Use of RNA polymerase as an enzymatic probe of nucleosomal structure

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ABSTRACT

Nucleosomes prepared from human placental nuclei and *Escherichia coli* DNA-dependent RNA polymerase (nucleoside triphosphate: RNA nucleotidyl transferase EC.2.7.7.6) form stable initiation complexes. This property is utilized as a probe of nucleosome structure. RNA polymerase initiation has been studied on purified nucleosomes, nucleosome cores, and nucleosomal DNA. The affinity of *E. coli* RNA polymerase for both nucleosome cores and monomers was 5-6 fold less than that found for nucleosomal DNA. No difference in apparent initiation K_m was found between cores and mononucleosomes. This suggests that initiation does not preferentially occur on the DNA tails of nucleosomes. Once initiated and allowed to form nascent RNA, these complexes are very stable to ionic strength changes. Under conditions in which free enzyme is inactivated with rifampicin, the enzyme in the complex retains activity as demonstrated by its ability to transcribe and reinitiate on both nucleosomes and free DNA. These complexes can be well resolved from free nucleosomes on preparative polyacrylamide gels and both can be eluted from gels for analysis of proteins and DNA sequence complexity. Studies using (125 I) labelled nucleosomes show that histones are retained in the initiation complex, and are not dissociated by the enzyme during initiation.

INTRODUCTION

Many lines of evidence have led to the view that chromatin exists in a highly ordered structure of regularly repeating subunits, called nucleosomes. The role, if any, of the nucleosome structure in the process of transcription of chromatin has not been established. However, the presence of messenger specifying sequences in nucleosomes has been demonstrated by hybridization of DNA complementary to cytoplasmic polyadenylated RNA isolated from chromatin subunits (1,2). Comparison of the kinetics and extents of hybridization of cDNA with nucleosomal DNA and with nuclear DNA demonstrate that most of the repetitive sequences and single copy sequences in mRNA are present in nucleosomes. These results suggest that the DNA in the nucleosomal structure may be transcribed in vivo.

Previous work from this laboratory (3) and elsewhere (4,5) has demonstrated that exogenous DNA-dependent RNA polymerase can transcribe isolated nucleosomes in vitro. These experiments also show that template restriction observed in purified or reconstituted chromatin is preserved at the nucleosomal level. This raises the question of whether there is a subpopulation of nucleosomes that, by virtue of their structural or compositional properties can form stable initiation complexes with a heterologous RNA polymerase. If a subset of the nucleosome population can form such complexes this property can be exploited as a probe of nucleosome structure. In this respect polymerase activity is similar to other enzymatic probes of chromatin structure such as micrococcal nuclease or DNAase I. Identification and purification of a nucleosome subset from actively transcribed genes has great significance for an understanding of gene transcription in eukaryotes. As a first step towards this goal, we show here that nucleosome-RNA polymerase complexes can be formed and separated from free nucleosomes. Some properties of these complexes are also described.

MATERIALS AND METHODS

Preparation of nucleosomes: Nucleosomes were prepared by micrococcal nuclease digestion of human placental nuclei prepared as described previously (6). The digestion was performed for 12 minutes at 37°C using 5 units of micrococcal nuclease (Worthington) per A₂₆₀ of DNA. After removal of particulate material, the digested chromatin was fractionated on a BioGel A 5M column equilibrated in 10 mM Tris-HCl (pH 7.9), 0.2 mM EDTA. The mononucleosome peak was pooled and concentrated using a collodion bag apparatus. Nucleosome cores were prepared by digesting the nuclei for 20 min. The cores were purified as described for the mononucleosome preparation.

¹²⁵I-labelling of nucleosomes: 30-40 µg of purified nucleosomes were labeled with ¹²⁵I using Enzobeads (Biorad). The reaction was started by the addition of Enzobeads, and was performed in a final volume of 250 µl containing 8 mM Tris-HCl (pH 7.9), 0.12% β-D-glucose, and 200-400 µCi of carrier free ¹²⁵I (Amersham). After 45 min at room temperature, unreacted ¹²⁵I was removed by G-50 Sephadex chromatography.

Formation of nucleosome - RNA polymerase transcription complexes:

Nucleosomes and Escherichia coli DNA-dependent RNA polymerase (7) at a 1:3 weight ratio were incubated at 37°C in 50 mM Tris-HCl (pH 7.9), 150 mM KCl 0.2 mM dithiothreitol, and 12 mM MgCl₂ containing 100 µM ATP, GTP, and CTP. After 10 min, 15 µCi of [³H]-ATP, [³H]-GTP, [³H]-CTP, and [³H]-UTP (specific

activity of each isotope is 16 Ci/nmol), 30 nmol of unlabelled UTP, and 30 μ g of rifampicin were added to give a total volume of 300 μ l. The polymerization was allowed to continue for 10 sec, and the reaction mix was quickly chilled and applied to a G-50 Sephadex to remove unincorporated nucleotides. The G-50 Sephadex column was equilibrated in either the reaction buffer or 1 mM Tris-HCl (pH 7.9) for enzyme activity measurements and gel electrophoresis, respectively, of the void material.

Enzyme activity measurements: Nucleosome transcription assays were performed in 50 mM Tris-HCl (pH 7.9), 150 mM KCl, 12 mM MgCl₂, 0.2 mM dithiothreitol with each of the four nucleotides in excess at 100 μ M and ³H-labelled as described above. Mononucleosome DNA transcription was performed as above, except that the KCl concentration was 50 mM. To determine the apparent initiation K_m for nucleosomes, nucleosome cores, and nucleosomal DNA, initiation was measured by allowing initiation complexes to form in the presence of three labelled nucleotides (GTP, ATP, and CTP) at various template concentrations. After 20 min, uninitiated RNA polymerase was inactivated by the addition of rifampicin; UTP was also added to allow any initiated chains to be completed. After 10 min, the incorporation into RNA was measured by either precipitation with 10% trichloroacetic acid, or by spotting aliquots on DE 81 filters (8). This technique allows one to measure relative differences in initiation (9). To determine absolute initiation levels, it is desirable to measure incorporation of [γ -³²P] NTP (10), a technique that is not usable with nucleosomes because of the presence of low levels of protein kinase activity in nucleosomal preparations (11). Template capacities were measured as previously described (3) except that the [³H]-UTP and rifampicin were added simultaneously after preincubation of the template for 90 sec with the three unlabelled nucleotides. The reaction was stopped by spotting aliquots on DE-81 filters as described above.

Gel electrophoresis: Following desalting on G-50 Sephadex, the reaction mixes were concentrated and applied to 0.5 x 13 cm polyacrylamide gels (3% polyacrylamide, 50 mM Tris-HCl (pH 7.9), 20% sucrose) and electrophoresed at 1 mA per gel for 16 h in 50 mM Tris-HCl (pH 7.9). Two millimeter slices were digested in NCS solubilizer and the radioactivity monitored. When [¹²⁵I] nucleosomes were used, radioactivity was measured in a Packard Gamma Spectrometer. SDS polyacrylamide gels were run as described by Laemmli and Favre (12). Preparative 3% polyacrylamide gels (1.5x13cm) were prepared as described above and run at 8.5 mA/gel for 18 h.

Elution of transcription complex from preparative gels: Gel slices contain-

ing mononucleosome-polymerase transcription complexes and uncomplexed mononucleosomes were labeled with [γ - ^{32}P]-ATP (prepared by the method of Johnson and Walseth, 1979 (13)) and polynucleotide kinase (New England Biolabs). The gel slices were incubated in 10 mM Tris-HCl, pH 7.9, 20 mM dithiothreitol and 2.5 mM MgCl_2 containing 20 μCi [γ - ^{32}P] ATP and 3 μl polynucleotide kinase (2.4 units). This was incubated 12 hr at 37°C . The slices were rinsed with 50 mM Tris, pH 7.9 just prior to electrophoresis.

Elution of labeled material from the gels was accomplished by a modification of the procedure of Karsnas and Roos (14). The material was eluted into a sucrose gradient as described except that the buffer used to make the gradient and the anode and cathode buffers was 150 mM Tris-HCl, pH 7.9. Electrophoresis was performed at 7 mA/gel for 18-44 hr. Recoveries were not improved by electrophoresis for longer than 24 hr. After electrophoresis, the gradients were fractionated from the top.

The ^{32}P -containing fractions were pooled, dialyzed against water, and digested with 100 $\mu\text{g/ml}$ proteinase K (E. Merck Biochemicals) for 30 min at 37°C . The digested samples were then extracted with phenol and sevag and precipitated overnight with 2 volumes of ethanol. The pellets were dried and redissolved in 100 μl water.

Nick translation of DNA: The extracted DNA was labeled by nick translation using a modification of the method of Balmain and Bernie (15). The nick translation reaction (100 μl , mixed on ice) contained 50 mM Tris-HCl, pH 7.9, 10 mM MgCl_2 , 10 mM dithiothreitol, 50 $\mu\text{g/ml}$ BSA, and the four deoxyribonucleoside 5'-triphosphates. Fifty μCi each of [^3H]dCTP (25 Ci/mmol), [^3H]dTTP (69 Ci/mmol), and [^3H]dATP (10 Ci/mmol), and 5 μM dGTP were present. An aliquot of the redissolved DNA (complexed or non-complexed mononucleosome) and 3 μl (14 units) DNA polymerase I (Boehringer) were added. The reaction mixture was incubated 4 hr at 16°C . The reaction was stopped by the addition of 0.3 ml water, and phenol extracted. The aqueous layer was chromatographed on a G-50 Sephadex column (0.9 x 55 cm) in 1 mM Tris-HCl, pH 7.9 to remove unincorporated triphosphates.

RESULTS

Nucleosome-E. coli RNA polymerase initiation complexes were formed to examine the structural features present in nucleosomes that may govern RNA polymerase-chromatin interactions. To minimize perturbations to nucleosomal structure, we used nascent RNA as a label for the transcription complexes formed in vitro. It is important to note that all complex formation

experiments were done under conditions of enzyme excess. The conditions of enzyme excess were established by titrating a fixed amount of nucleosomes or DNA with varying quantities of RNA polymerase holoenzyme. The solubility of nucleosomes in low ionic strength buffers is affected by as little as 2 mM MgCl_2 (6). The complex formation experiments, therefore, were done at 150 mM KCl (3) to avoid precipitation of nucleosomes with possible changes in structure. Figure 1 shows that after complex formation, removal of unincorporated nucleotides, and inactivation of free polymerase (3), the polymerase present in an enzyme-nucleosome complex can continue transcription when additional substrates are added. With no template addition (panel A), synthesis is linear for at least 50 min despite the large dilution resulting from G-50 Sephadex chromatography. Hence, the enzyme can complete the nascent chains and reinitiate synthesis on the nucleosomes in the mixture. If excess DNA is added (panel B), approximately 5-fold more synthesis is observed at each time point. If the reaction mixtures are desalted and analyzed on polyacrylamide gels, the profile shown in Figure 2 is obtained. The transcription complex labelled with nascent RNA appears as a bisphasic peak, and is well

Figure 1. Retention of activity by RNA polymerase in the nucleosome transcription complexes. *E. coli* RNA polymerase-nucleosome transcription complexes were formed as described in Materials and Methods. Unincorporated nucleotides were removed using G-50 Sephadex equilibrated in reaction buffer (50mM Tris-HCl (pH 7.9), 150 mM KCl, 12 mM MgCl_2). Labelled nucleotides (100 μM) were added to the void material and aliquots removed at various times. All points are corrected for zero time incorporation. (panel A=1171 cpm; panel B=916 cpm), which represents the ^3H incorporated initially into the initiation complex. A. RNA synthesis observed by isolated RNA polymerase-nucleosome complexes with no additions. B. RNA synthesis by RNA polymerase-nucleosome complexes with calf thymus DNA added (100 $\mu\text{g/ml}$).

Figure 2. Analysis of nucleosome-RNA polymerase complexes on 3% polyacrylamide gels. Gels, 0.5 x 14 cm. were run as described in Materials and Methods. Nucleotide incorporation into nascent RNA was determined following digestion of 2 mm slices with NCS solubilizer. The position of free monomer was determined by ethidium bromide staining.

resolved from uncomplexed mononucleosomes visualized by ethidium bromide staining. A peak of free RNA resulting from readthrough is observed near the end of the gel. Control experiments (data not shown) demonstrate that formation of the biphasic peak depends on the addition of polymerase and is not a result of nonspecific interaction between nucleotides and nucleosomes.

We have considered the possibility that *E. coli* RNA polymerase does not interact with intact nucleosomes, but rather transcribes free DNA in nucleosome preparations. The nucleosome preparations used in our experiments were checked carefully for the presence of free DNA before and after the formation of the transcription complexes. Figure 3 shows that the mononucleosomes used in these experiments contain no free DNA; furthermore, after complex formation, no free DNA is released (data not shown). The limit of detection of free DNA on the overloaded nucleosome gel is 5% of the A_{260} applied to the gel (3). Thus, it appears that the RNA polymerase does not destabilize the

Figure 3. Polyacrylamide (6%) gel electrophoresis of monomer preparation. Mononucleosomes used for formation of transcription complexes were analyzed for the presence of free DNA. Upper panel - 12 μ g of nucleosome monomer. Lower panel - 1.5 μ g of core and mononucleosomal DNA. F = fluorescence intensity from ethidium bromide staining. Electrophoresis was terminated early so any small DNA fragments would be detected.

nucleosomal structure to such an extent that free DNA appears in solution.

This finding does not exclude the possibility of transient dissociation of the histone core from the DNA during the transcription process. To eliminate this possibility, one must examine the transcription complex directly. Previous investigation of *E. coli* RNA polymerase-nucleosome interactions (3, 5) have strongly suggested that during formation of the initiation complex, no dissociation of core histones occurs. However, none of these investigations have examined isolated nucleosome-polymerase transcription complexes for the presence of histones. To detect histones, we wanted to label nucleosomes under the mildest possible conditions with minimal manipulations. Lactoperoxidase catalyzed iodination was the method of choice. Previous studies (16) have shown that lactoperoxidase catalyzed iodination is a sensi-

tive probe of histone conformation. Histone tyrosyl residues are not equally accessible to iodination in chromatin as compared to the free state. This reaction is useful for preparing highly labelled nucleosomes for inclusion as tracers in the initiation complex reactions. Figure 4 shows that all core histones are labelled with ^{125}I . Histones H2b and H4 appear to be most extensively labeled. H4 contains 3.9 moles per cent tyrosine, while H2a, H2b and H3 contain 2.3, 2.4 and 2.2 mole per cent tyrosine, respectively (17). More label is present in H2b than would be predicted from the amino acid composition. Analysis of the methylene blue stained gels showed that all histones are present in these gels in apparent equimolar amounts (data not shown). When tracer quantities of [^{125}I]-nucleosomes were included in a transcription complex reaction containing unlabelled nucleotides the results shown in Figure 5 were obtained. A biphasic peak that is coincident with that for nascent RNA (see Figure 2) was observed in the upper portion of the

Figure 4. SDS gel electrophoresis of [^{125}I]-nucleosomes. Nucleosomes were labeled with [^{125}I] by means of lactoperoxidase. The labelled material was run on a 17% SDS gel (Laemmli and Favre, 1973) in the presence of histone markers, stained with Coomassie blue and subjected to autoradiography. The left channel contains twice as much of the labelled sample as the right channel.

Figure 5. Polyacrylamide gel (3%) electrophoresis of RNA polymerase- $[^{125}\text{I}]$ -nucleosome transcription complexes. RNA polymerase-nucleosome initiation complexes were formed as described in Fig. 2 with the inclusion of tracer amounts of labelled nucleosomes. Electrophoresis was performed as described previously.

gel. In addition a peak of $[^{125}\text{I}]$ -mononucleosome was observed further down the gel. The labelled mononucleosome peak was coincident with unlabelled material, which suggests that iodination does not affect the electrophoretic properties of nucleosomes. These results demonstrate that nucleosomes remain intact following interaction with RNA polymerase and that histones are present in the transcription complex. The size of the observed ^{125}I peaks, however, may not represent the true ratio of mononucleosome-polymerase complex to uncomplexed mononucleosomes.

The above data show that nucleosome composition is not markedly changed during the initiation of transcription. However, they do not rule out the possibility that the observed initiation is occurring on the DNA tails of the 185 base pair mononucleosome. To examine this possibility, it was necessary to obtain a relative measurement of the affinity of RNA polymerase for mononucleosomes and cores in which the DNA tails have been removed. This was accomplished by template saturation studies under conditions in which reinitiation cannot occur. Transcription was initiated with three nucleotides, followed by the simultaneous addition of the fourth nucleotide and rifampicin. The total incorporated radioactivity is a measure of the amount of

initiation on each template. Since the template sizes are defined (mononucleosome, 185 base pairs; core, 145 base pairs) one can discuss the different template concentrations in terms of moles of DNA. By measuring initiation over a range of template concentration and constructing a double reciprocal plot, one can determine an apparent K_m for the initiation reaction on cores, mononucleosomes, and mononucleosomal DNA. Linear regression analysis was used to fit the points to a straight line. Correlations of greater than 0.95 were observed in each case. Table 1 shows that there is essentially no difference in apparent K_m of initiation between mononucleosomes and core particles. Thus, initiation does not occur primarily on the tails of the mononucleosome. The nucleosomal DNA control gives an apparent K_m 6.3-fold less than for the nucleosome preparation. Template capacity of mononucleosomes, mononucleosomal DNA, phosphorylated mononucleosomes and placental DNA were measured using excess polymerase and conditions which block reinitiation. As shown in Table 2, mononucleosomes have 11.3% of the template capacity of an equivalent amount of high molecular weight DNA; the template capacity of mononucleosomal DNA is 54.5% as compared with total DNA. Thus, the ratio of the template capacities of nucleosomal DNA to nucleosomes, 4.8, is in close agreement with the above K_m data and with previous work (5). Treatment of the mononucleosomes with polynucleotide kinase and ATP causes an increase in the template capacity of approximately 2 fold, while similar incubation with ATP resulted in less than 15% of the increase.

Mononucleosome-polymerase complexes and uncomplexed mononucleosomes were isolated from preparative gels by electrophoretic elution and the DNA from each was purified. In order to measure the amount of DNA in the nucleosomal fractions the material isolated from the gel was labeled with ^3H -dNTPs by nick translation under conditions where all components except DNA were present in excess. Thus, the incorporation of label reflects the relative amount of DNA in the nucleosomal fractions. The extent of labelling of uncomplexed mononucleosomal DNA was 7X that of the DNA from the mononucleosome-

Table 1. Affinity Constants for RNA-polymerase Binding to Various Templates

Template	Apparent K_m (mM)
1. Monomeric DNA	0.16
2. Nucleosome	1.0
3. Core-particle	0.83

Table 2. Template Capacity of High Molecular Weight DNA, Nucleosomes, Phosphorylated Nucleosomes, and Nucleosomal DNA

Template	Template Capacity (%)	
Placental DNA	100	---
Mononucleosomal DNA	54.5 ±	12.5
Mononucleosomes	11.3 ±	2.5
Phosphorylated Mononucleosomes	19.5 ±	7.3

Aliquots containing 5 μ g of DNA from each sample were used as template for 18 μ g of RNA polymerase as described in Materials and Methods. Phosphorylation in 5mM Tris, pH 7.9, 5mM DTT, 5mM MgCl₂, 150mM KCl, 5 μ M ATP, containing 2 units polynucleotide kinase at 37° for 30 min was performed immediately prior to template measurements.

polymerase complex. The results indicate that the mononucleosomes which complex with the polymerase represent 15% of the mononucleosome population.

DISCUSSION

There is a substantial body of evidence that transcribed genes are organized into nucleosomal structures (1,2,18). The nucleosomes present in transcriptionally active chromatin apparently are structurally different from those present in untranscribed regions. This difference has been shown most clearly by differences in DNase I sensitivity (19, 20, 21, 22). Preferential sensitivity to micrococcal nuclease has also been reported in some systems (23). *E. coli* RNA polymerase has been useful as a probe for chromatin structure in a number of systems. Experiments examining globin (24) and ovalbumin (25,26) gene transcription demonstrate that this enzyme preferentially transcribes the active regions of chromatin under conditions in which RNA-dependent RNA polymerization (27) and endogenous mRNA contamination are minimized.

Previous studies from this laboratory have demonstrated that the template restriction observed for chromatin is preserved after nucleosomes are prepared (3). Consequently, we decided to use *E. coli* RNA polymerase in our initial attempt to fractionate the transcriptionally active nucleosomes. As shown in Figures 1 and 2, the polymerase-nucleosome initiation complex can be isolated intact and can be separated from the free monomer. Additionally, the nucleosome composition remains intact during initiation as evidenced by the presence of core histones in the transcription complex (Figure 5). Our measurements

of the relative affinity of RNA polymerase for nucleosome, cores, and monomer DNA, respectively, (Table 1) support previous template capacity determinations as shown in Table 2 and previously reported (5). In addition they indicate that the differences observed are primarily at the level of the initiation of transcription. Under the conditions used, the retarding effect of nucleosomes on elongation rate (4) in the presence rifampicin would not account for the observed differences in apparent K_m .

An important question posed by this work is whether the nucleosomes which form initiation complexes with RNA polymerase represent a subset of the total nucleosomal population, or are they representative of the entire nucleosomal population. To answer this question, it is necessary to develop preparative methods for isolating the transcription complexes free of uncomplexed monomer. We have found that preparative gel electrophoresis followed by electrophoretic elution onto sucrose gradients allows the isolation of both the complex and the free mononucleosome. From our results we estimate that 15% of the mononucleosomes can form stable initiation complexes with RNA polymerase. Further characterization of the DNA and proteins in the transcription complex and in the uncomplexed mononucleosomes is in progress and should give more insight into the nature of the nucleosome-polymerase complex.

Several laboratories have shown that nucleosomes from actively transcribed regions are more sensitive to DNase I digestion (28,29,30). This sensitivity reflects an altered nucleosome structure and chemical composition (29) which maybe required for transcription. The binding of heterologous polymerase may reflect similar properties of a nucleosomal subset. Since it is unlikely that the bacterial RNA polymerase recognizes true promoter sites on human nucleosomes, we suggest that polymerase may be recognizing significant differences in physical or chemical properties of a portion of the nucleosome population.

In initial experiments to estimate the proportion of nucleosomes in the complex we treated nucleosomes with γ - ^{32}P -ATP and polynucleotide kinase to label the 5' termini of nucleosomal DNA. This procedure was unsatisfactory for two reasons. First, after treatment about one-third of the acid insoluble radioactivity became resistant to DNase I digestion and sensitive to protease digestion. Previous work has shown that endogenous protein kinase activity in Hela nucleosomes labels histones and nonhistone proteins when exposed to γ - ^{32}P -ATP (11). Second, the template capacity of treated nucleosomes with *E. coli* RNA polymerase is at least 2 fold higher

(Table 2). These results indicate substantial structural modification of the nucleosomes during the kinase reaction. This led us to use ^{125}I -labeling of the nucleosomes as a tracer method. Lilley et al, (31) used nucleosomes treated with polynucleotide kinase to study nucleosome-polymerase interaction and found that there is dissociation of nucleosomes to free DNA. They concluded that nucleosomal transcription experiments actually involved the free DNA released from nucleosomes. Our results described above indicate that nucleosomal dissociation is a consequence of the polynucleotide kinase treatment used by Lilley et al (31).

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