Sequence specific interaction of the chromosomal proteins with DNA

Dorothy Tuan^{*}, Christopher Chetsanga^{**} and Paul M. Doty

Department of Chemistry, Harvard University, Cambridge, MA 02138, USA

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ABSTRACT

Calf thymus chromatin was partially deproteinized by 0.6-1 M NaCl extraction. From the shape of the temperature derivative plot of its melting curve, the DNA in each chromatin was resolved into regions of exposed DNA, and DNA still complexed with proteins. The partially deproteinized chromatin was capable of directing *in vitro* RNA synthesis in an amount proportional to the fraction of exposed DNA. The *in vitro* RNA transcript was hybridized to denatured calf DNA under hybridization conditions where only RNA's transcribed from repetitive DNA sequences were able to form hybrids. From the results, it is deduced that the *in vitro* RNA transcripts of 0.6 M NaCl-extracted chromatin after the removal of HI histones plus one-quarter of the non histones may be transcribed from repetitive DNA sequences and those of 1 M NaCl-extracted chromatin contain more transcripts from the unique DNA sequences.

INTRODUCTION

The HI histone exhibits several structural and functional features which distinguish it from the other histones: the HI histone has been implicated in the condensation of chromatin (1,2), and phosphorylated, it may serve as a trigger for cell division (3); in active chromatin, the HI histone is depleted (4,5); it contains a very high proportion of basic amino acids, approximately 25% of the molecule is composed of lysine residues; despite its very basic charge, it is the most readily removed of all histones and can be dissociated from chromatin with 0.6 M NaCl (6). In the primary subunit structure of chromatin (7,8, and for review, 9), the HI histone is not found in the octamer histone core associating tightly with around 150 base pairs of DNA to form the basic subunit (10-14); rather, it is found in association with

40-50 base pairs of DNA located in the periphery of the subunit (15) or between neighboring units in the linker region (16,17), with lengths much longer than 50 bases (14).

In an effort to find out if the DNA associated with HI possesses characteristics different from that of DNA associated with the other histones, calf thymus chromatin was extracted with increasing concentrations of NaCl solution such that histones were removed in a stepwise manner to expose the underlying DNA; the resulting partially deproteinized chromatins were studied by thermal denaturation and in vitro RNA synthesis. The RNA transcripts were purified, and hybridized to calf DNA immobilized on membrane filters. Finally, the unique-repetitive sequence content of the RNA was deduced from a comparison of the hybridization level of such RNA and the template activity of the chromatin whence the RNA originated.

MATERIALS AND METHODS

Preparation of chromatin: Calf thymus chromatin was isolated from the frozen tissue. Purified chromatin, after sedimenting through 1.7 M sucrose, was dispersed into 0.01 M Tris, pH 8, either by homogenization which does not cause rearrangement of chromosomal proteins (16,19) or by sonication (2). Homogenized chromatin was used in the present work with the only exception of thermal denaturation of intact chromatin, where sonicated chromatin was used. To avoid intrinsic hydrolytic degradation of the chromosomal proteins, freshly isolated chromatin was used within 6 days of preparation, during which the protein to DNA ratio of chromatin remains constant (2).

Stepwise dissociation of histones from chromatin: Three concentrations of NaCl solutions (0.6 M, 0.8 M, and 1 M) were used to dissociate histones from chromatin. The method was essentially that of Olenbusch et al. (6) with minor modifications. The

partially deproteinized chromatin was sedimented from dissociated proteins in NaCl solutions by centrifugation at 39,000 rpm for 24 hours. To avoid co-sedimentation of the dissociated protein aggregates with the sedimenting partially deproteinized chromatin, 1.7 M sucrose in the same concentration of NaCl served as cushions. At the end of centrifugation, the supernatants containing the dissociated proteins were carefully siphoned off and discarded. The pellets were dissolved in the same concentration of NaCl solutions from which they had been pelleted; the NaCl was then dialyzed out with three changes of 10X volume of 0.01 Tris, pH 8. Intact chromatin in 0.01 Tris, pH 8, which had undergone similar procedures as the partially deproteinized chromatin served as control sample.

Compositional analysis: Macromolecular content (DNA, histones and non-histones) of chromatin and the deproteinized chromatin were determined as previously described (18).

Thermal denaturation: Intact chromatin, 0.6 M, 0.8 M, 1 M NaCl extracted homogenized chromatin and completely deproteinized chromatin-free DNA were dissolved in 0.01 M Tris, pH 8, ($A_{260} \text{ m}\mu = 0.5-0.60 \text{ D./ml}$) and heated at a rate of 0.5°C/min. in a Gilford multiple sample recording spectrophotometer. Intact sonicated chromatin was used instead of homogenized chromatin because of the anomalous melting behavior of the latter (2). Derivative melting profiles were obtained from the slope of the melting profile: $\frac{d A_{260}(T)}{dT} = \frac{A_{260}(T+1) - A_{260}(T-1)}{2}$ v.s.T. The melting temperature T_m of each cooperative melting band is the temperature of maximum $\frac{dA}{dT}$. Fractional area under each melting band was obtained by integrating $\frac{d A_{260}(T)}{dT}$ from T_{initial} to T_{final} . For band I: $T_i = 55^\circ\text{C}$, and $T_f = 68^\circ\text{C}$; for band II: $T_i = 68^\circ\text{C}$, and $T_f =$

74°C; for band III: $T_i = 74^\circ\text{C}$, and $T_f = 79^\circ\text{C}$; for band IV: $T_i = 79^\circ\text{C}$, and $T_f = 95^\circ\text{C}$.

Template assay: The incubation mixture for in vitro RNA synthesis contained 10 μM of MgCl_2 , 0.25 μM MnCl_2 , 0.1 μM each of the NTP's, and 10 units (1 unit = 1 μM of AMP incorporation per 10 min. at 37°C) of F_5 *E. coli* RNA polymerase (20). Radioactive label was either ^{14}C -ATP or ^{14}C -GTP with a specific activity of 0.5 $\mu\text{C}/\mu\text{M}$. 2-12 μg of free DNA or DNA in chromatin were added to the reaction mixture to serve as the template for RNA synthesis. Total volume of the reaction mixture was 0.25 ml and incubation was at 37°C for 10 min. The reaction was terminated by the addition of 0.2 ml of 50% TCA. The acid insoluble material was collected on membrane filters and counted in a Beckman LS-250 scintillation system. To check the RNAase activity of chromatin, 20 μg of ^{14}C -labeled RNA was incubated in the presence of 1 μg of commercial ribonuclease or in the presence of 20 μg of chromatin for periods of 10 minutes to 24 hours. The radioactivity of the incubated RNA was similarly counted.

Bulk RNA synthesis for hybridization was carried out in a template assay system scaled up to 40-fold in both volume and ingredient content. ^3H -labeled ATP was the radioactive carrier. Approximately 1 mg of DNA in the form of pure DNA or chromatin was used as the template. Incubation was for one hour at 30°C. The RNA was purified by phenol extractions, DNAase treatment and finally ethanol precipitation. The ethanol-precipitated RNA was dissolved in 2 ml of 0.01x SSC and dialyzed against 3-4 changes of the same buffer. Concentration of the RNA was determined by A_{260} $\text{m}\mu$ (21). To obtain the specific activity of the RNA, a portion of the RNA solution was spotted onto filters and counted.

Hybridization experiments: Alkaline-denatured calf thymus DNA was applied to nitrocellulose filters in an amount of 2-10 μ g DNA per filter (22). To ensure easy monitoring of DNA retention, the DNA was methylated with ^{14}C -dimethyl-sulfate by a modified method of Smith, Armstrong and McCarthy (23). Incubation mixture contained 25-200 μ g of ^3H -labeled RNA transcribed from calf thymus DNA or chromatin, 2x SSC and 30% of formamide and incubation was for 24 hours at 37°C (24). At the end of incubation, filters were treated with RNAase, washed with 2x SSC, dried and counted in a scintillation counter. E. coli DNA-impregnated filters or blank filters were included in hybridization reactions to monitor background counts nonspecifically adsorbed to the filters.

Hybridization competition experiments were performed in two steps. First, two DNA filters were equilibrated, under conditions specified above, with a saturating amount of ^3H -labeled RNA; one filter was switched after 24 hours to a second incubation mixture containing a saturating amount of ^3H -labeled competing RNA and incubated for another 24 hours. Additional hybrid was then calculated from the difference in radioactivity of the two filters.

RESULTS

Composition of whole and partially deproteinized chromatin: The protein to DNA ratio of intact as well as 0.6 M-1 M NaCl extracted chromatin is presented in Table I. Approximately 15% of total protein originally present in chromatin is removed by each step of extraction; thus after 1 M NaCl extraction, roughly 45% of the total protein is removed from chromatin. For 0.6 M NaCl extracted chromatin, the protein to DNA ratio is independent of the method of preparation; whether ultracentrifugation as described in this report, or gel filtration (25) was used to achieve separation of

TABLE I
Compositional Analysis of Chromatins

Sample ^(a)	histone/DNA	nonhistone/DNA	total protein (hist+nonhist)	lysine+arginine/nucleotide ^(b) histone nonhistone	% protein left
Ch	1.06 ± 0.05	0.391 ± 0.06	1.45 ± 0.11	0.73 0.14	100%
Ch ^{0.6}	0.864 ± 0.026	0.303 ± 0.006	1.17 ± 0.09	0.64 0.11	81%
Ch ^{0.8}	0.713 ± 0.013	0.275 ± 0.06	1.0 ± 0.07	0.53 0.1	69%
Ch ¹	0.673 ± 0.013	0.263 ± 0.07	0.8 ± 0.08	0.4 0.09	55%

(a) Ch is chromatin and Ch^{0.6}, Ch^{0.8}, Ch¹ are chromatin extracted with 0.6, 0.8, and 1 M NaCl.

(b) The amino acid composition of the histones was from Murray (1964) and that of the non-histone from Spelsberg and Hnilica (1969).

the dissociated protein, the same protein to DNA ratio was obtained. For 0.8 M or 1 M NaCl extracted chromatin, the protein to DNA ratio, however, varies with the means of protein separation: ultracentrifugation yields chromatin richer in protein content. This will be discussed later.

From the above compositional analysis and previous gel electrophoresis study (6), it can be shown that 0.6 M NaCl removes the lysine-rich HI histones completely, and roughly one-quarter of non-histone proteins; thus the 0.6 M NaCl extracted chromatin contains the arginine histones (H2A, H2B, H3, and H4) but no HI; 0.8 M to 1M NaCl removes increasing amounts of the arginine histones in addition to all of HI histones, but little non-histone proteins. Thus, the 0.8 M and 1 M NaCl extracted chromatin contain not only no HI but also decreasing amount of the arginine histones.

Thermal denaturation profiles of whole and partially deproteinized chromatin: The melting profiles of intact chromatin, partially deproteinized chromatin and completely deproteinized chromatin, together with their temperature derivative plots are presented in Figure 1. The derivative plots reveal four cooperative melting bands: T_m of band I is 65°C , of band II, 74°C , of band III, 79°C and of band IV, 82°C . Each band represents a thermal phase transition of a segment of DNA whose phosphate groups have been neutralized to different extents by the positive charges on the associated proteins. Thus, band IV with a T_m of 82°C is due to a DNA-histone complex with a lysine plus arginine/nucleotide ratio of 1.5 (26) and band III with a lower T_m is due to a DNA-histone complex of a ratio smaller than 1.5. Band I with a T_m of 65°C is due to free DNA (Fig. 1e). Band II is due in part to non-histone protein binding to DNA and in part to weak coupling of melting at the boundaries between free DNA and histone-bound DNA regions or to small gaps of free DNA between adjacent histone-bound segments (27).

From the fractional area under each melting band, it is possible to estimate the fraction of DNA having that specific thermal stability. Table II summarizes the partition of DNA among melting bands for chromatin and the partially deproteinized chromatins. The area under band I of intact chromatin, due to the ambiguous shape of the derivative curve around this area, cannot be accurately assessed and shows considerable variation. With 0.6 to 1 M NaCl extraction, the fractional area under band I increases as more protein is dissociated from chromatin, while that under bands III and IV decreases by a nearly equal amount; the area under band II remains rather constant. Accompanying the drops in

Figure 1a

Figure 1b

Figure 1d

Figure 1c

Fig. 1: Melting profiles of whole and partially deproteinized chromatin and their derivative plots: (a) chromatin; (b) 0.6 M NaCl extracted chromatin; (c) 0.8 M NaCl extracted chromatin; (d) 1 M NaCl extracted chromatin; (e) free DNA.

bands III and IV with 0.6-1 M NaCl extraction, the area under band I (the percentage of free DNA segments) increases from 12.2% for intact chromatin to 28% for 0.6 M chromatin, 39% for 0.8 M chromatin and finally 45% for the 1 M NaCl extracted chromatin.

TABLE II
Fractional Area Under Each Melting Band

	<u>band I</u>	<u>band II</u>	<u>band III</u>	<u>band IV</u>
Whole chromatin	12.5 $\pm$ 2.5%	25.2 $\pm$ 1%	17 $\pm$ 2%	45.3 $\pm$ 0.8%
0.6 M chromatin	28 $\pm$ 2%	20.5 $\pm$ 0.5%	13 $\pm$ 3%	35 $\pm$ 1%
0.8 M chromatin	40 $\pm$ 3%	20 $\pm$ 2%	11%	30%
1 M chromatin	45%	20%	12%	22%
DNA	100%			

The area under band II remains around 20% for all the deproteinized chromatins.

Template activities: The template activity of various chromatins were measured by radioactive ATP incorporation under the condition that the template was the limiting factor for ribonucleotide incorporation (Fig. 2a). Using free DNA template as the standard of comparison (100%), the template activity of the same amount of DNA in chromatin is 18%, in 0.6 M NaCl extracted chromatin, 22%, in 0.8 M chromatin, 33%, and in 1 M chromatin, 38% (Table III). The increase in template activity is not the result of the gradual removal of ribonuclease activity from chromatin because intact calf thymus chromatin isolated by the present procedure contains negligible amounts of ribonuclease activity. When radioactively labeled RNA was incubated with chromatin for up to 24 hours at 37°C, little radioactivity was lost; the same amount of labeled RNA incubated in the presence of ribonuclease was almost completely digested within 10 minutes of incubation.

To obtain the inherent capability of the templates to direct RNA synthesis under the condition of infinite excess of polymerase and substrates - an experimental condition logistically difficult to provide - the rates of RNA synthesis in Figure 2a were further analyzed by procedure similar to that of the Michaelis

Fig. 2: (a) Template activity of the chromatins measured by ^{14}C -AMP incorporation; (b) Lineweaver-Burk reciprocal plot of data in (a) abscissa: $1/\text{DNA}$ ($1/\mu\text{g}$); ordinate: $1/\text{RNA syn.}$ ($1/\text{m}\mu\text{m AMP}$).

and Menten equation: $v = V_{\text{max}} \cdot A / K + A$, where v is the rate of RNA synthesis; V_{max} , the rate of RNA synthesis in the presence of infinite template; and K , the dissociation constant of the polymerase-template complex. Differentiation of the above equation with respect to A and taking the limit as A approaches zero yields the inherent template capability: $\lim_{A \rightarrow 0} (dv/dA) = V_{\text{max}}/K$. (28). The V_{max}/K for various chromatins and free DNA can be graphically obtained from the reciprocal of the slopes of the Lineweaver-Burk plot of the data in Figure 2a (Fig. 2b). The percent V_{max}/K for various chromatins, using free DNA as the standard (100%), are al-

Table III
The Template Activity of Chromatins and Free DNA

	template % (a)	V_{max}/K % (b)	yield of RNA amount synthesized RNA/ 100 μ g DNA template % (c)
free DNA from chromatin	100%	100%	90%
chromatin	$18.3 \pm 1.1\%$	12%	13%
0.6 M chromatin	$22.4 \pm 1.7\%$	21%	19%
0.8 M chromatin	$33.4 \pm 1.9\%$	32%	
1 M chromatin	$38.4 \pm 2\%$	37%	34%

(a) Percentage template activity of each sample was obtained from the ratio of μ m AMP incorporation on various chromatins to the μ m of AMP incorporation on the same amount of free DNA. Template concentrations of DNA were 4, 6, 8, 10 μ g in 0.25 ml of incubation mixture. The presented percentages were from two sets of chromatin preparations, hence each value is the average of 8 numbers.

(b) V_{max}/K is obtained from the slope of the Lineweaver-Burk reciprocal plot of Fig. 2a. The straight line intersects the ordinate at $1/V_{max}$, and the abscissa at $-1/K$.

(c) The yield of RNA is calculated from the amounts of AMP and GMP incorporation in Fig. 2a and Fig. 3 at template concentrations below 3 μ g. Assuming symmetrical modes of RNA synthesis: (AMP) = (UMP), (GMP) = (CMP), the total amount of synthesized RNA can be calculated. This number divided by template concentration then gives the percentage yield of RNA synthesis.

so presented in Table III. Except for intact chromatin, the graphically obtained inherent template capability agrees well with the experimentally obtained values.

For intact chromatin the K and V_{max} , obtained from the intercept on the abscissa ($-1/K$) and ordinate ($1/V_{max}$) in Figure 2b, are different from those of the free DNA. This indicates that the chromatin template binds polymerase less strongly than free DNA (29) and that the rate of transcription is one-quarter that of free DNA. Intact chromatin has been shown to have one-tenth as many enzyme binding sites, directing RNA synthesis at rates half to one-third that of the free DNA (30). The K values of the partial chromatins and free DNA are, however, similar (Fig. 2b), indicating that the nature of the polymerase binding sites are alike; the rates of RNA synthesis directed by the partial chromatins (V_{max}) are intermediate between free DNA and whole chromatin.

In Figure 3, radioactive GTP of the same specific activity re-

placed ATP as the radioactivity carrier in the incubation mixture. When free DNA served as template, the levels of radioactive GMP or AMP incorporation into RNA were high and reflected the base composition of calf DNA (31), as has been reported (32). This indicates that the mode of *in vitro* RNA synthesis directed by DNA is symmetrical. When chromatin or the partially deproteinized chromatin served as template, the amount of radioactive GMP incorporation was, however, less than the radioactive AMP incorporation. If one assumes that the partial chromatin, as well as intact chromatin (33,34,35), also directs RNA synthesis symmetrically, the total amount of RNA transcripts from various templates can then be calculated from the amount of AMP and GMP incorporation. The yields of RNA from 100 μ g DNA template, also presented in Table III, agree well with the intrinsic template activities estimated from V_{max}/K .

Fig. 3: Template activity of the chromatins measured by ^{14}C -GMP incorporation.

Hybridization experiments: Hybridization plateaus of RNA transcripts from chromatin, partially deproteinized chromatin and free DNA are presented in Figure 4. The RNA from chromatin hybridizes to denatured DNA to a saturating level of 11%, the RNA from 0.6 M chromatin to a level of 17%, RNA from 0.8 M chromatin to a level of 20%, RNA from 1 M chromatin to a level of 24% and finally RNA from free DNA to a level of 27% (Table IV) which, when compared to a hybridization plateau of 5-10% reported by others (36,37), is high and may be a result of the presence of formamide in the hybridization mixture which selectively stabilizes DNA-RNA hybrids (38), thus diminishing the possibility of duplex formation between complementary RNA strands. To see if each increment in hybridization levels represents the revelation of new DNA sequences, competition hybridization experiments were carried out. When the competing RNA was transcribed from template deproteinized by a higher salt concentration, more hybrids are formed; no additional hybrids are formed when the competing RNA was transcribed from templates extracted with lower salt concentrations (Table IV). Furthermore, the amount of additional hybrids obtained from the competition experiment agrees with the difference in saturation hybridization levels (Table IV).

The results in Tables I, II, III, and IV can be summarized in Table V: as increasing amounts of proteins are removed from chromatin by salt extractions (column 1), a higher percentage of DNA in chromatin melts like free DNA (column 2), the template activity of such chromatin as well as the yield of RNA presented as percentage DNA template (column 3) changes in accordance with the percentage of exposed DNA, hybridization plateaus (column 4) of RNA transcribed from these templates also increase, al-

Fig. 4: Saturation plateau of hybridization. RNA transcribed from: chromatin (o); 0.6 M NaCl extracted chromatin (□); 0.8 M NaCl extracted chromatin (x); 1 M NaCl extracted chromatin (Δ); free DNA (●).

Table IV
Saturation Hybridization and Competition Hybridization

	% saturation hybrid µg RNA hybridized/µg DNA	competition ^(a) hybridization	additional hybrids
Ch	11.1 ± 0.4%		
Ch ^{0.6}	16.8 ± 1.1%	Ch → Ch ^{0.6}	6.8 ± 0.5%
Ch ^{0.8}	20.4 ± 0.5%		
Ch ¹	24 ± 1%	Ch ^{0.6} → Ch ¹	8.3 ± 0.7%
DNA	26.6 ± 1.4%	Ch ^{0.6} → DNA	10.2 ± 0.4%
		DNA → Ch	0
		DNA → Ch ^{0.6}	0

(a) Ch → Ch^{0.6}: competition experiment in which DNA filters were first equilibrated in saturating amount of RNA transcribed from Ch, and then switched to a second vial containing saturating amount of RNA transcribed from Ch^{0.6}.

though on a depressed scale. Should all of the transcribed RNA be able to hybridize to DNA, the hybridization plateau (percent DNA in hybrid) would be equivalent to the total amount of synthesized RNA in column 3. But the present hybridization conditions can pick up only RNA transcribed from repetitive RNA sequences

Table V Summary of Tables I, II, III, and IV

	(1) % total protein removed	(2) % free DNA (melting band I)	(3) % yield of RNA (RNA/100 μ g DNA template) (c)	(4) % hybridization (% of repetitive DNA sequences) (a)	(5) yield of RNA minus % hybridization (% of nonrep. DNA sequences) (b)
Ch	0%	13%	13%	11%	2%
Ch ^{0.6}	15%	28%	19%	17%	2%
Ch ^{0.8}	27%	40%		20%	
Ch ¹	42%	45%	34%	24%	10%
DNA	100%	100%	90%	27%	63%

(a) Only RNA transcribed from the repetitive DNA sequences are picked up by the present experimental condition.

(b) The difference between the template activity and the experimental hybridization value (the latter being a measure of the repetitive DNA sequences) would approximate the percentage of nonrep. DNA sequences present in the exposed DNA segments of each chromatin.

(c) Third column in Table III.

(36,37,39). The hybridization plateau (column 4) may thus be a measure of the amount of RNA transcribed from repetitive DNA sequences, and with assumptions which will be discussed later, the difference of RNA yield (column 3) and hybridization plateau (column 4) then provides a rough estimate of the amount of RNA transcribed from nonrepetitive DNA sequences (column 5 in Table V).

DISCUSSION

By analysis of the shape of derivative melting profiles, the DNA in intact chromatin is resolved into four regions: exposed DNA (melting band I), DNA complexed to non-histones (melting band II), and DNA covered by histones to different extents (melting bands III and IV). In partially deproteinized chromatin, more exposed DNA appears as the result of dissociation of histone and non-histone proteins. It can be argued that the exposed DNA segments in partially deproteinized chromatins do not represent the original sites from which histones are removed but are artifacts of extensive histone exchange and rearrangement during NaCl extraction (40). Histone exchange and rearrangement occur, how-

ever, only at NaCl concentrations where a specific class of histones are partially dissociated from DNA such that the dissociated histone molecules, in kinetic equilibrium with the still associated ones, are able to engage in the exchange or rearrangement process; at NaCl concentrations where a special class of histones is completely dissociated or not dissociated at all, histone exchange and rearrangement apparently cannot take place.

When chromatin is dissolved in 0.6 M NaCl, the binding of the lysine-rich H1 histones to DNA is greatly diminished so that they are completely dissociated, while the arginine histones still bind DNA with sufficient strength (41), so that none of them are dissociated; at this salt concentration, neither the lysine-rich nor the arginine histones are able to engage in exchange and rearrangement. The bare DNA regions in 0.6 M NaCl-extracted chromatin thus cannot be the product of histone exchange and rearrangement. In support of this, the protein to DNA ratio in 0.6 M chromatin, as mentioned in the Result section, is independent of the means (gel filtration or ultracentrifugation) by which the dissociated protein is separated from the chromatin; if exchange of proteins occurred in 0.6 M NaCl, gel filtration would have yielded a partial chromatin of lower protein to DNA ratio than ultracentrifugation. Furthermore, when lysine-rich histones are selectively dissociated from whole chromatin not by 0.6 M NaCl extraction but by their affinity for added tRNA in low salt where no histone exchange or rearrangement can take place, the resulting partially deproteinized chromatin contains free DNA and protein-covered DNA regions in percentages comparable to those in the 0.6 M NaCl-extracted chromatin (42).

In 0.8 M NaCl, when the lysine-rich histone is completely dis-

sociated from DNA, the binding of the arginine histones to DNA begins to be weakened and some are dissociated, exchange or rearrangement of the arginine histone may ensue. In view of the preservation of specific gene transcription in chromatin which has been reconstituted from chromosomal proteins and DNA by dialysis in gradient salt solutions of 2 to 0.4 M (43,44,45), arginine histones dissociated from DNA in melting bands III or IV will be able to recombine with DNA in regions from which they are dissociated.

The concomitant increase of percentage template activity with the percentage of exposed DNA in chromatins suggests that the template for in vitro RNA synthesis may be the exposed DNA zones, for the DNA in melting bands III and IV decreases in size with increasing concentrations of NaCl extraction and has been shown to be inactive in transcription (28,49), and the DNA in melting band II remains rather constant in amount for all chromatins, although there is no direct evidence to exclude the DNA in Band II from the template region. The kind of DNA in Band II, since its percentage remains rather constant for all chromatins, may either remain the same, or in a state of steady flux, e.g., as some DNA in Band II becomes Band I DNA, equal amounts of DNA in Band III and IV become Band II DNA. In the former case, the additional transcripts will be from DNA in Band I; in the latter, the additional transcripts will be from DNA in Bands I and II, both of which appear as results of deproteinization.

From the value of K, the dissociation constant of template-polymerase complex (Fig. 2b), the template active regions of partially deproteinized chromatin show the same polymerase binding strength as free DNA. The DNA in melting band I bears resemblance

- to free DNA, for it not only exhibits the same T_m as free DNA, but also bands at the same position in CsCl density gradients (42). Because the percentage template activity of the partially deproteinized chromatin is generally smaller than the percentage amount of DNA in Band I, not all of the free DNA in partially deproteinized chromatin can direct RNA synthesis. The discrepancy seems to occur in 0.6 M chromatin, in which 15% additional DNA is revealed in Band I, yet the increase in template activity is 6-7% (Table V). For 1 M chromatin, the increase in template activity seems in keeping with the incremental amount of free DNA.

In DNA-RNA hybridization experiments, the transcribed RNA molecules should theoretically be able to anneal to their primer DNA and yield a hybridization level identical in numerical value to the RNA yield of the chromatin. But the present experimental condition of hybridization is such that only RNA transcribed from the repetitive DNA sequences with redundancy in the range of $>10^3$ copies per genome is able to form hybrids (37,47), those from the unique sequences are not picked up (37). If one assumes that the DNA template in partially deproteinized chromatin is transcribed with relatively uniform rates of 1 to 4 times the rate of chromatin transcription (Fig. 2b), e.g., no region is transcribed at much faster rates (10 times or more) than others, the hybridization level may also represent the repetitive sequence in the template. The hybridization level, however, represents the maximum amount of repetitive sequences in the template because of possible mismatching of RNA to repetitive DNA with similar sequences as the template. Assuming that such mismatching does not occur extensively relative to perfect hybrid formation, the difference of total RNA and hybridization level then provides a mini-

mum estimate of the nonrepetitive DNA sequences present.

When intact chromatin serves as template for RNA synthesis, the yield of RNA per 100 μ g DNA is 13 μ g (column 3 in Table V); of this, 11 μ g can hybridize to 100 μ g of denatured DNA immobilized on membrane filters (column 4), and may be transcribed from repetitive DNA sequences; the remaining 2 μ g may thus be from nonrepetitive sequences (column 5). A weight ratio of repetitive to nonrepetitive transcripts is thus around 5. A ratio of 4 for both in vitro and in vivo transcripts of chromatin has been estimated from solution hybridization of RNA transcripts to cDNA's made from poly-A⁺ nuclear RNA's previously fractionated into repetitive and nonrepetitive sequences (35). Furthermore, this ratio has been shown by cDNA-bulk DNA hybridization to reflect at least in part the gene dosage of template DNA (35). Our ratio of 5 thus represents an overestimation of repetitive RNA transcripts by about 5%. For free DNA, the yield of RNA transcripts is 90 μ g per 100 μ g DNA template, and 27 μ g can hybridize to DNA and may be from the repetitive DNA sequences. The percentage of repetitive and nonrepetitive RNA sequences transcribed from free DNA also reflect the sequence composition of calf DNA determined by renaturation kinetics (48). For 0.6 M NaCl-extracted chromatin, the yield of RNA per 100 μ g DNA is 19 μ g; of this, 17 μ g can hybridize to 100 μ g of denatured DNA and may be from repetitive DNA sequences; the remaining 2 μ g may then be from nonrepetitive DNA sequences. For 1 M NaCl-extracted chromatin, the yield of RNA is 34 μ g and a maximum of 24 μ g of this may be transcribed from repetitive DNA sequences while the remaining 10 μ g are from nonrepetitive DNA sequences.

In 0.6 M NaCl-extracted chromatin after the dissociation of HI plus a quarter of the non-histones, approximately 15% DNA is expo-

sed, the increment in template activity as compared to that of chromatin is, however, only 6%; thus a significant portion of the exposed DNA may be inactive for in vitro RNA synthesis. The additional transcripts from the template-active portion can hybridize to DNA immobilized on filters and may thus be transcribed from repetitive DNA. From 1 M chromatin to free DNA, the percentage increment in template activity is in keeping with the percentage of additional exposed DNA (55%), yet few additional transcripts from free DNA can hybridize to DNA. Judging from the sequence contents of transcripts from 1 M chromatin and free DNA, the majority of the nonrepetitive DNA sequences in free DNA may still be masked in 1 M chromatin and remain associated with the residual arginine histones and the non-histones. The method employed by the present study for the estimation of nonrepetitive sequence content is, however, indirect and involves assumptions in relation to the mechanism of in vitro transcription and hybridization; the results thus need confirmation from a more direct method such as solution hybridization of excess RNA transcripts with labeled single copy DNA.

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*Present address:- Department of Hematology - Oncology, Children's Hospital Medical Center, 300 Longwood Avenue, Boston, MA 02115, USA

** Present address:- Biochemistry Program, Department of Natural Sciences, University of Michigan, Dearborn, MI 48128, USA

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