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The nature of the interaction of nucleosomes with a eukaryotic RNA polymerase II

D.M.J.Lilley, M.F.Jacobs and M.Houghton

Departments of Biophysics and Biochemistry, Searle Research Laboratories, Lane End Road, High Wycombe, HP12 4HL, UK

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ABSTRACT

The integrity and stability of nucleosomes under transcription assay conditions has been found to depend on concentration and ionic environment. Rifamycin AF/013, a commonly used inhibitor of initiation, is particularly effective in destabilisation of nucleosomes. Intact nucleosomes are refractory to transcription by wheat RNA polymerase II, the histone core preventing initiation. Template titration suggests that the polymerase can, however, bind to nucleosomes, and a 15-16S complex has been observed on sucrose gradients. DNase I digestion of polymerase-nucleosome incubations indicates that whilst histone is still present in the complex, the nucleosome conformation is altered resulting in enhanced nucleolysis at sites near the DNA centre but reduced overall kinetics of digestion.

INTRODUCTION

There is increasing evidence that active eukaryotic genes are complexed with histones and possibly other proteins, (for reviews see 1-3). Active gene sequences are found in nucleosomal fractions from nuclease digestions (4,5), and electron microscopy of active non-ribosomal transcription units shows 'beads' of nucleosomal appearance between nascent ribonuclear protein strands (6).

The structure of the nucleosome is now known in broad detail at the molecular level (7,8). A core of eight histone molecules is surrounded by almost two turns of DNA comprising 145 base pairs. For RNA polymerase II binding, initiation and elongation, local strand separation of DNA is involved, which is topologically not possible for twisted and surface bound DNA. Some disruption of the structure is therefore likely during transcription and various proposals have been made (9,10). One can consider certain limiting possibilities. Histones may be transiently displaced from DNA to allow passage of polymerase; a process which is not likely to be of great efficiency. Alternatively, the structure may deform to allow some

local rotation about the twist axis without histone displacement. A third possibility is that the histone core may translocate along the DNA ahead of the polymerase molecule. The enhanced DNase I sensitivity of active genes (11,12) indicates the presence of some local structural perturbation, which need not reside at the single nucleosome level. In vitro testing of these models is difficult. Transcription has been observed on mini chromosomes (13-15), at a low rate with respect to free DNA, and nascent RNA strands visualised by electron microscopy. Williamson and Felsenfeld (16) have similarly observed transcription on T7 DNA reconstituted with histone. Whilst the results appear to exclude histone displacement, core 'sliding' is difficult to eliminate by these experiments. The use of single nucleosome preparations prevents undetected histone sliding and two investigations have been made (17,18). However, microscopy is unrevealing in this system, and the possibility of polymerase independent dissociation to free DNA from which transcription is observed should be excluded before definite conclusions may be made.

In this work we have studied the interaction between a eukaryotic RNA polymerase II with single nucleosomes. We have observed that at the low nucleosome concentrations used to achieve enzyme excess for nucleosome transcription experiments, dissociation to free DNA is considerable, irrespective of the presence of polymerase. When allowance has been made for this DNA no transcription is attributable to intact nucleosomes, the reason being failure to initiate. Nevertheless, the RNA polymerase binds to the nucleosome. We have used DNase I digestion of the resulting complex in an attempt to identify any inherent ability of wheat RNA polymerase II to alter nucleosome conformation.

EXPERIMENTAL

Nucleosome Preparations

145 bp core particles: Nuclei from 12 ml of chicken blood were lysed in 40 ml of 10mM Tris pH 7.5 and histone H1 was removed from the resulting chromatin by washing in 0.6M and 0.65M NaCl. Ca^{++} was added to 0.75mM and the chromatin digested using 40 units/ml micrococcal nuclease (Porton) for 1 min at 37^{o} C. Digestion was terminated by addition of EDTA to 5mM at 0^{o}C and the digest fractionated on a 10-45% zonal sucrose gradient as described previously (7). The monomer peak contained predominantly 145 bp core particles, see Figure 1.

160 bp nucleosomes: These nucleosomes with longer DNA length were prepared

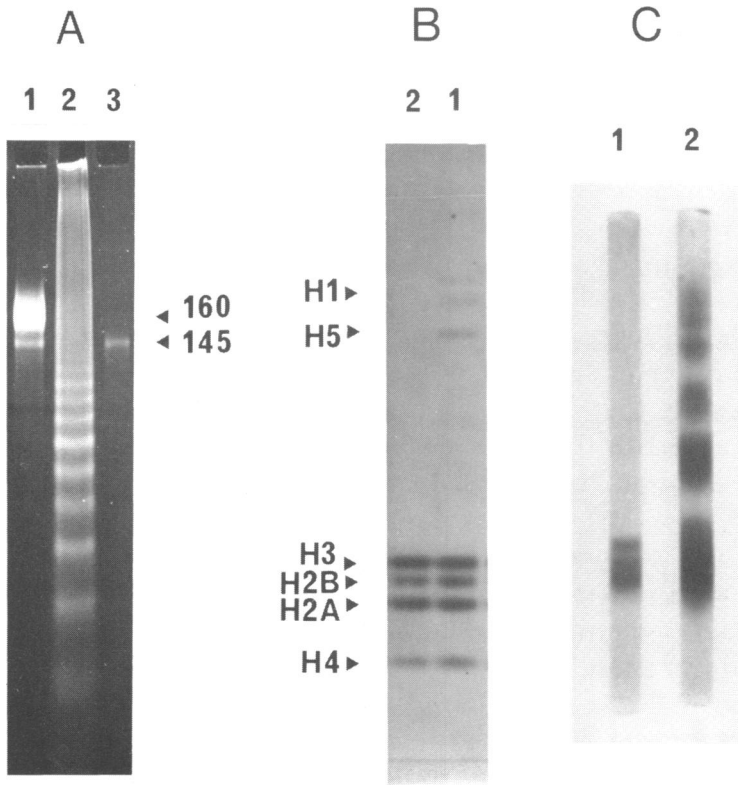

Figure 1. Characterisation of nucleosomes used for transcription and binding experiments.
(A) Gel electrophoresis under denaturing conditions of (1) 160 bp nucleosomal DNA, note the small proportion of 145 bp DNA also present, (2) fragments derived by DNase I digestion of chromatin for calibration, (3) 145 bp nucleosomal DNA.
(B) 15% SDS gel electrophoresis of histones from 160 bp nucleosomes (1) before complete removal of H1, (2) after H1 removal.
(C) 7.5% SDS gel electrophoresis of histones from (1) unmodified nucleosomes, (2) DMS crosslinked nucleosomes.

by digestion of chromatin containing H1 for 6 min, using 150 units/ml micrococcal nuclease. The monomer peak from the zonal gradient was concentrated by Amicon ultrafiltration to A_{260} = 40, then dialysed extensively against 0.65M NaCl in 10mM Tris pH 7.5. H1 was then removed by placing on a Biorad P100 column equilibrated with 40mM NaCl in 10mM Tris pH 7.5. The nucleosome fraction eluted with the column void and contained predominantly 160 bp nucleosomes with a small proportion of 145 bp core particles and was

refractionated on a 5-27.7% sucrose gradient. No H1 remained at this stage, see Figure 1.

Nucleosome kinasing: 160 bp and 145 bp particles were radioactively labelled at their 5'-termini using T4 polynucleotide kinase (Biolabs) and 3000 Ci/mmol [γ-^{32}P] ATP (Radiochemical Centre, Amersham) (19,20). Labelled nucleosomes were purified by centrifugation at 2°C on 5-27.7% isokinetic sucrose gradients in 10mM Tris pH 7.8, 0.7mM EDTA containing 50μg/ml bovine serum albumin to aid nucleosome stability.

Nucleosome crosslinking: 160 bp nucleosomes were crosslinked by 5 mg/ml dimethyl suberimidate (DMS) (Pierce Chemicals), in 100mM sodium borate pH 9.0 for 20 min at 20°C. The crosslinked particles were then dialysed extensively against 10mM Tris pH 7.8, 0.7mM EDTA at 4°C.

DNA was purified from nucleosome preparations by pronase digestion and phenol extraction. DNA concentrations were measured spectrophotometrically.

Transcription Conditions

Wheat germ RNA polymerase II was obtained from Miles Research Products, prepared using polyethyleneimine precipitation (21). This preparation is of high purity and minimal nuclease activity as assayed using supercoiled DNA. In most transcription experiments polymerase and template were preincubated for 20 min at 25°C in 40mM Tris pH 7.9, 1mM DTT, 1mM $MnCl_2$, 0.02mM EDTA, 50 μg/ml ovalbumin, 5% glycerol and 20mM $(NH_4)_2SO_4$. Transcription was initiated by addition of nucleoside triphosphates, 0.4mM ATP, GTP, CTP and 0.05mM UTP containing 1 to 5 μCi [α-^{32}P] UTP per 25μl incubation volume. Incubations were typically 25-50μl containing 2μg/ml template and 20 units/ml RNA polymerase. 1 unit catalyses the incorporation of 1 nmole UTP into RNA in 10 min at 25°C using native calf thymus DNA. Aliquots removed after required times were spotted on 3MM paper circles (Whatman), repeatedly washed in 5% TCA containing 3% sodium pyrophosphate, dried and counted by standard scintillation counting.

Rifamycin AF/013 (22,23) was a gift from Dr. C.J. Chesterton, and highly purified ovalbumin was a gift from Dr. J.S. Emtage.

Gel Electrophoresis

Whole particle gels were prepared according to Todd and Garrard (24). Gel slabs containing 2.5% polyacrylamide, 0.5% agarose in 6.4mM Tris pH 8, 3.2mM sodium acetate, 0.32mM EDTA were run with recirculation of buffer at 4°C for 3 hr. Samples were loaded in 15% sucrose containing bromophenol blue.

Native nucleosomal DNA was examined on 3.5% polyacryamide gels (25) using 40mM Tris pH 7.2, 20mM sodium acetate and 1mM EDTA. DNA samples were loaded

in 7% ficoll 70C (Sigma) and run at 50v for 16 hr.

Single stranded nucleosomal DNA and RNA transcripts were electrophoresed on 12% polyacrylamide-7M urea gels (26) for 16 hr at 90v. DNA or RNA samples were denatured by heating at 100°C for 2 min with deionised formamide followed by application in ficoll containing bromophenol blue and xylene cyanol FF. RNA length calibration was according to Maniatis (26) using marker dye positions, checked using 5S RNA, and we estimate accuracies to within 5 nucleotides in the 160 nucleotide region.

Gels containing ^{32}P labelled DNA or RNA were autoradiographed using Kodak XH1 film for 1 to 5 days. Developed films were scanned using a Joyce-Loebl densitometer.

Density Gradient Centrifugation

Incubated ^{32}P labelled DNA and nucleosomes were examined on 5-27.7% isokinetic sucrose gradients in 10mM Tris pH 7.8, 0.7mM EDTA using a Beckman SW41 rotor at 38K rpm for 17 hr at 2°C.

Nucleosome-polymerase complexes were layered onto 15-33.5% sucrose gradients in 5mM Tris, pH 7.9, 1mM DTT, 1mM Mn^{++}, 20mM $(NH_4)_2\ SO_4$ and 50μg/ml BSA. Unincubated nucleosomes and their DNA were layered onto identical gradients as sedimentation standards. Gradients were centrifuged at 40K rpm at 2°C for 20 hr in a Beckman SW41 rotor. 0.35 ml fractions were collected and radioactivity estimated by Čerenkov photon counting.

DNase I digestions

[5' - ^{32}P] labelled nucleosomes and nucleosome polymerase complexes at 0.25 to 0.5μg/ml DNA were digested with 0.05 units/ml DNase I (Worthington) at 25°C in 40mM Tris, pH 7.9, 1mM Mn^{++}, 1mM Mg^{++}, 1mM DTT, 20mM $(NH_4)_2\ SO_4$ and 50 μg/ml BSA. Digested DNA was extracted using pronase and phenol/chloroform, ethanol precipitated in the presence of 20μg/ml tRNA as carrier and denatured in formamide. The fragments were electrophoresed on 12% polyacrylamide-7M urea gels (26).

RESULTS

Nucleosome Stability

Nucleosome preparations are normally studied at concentrations in the 10-0.1 mg/ml range, at which they appear to be very stable. Transcription studies are performed at much lower concentrations, where stability may be reduced. To investigate this possibility we have incubated nucleosome preparations under conditions of concentration, buffer composition and ionic

strength equivalent to those used in transcription experiments, followed by examination of integrity using gel electrophoresis and sucrose gradient centrifugation. In order to detect DNA and nucleosomes at these concentrations (~1 μg/ml DNA) we have used radioactively labelled nucleosomes prepared using polynucleotide kinase and [γ-^{32}P] ATP (20).

Figure 2 shows an autoradiograph of a polyacrylamide gel of nucleosomes which have been incubated at 25°C in 40mM Tris pH 7.9, 1mM Mn^{++} and various ionic strengths, ovalbumin and inhibitor concentrations. It is apparent that under all conditions some degradation to free DNA has occurred. Tracks 1-3 and 4-6 show the effects of raising the ionic strength with ammonium sulphate, and it is clear that elevation of ionic strength results in further degradation of structure. At 150mM, ammonium sulphate conversion to free DNA is

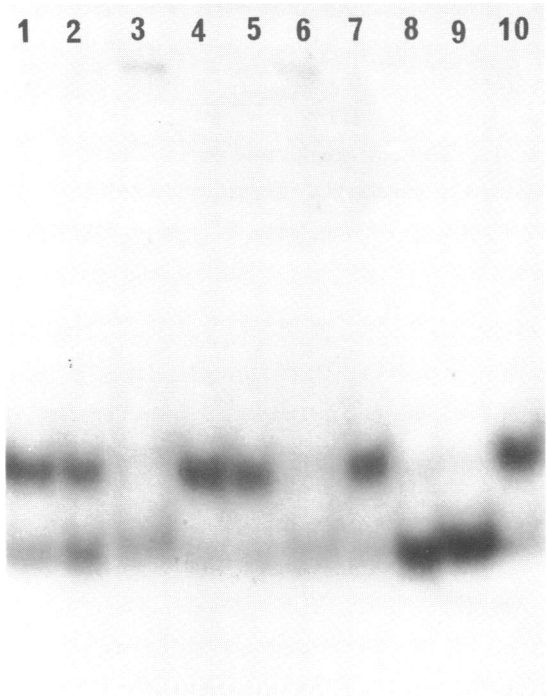

Figure 2. Autoradiograph of acrylamide gel of kinased nucleosomes following 20 min incubation under transcription conditions at 25°C, 40mM Tris pH 7.9, 1 mM $MgCl_2$. Tracks 1-3, 20, 50 and 150 mM ammonium sulphate respectively, no ovalbumin; tracks 4-6, 20, 50 and 150 mM ammonium sulphate respectively, 50 μg/ml ovalbumin; tracks 7-10, no inhibitor, 50 μg/ml, 250 μg/ml rifamycin AF/013 and 20 μg/ml rifampicin respectively, 20mM ammonium sulphate, no ovalbumin, 5% dimethyl formamide.

almost total. Comparison of tracks 1-3 with tracks 4-6 indicates that the inclusion of 50μg/ml ovalbumin confers a partial stabilisation of the nucleosomes, which is particularly evident at 50mM ammonium sulphate (compare tracks 2 and 5). We have observed analogous stabilisation using bovine serum albumin in place of ovalbumin. The degradation of nucleosomes thus appears to be a function of concentration, ionic strength and, to a limited extent, protein concentration. We have also noted that the time of storage is important, the proportion of free DNA generated increasing with the age of preparations. 145 bp core particles seem to have greater stability than 160 bp particles, see Figure 4. Tracks 7-9 show that there is also a pronounced destabilisation by rifamycin AF/013. This does not result from the dimethyl formamide in which the inhibitor is dissolved, shown in track 7, but must be an effect of the rifamycin itself. Concentrations of 50 or 250μg/ml (tracks 8 and 9) appear equally effective in inducing the degradation, but in contrast 20μg/ml rifampicin (track 10) has no effect. Rifamycin AF/013 prevents initiation of eukaryotic class II polymerases as a function of polymerase concentration (22). Under our conditions, at concentrations of rifamycin above 50μg/ml no reinitiation occurs, but below this concentration reinitiation is significant. Thus it is not possible to prevent reinitiation in this system without displacing histones from nucleosomes. The use of heparin as an initiation inhibitor is also undesirable as this is known to result in structural damage to chromatin (27). We have obtained equivalent results to these using centrifugation on isokinetic sucrose gradients.

Template Restriction

A typical time course of RNA synthesis by wheat germ RNA polymerase II on 160 bp nucleosome and 160 bp DNA templates is shown in Figure 3. It is apparent that the presence of histone results in considerable template restriction. From the results in the previous section it is clear that there will be a proportion of histone free DNA in the incubations containing nucleosomes and hence the question arises whether this free DNA accounts for all the observed transcription.

We have therefore performed parallel incubations in which we have attempted to estimate the proportion of free DNA generated from kinased nucleosomes by sucrose density gradient centrifugation, and made comparisons with template restriction observed in transcription incubations. Figure 4 shows the results of isokinetic gradient centrifugation of 145 and 160 bp nucleosomes incubated in the absence of RNA polymerase for the same conditions of buffer, temperature and time as the transcription preincubations. [5' - ^{32}P] nucleosomes

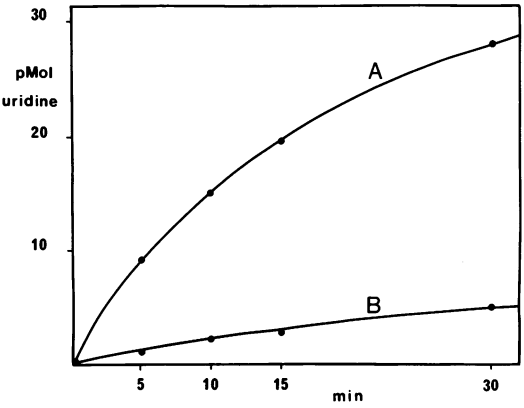

Figure 3. Time course of RNA synthesis from nucleosomes (B) and their DNA (A) in the absence of initiation inhibitors. Transcription of 0.8 µg/ml total DNA by 17 units/ml RNA polymerase under standard conditions.

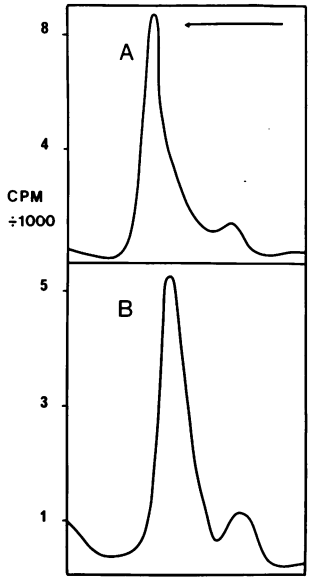

Figure 4. 5-27.7% isokinetic sucrose gradients of (A) 145 bp and (B) 160 bp nucleosomes after 20 min incubation at 25°C in 40mM Tris pH 7.9, 1mM $MnCl_2$, 15mM ammonium sulphate and 50 µg/ml ovalbumin. Relative concentrations of [5'-^{32}P] kinased nucleosomes and DNA extracted from them were measured by radioactive counting, but were approximately 0.6 µg/ml. The arrow indicates the direction of sedimentation.

were used in order to detect DNA on gradients and to enable accurate measurement of relative concentrations. Small 5S DNA peaks are seen in addition to the main 11S nucleosome peaks, integration of which enables the estimation of free DNA percentages. Whilst a small proportion of RNA synthesis from intact nucleosomes cannot be excluded by these data, it is clear that the bulk of observed transcription may be accounted for by free DNA. The agreement between free DNA proportion and nucleosome transcription is within 6% and the experimental error is probably not less than this.

In support of this we note that all attempts to stabilise nucleosome preparations result in increased template restriction. Inclusion of 50 μg/ml ovalbumin into these incubations, which we have demonstrated stabilises nucleosomal structure, markedly reduced RNA synthesis from nucleosome preparations but not from their DNA.

Transcript Length

We have examined RNA transcript length from predominantly 160 bp nucleosomes and DNA, both in the presence and absence of reinitiation. Wheat germ RNA polymerase II was incubated with nucleosomes for 20 min, followed by addition of nucleoside triphosphates including [α-^{32}P] UTP. If reinitiation was to be inhibited, rifamycin was added to 250 μg/ml after 10 sec. We have found that this interval is required for stabilisation of initiation complexes, but is too short to result in significant reinitiation.

Figure 5a shows an autoradiograph of the transcripts synthesised from 160 bp nucleosomes and DNA, electrophoresed under denaturing conditions. Aliquots were removed from the incubations at short times following addition of nucleotides and rifamycin. The major RNA transcripts are present in a discrete region of the gel, and length calibration using bromophenol blue and xylene cyanol marker dyes (26) indicates that the major transcript is 165 ± 4 nucleotides in length, with a minor component of 150 nucleotides. These

Template	pMol uridine incorporated x10	% Nucleosome/DNA transcription	% free DNA generated
160 bp nucleosome	1.54		
DNA	6.14	25	19
145 bp nucleosome	1.60		
DNA	9.18	17	14

Table 1. Comparison of RNA synthesis from nucleosomes and DNA with proportions of histone free DNA generated by incubation in transcription buffer.

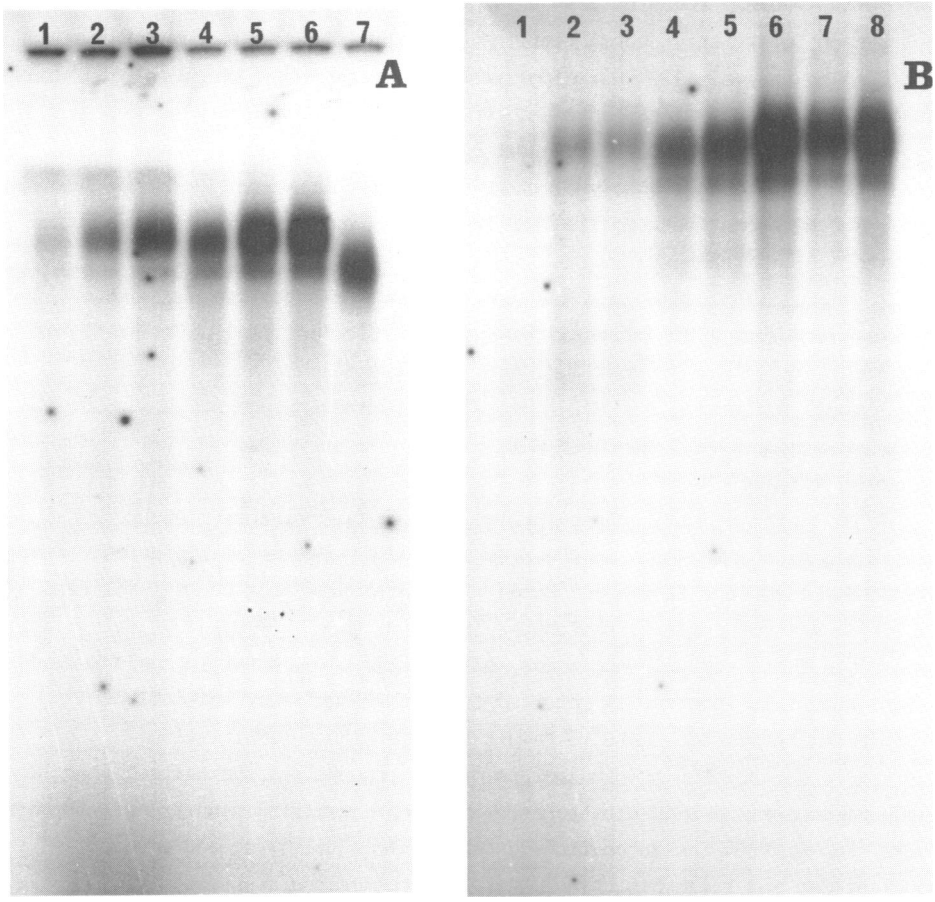

Figure 5. Gel electrophoresis of transcripts synthesised from nucleosome preparations and their DNA.
(A) 250 μg/ml rifamycin AF/013 added 10 sec after addition of nucleosides triphosphates. 2.5 μg/ml 160 bp nucleosomes (tracks 1-3), 1.2 μg/ml 160 bp DNA (tracks 4-6) and 1.2 μg/ml 145 bp DNA were preincubated for 20 min at 25°C with polymerase under standard conditions, RNA synthesis was initiated by addition of nucleotides, including [α-^{32}P] UTP, and aliquots removed at 30 sec (tracks 1 and 4), 1 min (tracks 2 and 5) and 3 min (tracks 3,6 and 7). After denaturation in formamide these were electrophoresed (26) and the gel autoradiographed. Equal radioactive loadings were attempted, though this was not possible for the early nucleosome time points.
(B) No rifamycin added. The basic protocol was as described for (A). Tracks 1-4, 160 bp nucleosomes 30 sec, 1, 5 and 10 min; tracks 5-8, 160 bp DNA, 30 sec,1, 5 and 10 min.

correspond to full length transcripts of the two DNA lengths present in the template. Transcription of a 'trimmed' preparation of 145 bp DNA, shown in track 7, results in synthesis of the shorter RNA only. These results indicate that the majority of initiation events are at the ends of the DNA. Micrococcal nuclease would not be expected to make consistently flush ended cleavages and we have observed previously (28) that short single-stranded projections can serve as pseudo promoters for initiation of wheat germ polymerase II. Very similar transcript lengths are observed when the transcription is performed without rifamycin addition, shown in Figure 5b. Greater smearing is observed since reinitiation can occur and thus later aliquots will contain uncompleted RNA chains. The kinetics of RNA synthesis are similar both for nucleosome and DNA solutions, with and without rifamycin. Some full length transcripts are present after 30s elongation, corresponding to an elongation rate of at least 5 base/s. If nucleosomes were transcribed the rate might be expected to be slower than that for the DNA. No obvious 'slow' fraction is evident for incubation of relatively intact (i.e. no rifamycin present) nucleosomes, suggesting that the RNA is synthesised on free DNA only.

Quantitation of Relative Initiation

Since the predominant RNA species synthesised are full length transcripts we can estimate the relative initiation frequencies for nucleosome and DNA preparations. Following rifamycin addition, initiated chains are completed resulting in a plateau of uridine incorporation, the level of which is proportional to the number of initiation events.

The results of such an experiment are shown in Figure 6, where it can be seen that plateaux of RNA synthesis are reached in 1-2 min for both templates. Since elongation is performed in the presence of 250μg/ml rifamycin, at this stage of the experiment both templates are effectively 100% DNA. Confirmation of this is indicated by the black squares in this figure, which represent uridine incorporation in an analogous experiment where Sarkosyl NL 30 was added to 0.1% at 3 min. This results in little or no additional incorporation. Sarkosyl is known to remove histones but not initiated polymerase molecules (29,30) and hence excludes the possibility of a fraction of 'rifamycin resistant' nucleosomes with initiated polymerases prevented from elongating. The time taken to reach a plateau places a lower limit on the elongation rate on DNA of 2 bases/sec. Comparison with the rate measured from the appearance of full length transcripts on gels suggests that there may be a range of elongation rates, possibly dictated by base composition.

It is clear from these results that the number of initiation events is

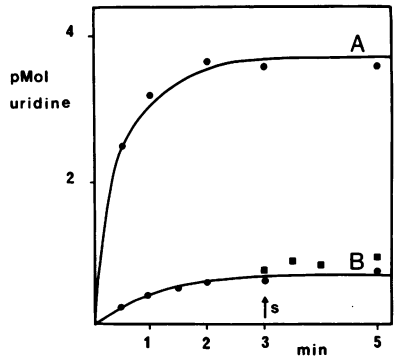

Figure 6. Time course of RNA synthesis from 160 bp nucleosome preparation (B) and its DNA (A), with reinitiation inhibited. 20 min preincubation under standard conditions, with rifamycin AF/013 added to 250 µg/ml 10 sec after nucleotide addition. In a duplicate experiment, sarkosyl NL-30 was added (point S) after 3 min and RNA synthesis measured (black squares)

lower for nucleosome solutions than for the corresponding histone free DNA. Absolute numbers of initiation events may also be calculated from these data, knowing the number of DNA molecules in the incubations. For DNA this corresponds to a single initiation per 3 to 5 DNA molecules. Our studies of plasmid templates (28) indicate that wheat germ polymerase II has a marked preference for initiation on single stranded DNA, including 3' polydeoxycytidine extensions, and hence this fraction probably represents those molecules in which nuclease cleavage has left a protruding 3' end of sufficient length to allow initiation.

Effect of Chemical Crosslinking

Dimethyl suberimidate reacts with histone amino functions resulting in covalently crosslinked species (31). If nucleosomes are transcribed without histone displacement it is likely that some structural rearrangement is a prerequisite which would be thwarted by the crosslinking. We have therefore attempted the transcription of DMS crosslinked nucleosomes, comparing the results with uncrosslinked material and DNA which had been subjected to identical procedures. The results are given in Table 2. It is clear that both in the presence and absence of reinitiation events, crosslinking has no effect upon the transcription of nucleosomes which is greater than experimental error,as judged by the effect on DNA transcription. Two conclusions are possible. Nucleosomes may be transcribed in a way which is not affected by histone crosslinking, an unlikely conclusion in view of the

Template	Rifamycin μg/ml	pMol Uridine Incorporated Uncrosslinked	Crosslinked	Template restriction (uncrosslinked)
DNA	0	7.25	6.53	
Nucleosome	0	1.23	1.09	0.17
DNA	250	1.52	1.33	
Nucleosome	250	0.35	0.43	0.23

Table 2. Comparison of transcription of crosslinked and uncrosslinked nucleosomes, and their DNA. Transcription of 0.01 μg DNA for 10 min, following 20 min preincubation of polymerase and template at 25°C.

constraints on the unwinding of nucleosomal DNA. A more likely explanation, and one more in accord with other results presented here, is that all the observed transcription results from histone free DNA.

The results shown in Table 2 also indicate that there is minimal effect of the elongation conditions, i.e. presence of nucleoside triphosphates and the resulting possibility for elongation upon nucleosome stability. The template restriction values observed with rifamycin are a measure of relative numbers of initiation events following preincubation. Those calculated in the absence of rifamycin will represent an average value for the complete elongation phase. Thus if new initiation sites were being created at this stage, greater RNA synthesis would be expected from the nucleosome incubations. This is not the case.

Template Titration

The results presented above strongly suggest that the bulk of transcription observed from nucleosomal preparations occurs on free DNA generated from nucleosomes degraded by the incubation conditions, and that therefore the intact nucleosomes are refractory to transcription by this polymerase. There are several possible reasons for this. First, there could be no interaction between the RNA polymerase and nucleosomes. Second, RNA polymerase may bind to the nucleosome but be prevented from initiating successfully. Third, initiation may occur but the histone core may prevent elongation. As a variant of the latter explanation, elongation may be possible but at a reduced rate than on histone free DNA. This last possibility is excluded by the observed kinetics of RNA synthesis above.

These possibilities may be distinguished by studying the number of initiation events as a function of template concentration at fixed RNA polymerase concentration (32). This method takes advantage of the known free

DNA component of the nucleosome preparation, and studies competition between the two species. A variable quantity of the template is preincubated with a fixed amount of RNA polymerase followed by addition of nucleotides and rifamycin. Initiated complexes are then allowed to finish elongation and RNA synthesis measured. Template concentrations considered are total concentrations, i.e. DNA and nucleosomal DNA for the nucleosome preparations.

The results from this experiment are shown in Figure 7. For pure DNA a plateau is reached as the quantity of DNA present in the incubation saturates available RNA polymerase. The position of the corresponding curve for the nucleosome preparation may be predicted on the basis of the above possibilities. If there is no interaction between polymerase and intact nucleosomes, then the available DNA template is merely effectively more dilute; RNA synthesis should plateau at the same level of uridine incorporation but at higher total DNA concentration. If binding, but no initiation occurs, then nucleosomes will compete non-productively for polymerase molecules; RNA synthesis will plateau at a lower level but at a similar template concentration. Finally, if initiation has occurred, then addition of rifamycin removes histone and the elongation species is indistinguishable from the pure DNA complex; in this case the template titration curve will be identical with that for pure DNA. The experimental result, shown in Figure 7, is in close agreement with the second possibility, i.e. the polymerase is bound to the nucleosomes in the preincubation phase, but the histone core prevents true initiation as nucleotides are added. The ratio between plateau levels for DNA

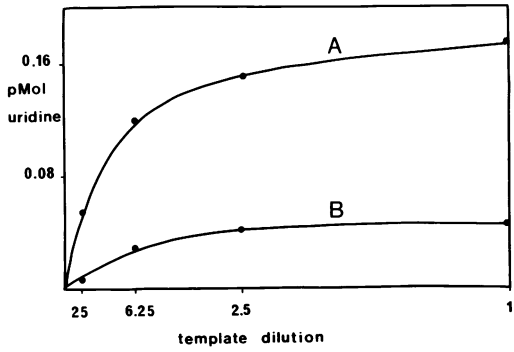

Figure 7. Template titration using a constant polymerase concentration with varying dilutions of 160 bp DNA (A) and 160 bp nucleosome (B) preparations. Unit dilution corresponds to 25 µg/ml total DNA, and polymerase concentration was maintained at 1 unit/ml. After a 20 min standard preincubation with polymerase, nucleotides were added followed by rifamycin AF/013 to 250 µg/ml at 10 sec. Uridine incorporation was measured after a further 10 min at 25°C.

and nucleosome preparations will be a function of both the proportion of free DNA present, and the relative binding constants for polymerase with nucleosomes (K_N) and DNA (K_D), i.e.

$$\frac{[\text{Nsome - Pol}]}{[\text{DNA - Pol}]} = \frac{[\text{Nsome}]}{[\text{DNA}]} \cdot \frac{K_D}{K_N} \quad \text{where } K_{Tem} = \frac{[\text{Pol}]\,[\text{Template}]}{[\text{Template - Pol}]}$$

Comparison of relative plateau levels measured in this experiment with those from Figure 6, i.e. relative initiation numbers when in enzyme excess, shows that the binding constants for DNA and nucleosome are very similar. The binding mechanisms may therefore also be similar, with the histone core preventing initiation for stereochemical reasons.

A polymerase-nucleosome complex

Figure 8 shows the sucrose gradient profiles of 160 bp DNA, 160 bp nucleosomes and 160 bp nucleosomes preincubated with wheat RNA polymerase II. 5 and 11S peaks of radioactivity are observed for DNA and nucleosomes respectively, but incubation of polymerase with nucleosomes results in a new broader peak of mean sedimentation coefficient 15-16S. Since we know that these conditions do not result in a large scale removal of histone from DNA, an assumption

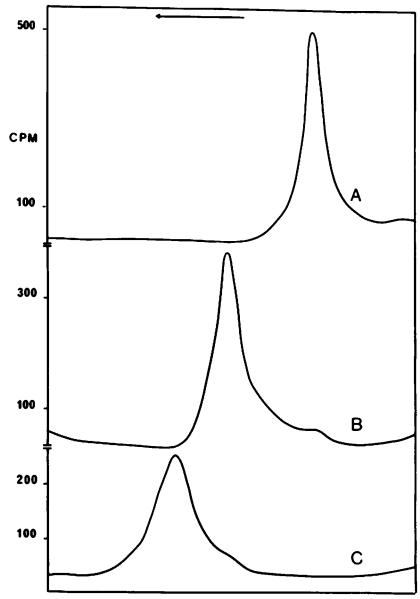

Figure 8. Sedimentation of [5'-^{32}P] 160 bp DNA (A), nucleosomes (B) and nucleosomes preincubated with RNA polymerase (C) on 15-33.5% isokinetic sucrose gradients in standard transcription buffer. The arrow indicates the direction of sedimentation.

substantiated by later DNase I digestion data, we conclude that the new peak contains a complex of nucleosome and RNA polymerase. We have observed similar peaks using a variety of nucleosome preparations and incubation conditions. Purified eukaryotic RNA polymerases II sediment at around 15S (33) and as wheat germ polymerase has a molecular weight typical of general eukaryotic polymerases (34) its $S_{20,w}$ is probably very similar. We might therefore expect a complex of polymerase and nucleosome to have a sedimentation coefficient in excess of the 15-16S observed. It is possible that the complex may possess an extended geometry with a high frictional coefficient, or perhaps more likely that the binding may represent a dynamic equilibrium. The latter would result in peak broadening together with a mean sedimentation coefficient between that expected for a complex and that of the nucleosome. That is just the result obtained in our experiments. The possibility that DNA-binding proteins other than RNA polymerase are responsible for the observed complex formation is unlikely since the enzyme is very pure as judged by SDS acrylamide gel electrophoresis, and the stoichiometry required for complex formation is of the same order as that required for maximal transcriptional activity. Also, nucleosome-polymerase interaction is a required conclusion of our previous template titration studies.

In order to obtain conformational information we have used the technique of DNase I digestion of [5' - ^{32}P] labelled nucleosomes (20,35-37). Nucleosome and nucleosome-polymerase incubations were digested by DNase I followed by separation of DNA fragments under denaturing conditions and autoradiography. Only those fragments still possessing an original ^{32}P labelled 5' terminus appear on the autoradiograph, and the technique has been shown to be a very sensitive probe of DNA conformation in the nucleosome. The result of this experiment with 160 bp nucleosomes is shown in Figure 9a. The tracks on the left (1-4) are the result of increasing DNase I digestion of nucleosomes incubated under polymerase binding conditions, but without RNA polymerase itself present. The expected pattern of unequal intensities is observed, shown also as a scan in Figure 9b. The pattern is qualitatively similar to those published for 145 bp nucleosomes (20,35-37) but is shifted to higher multiples of 10 nucleotides, as might be expected for the longer DNA particles. Further interpretation is, however, complicated by this fact, since we do not know how the 'extra' spacer DNA is distributed. It may be symmetrically, asymmetrically or randomly arranged and, if not symmetric, kinasing may itself be partially asymmetric. Nevertheless, when we compare these results with those in the right hand tracks (5-7), exactly equivalent digestions where

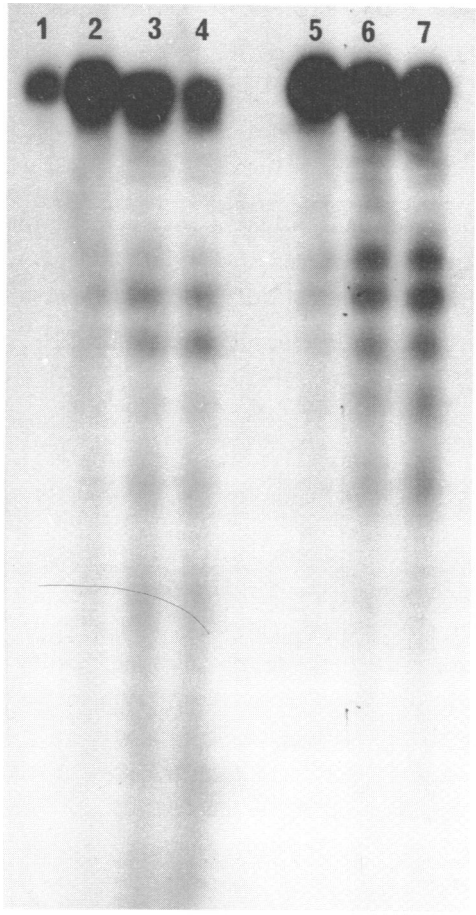

Fig A

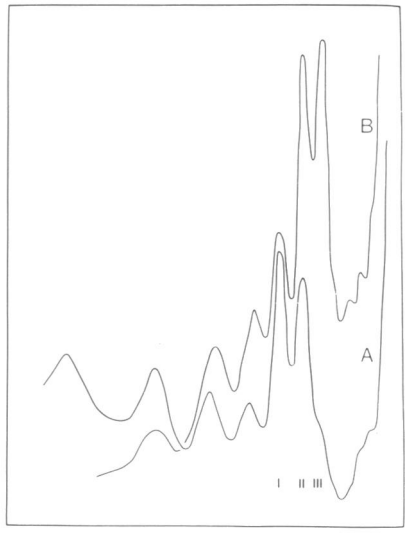

Fig B

Figure 9. DNase I digestion of [5'-^{32}P] 160 bp nucleosomes and nucleosome-RNA polymerase incubations.
(A) Autoradiograph of DNA fragments electrophoresed under denaturing conditions. Track 1, undigested nucleosomes; tracks 2-4, nucleosomes digested for 1, 5 and 10 min respectively; tracks 5-7, nucleosome-polymerase incubations digested for 1, 5 and 10 min respectively.
(B) Autoradiograph scans from steady state tracks from (A). (A) 160 bp nucleosomes, (B) 160 bp nucleosome-polymerase incubations. Bands I, II and III correspond to fragments of 50, 60 and 70 nucleotides respectively.

polymerase has been included, interesting differences are apparent. Under our conditions it appears that a steady state of digestion is reached after a few minutes' incubation such that the relative intensities of bands does

not change with time of digestion except for reduction in intensity of the full length 160 nucleotide band. This is true for nucleosomes with and without RNA polymerase. At much longer times, or with increased DNase I quantities, the 160 nucleotide band is lost, but the 'uneven ladders' are produced right up to this point, indicating that all the DNA is in a nucleosomal like conformation.

Comparison of the overall kinetics of digestion indicates that prior incubation of nucleosomes with RNA polymerase reduces accessibility to DNase I. This could arise from steric protection due to the bulk of the polymerase, or to a change induced in the nucleosome itself, or both. The relative steady state intensities of bands is also altered, shown clearly in the scans in Figure 9b. In particular, the band at 70 nucleotides, almost absent when polymerase was omitted, becomes very intense in the polymerase incubations. Increased intensity in a given band may arise from higher frequency of cleavage at that site, or from reduced cleavage at one nearer the 5'-terminus. In spite of the absence of lower multiples for the complex, consistent with protection by polymerase binding at the terminus, the latter explanation appears to be unlikely to be correct for two reasons. Firstly, such effects would appear at all cleavage points (note the 80 nucleotide position remains uncleaved), not predominantly at a single position, unless there is a cooperative effect directing the nuclease from the 70 nucleotide site to a lower position, which is itself unlikely. Secondly, examination of the known shape and dimensions of the nucleosome (7,8) indicates that the central 70 nucleotide position will be within ~20Å of the 5'-termini. Thus protection conferred against cleavage at the lowest multiple sites by polymerase binding at a 5' end would be expected to reduce digestion kinetics at the 70 nucleotide position also. If, however, binding alters the nucleosome conformation, the spatial separation between these sites might increase, as well as changing the local DNA environment at 70 nucleotides. These results suggest, therefore, that interaction with RNA polymerase is altering the DNA conformation of the nucleosome. The strong 70 nucleotide band further suggests, though does not prove, that the nucleosome may be symmetrically 'opened' by the polymerase, thus exposing a new cleavage site at the particle centre, subject to uncertainty in the location of the symmetrical centre of a 160 bp nucleosome.

We have checked the effect of the wheat polymerase II on the DNase I digestion under identical incubation conditions of nucleosomal DNA free of histone. Whilst the polymerase affords the DNA some protection to nucleolysis, the fragment patterns are markedly different from those of

nucleosome-polymerase incubations, further confirming that the histones do remain associated with the DNA under the conditions used. The bands are more diffuse than those seen from nucleosomes, the major fragment being 30-40 nucleotides in length. This probably represents sections of DNA protected by interaction of the termini with the polymerase active centre.

DISCUSSION

The results presented here indicate that whilst single nucleosome preparations are of high stability above 50 µg/ml, at which concentrations biophysical studies are generally performed, below this their integrity becomes concentration dependent, and free DNA is detectable. Similar dilution dependence is well known for some enzyme activities, for example RNA polymerases, and the observed protection against degradation by inclusion of ovalbumin is also typical of such processes. The nature of nucleosome breakdown is unclear. Badly dissociated preparations show no evidence of either nucleolysis or proteolysis, i.e. covalent bond breakages are not involved.

Clearly the demonstrated generation of free DNA in nucleosome preparations at low concentration has important ramifications for the interpretation of transcription experiments; in particular, the observed nucleosome disruption caused by rifamycin AF/013. We have concluded here that RNA synthesis from 160 and 145 bp nucleosome solutions arises only from the free DNA component, when a eukaryotic RNA polymerase is used. This is based on the following evidence:

1. The proportion of free DNA generated on incubation without RNA polymerase can account for the observed levels of RNA synthesis and numbers of initiation events. Any modifications resulting in greater stabilisation of the nucleosomes reduces observed transcription correspondingly.
2. Template restriction is not reduced by the presence of rifamycin, indicating that the polymerase does not itself induce histone displacement.
3. Chemical crosslinking of the nucleosome histone core has no effect upon the levels of RNA synthesis. Rearrangement of histone cores is not therefore involved in the observed transcription.
4. The kinetics of RNA synthesis are the same for both nucleosomes and their DNA. No significant 'slow' fraction is observed for the former. Either displacement or rearrangement of histone cores would probably require activation energy resulting in reduced rates of transcript synthesis.

5. Competition experiments between free DNA and nucleosomes indicate that whilst RNA polymerase binds to nucleosomes it is unable to initiate.

In previous studies of transcription of whole chromatin it has been difficult to estimate structural damage at the nucleosome level, and there have been few investigations of interactions between purified RNA polymerases and single nucleosomes. Two reports have recently appeared (17,18) in which the authors conclude that E.coli RNA polymerase can initiate on and transcribe through single nucleosomes. Whilst the bacterial polymerase may be able to initiate on nucleosomes, even though it has not evolved to transcribe histone-bound DNA, transcription of free DNA has not been rigorously excluded in these systems. Two investigations (38,39) of transcription by class II RNA polymerases of DNA reconstituted with histones have shown increasing template restriction as histone is added, presumably due to reduced initiation as the DNA becomes histone-bound. It therefore seems likely that nucleosome structure is refractory to RNA synthesis by purified eukaryotic RNA polymerases II. In our system end initiation occurs on DNA but is prevented by an adjacent histone core. Further conclusions can only be speculative for two main reasons. Firstly the structure and possibly even the existence of eukaryotic promoters is an area of considerable uncertainty, but end initiation on DNA may be a rather poor model for in vivo events. Secondly purified polymerases may be deficient in some respect for initiation. Subject to these reservations we note that the results may indicate that initiation regions may differ from bulk chromatin in their nucleoprotein structure.

Despite the lack of initiation, the evidence presented in this paper indicates that there is an interaction between isolated nucleosomes and wheat germ RNA polymerase II. The perturbation of DNase I digestion patterns implies that the whole nucleosome structure is involved in the complex formation. We have previously demonstrated (28) that this polymerase, in common with mammalian and lower eukaryote RNA polymerases II (40,41), shows a preference for binding to single-stranded sections or ends of DNA, which is consistent with the full length transcripts observed from nucleosome-derived DNA. Pronounced effects are nevertheless seen at the centre of the nucleosomal DNA, suggesting a possible conformational transition.

Conformational transitions in nucleosomes and chromatin have been the subject of considerable recent interest. Several intermediate conformations have been postulated on subjection of nucleosomes to a range of ionic strengths (42,43) including the observation by electron microscopy of 'half-nucleosomes' (44). Dubochet and Noll have proposed (45) the existence of a

central hinge region allowing nucleosomes to alter their geometry about their centres in response to crystal packing forces. Analogous equilibria in isolated histone cores have been proposed (46), and Martinson (47) has shown the disruption of H2B-H4 contacts at low ionic strength. A reversible chromatin transition as a function of pH has been deduced from fibre X-ray diffraction studies (48) which, together with other hydrodynamic, spectroscopic and EM investigations supports the general idea of a potential conformational flexibility of the nucleosome. Since the bulk of available evidence supports a view (9,10) of histones remaining on active genes during transcription, some disruption of the structure is likely to be a requirement in order to achieve DNA strand separation. The results presented here indicate that some ability for this may reside in the polymerase itself. Active genes are clearly conformationally distinct in some respect, since they exhibit enhanced DNase I digestion kinetics (11). This may not be due to the actual presence of RNA polymerase since the property is retained in relatively dormant erythrocytes (11), hormone withdrawn oviduct cells (49) and is independent of transcriptional levels (50). This is supported by our observation that whilst the pattern of nucleosomal DNA cleavage is altered by interaction with polymerase, the kinetics are actually slowed. Thus gene activation appears to involve steps independent of the direct presence of RNA polymerase. However, the ability of the polymerase to alter nucleosome structure may be a part of the overall mechanism of class II transcription in eukaryotes.

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