Effect of Estrogen on Gene Expression in the Chick Oviduct

KINETICS OF INITIATION OF IN VITRO TRANSCRIPTION ON CHROMATIN*

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MASA KI HIROSE, MING-JER TSAI, AND BERT W. O’MALLEY

From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77025

The kinetics of initiation of in vitro RNA synthesis by *Escherichia coli* RNA polymerase on the chromatin template of chick oviduct was examined and compared to initiation on the template of deproteinized chick DNA. The formation of rapidly starting, highly stable binary complexes (RS complexes) between RNA polymerase and chromatin was an apparent first order process with a half-time \( t_{1/2} \) of 9.4 min. By contrast, the \( t_{1/2} \) of formation of RS complex on chick DNA was 1.3 min. The apparent first order rate constants of RNA chain initiation from preformed RS complexes were 0.57 s\(^{-1}\) for chromatin and 0.98 s\(^{-1}\) for DNA. Thus, although the formation of RS complex on chromatin was much slower than on DNA, the actual rate of RNA chain initiation on chromatin was similar to that on DNA. The effects of temperature on the formation of RS complex were examined. On chick DNA, both the rate of formation and the amount of RS complex formed decreased as the temperature of preincubation was lowered from 37° to 0°. On chromatin, the \( t_{1/2} \) of formation of RS complex was independent of temperature, and the amount of complex formed was only slightly affected at changing temperatures. The kinetics of initiation on chromatin is consistent with the hypothesis that the rate-limiting step in the formation of RS complex is dissociation of RNA polymerase from nonspecific interaction with chromosomal proteins. Furthermore, chromosomal proteins seem to interact with initiation sites to facilitate the opening of the DNA strands required for RS complex formation.

Isolated chromatin has commonly been used as a template for exogenously added RNA polymerase to study the transcriptional activity of a given tissue. RNA polymerase from *Escherichia coli* is able to transcribe specific messenger RNA sequences from chromatin isolated from a tissue actively synthesizing the mRNA tested, but not from tissues which do not synthesize the particular mRNA in question (1-4). Thus, the transcription of isolated chromatin by *E. coli* RNA polymerase is a popular and appropriate measure for studying specific gene regulation in vitro. Recently we have developed an assay which specifically measures the number of RNA initiation sites available to RNA polymerase in isolated chromatin (5). Using this assay, it was demonstrated that an increase occurs in the number of initiation sites of RNA polymerase in oviduct chromatin after administration of estrogen to the immature chick (6). This increase correlates well with changes in the levels of nuclear RNA (7-9), endogenous RNA polymerase activity (10, 11), chromatin template capacity (10-12), and the level of mRNA for a specific induced protein, ovalbumin (9, 13-17). We have also recently suggested that following estrogen administration, the hormone-receptor complex may directly interact with chromatin to mediate the opening of new initiation sites for gene transcription (17). Although chromatin initiation sites for RNA synthesis may play a major role in gene regulation, the interaction of RNA polymerase with chromatin and the structure of these initiation sites are not well understood. We have, therefore, studied in detail the kinetics of RNA initiation as a probe of the structure of initiation sites for RNA polymerase in chromatin.

Chamberlin and co-workers have examined the initiation of RNA synthesis using *E. coli* RNA polymerase and bacteriophage T7 DNA as a model system (18-24). The initiation of RNA synthesis by RNA polymerase can be separated into two general processes: binding of the enzyme to a specific site on the DNA at which RNA initiation can occur and the enzymatic formation of a phosphodiester bond between two ribonucleoside triphosphates.

The formation of RNA polymerase-DNA complexes capable of initiating RNA synthesis was suggested to occur through several intermediate steps:

\[
\text{Nonspecific complex} \xrightarrow{(1)} \text{I complex} \xrightarrow{(2)} \text{RS complex} \]

When RNA polymerase is incubated with DNA, the enzyme is initially bound randomly and reversibly to DNA to form a nonspecific complex (Step 1) (19). If such binding occurs at or
near a true initiation site for RNA synthesis, an initial preinitiation complex (I complex) is formed (Step 2) (19). This complex must undergo a transition to form a highly stable complex (RS complex) which can rapidly initiate RNA synthesis in the presence of ribonucleoside triphosphates (Step 3) (21, 24). The equilibrium between I and RS complex is very sensitive to temperature and ionic strength (23, 24). The conversion from I to RS complex has been postulated by Chamberlin and co-workers (21, 24) to involve local opening of the DNA duplex structure. E. coli RNA polymerase is very sensitive to inhibition by rifampicin when the enzyme is not bound to DNA. However, when the RNA polymerase is bound in the RS complex, the enzyme is 2 orders of magnitude less sensitive to rifampicin than the free enzyme (20). Therefore, the rate constant of formation of the RS complex can be determined by measuring the RNA polymerase activity in the presence of a concentration of rifampicin sufficient to completely inactivate free enzyme, but which does not greatly affect enzyme bound in the RS complex.

RNA polymerase in the RS complex can rapidly initiate RNA chain synthesis in the presence of the four ribonucleoside triphosphates. Once the first phosphodiester bond is formed, the RNA polymerase is completely insensitive to rifampicin (25, 26). When rifampicin and the four ribonucleoside triphosphates are added simultaneously to the RS complex, the attack by rifampicin and the formation of the first phosphodiester bond are challenging reactions. Thus the rate constant of the formation of the first phosphodiester bond can be determined by measuring RNA polymerase activity in the presence of varying concentrations of rifampicin (22).

In this report, both rate constants for the formation of the RS complex and for RNA chain initiation from preformed RS complexes were determined on the templates of chick DNA and chick oviduct chromatin. The results of kinetic measurements for the initiation of RNA synthesis suggest that the rate-limiting reaction in chromatin transcription is dissociation of the enzyme from nonspecific binding interactions with chromosomal proteins. We also postulate that the initiation sites in chromatin are held in a conformation which greatly facilitates opening of the DNA duplex to form the RS complex.

**EXPERIMENTAL PROCEDURE**

**Materials**—Ribonucleoside triphosphates were obtained from P-L Biochemicals. [14C]UTP (14 Ci/mmol) was purchased from Schwarz/Mann. Rifampicin was obtained from Calbiochem and heparin was obtained from Sigma Chemical. E. coli paste (strain K-12) was purchased from Grain Processing Co. All other chemicals were reagent grade and were obtained from J. T. Baker or Fisher. E. coli RNA polymerase, which contained greater than 70% equivalency of σ factor, was isolated by the method of Burgess (27) as modified by Bautz and Dunn (28), and stored at -20°C in buffer containing 0.1 M Tris-HCl, pH 7.9, 0.1 mM MgCl₂, 0.1 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM dithiothreitol, and 50% glycerol at a concentration of 10 mg/ml. Chick DNA was isolated from chick oviduct as reported previously (29).

**Preparation of Chromatin**—Oviducts were removed from chicks which had received daily injections of 5 mg of estradiol-17β for 2 days, rinsed in cold saline (0.9% NaCl solution), and stored at -110°C prior to processing. Chromatin was isolated according to the protocol of Tsai et al. (30). Isolated chromatin was analyzed as described earlier (30) and stored at -20°C in 0.2 M Tris-HCl, pH 7.5 and 0.1 mM EDTA. The chromatin preparation used for these studies was found to have 0.980 mg/ml of DNA, 1.65 mg/ml of histones, and 1.33 mg/ml of non-histone proteins.

Dechelated chromatin (DNA-AP₂-AP₃-AP₄) and reconstituted histone DNA complex were prepared according to the procedure of Spelsberg et al. (30). Histone-DNA complex was obtained by reconstituting chick DNA and the histone fraction at a mass ratio of 1:0.

Assay for RNA Polymerase Activity—E. coli RNA polymerase was diluted with 1 mg of bovine serum albumin dissolved in 50 mM Tris-HCl, pH 7.9 and incubated with chick DNA or chick oviduct chromatin. The preincubation mixture contained 9.25 mM Tris-HCl, pH 7.9, 0.25 mM ammonium sulfate, 2.5 mM 2-mercaptoethanol, and 0.5 mg/ml of bovine serum albumin. Unless otherwise specified, RNA polymerase was present at 1.12 μg/ml with 6.0 μg/ml of DNA or at 28 μg/ml with 20 μg/ml (as DNA) of chromatin. At these enzyme/DNA ratios, the specific initiation sites on either template were almost completely saturated with RNA polymerase (5). After the preincubation, RNA synthesis was initiated by mixing the preincubation mixture and 0.25 volume of the substrate mixture which contained 0.2 μCi/ml of [3H]UTP and 0.75 μm of each of ATP, CTP, GTP, and UTP in either the presence or absence of rifampicin. After incubation at 37°C for 0 to 15 min, RNA synthesis was terminated by the addition of 0.15 ml of 0.2 M EDTA, pH 7.9 and cooling at 0°C. Bovine serum albumin was added to give 150 μg of total protein, followed by approximately 5.0 ml of cold 5.0% trichloroacetic acid containing 0.01 M sodium pyrophosphate. After standing for 15 min at 0°C the solution was centrifuged at 700 g for 15 min. The pellet was dissolved in 0.15 ml of cold 0.2 M NaOH and immediately reprecipitated with cold trichloroacetic acid. The precipitate was collected on glass fiber filters (Reeve Angel 934 AH) and washed with 30 ml of the cold trichloroacetic acid. When high concentrations of rifampicin were used, rifampicin was removed by washing the filters twice with 1.0 ml of cold 40% ethanol containing 5% trichloroacetic acid in order to avoid the serious quenching effect of this compound. The amount of radioactivity present was determined as previously described (5).

**RESULTS**

**Time Course of RNA Synthesis**—The time course of RNA synthesis by Escherichia coli RNA polymerase on either chick oviduct DNA or chromatin was first examined in order to determine the incubation conditions to be used in later experiments. After binary complexes between RNA polymerase and the template were formed, RNA synthesis was initiated by the addition of the four ribonucleoside triphosphates in either the presence or absence of the initiation inhibitor, rifampicin (Fig. 1). For chick DNA, the time course of RNA synthesis was linear for at least 1.5 min in the presence or absence of rifampicin. However, under our assay conditions, the release of enzyme from DNA and the reinitiation of RNA chain synthesis after short elongation is slower than the elongation rate itself. Following initiation of the first round of RNA synthesis, it takes some time before a second round of RNA synthesis can begin. Therefore, the time course of RNA synthesis on DNA in the absence of rifampicin slows after 7 to 8 min. On chromatin, however, a reasonably linear relationship was not observed even at this short incubation period. This result is consistent with the observation that the size of RNA transcribed in vitro from chick oviduct chromatin is more heterogeneous (31). Even after 1.5 min a significant amount of reinitiation occurs in the absence of rifampicin when chromatin is transcribed. The observation of reinitiation in the absence of rifampicin will be discussed in more detail later.

In the presence of rifampicin, the level of RNA synthesis...
reached a plateau after 6 to 9 min of incubation for either template. Further incubation to 15 min did not result in any reduction in the amount of ribonucleotide incorporated into RNA. Since the elongation rate for *E. coli* RNA polymerase on DNA and chromatin and the average product size are not drastically different, it is expected that 6 to 9 min represents the length of time required for the longest RNA chains to be synthesized on either template. Thus, it is unlikely that RNase activity in the chromatin influences the overall incorporation of UMP into an acid-insoluble form within 15 min of incubation (although the actual size of RNA may be greatly reduced).

**Formation of RS Complex—**Binary complexes capable of rapidly initiating RNA synthesis (RS complex) were formed by preincubating *E. coli* RNA polymerase with chick oviduct DNA or chromatin. After preincubation for varying lengths of time, the amount of RS complex was assayed by measuring RNA polymerase activity following the simultaneous addition of the four ribonucleotides and rifampicin (Fig. 2). The formation of RS complexes on DNA reached a maximal level of 15 min of preincubation, whereas on chromatin the maximum was not reached until 40 min.

As shown in the insets to Fig. 2, semilogarithmic plots of the maximum level of RNA synthesis minus the level of RNA synthesis at each preincubation time versus the time of preincubation were linear on both templates. This result would only be expected if the formation of RS complexes occurs via a first-order process. The half-time of formation of the RS complex (t½) was 1.3 min on chick DNA. On the other hand, the t½ for formation of the RS complex on chromatin was 9.4 min or approximately 7 times slower than on DNA.

The theoretical maximum level of RNA synthesis (RS max) was obtained by extrapolation of the semilogarithmic plots (insert to Fig. 2) to the intercept on the ordinate.

The RS max on chromatin was approximately 5% of that determined for DNA, when normalized to an equivalent amount of enzyme. This result is consistent with the previous observation that 70% of *E. coli* RNA polymerase bound to chick DNA could initiate RNA synthesis in the presence of rifampicin, but only 3 to 6% of the enzyme bound by chick oviduct chromatin could initiate RNA synthesis (5). The observable difference is primarily due to the limited number of specific initiation sites present on chromatin, 1/10 to 1/20 of that found on DNA. However, the number of nonspecific sites on chromatin is at least as great or greater than that of DNA due to nonspecific interaction between RNA polymerase and chromatin proteins.

The slower rate of RS complex formation on chromatin as compared to DNA might possibly be attributed to a gradual change in the chromatin structure during the preincubation period, such as the dissociation of chromosomal proteins. Such a change could potentially lead to the uncovering of new initiation sites for RNA polymerase during the preincubation period. To test this possibility, chromatin was incubated without RNA polymerase for varying lengths of time. This pretreated chromatin was preincubated for 15 min with RNA polymerase and the amount of RS complex present measured on a standard assay. The level of RNA polymerase activity (Fig. 2B, open triangles) was constant for chromatin pretreated for periods up to 60 min and closely corresponded to the activity of chromatin which was preincubated for 15 min with RNA polymerase in the absence of pretreatment. These observations strongly indicate that no artifactual modification of the chromatin structure occurs during 60 min of preincubation.

The possibility that endogenous RNA polymerase activity in the chromatin might contribute to the level of rifampicin-
resistant RNA polymerase activity was also examined. RNA synthesis in the absence of any added exogenous E. coli RNA polymerase (closed triangles) was found to be much lower than the activity in the presence of exogenous RNA polymerase. Thus, the endogenous RNA polymerase activity in this preparation did not contribute significantly to the total level of RNA synthesis and the $t_n$ measured actually represents the formation of RS complexes between E. coli RNA polymerase and chromatin.

**Determination of Rate Constant of RNA Initiation**—The theoretical basis for determination of the rate constant of RNA chain initiation, as developed by Mangel and Chamberlin (22) utilizing E. coli RNA polymerase and bacteriophage T, DNA, is expressed in the following equation:

\[
\frac{C_i}{C^*} = \frac{R_i[R]}{k^*} + 1
\]

Where $C_i$ represents the total concentration of RS complex present at the time of addition of ribonucleotide and rifampicin; $C^*$ represents the concentration of RS complexes which are able to initiate RNA synthesis at a given concentration of rifampicin, $R$; $k_2$ represents the second order rate constant of rifampicin attack; and $k^*$ represents the apparent first order rate constant for RNA chain initiation at a fixed concentration of ribonucleotide. $C_i/C^*$ should be proportional to the ratio of RNA polymerase activity in the absence of rifampicin ($V_0$) to that in the presence of rifampicin ($V^*$). Thus, the slope of the plot of $V_i/V^*$ versus the concentration of rifampicin, the value of $k_2/k^*$ can be obtained. If $k_2$ is separately analyzed, it is then possible to obtain the rate constant for RNA chain initiation, $k^*$.

Prior to the determination of $k^*$, therefore, it was necessary to measure the second order rate constant of rifampicin attack on RS complexes between E. coli RNA polymerase and both chick DNA and chromatin. Enzyme was first preincubated with DNA for 15 min or with chromatin for 40 min to allow formation of the RS complex. After preincubation, rifampicin was added and incubated with the preformed RS complexes for varying lengths of time. RNA synthesis was then initiated by the addition of the four ribonucleotides and the level of RNA polymerase activity remaining determined. As shown in Fig. 3, a semilogarithmic plot of RNA polymerase activity versus the time of incubation of RS complex and rifampicin was linear for a fixed concentration of rifampicin on both DNA and chromatin. The plots of several different rifampicin concentrations intersect at the same point on the ordinate for both templates.

This is expected, since at zero time, no RS complexes should be inhibited by rifampicin. However, these levels do not intersect with the plot of RNA polymerase activity obtained in the absence of rifampicin, as theoretically predicted. The intersection point in the presence of rifampicin was 70% of that in the absence of inhibitor with chick DNA and only 25% for chick oviduct chromatin. The most likely explanation for this discrepancy is that in the absence of rifampicin, reinitiation occurs even within 90 s of incubation. Such reinitiation could not occur in the presence of rifampicin. Therefore, the level of RNA synthesis in the absence of rifampicin would be artificially high and not reflect the true number of RS complexes at zero time. This problem is greater for chromatin than for DNA as suggested by Fig. 1. It is likely due to the greater heterogeneity of RNA size during chromatin transcription and the presence of a large percentage of the RNA polymerase molecules (about 95%) which are not in RS complexes, but which might initiate RNA synthesis during a 90-s incubation without inhibitor.

The pseudo-first order rate constant of rifampicin inhibition of RS complexes was calculated from the slopes of each plot derived from a single rifampicin concentration. If these pseudo-first order rate constants are then plotted versus the rifampicin concentration, as shown on the inset to Fig. 3, the actual second order rate constant of rifampicin attack can be determined. From the slopes of these plots, the values of $k_2$ were calculated to be $2.3 \times 10^6$ s$^{-1}$ M$^{-1}$ for chick DNA and $0.8 \times 10^6$ s$^{-1}$ M$^{-1}$ from chick oviduct chromatin. The enzyme bound to initiation sites on the chromatin was approximately 3 times more resistant to rifampicin than that bound to initiation sites on DNA.

The measurement of the $k_2$ value for both chick DNA and chromatin allows the determination of the rate constant of RNA chain initiation, $k^*$, on these two templates. As in the previous experiment, enzyme and templates were preincubated to allow maximum formation of RS complex. RNA synthesis was subsequently initiated by the addition of the four ribonucleoside triphosphates and varying concentrations of rifampicin. Fig. 4 shows the plots of the ratio of RNA polymerase activity in the presence of rifampicin, $V^*$, to that in the absence of rifampicin, $V_0$ versus the concentration of rifampicin. As shown in the inset, a plot of the reciprocal of this ratio, $1/V^*$ versus rifampicin concentration was linear on both DNA and chromatin, as predicted by Equation 1. The intercepts on the ordinates for these plots, however, which should theoretically be 1.0 according to Equation 1, are 1.4 for chick DNA and 4.0 for chromatin. These values, as discussed for Fig. 3, probably are artificially high due to secondary initiations in the absence of rifampicin during the 90-s incubation.
level of true initiation sites (at the time of addition of ribonucleotide and rifampicin) should be 70% of the RNA polymerase activity in the absence of rifampicin for DNA and only 25% for chromatin. The true concentration of RS complex, C", can be expressed as:

\[ C" = fC* \]  

(2)

where \( C* \) is the observed concentration of RS complex determined in the absence of rifampicin and \( f \) is the reciprocal of the intersect on the ordinate of the plot of \( V_J/V^* \) versus rifampicin concentration. The Equation 1 can, thus, be modified to:

\[ C" = \frac{k[R]}{k^*} + \frac{1}{f} \]  

(3)

Consequently, using this correlation factor, \( f \), and the previously determined values of \( k \), it is possible to calculate \( k^* \) from the slopes of the plots of \( V_J/V^* \) versus concentration of rifampicin. Apparent \( k^* \) values obtained in this manner were 0.98 s" for chick DNA and 0.57 s" for chromatin at a ribonucleotide triphosphate concentration of 0.15 mM. The rate constants of RNA chain initiation for RS complex of *E. coli* RNA polymerase with 5.0 pg of the chick oviduct chromatin in 0.2 ml of the preincubation mixture. After 15 min at 37°, RNA synthesis was initiated by the simultaneous addition of 0.65 ml of the ribonucleoside triphosphate mixture containing various concentrations of rifampicin as indicated, and continued at 37° for 90 s. B, RS complex was formed by preincubating 7.0 pg of RNA polymerase with 5.0 pg of the chick oviduct chromatin in 0.2 ml of the preincubation mixture at 37° for 40 min. Other conditions were the same as in A.

The question of the rate-limiting step of RS complex formation was first examined utilizing chick DNA as template. It was suggested by the results shown in Fig. 2 that the formation of RS complexes proceeds via first order kinetics. If this is true, the \( t_h \) of RS complex formation should not change when the enzyme and DNA concentration are varied at a fixed ratio. When the \( t_h \) was determined as shown in Fig. 2, the following results were obtained for DNA: 1.3 min for 5.6 \( \mu \) g/ml of enzyme and 3.0 \( \mu \) g/ml of DNA; 1.3 min for 11.2 \( \mu \) g/ml of enzyme and 6.0 \( \mu \) g/ml of DNA; and 1.2 min for 22.4 \( \mu \) g/ml of enzyme and 12.0 \( \mu \) g/ml of DNA. These results indicate that the formation of RS complex on chick DNA occurs by a first order process. Therefore, the possibility that the rate-limiting step in RS complex formation is the formation of I complex (Step 2), which should occur by a second order process, can be ruled out.

It was still necessary to distinguish between the dissociation of enzyme from nonspecific binding sites (Step 1) and the transition from I to RS complex (Step 3), which should both occur by first order process. In order to differentiate between these two steps, the effects of ionic strength on the \( t_h \) of RS complex formation was examined. Lowering the ionic strength in the preincubation mixture should destabilize the DNA duplex structure. Thus, if Step 3 was rate-limiting, the transition from I to RS complex would be expected to be facilitated at lower ionic strengths. The results of experiments in which the concentration of ammonium sulfate was varied during the preincubation period, but kept constant during RNA synthesis are shown in Table I. The \( t_h \) of RS complex formation on chick DNA was indeed affected by lowering the ionic strength, however, the change was the opposite of that predicted from the known effect of ionic strength on DNA duplex structure. It should also be noted that the ratio of RS max in the presence of ammonium sulfate to that in the absence of salt was 1.1, indicating that the maximum level of RS complex was not affected by ionic strength. These observations suggest that at 37° the rate-limiting step in the formation of RS complex on DNA is not the conversion of I to RS complex, but rather the dissociation of enzyme from nonspecific binding sites. The presence of higher ionic strengths might interrupt charge interactions between RNA polymerase and nonspecific binding sites on the DNA, thus facilitating the dissociation of
enzyme from these nonspecific sites. This would account for the lower $t_a$ observed at the higher concentration of ammonium sulfate.

**Rate-limiting Step of RS Complex Formation on Chromatin**—The formation of RS complexes on chromatin is 7 times slower than on DNA. Due to the presence of chromosomal proteins, the determination of rate-limiting step of RS complex formation on chromatin is more complicated than for DNA. Four possible explanations were considered: (a) the rate-limiting step is the dissociation of enzyme from nonspecific binding sites in chromatin and the nature of these nonspecific sites is the same as for deproteinized DNA (i.e., charge interaction between DNA and RNA polymerase). The ratio of specific to nonspecific binding sites on chromatin, however, is much lower than for the DNA. Therefore, the possibility of RNA polymerase encountering a specific initiation site is decreased and the $t_a$ of formation is higher on chromatin than on DNA. (b) The rate-limiting step is the dissociation of enzyme from nonspecific binding sites; however, the nonspecific binding is mainly due to a reversible interaction between enzyme and chromosomal proteins. If the rate of dissociation of enzyme from these chromosomal proteins were slower than for nonspecific DNA binding sites, the $t_a$ would be increased for chromatin compared to DNA. (c) The rate-limiting step is the formation of I complex. The presence of chromosomal proteins somehow retards the rate of this formation compared to that for deproteinized DNA. (d) The rate-limiting step is the conversion of I complex to RS complex. The presence of chromosomal proteins somehow retards this conversion, resulting in a higher $t_a$ for chromatin than for DNA.

The most simple of these possible explanations to eliminate is (c), that formation of I complex is rate-limiting. As was the case for chick DNA, the time course of formation of RS complex on chromatin (Fig. 2) suggested that the process was a first order reaction. Furthermore, varying concentrations of enzyme and chromatin (as DNA) at a fixed ratio did not affect the $t_a$ of formation: 9.4 min for 28 µg/ml of enzyme and 20 µg/ml of chromatin and 9.5 min for 56 µg/ml of enzyme and 40 µg/ml of chromatin. These results suggest that RS complex formation is a first order process on chromatin, as was seen for DNA, and thus, the formation of I complex could not be the rate-limiting step.

The possibility that (a) is the explanation for the slower $t_a$ of RS complex formation on chromatin as compared to DNA can also be eliminated. Indeed, the ratio of $RS_{max}$ on native chromatin to chick DNA was 0.046. The ratio of $RS_{max}$ between two templates should be approximately equal to the ratio of RNA initiation sites available, since the size of RNA synthesized for 90 s is not expected to be very heterogeneous, and the $k^*$ and elongation rate of RNA polymerase is similar for chromatin and DNA. Therefore, the ratio of specific to nonspecific sites on chromatin is much lower than for DNA, as predicted by possibility (a). However, no significant effect of ionic strength on the $t_a$ was observed, as had previously been shown for DNA (Table I). Such an effect would be expected if nonspecific binding involved interactions between the DNA in chromatin and RNA polymerase. To further investigate possibility (a), dehistonized chromatin and reconstituted histone-DNA complex were used as templates. As shown in Table I, the $RS_{max}$ for these two modified chromatins were similar to DNA, and therefore, the specific to nonspecific site ratio should be greatly increased over native chromatin and might even be higher than DNA. However, the $t_a$ values determined for the modified chromatins still remained slower than the $t_a$ on DNA. Even though these modified chromatins resemble DNA in terms of the number of available initiation sites, the $t_a$ of RS complex formation is more like that observed on native chromatin. These results suggest that possibility (a) can be ruled out as the correct explanation.

The ratio of $RS_{max}$ in the presence of salt to that in the absence of salt was greater than 1.0 for any chromatin tested. Although the higher salt is known to stabilize the DNA structure, the maximum level of RS complexes formed was not decreased at the high salt concentration. Therefore, the equilibrium between I and RS complex on chromatin is not disturbed by ionic strength. Unfortunately, it is difficult to maintain chromatin structure under higher salt concentrations which might effectively decrease the $RS_{max}$. Therefore, the effect of temperature on the formation of RS complex was also examined.

**Temperature Dependency of Formation of RS Complex**—The temperature dependency of the formation of RS complex was examined by varying the temperature of preincubation of RNA polymerase and template, followed by incubation for 15 min at 37°C. Longer incubation periods were used for these particular experiments so that the effect of temperature on the elongation rate during the period of warming to 37°C would be negligible. The $t_a$ values obtained using 15-min incubations for RNA synthesis were very similar to values obtained with 90-s incubations (cf. Tables I and II).

The effect of temperature on RS complex formation was first examined on chick DNA (Fig. 5). The $RS_{max}$ observed for DNA decreased dramatically as the preincubation temperature was lowered from 37°C to 0°C. This observation is consistent with the results of analogous experiments utilizing bacteriophage T7 DNA and E. coli RNA polymerase (24). The $t_a$ of RS complex formation on DNA increased remarkably as the temperature of preincubation was lowered. The effect of temperature on $t_a$ resembled the mirror image of the temperature dependency of $RS_{max}$. Temperature is well known to have a dramatic effect on the stability of DNA structure with higher temperatures favoring destabilization of the DNA duplex. We interpret the strong temperature dependency of $RS_{max}$ as indicating that local opening of the strands of DNA is necessary for RS complex formation. The temperature dependency of $t_a$ suggests that at lower temperature the conversion from I to RS complex, which should be highly dependent on temperature, is

| Template                  | $t_a$ (min) | Ratio of $RS_{max}$  
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<td>0°C</td>
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<td>Chick DNA</td>
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<tr>
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<td>Chick oviduct chromatin*</td>
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<td>Native</td>
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<td>9.4</td>
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<td>Dehistonized</td>
<td>17.0</td>
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<td>Reconstituted (DNA + histones)</td>
<td>19.0</td>
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*Isolated from diethylstilbestrol-treated oviduct.
the preincubation mixture. After various time intervals, 0.05 ml of the
RNA was preincubated with chick oviduct chromatin (5.0 µg) in 0.2 ml of
the ribonucleoside triphosphate mixture containing 0.2 mg/ml of rifampicin. RNA synthesis was continued at 37°C for 15 min. B, RNA polymerase (7.0 µg) was preincubated with chick oviduct chromatin (5.0 µg) in 0.2 ml of
the ribonucleoside triphosphate mixture containing 0.2 mg/ml of rifampicin was added to the preincubation mixture. RNA was synthesized at 37°C for 15 min. RSmax and tH were determined as shown in the insets in Fig. 2 on both templates of DNA and chromatin.

gradually becoming the rate-limiting step. As described above, at 37°C the dissociation of enzyme from nonspecific sites was found to be rate-limiting.

By contrast, when chick oviduct chromatin was used as template, the effects of temperature on RS complex formation were quite different from those observed on deproteinized DNA. The temperature dependency of RSmax on chromatin was relatively poor compared to DNA. Thus, the conversion from the closed to the open form of the DNA at the initiation site is not as highly dependent on temperature for chromatin as that seen for DNA. Furthermore, the tH for RS complex formation on chromatin was completely independent of preincubation temperature. Therefore, the conversion of I to RS (d) which is highly temperature-dependent, is not the rate-limiting step in RS complex formation throughout the temperature range tested.

In order to further investigate the DNA structure at the RNA initiation site on chromatin, the temperature dependencies of RSmax and tH at 0°C and 37°C were examined for several templates (Table II). On native oviduct DNA, there was a large temperature dependency for both the RSmax and tH of RS complex formation. On denatured chick DNA, however, RSmax and tH showed little temperature dependency. This is most likely due to the fact that no opening of the DNA strands is necessary for formation of the RS complex on denatured DNA, and thus, the process of RS complex formation is not greatly affected by temperature. On chromatin, the dependency of RSmax and tH were similar to that for denatured DNA (Table II). This is consistent with the hypothesis that certain chromosomal proteins are able to facilitate the conversion from I to RS complex in the chromatin, making this conversion less dependent on temperature. Modified chromatins displayed temperature dependencies which were intermediate between native and denatured DNA. Thus, as chromosomal proteins are removed, the RS complexes formed become more similar in nature to those found on native DNA. Taken together, these results indicate that the rate-limiting step in RS complex formation on native chromatin at any temperature tested is the dissociation of the enzyme from nonspecific interactions with chromosomal proteins. Thus, explanation (b) is the most likely candidate for understanding why the tH of the RS complex formation on chromatin is slower than that for DNA.

Effect of 1-Anilino-8-naphthalene Sulfonate on tH.—We have suggested that interactions between RNA polymerase and chromosomal proteins play a major role in determining the rate of RS complex formation. Since varying ionic strength had little effect on the tH for RS formation in chromatin (Table I), the interaction between enzyme and chromosomal proteins may be hydrophobic in nature. To test this possibility, the effects of ANS on the tH of RS complex formation were examined. ANS is known to bind to hydrophobic regions of proteins, and thus, should be able to interfere with hydrophobic interactions between proteins. The tH was measured in the presence of ANS at concentrations which had little or no effect on RNA polymerase activity. Conditions were the same as described in Fig. 2, except that the bovine serum albumin concentration was reduced to 40 µg/ml. As shown in Fig. 6, the tH of RS complex formation was not changed for DNA. On chromatin, however, the tH was significantly decreased by the presence of ANS. At 0.75 mM ANS, the amount of ANS bound to chromatin was 0.37 µmol/mg (as DNA), whereas only 0.04 µmol/mg was bound to DNA. It appears likely that ANS interrupts hydrophobic interactions between RNA polymerase and chromosomal proteins, and thus, facilitates the formation of RS complexes between enzyme and chromatin.

Discussion

The initiation of RNA synthesis is a two-step process. The first step involves the binding of RNA polymerase to an initiation sequence in the DNA and the conversion of the enzyme-DNA complex at the initiation site into a form capable of rapidly initiating RNA synthesis. The second step is the actual initiation of RNA synthesis via the formation of the first phosphodiester bond between two ribonucleoside triphosphates. In this paper, we have compared the process of RNA initiation by E. coli RNA polymerase on the templates of chick DNA and chick oviduct chromatin.

The method utilized to study the process of RNA initiation was developed by Chamberlin and co-workers (18–24) to study...
in detail the initiation process on bacteriophage T5 DNA. This method involves challenging a mixture of RNA polymerase and DNA with the simultaneous addition of the four ribonucleoside triphosphates and rifampicin. Under these conditions, enzyme molecules which are bound at initiation sites and have undergone the conversion to the opened form of binary complex (RS complex) are capable of rapidly initiating RNA synthesis. These enzyme molecules escape rifampicin inhibition with a high efficiency. Conversely, RNA polymerase bound at nonspecific sites or in the closed form of preinitiation complex are not capable of rapidly initiating RNA synthesis and are inhibited by the rifampicin. The challenge technique, thus, specifically measures enzyme molecules in the open form of preinitiation complex and both the rate constants of formation of RS complexes and of RNA chain initiation from preformed RS complexes can be determined.

The apparent first order rate constant of RNA chain initiation ($k^*$) at 0.15 mM ribonucleoside triphosphate was found to be 0.98 s$^{-1}$ for chick DNA, which corresponds to a $t_n$ for RNA chain initiation of 0.7 s. This rate constant is approximately the same as that determined for E. coli RNA polymerase and bacteriophage T5 DNA at an equivalent ribonucleotide concentration (22). On chick oviduct chromatin, the $k^*$ was determined to be 0.57 s$^{-1}$, which gives a $t_n$ for RNA chain initiation of 1.2 s. Thus, the $k^*$ for RNA initiation by E. coli RNA polymerase is slightly slower on chromatin than on DNA.

The rate of formation of RS complex between RNA polymerase and either chick DNA or chromatin is remarkably slow compared to the process of RNA chain initiation. On DNA, the $t_n$ for RS complex formation was 1.3 min whereas on chromatin, the $t_n$ of formation was 9.4 min. Furthermore, the maximum level of RS complex which could be formed in the case of chromatin (RS$_{max}$) was only about 5% of that observed for DNA. Thus, while DNA and chromatin only varied slightly in terms of their relative rate constants of RNA chain initiation, the formation of RS complexes on the two templates was dramatically different.

In determination of the $k_1$ of rifampicin attack and the $k^*$ of RNA chain initiation, RNA polymerase activity determined by extrapolation to zero concentration of rifampicin was 70% of the actual level measured in the absence of rifampicin for DNA. On chromatin, the level of RNA polymerase activity at the ordinate intersection point was only 25% of that in the absence of rifampicin. The explanation suggested for this discrepancy was that secondary initiations from a single initiation site occur during the 90-s incubation resulted in the level of activity in the absence of rifampicin to be artificially high. If this is the case, on DNA an average of 1.4 initiation events per initiation site is occurring in 90 s, while on chromatin the level of initiation events per site is 4 over this same time span. Previous results from this laboratory demonstrated that only about 4% of the RNA polymerase bound to chromatin can initiate RNA synthesis in the presence of 40 µg/ml of rifampicin. Thus, even if every initiation site is utilized four times during a 90-s initiation in the absence of rifampicin, a large portion (about 84%) of the RNA polymerase molecules bound to chromatin at saturation levels cannot initiate RNA synthesis. Such RNA polymerase may be bound tightly to chromosomal proteins or perhaps at initiation sites on the DNA where RNA synthesis is blocked by the presence of protein.

In order to interpret the temperature dependency of RS complex formation, we have attempted to derive a simple kinetic formula for the conversion from I complex to RS complex. It was strongly suggested that a local opening of the double-stranded structure of DNA is essential for the transition from I to RS complex (21, 24, 32, 33). To adequately explain the temperature dependency of the $t_n$, of RS complex formation and RS$_{max}$ on chick DNA, we have used a model which subdivides the I to RS conversion into two steps:

```
\begin{align*}
\text{I-complex} & \quad k_1 \quad \frac{k_2}{k_1} \\
\text{I'-complex} & \quad k_2 \\
\text{RS-complex} & \quad k_1 \\
\end{align*}
```

The structure of the I and RS complexes in this model are the same as in the model proposed by Chamberlin (21). The intermediate step, I' complex, involves a structure in which the strands of DNA are already destabilized at the initiation site, but in which RNA polymerase is not yet located between the DNA strands to form the highly stable RS complex. The transition from I to I' is dependent on the intrinsic $T_m$ of the DNA in the region of the initiation site. Since the $T_m$ of DNA is highly sensitive to temperature, the equilibrium between I and I' should be dramatically affected by changes in temperature. At 37°C, the ratio of I' to I may be extremely low. If the equilibrium between I and RS favors the formation of RS, however, a significant concentration of RS complex should be formed. It is considered to be a reasonable assumption that the equilibrium between I and RS is much less temperature dependent than that between I and I', since only the latter involves local denaturation of the DNA strands.

Using this model and the knowledge that for limited regions of DNA the rate of interconversion between closed and open forms is very fast (34, 35), we are able to derive the following equation which describes the relationship between RS complex formation and RS$_{max}$.

$$t_n = \frac{0.69}{k_c} \left(1 - \frac{RS_{max}}{[I]\_{eq}}\right) \quad (4)$$

From the equation it is possible to predict the effect of temperature on the relationship between the observed $t_n$ and RS$_{max}$, which would be expected if the conversion from I to RS complex was the rate-limiting step in RS complex formation. Mangel and Chamberlin (24) demonstrated that the formation of I$_0$ complexes between E. coli RNA polymerase and T5 DNA is not very dependent on temperature. Although we have no direct data on the temperature dependency of the formation of I$_0$ complexes between E. coli RNA polymerase and the templates we used, we assume that the results for T5 DNA should be applicable to our system. If this is true, the temperature dependency of the term RS$_{max}/[I]_{eq}$ from Equation 4 should be proportional to the temperature dependency of the observed $t_n$ as measured in Fig. 5. With the above assumptions, Equation 4 predicts that the temperature dependency of $t_n$ should be inversely related to that of RS$_{max}$. As can be seen in Fig. 5A, this is indeed the case for chick DNA. The correspond-
ence between the experimental results and theoretical expression suggests that at lower temperatures the conversion from $I$ to $RS$ complex becomes the rate-limiting step in the formation of $RS$ complex between native DNA and RNA polymerase.

On chick oviduct chromatin, however, the $t_{1/2}$ of $RS$ complex formation was completely independent of the temperature while $RS_{\text{max}}$ was only weakly dependent on temperature. This suggests that on chromatin the transition from $I$ to $RS$ complex is not rate-limiting for the formation of $RS$ complex even at lower temperatures. The rate-limiting step is most likely the dissociation of DNA polymerase from nonspecific interactions with chromosomal proteins. The majority of these nonspecific interactions appear to be due to hydrophobic interactions as suggested by the effect of ANS on the $t_{1/2}$ of $RS$ complex formation.

Recent work by Richardson (36) on the formation of stable binary complexes between $E. coli$ RNA polymerase and phage PM2 DNA may help in understanding the structure of initiation sites on chromatin. The amount of heparin-resistant complexes which could be formed on form II DNA (nicked, no superhelical twists) was 50% of that on form I DNA (~50 superhelical turns/molecule). In addition, the formation of heparin-resistant complexes on form II DNA was much slower than on form I DNA. Thus these two parameters vary inversely with increasing superhelicity of the DNA. Since superhelical DNA is known to have a lower activation energy for local opening of the DNA strands, this situation appears to be analogous to that observed when chick DNA was preincubated at increasing temperature (Fig. 5). The results are consistent with Equation 4 which was derived from our model for the $I$ to $RS$ conversion. Thus, the rate-limiting step for the formation of $RS$ complex on phage PM2 form II DNA appears to be the $I$ to $RS$ transition.

Richardson (36) also demonstrated that the transition temperature for the formation of heparin-resistant RNA polymerase activity on form II PM2 DNA was significantly higher than that for form I DNA. This is consistent with the fact that the activation energy for local melting of superhelix DNA is lower than that of relaxed DNA.

It is likely that initiation sites for RNA synthesis in chromatin may also be in a state in which the activation energy for opening of the DNA strands is greatly reduced from that of deproteinized DNA. These sites could be superhelical in nature, as such regions have been postulated to occur in chromatin structure (37). Alternatively, chromosomal proteins present at or near the initiation site could directly interact with DNA to facilitate the opening of the DNA strands. The presence of initiation sites in chromatin in which the activation energy for melting of the DNA strands is reduced appears consistent with the observed lack of temperature dependency of the $t_{1/2}$ of $RS$ complex formation and the shallow temperature dependency of $RS_{\text{max}}$. If this model is correct, it would imply that chromosomal proteins play an important role in determination of functional initiation sites for RNA synthesis in chromatin. The use of a prokaryotic RNA polymerase to study the initiation of RNA synthesis on eukaryotic chromatin may be questioned. Evidence from this laboratory, however, indicates that $E. coli$ RNA polymerase and hen oviduct RNA polymerase II utilize the same set of initiation sites in chromatin (38).

At the present time, it is not possible to prove that these in vitro initiation sites are identical with 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. Future studies will be directed towards determining the existence of specific groups of chromosomal proteins which may exert such positive effects on gene expression in chromatin.

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Kinetics of Initiation of Transcription on Chromatin


M Hirose, M J Tsai and B W O'Malley


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