Transcription of nucleosomes from human chromatin

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ABSTRACT

Nucleosomes (chromatin subunits) prepared by micrococcal nuclease digestion of human nuclei are similar in histone content but substantially reduced in non-histone proteins as compared to undigested chromatin. Chromatin transcription experiments indicate that the DNA in the nucleosomes is accessible to DNA-dependent RNA polymerase in vitro. The template capacities of chromatin and nucleosomes are 1.5 and 10%, respectively, relative to high molecular weight DNA, with intermediate values for oligonucleosomes. Three distinct sizes of transcripts, 150, 120 and 95 nucleotides in length, are obtained when nucleosomes are used as templates. However, when nucleosomal DNA is used as a template, the predominant size of transcripts is 150 nucleotides. When oligonucleosomes are used as templates longer transcripts are obtained. This indicates that RNA polymerase can transcribe the DNA contained in the nucleosomes.

INTRODUCTION

Many lines of evidence have led to the view that chromatin exists in a higher ordered structure of regularly repeating subunits, called nucleosomes. Electron microscopic studies of undigested and partially digested chromatin show a "beads on a string" appearance due to the tandem arrangement of these structures (1-5). As Van Holde et al. (2) originally observed, the nucleosomal "cores" are connected by about 40-60 base pairs of the continuing double-stranded DNA, although the interparticle DNA length may vary among different species (6). The DNA in the nucleosomes appears to be tightly folded around an octameric core containing two molecules each of the histones H2A, H2B, H3 and H4, and the integrity of these histones is essential for the folded subunit structure (7,8). Formation of the nuclease resistant subunit structures from purified viral, bacterial, or eukaryotic DNA and the four histones (3) indicates the absence of nucleotide sequence specificity in the interaction between DNA and the histone octamer.

The role, if any, of the repeating subunit 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 with DNA isolated from chromatin subunits (9,10). 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. In this report, we present evidence supporting this possibility by showing that at least some nucleosomes can be transcribed in vitro.

MATERIALS AND METHODS

Isolation of chromatin and nucleosomes: Fresh human placentas were minced, discarding fatty tissue, washed in SSC (0.15 M NaCl, 0.015 M Na₃ citrate, pH 7.0), frozen on dry ice and stored at -20° C until used. Unless specified all subsequent operations were carried out at 4° C. Approximately 40 g of tissue were minced and homogenized in 200 ml of SSC containing 5 mM NaHSO₃ and 0.2% NP-40 (Shell Co.) in a Waring Blender at low speed for 2 min. Then 200 ml of SSC containing 5 mM NaHSO₃ were added and the mixture was homogenized at high speed for 1 min. The homogenate was filtered twice through 4 layers of cheese cloth and twice through 2 layers of Miracloth. The filtrate was centrifuged at 1500 xg for 15 min in an SS-34 Sorvall rotor. The pellet was washed twice with 0.5 x SSC and once with 5 mM Tris-HCl, pH 7.8. The final pellet was resuspended in 0.5 mM Tris-HCl (pH 7.8), allowed to swell on ice at least 30 min. and centrifuged at 12,000 xg for 30 min. This step was repeated at least twice to obtain a gelatinous pellet of crude chromatin. Lysis of nuclei from placenta is difficult as compared to nuclei of human lymphocytes and calf thymus. The crude chromatin pellet was resuspended in 25 ml of 0.5 mM Tris-HCl (pH 7.8) and 50 ml of 1.7 M sucrose in 0.5 mM Tris-HCl (pH 7.8). Twenty-five ml aliquots of this suspension were layered onto 10 ml 1.7 M sucrose and centrifuged at 50,000 xg for 1.5 hr in an SW-27 rotor. The band at the top of the 1.7 M sucrose was collected, suspended in 100 ml of 5 mM Tris-HCl (pH 7.8), and centrifuged for 30 min at 12,000 xg. The pellet was dissolved in 50 ml of 5 mM Tris-HCl (pH 7.8), and dialyzed overnight against the same buffer. The chromatin was sheared in a Virtis "45" for 60 sec at 40 volts and stirred gently for one hour. The solution was centrifuged at 2000 xg for 10 min, the negligible pellet obtained was discarded and the supernatant was used as purified

solubilized chromatin. Nuclei prepared as previously described (11) were suspended in 5 mM phosphate buffer (pH 6.8) containing 0.1 mM CaCl_2 and incubated at 37°C with micrococcal nuclease (Worthington Biochemicals Corp., NFCP grade) at a concentration of 10 units/ A_{260} . The reaction was stopped by adding 0.1 M EDTA to a final concentration of 5 mM and cooling the reaction mixture in an ice bath. The reaction mixture was dialyzed against 10 mM Tris-HCl (pH 7.8) containing 0.2 mM EDTA, and then centrifuged at 12,000 $\times g$ for 30 min. The supernatant was applied to Bio-Gel A-5 m column in order to separate nucleosomes from di- and oligonucleosomes (11).

Circular dichroism measurements: The circular dichroism spectra were measured on a Durrum Jasco CD-SP spectrophotometer at room temperature. The concentration in each sample was such that the equivalent DNA concentration was always in the range of 50–80 $\mu\text{g/ml}$ in 10 mM Tris-HCl (pH 7.8). The values of $\Delta\epsilon$, calculated at 2 nm intervals were plotted against wavelength.

DNA isolation: DNA was purified from fresh human placenta by the method of Kirby and Cook (12). Nucleosomes were treated with pronase and then phenol extracted to obtain purified nucleosomal DNA. All phenol steps used freshly distilled phenol saturated with 0.1 $\times$ SSC, 0.2% 8-hydroxy-quinoline, with the pH of the aqueous phase adjusted to 7.0.

Chemical composition: DNA was determined by the diphenylamine reaction (13) with calf thymus DNA as the standard. RNA was determined by UV absorption after alkali treatment (14), with yeast RNA as the standard. Histones were extracted from chromatin with 0.4 N H_2SO_4 at 4°C. Nonhistone proteins were obtained from the acid insoluble residue of chromatin as the alkali-soluble material. The protein contents were determined by the method of Lowry, *et al.* (15), with bovine serum albumin (Fraction V) as the standard.

Polyacrylamide gel electrophoresis: Three percent composite cylindrical gels [2.5%, acrylamide (19:1, acrylamide:bisacrylamide) and 0.5% agarose made in running buffer] were run to analyze the pooled nucleosomes from the A-5 m column. The length of the DNA from mono- or oligonucleosomes was determined on 5.5% composite cylindrical gels [5% acrylamide (19:1, acrylamide:bisacrylamide) and 0.5% agarose made in running buffer]. The restriction fragments of PM2 DNA generated by the restriction enzyme, Hae III, were run on a parallel gel; the running buffer was 40 mM Tris-acetate (pH 7.8), 20 mM sodium acetate and 3 mM EDTA. Bromophenol blue was added to indicate the extent of migration in the gel. The gels were run at a constant current of 2 mA per tube. The gels were stained in a 2 $\mu\text{g/ml}$

ethidium bromide solution in running buffer for 30 minutes and then scanned in an Aminco Bowman fluorospectrophotometer; the exciting wavelength was 510 nm and the fluorescence was measured at 590 nm.

Template capacity of nucleosomes: DNA-dependent RNA polymerase was extracted from late log *Escherichia coli* B cells purchased from Miles Laboratories purified according to the procedure of Bautz and Dunn (16). The enzyme preparation contains the sigma subunit, is of high purity, as demonstrated by electrophoresis on SDS polyacrylamide gels, and has a specific activity of 270 units/mg protein. One unit of enzyme activity is equal to one nmole of ^3H -UMP incorporated in 10 min at 37°C using equimolar concentrations of ribonucleoside triphosphates. The polymerase has no detectable DNase contamination, as judged by nicking of closed circular PM2 DNA (this assay can detect as little as 4 ng/ml of DNase I), and no RNase activity on ^{14}C -18S rRNA as analyzed by agarose-urea polyacrylamide gels. The standard reaction mixture (0.25 ml) for assaying template activity contains: 80 mM Tris-HCl (pH 7.8), 150 mM KCl, 12 mM MgCl_2 , 4.8 mM 2-mercaptoethanol, 0.32 mM each CTP, GTP, ATP, and ^3H -UTP (123 Ci/mole), 4 units (15 μg) of polymerase, and 5 μg of template. Nucleosomes, oligonucleosomes and chromatin are completely soluble under the assay conditions (11). Rifampicin (10 μg) was added after 1 min at 37°C to prevent reinitiation and transcription was allowed to continue for 10 min at 37°C . The reaction was stopped by quickly cooling the reaction mixture to 0°C . Two drops of 1 mg/ml bovine serum albumin and 2 ml 5% trichloroacetic acid containing 0.01 M sodium pyrophosphate were added. The precipitate was collected and washed on glass fiber filters which were dried and the radioactivity counted in a liquid scintillation counter. Under these assay conditions RNA synthesis is maximal after 7-8 min incubation (17).

Purification of RNA transcripts and RNA size analysis: RNA was synthesized in a reaction mixture (1 ml) containing 15 units (60 μg) of polymerase, 20 μg template, 80 mM Tris-HCl (pH 7.8), 150 mM KCl, 12 mM MgCl_2 , 4.8 mM 2-mercaptoethanol, 0.32 mM each of GTP, ^3H -CTP (110 Ci/mole), ^3H -ATP (110 Ci/mole), and ^3H -UTP (123 Ci/mole). Reaction conditions were as described above. The reaction was terminated by cooling to 0°C , then treated for 15 min at room temperature with 10 $\mu\text{g}/\text{ml}$ RNase-free DNase I (Worthington Biochemicals). Subsequently, the mixture was incubated 30 min at room temperature with 30 $\mu\text{g}/\text{ml}$ pronase followed by addition of KCl and SDS to a final concentration of 0.4 M and 1%, respectively. The RNA was deproteinized by 1 phenol extraction at 60°C (1 min) and 3 extractions with an equal

volume of chloroform: isoamyl alcohol (24:1). The RNA was separated from free nucleoside triphosphates by Sephadex G-50 column chromatography. The resulting fractions containing labeled RNA were pooled and lyophilized to approximately 0.1 ml. Twenty microliters of RNA solution were run on 5% polyacrylamide, 98% formamide gels (18). The gels, including 2 parallel gels, one containing 5S rRNA and 4S RNA, and the other containing bacteriophage PM 2 DNA digested by restriction enzyme HAE III, were electrophoresed at 2 ma/gel in 0.025 M Na-phosphate, pH 6.8 for 2 hr at room temperature. The gels were sliced (2mm/slice), incubated in NCS at 50° C overnight and counted in scintillation counter after adding 10 ml of scintillation fluid.

RESULTS

We have developed a method for preparation of chromatin from human placental nuclei which has a very low template capacity. Chromatin prepared by banding in sucrose step gradients has an A_{320}/A_{260} ratio of less than 0.05, indicating very little chromatin aggregation. The physical and chemical properties of this chromatin are similar to those of chromatin prepared by other methods (19-21). The principal advantage of this technique is that the chromatin is not contaminated with unbroken nuclei. If the nuclear preparation is good and the nuclei are allowed to swell in hypotonic Tris-HCl buffer (pH 7.8), efficient lysis of nuclei occurs. On the other hand, if nuclei are contaminated with cytoplasmic material, they tend to aggregate; if one attempts to lyse such aggregates, complete lysis does not occur and some intact and partially lysed nuclei get entrapped in the chromatin. Under these conditions, chromatin sediments through the 1.7 M sucrose cushion and is recovered as a gelatinous pellet. Light microscopic analysis of such a pellet has always revealed the presence of contaminating nuclei (data not shown). On the other hand, chromatin obtained from complete lysis of a nuclear preparation in hypotonic Tris-HCl buffer (pH 7.8) bands at the interface, instead of pelleting through the 1.7 M sucrose cushion. This chromatin goes into solution very easily and has a protein to DNA ratio of 1.6:1 (Table 1). We have used this method successfully for preparation of chromatin from other human tissues (10).

Nucleosomes were prepared by digestion of human placental nuclei with micrococcal nuclease in 5 mM phosphate buffer (pH 6.7) and 0.1 mM CaCl_2 (figure 1). The digestion was stopped by addition of EDTA to 5 mM and cooling in an ice bath when about 25-30% of the original A_{260} units were

TABLE 1. Composition of Chromatin and Nucleosomes from Human Placenta

	DNA	RNA	PROTEIN	
			HISTONES	NON-HISTONES
Chromatin	1.00	0.11	1.28	0.31
Nucleosomes	1.00	--	1.27	0.06

Kinetics of Digestion of Nuclei by Microccal Nuclease

Figure 1: Kinetics of nuclear digestion by micrococcal nuclease. Purified nuclei were suspended in 5 mM sodium phosphate buffer (pH 6.8), 0.1 mM CaCl₂ to a final A₂₆₀=40. Micrococcal nuclease was added to a concentration of 10 units per A₂₆₀. Digestion was carried out at 37°C. Aliquots of the reaction mixture were taken at various times and added to 1 ml of ice cold 1 M NaCl-10% perchloric acid solution and allowed to stand in an ice bath with intermittent shaking. The samples were centrifuged and the fraction of acid soluble material determined by absorbance at 260 nm.

made acid soluble. At this stage of digestion, only a portion of the acid insoluble material is mononucleosomes. The nucleosomes were separated from oligonucleosomes by gel filtration on Bio-Gel A-5 m as described elsewhere (11). Fractions were pooled to give four samples with differing proportions

of nucleosomes and oligonucleosomes as shown in Table 2. We refer to sample 1 as nucleosomes, since it is 90% mononucleosomes.

The circular dichroism (C.D.) spectrum in the 250-320 nm region provides a rapid estimation of the quality of nucleosome preparations, since proteins contribute very little to the C.D. spectrum in this region. Hence, the conformational changes occurring in DNA due to histone interaction can be detected by following the changes in the C.D. spectrum. In the C.D. spectrum of the nucleosomes (data not shown) there is a negative dip in the spectrum at 295 nm, while DNA which is in the B-form has a positive value at this wavelength. The chromatin spectrum, which lies between B-form DNA and the nucleosome spectra, can be constructed by linear combination of the spectra for A-, B-, and C-form DNA (22).

Human placental nucleosomes contain similar amounts of histones but substantially less non-histone proteins than undigested chromatin relative to DNA (Table 1). The length of nucleosomal DNA was estimated by electrophoresis on 5.5% composite gels using Hae III restricted fragments of bacteriophage PM2 DNA as markers (figure 2). In this gel system, nucleosomal DNA migrates as a major sharp band 185 base pairs in length and two minor bands at 155 and 370 base pairs. The 155 base pair band may correspond to an intermediate digestion product of mononucleosomes while the 370 base pair band probably arises from dimers in the nucleosome preparation. Since the nucleosomes were to be used in transcription experiments, the possibility

TABLE 2. Template Capacity Measurements of High Molecular Weight DNA, Nucleosomes, Oligonucleosomes, and Nucleosomal DNA

TEMPLATE	COMPOSITION (%)				TEMPLATE CAPACITY (%)
	Monomer	dimer	trimer	tetramer	
DNA					100
Chromatin					1.5
Nucleosome	90	10			10
Nucleosomal DNA					19
Sample II	6	94			9
Sample III		7	93		6
Sample IV		5	21	74	2

Figure 2: Length of nucleosomal DNA. The DNA was purified by phenol extraction and electrophoresed on 5.5% composite cylindrical gels. Fluorescent scans were obtained after staining the gels with 2 $\mu\text{g}/\text{ml}$ ethidium bromide. (A) PM2 DNA fragments generated by the Hae III restriction enzyme used as markers. (B) Nucleosomal DNA.

that nucleosomes might be contaminated with free DNA was examined. Figure 2 shows that >95% of the DNA extracted from nucleosomes is in the 155 and 185 base pair bands. DNA of this length can be resolved from nucleosomes by electrophoresis on 3% composite gels. No DNA was detected in the gel containing nucleosomes alone (figure 3a) while the gel containing nucleosomes and purified nucleosomal DNA showed distinct bands (figure 3b). The limit of the detection of free DNA on the overloaded nucleosome gel is 5% of the A_{260} applied to the gel. From these data we conclude that the nucleosomes contain very little, if any, free DNA.

Using excess *Escherichia coli* DNA-dependent RNA polymerase and conditions which block reinitiation of transcription, the template capacities

Figure 3: Gel electrophoresis of nucleosomes and nucleosomal DNA. Nucleosomes and nucleosomes plus nucleosomal DNA were run on parallel, cylindrical 3% composite gels. Fluorescent scans were obtained after staining the gels with 2 $\mu\text{g}/\text{ml}$ ethidium bromide.

(A) 12 μg of nucleosomes

(B) 3 μg of nucleosomes + 3 μg of nucleosomal DNA.

of placental chromatin, nucleosomes and nucleosomal DNA were assayed and compared to that of placental DNA under the same conditions (Table 2). Placental chromatin has 1.5% of the template capacity of an equivalent amount of high molecular weight DNA; the template capacity of nucleosomes (sample I) is 10% when compared to that of high molecular weight DNA. As indicated in Table 2, the template capacities of samples II, III, and IV were also measured. Sample II is composed of 94% dimers and has a template capacity of 9% that of high molecular weight DNA. Sample III with a composition of 93% trinucleosomes and 7% dinucleosomes has 6% template capacity. Sample IV which is a mixture of 74% tetra-, 21% tri- and 5% dinucleosomes, has a template capacity of 2%. Thus the template capacity decreases as the number of nucleosomes in the chain increases.

In order to establish that the DNA in the nucleosomal structure is

transcribed, the chain lengths of the RNA transcripts of both nucleosome and nucleosomal DNA were examined by polyacrylamide gel electrophoresis under denaturing conditions. The transcripts of three different nucleosome preparations each contain at least three classes of molecules with chain lengths of 150, 120 and 95 nucleotides (figure 4a). The extent of contamination with dinucleosomes is reflected in the transcripts as a peak at 285 nucleotides (figure 4a). When nucleosomal DNA is transcribed, the major class of transcripts is 150 nucleotides in length (figure 4B), with decreased amounts

Figure 4: Size of nucleosome and nucleosomal DNA transcripts, RNA was synthesized *in vitro* and loaded onto 5% polyacrylamide, 98% formamide gels. Markers RNAs, 4S and 5S rRNA, were run on parallel gels.
 (A) RNA synthesized using nucleosomes as a template.
 (B) RNA synthesized using nucleosomal DNA as a template.

of the 120 and 95 nucleotide long transcripts. Transcripts synthesized from oligonucleosomes result in RNA with chain lengths much longer than nucleosomal transcripts (figure 5), with the majority of the RNA greater than 200 nucleotides long. These data show that transcription can proceed from one nucleosome to another along the length of the DNA in the oligonucleosome.

In order to establish that the DNA contained in nucleosomes is available for transcription it is necessary to examine the possibility that the transcription observed could be due to contamination of the nucleosome preparation with free DNA. This possibility seems unlikely for several reasons: (a) all nucleosome preparations are purified by gel filtration, (b) purified nucleosomal DNA is not nicked as shown by electrophoresis on denaturing gels (23) as would be expected of free DNA which had survived nuclease digestion, (c) using 3% composite gels we do not see free nucleosomal DNA contamination

Figure 5: Size of oligonucleosome transcripts. RNA was synthesized for 40 minutes. The reaction mix was then applied to Sephadex G-50 to separate the transcript from unincorporated nucleotides. The material eluting in the void volume was pooled, treated with DNase I and pronase, and extracted as described in Materials and Methods. The purified material was concentrated by lyophilization and co-electrophoresed with markers on 14 cm 5% polyacrylamide - 98% formamide gels.

(figure 3a); this gel system can resolve free nucleosomal DNA since it migrates faster than intact nucleosomes and (d) the amount of free DNA template needed to account for the 56 pmoles of ^3H -UTP incorporated in the nucleosome transcription experiments (Table 2) would be 2.7 μg DNA. This would correspond to 53% of the material applied in the gel electrophoretic analysis of nucleosomes (figure 3a).

We considered the possibility that the nucleosome is disassociated during a transcriptional event. To test this possibility a 1:1 mixture of mono-nucleosomes and radioactive HeLa DNA was transcribed by *E. coli* RNA polymerase. The mixture was then digested exhaustively with micrococcal nuclease and the amount of nuclease resistant HeLa DNA determined. The amount of nuclease resistant HeLa ^3H -DNA was not different following incubation with or without nucleosomes, RNA polymerase, or nucleosomes plus polymerase. These results suggest that the histone core is not removed during transcription.

DISCUSSION

The present view of chromatin is that the DNA is packed into a regularly repeating series of subunits with a repeat length of about 200 base pairs. This subunit model of chromatin structure has a number of attractive features. For example, it allows for the packaging of long strands of DNA into smaller units (nucleosomes) (7,24) which appears to be a requirement of chromatin structure. Initially it was suspected that the portion of DNA available for transcription was not contained in the nucleosome structure, and some electron microscopic evidence supporting this view was obtained (25). Evidence that this might not be the case was obtained by Lacy and Axel (9) and Kuo *et al* (10), who demonstrated that sequences represented in cellular RNA existed in nuclease resistant subunits. This apparent paradox can be explained by the recent electron micrographs of chromatin-associated fiber arrays from milkweed bug by Foe *et al*, (26). In agreement with Miller and Hamkalo (25) and Franke *et al*. (27), Foe *et al*. (26) found that the active transcriptional units of ribosomal chromatin do not contain repeating nucleosomal structures. However, in milkweed bug embryo, a class of active transcriptional units of non-ribosomal chromatin was found to contain a repeating nucleosomal structure.

In the work presented here, we show that at least three distinct sizes of transcripts are synthesized *in vitro* when nucleosomes are used as templates. However when nucleosomal DNA is used as a template, the transcripts

are similar in sizes to those obtained from nucleosomes, but a different proportion of DNA molecules are in each chain length class. The predominance of 150 nucleotide long RNA in both cases indicates that initiation of transcription is occurring at the termini in most instances. The presence of 60, 95 and 120 nucleotide RNAs suggests that either there are regions where elongation is prevented or hindered, or that initiation is occurring within the nucleosomal structure. The results of oligonucleosome transcription appear to support the latter hypothesis. The size distribution of the oligonucleosome transcripts indicates that RNA polymerase can read through nucleosomal structures, and probably initiate transcription within them as well.

The strong association of DNA with the histone-octameric core of the nucleosome as indicated by thermal stability measurements (27) suggests that it is unlikely that the nucleosome disintegrates during and reassociates following a transcriptional event. Transcription of the SV-40 minichromosome by *E. coli* RNA polymerase resulted in no apparent structural alterations (29). An intriguing possibility is that some symmetry in the histone-pair associations in the core would allow opening of the nucleosome for transcription of the stronger DNA - histone interactions. A model of nucleosome structure based upon two symmetrically paired half-nucleosomes has been proposed by Weintraub *et al.* (28).

We find that the template capacity of human placental chromatin prepared by the banding procedure is only about 2% that of free DNA, while the values reported by Marushige and Bonner (29), Sawada *et al.* (30), Tsai and Saunders (31) on chromatins from other tissues are considerably higher, 5-10%. We have obtained this value consistently in numerous independent preparations. One explanation for the low template capacity is that chromatin purified by the banding technique contains no contaminating nuclei, which may contain histone specific proteases.

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