone proteins are essential for maintaining the characteristics inherent to RNA chain initiation on chromatin. Spelsberg et al. (26) have fractionated the non-histone chromatin proteins into four fractions (AP₁, AP₂, AP₃, AP₄) having different electrophoretic gel banding patterns. This fractionation method has proved to be useful in defining the acceptor sites for hormone-receptor complexes in the nuclear chromatin (26). By adapting this procedure we have analyzed the importance of each fraction in determining the type of initiation process observed with reconstituted chromatin proteins. Apparently from Fig. 5 and Table II, the absolute quantity of non-histone proteins is important in changing the "DNA-type" initiation pattern to a "chromatin-type" initiation pattern.

In contrast to other histone proteins, it has been previously reported that removal of F₁ histone does not greatly affect the chromatin template activity (41, 42). Our results are consistent with these previous studies. Removal of F₁ only slightly increased the level of RNA chain initiation without changing the temperature dependence for formation of stable preinitiation complexes. However, removal of either arginine-rich histones (F₂⁺ₐ + F₃) or moderately lysine-rich histones (F₂⁺ₐ + F₃) completely eliminated or masked chromatin-type initiation. The total level of RNA synthesis reached was similar to that observed with deproteinized DNA template. These results are consistent with the current thinking that the basic structure of chromatin is composed of Y bodies which contain arginine-rich and moderately lysine-rich histone (43, 43a). On removing any of these histones the basic structure of chromatin is lost. This change appears to be accompanied by a loss of the characteristic "chromatin-type" pattern for RNA chain initiation.

The fidelity of reconstitution is very important for interpreting the effects of chromatin proteins on transcription in vitro. The results of Stein et al. (44) suggested that reconstituted chromatin was identical to native chromatin in terms of the protein/DNA ratios, electrophoretic patterns of histones and non-histones, circular dichroism spectra, binding of reporter molecules, and template activities. However, before native and reconstituted chromatin can be shown to be identical, many more functional studies of chromatin structure should be carried out. The results reported in this paper and the observations that both native and reconstituted chick oviduct chromatin (45) and erythrocyte chromatin (46) synthesize similar yields of ovalbumin and globin mRNA molecules, respectively, are further support for the fidelity of reconstitution.

The use of a prokaryotic RNA polymerase to study the initiation of RNA synthesis on eukaryotic chromatin also may be questioned. However, the results with hen oviduct and calf thymus RNA polymerase II (Table I) suggested that eukaryotic RNA polymerase behaved similarly to the bacterial RNA polymerase with regard to the temperature dependency for formation of stable preinitiation complexes. Recent evidence from this laboratory indicates that E. coli RNA polymerase and hen oviduct RNA polymerase II utilize similar sets of initiation sites in chromatin (35). Briefly, the experiments were carried out by preincubating chromatin with saturating amounts of both E. coli and homologous RNA polymerase. RNA synthesis was initiated by the addition of nucleoside triphosphates and the initiation inhibitor, rifamycin AF013. In this experiment, we would expect that the level of RNA synthesis would be equal to the sum of individual RNA polymerases if they used separate initiation regions. On the other hand, the level of RNA synthesis should be one-half the sum of that observed with the individual enzymes if these two enzymes competed with each other for a given region of chromatin during the preincubation. The results obtained showed that E. coli RNA polymerase competed with oviduct RNA polymerase II when chromatin was used as template. In contrast, the majority of the initiation sites on pure DNA which were utilized by the oviduct RNA polymerase II were observed to be different from those utilized by the E. coli enzyme.

At the present time, it is not possible to prove that these in vitro initiation sites are identical to the actual initiation sites utilized in vivo. Nevertheless, the ability of different RNA polymerases to utilize common sites would be consistent with the hypothesis that chromosomal proteins are major determinants of initiation sites for RNA polymerase in chromatin.

The lack of a sigmoidal temperature-dependent transition for the formation of preinitiation complexes for both single strand DNA and chromatin is consistent with the hypothesis that promoter regions of chromatin are in a partially destabilized form. We have recently isolated RNA polymerase II from

| Table II | Protein content and RNA synthesis from reconstituted protein-DNA complexes |
|-----------------------|-----------------------------|---------------------|---------------------|
|                      | Histone/DNA | Non-histone/DNA | RNA synthesis 37°C/0°C | [H]JUMP incorporated at 37°C |
| **Experiment A**     |              |                  |                         | cpm                          |
| Chromatin A          | 1.47         | 1.10             | 1.6-2.0                | 2,200                         |
| (Histone·Ap₁)        | 1.27         | 0.95             | 1.8                    | 2,200                         |
| (Histone·Ap₁·DNA)    | 1.29         | 0.72             | 2.5                    | 3,800                         |
| (Histone·Ap₁)        | 1.25         | 0.60             | 3.4                    | 5,100                         |
| **Experiment B**     |              |                  |                         | cpm                          |
| (Histone) Ap₁       | 1.02         | 1.01             | 1.9                    | 3,400                         |
| (Histone·Ap₁·DNA)    | 0.99         | 0.60             | 3.7                    | 4,400                         |
| (Histone·Ap₁·DNA)    | 0.92         | 0.52             | 5.0                    | 7,500                         |
RNA polymerase since the eukaryotic enzyme prefers single strand DNA. However, on chromatin the level of RNA synthesis achieved by oviduct RNA polymerase II is the same as that observed when using *E. coli* RNA polymerase. These results once again are consistent with the hypothesis that the promoter sites present in chromatin DNA are at least partially destabilized. It is unfortunate that the complex nature of chromatin and the lack of suitable genetic mutants in eukaryotic cells force investigators to take indirect approaches to study transcription. Although the studies summarized in the present manuscript provide new information relative to this important process, they are of necessity an example of such an indirect approach. Currently we are in the process of purifying oviduct tissue. The enzyme transcribes deproteinized, destabilized. It is unfortunate that the complex nature of chromatin sites for RNA synthesis

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