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Abstract

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THERMAL DENATURATION AND TEMPLATE ACTIVITIES OF RECONSTITUTED DNA-HISTONE COMPLEXES

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Abstract. Reconstituted complexes of DNA with histone were prepared by salt-and-urea step gradient dialysis. The DNA was complexed with histone H1, with the combination of the other four histones H2A, H2B, H3 and H4, and with whole histones. These DNA-histone complexes were purified by Bio-Gel column chromatography, and the weight ratio of histone-to-DNA was determined in each complex. The thermal denaturation profile and nuclease digestion pattern of DNA-histone H2A, H2B, H3 and H4 complex were compatible with those of the polynucleosome structure of chromatin. The template activities for transcription were compared in these DNA-histone complexes by separately measuring initiation reaction and chain elongation. The binding of histone H1 to DNA strongly inhibited the initiation, while the binding of the combination of the other four histones to DNA partially inhibited the initiation and chain elongation. The binding characteristics are discussed with regard to the role of histone H1 and the other four histones in chromatin structure and template activity.

Among the many approaches to the study of the role of histone, correlative studies on the physicochemical properties and template activities of reconstituted complexes of DNA with histones appear to be of prime importance. Investigations into thermal denaturation have been reported on reconstituted complexes of DNA with whole histones, individual histone, and some combinations of individual histone species (1-8). However, studies of template activity for transcription of reconstituted DNA-histone complexes have led to a variety of contradictory conclusions. For example, strong inhibition of template activity was observed by binding of lysine-rich histone H1 (9, 10), histones H1b and H2B (11), arginine-rich histone H4 (3) and all histone species (3).

Recent studies have indicated that in native chromatin, four histone species other than histone H1 exist as an octameric complex $(H2A \cdot H2B \cdot H3 \cdot H4)_2$ (12, 13). The fundamental structure of chromatin fibers has been shown to be a regular arrangement of globular particles connected by short stretches of DNA

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filaments, like beads on a string (14, 15). In the repeating globular particle (nu body or nucleosome), DNA was shown to be complexes with four histones $(H2A \cdot H2B \cdot H3 \cdot H4)_2$ (16–18). However, the relation of the repeating subunit structure has not been clarified with template activity and with control of gene expression.

By electron microscopic autoradiography of transcription on isolated chromatin fibers, we have provided direct evidence that the transcriptionally active regions of chromatin are not the very long stretches of free DNA but the extended chromatin fibers consisting of DNA bound with histone and nonhistone proteins (19). These regions exhibited a regular or irregular repeating subunit structure with or without visible short stretches of DNA filaments (19).

In the present communication, we report on the thermal denaturation profiles and template activities of reconstituted complexes of DNA with histone H1, with combination of the other four histones H2A, H2B, H3 and H4, and with whole histones. The template activities were analyzed under conditions which permitted separate measurements of the initiation reaction and chain elongation reaction of transcription.

MATERIALS AND METHODS

Preparation of chromatin, DNA and histones. Chromatin was prepared from quick-frozen calf thymus by the method of Bonner *et al.* (20). DNA was isolated from this chromatin by the method of Marmur (21). Whole histones, histone H1 and the mixture of the histones H2A, H2B, H3 and H4 were prepared by the method of Johns (22). Histone species were analyzed by electrophoresis on 15% polyacrylamide-2.5 M urea-0.9 M acetic acid gels, pH 2.7, prepared according to the method of Panyim and Chalkley (23).

Reconstitution of DNA with histones. Complexes of DNA with histone H1, with the other four histones and with whole histones were prepared as follows. DNA and histones were each dissolved at a concentration of 300 μg/ml in medium consisting of 2 M NaCl, 5 M urea, 10 mM $NaHSO_3$ and 10 mM Tris-HCl, pH 7.5, and mixed at a DNA to histone ratio of 1 : 1. The DNA-histone mixtures were dialyzed against the same medium with a decreasing NaCl concentration : 2.0 M, 1.0 M, 0.6 M, 0.15 M and to 0 M. The DNA-histone complexes thus formed were passed through Bio-Gel A-15m columns in the final dialyzing medium. The fractions containing DNA-histone complexes were separated from fractions containing unbound histones. The DNA-histone complex fractions were collected and further dialyzed against 1 mM Tris-HCl, pH 7.5 to remove urea.

Determination of DNA and histone in DNA-histone complexes. Isolated DNA was determined by ultraviolet absorption at 260 nm using E mg of 20. DNA content of the DNA-histone complexes was determined by the diphenylamine method (24) using calf thymus DNA as the standard. Protein content of the isolated histone preparations and of the DNA-histone complexes was deter-

mined according to Lowry *et al.* (25) with bovine serum albumin as the standard.

Thermal denaturation. Thermal denaturation spectra of DNA-histone complexes were recorded with a Hitachi EPS-3T autorecording spectrophotometer. The samples were dialyzed against 0.25 mM EDTA, pH 8.0, adjusted to give A_{260} of 0.3-0.6, degassed under vacuum, and heated at the rate of 0.5°C per min in a stoppered cuvette of 1.0 cm light-path equipped with a thermocouple in the cuvette and with a thermo-regulatory circulating oil bath. The reference cuvette contained the same medium and was heated simultaneously. The DNA-histone complexes were in a soluble state, and the light scattering of the DNA-histone complexes before and after melting was negligible as judged from the absorption at 320 nm.

Computer analysis of thermal denaturation spectra. The experimental error (noise) of the thermal denaturation spectra was corrected by a subroutine program (smoothing) on a computer (NEAC 2200/500). The corrected data were subsequently differentiated by the subroutine program, the analyzed data normalized, and the derivatives finally printed and plotted in chart form. The derivatives of hyperchromicity (Δh) at temperature T were calculated by the equation, $\Delta h_T/\Delta T=(h_{T-2}-4h_{T-1}+3h_T)/2$, where coefficients 4 and 3 are mathematical weights.

Limit digestion of chromatin and reconstituted DNA-histone complexes with staphylococcal nuclease and analysis of DNA fragments by polyacrylamide gel electrophoresis. Chromatin and reconstituted DNA-histone H2A, H2B, H3 and H4 complex were partially digested with staphylococcal nuclease (300 units/ml) (Worthington; 6,000 units/mg) for 30 sec. at 37°C as described (26). Each digested product was extracted with a mixture of chloroform and isoamylalcohol, and subjected to 2.5% polyacrylamide gel electrophoresis for 1 hr at 5 mA/tube. The gels were stained with ethidium bromide.

Template activity measurement. Nucleoside triphosphates and *E. coli* RNA polymerase [nucleoside triphosphate RNA nucleotidyltransferase (EC2. 7. 7. 6), 200 to 400 units/mg] were purchased from Boehringer Manheim and [^{3}H]UTP (10-30 Ci/mmol) from Radiochemical Center, Amersham. The incubation mixture contained in 0.2 ml: 40 mM Tris-HCl, pH 7.9; 8 mM $MgCl_2$; 2 mM $MnCl_2$; 0.1 mM dithiothreitol (DTT); 50 mM KCl; 0.4 mM each of ATP, GTP, and CTP; 1 μCi of [^{3}H]UTP with 0.04 mM UTP; 1 μg of DNA or various DNA-histone complexes containing 1 μg of DNA; and 1 unit of *E. coli* RNA polymerase. One unit is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of labeled AMP into an acid-insoluble product in 10 min at pH 7.9 and 37°C, using calf thymus DNA as template. The reaction was started by adding RNA polymerase. After incubation at 37°C for 15 min, the reaction was stopped by adding 0.1 ml of bovine serum albumin solution (1 mg/ml) and 5 ml of cold 7% trichloroacetic acid containing 0.1 M sodium pyrophosphate, and the precipitates were collected on glass-fiber filters (Whatman GF/C), washed with 25 ml of 7% trichloroacetic acid containing 0.1 M sodium pyrophosphate and dried. The trichloroacetic acid insoluble radioactiv-

ities on the filters were counted in PPO-POPOP-toluene scintillator by a Packard liquid scintillation counter.

Transcription without reinitiation. For the initiation reaction of transcription and chain elongation, the technique of Hyman and Davidson (27) was used. *E. coli* RNA polymerase and template were incubated at 37°C in 1.0 to 0.5 ml of reaction medium containing 10 mM Tris-HCl, pH 7.9, 2 mM $MnCl_2$, 0.1 mM DTT, 0.08 mM each of ATP and GTP, and 1 or 2 μCi of [^{3}H]UTP with 0.008 mM UTP. CTP was omitted. This initiation reaction, usually for 15 min, was stopped by adding $(NH_4)_2SO_4$, 0.4 M in final concentration. This step is important to prevent further chain initiation by enzyme molecules (26–28). Propagation in high salt concentration without reinitiation was then started by adding CTP, 0.08 mM in final concentration, and $MgCl_2$, 8 mM in final concentration. Incorporation of [^{3}H]UMP into RNA was determined at various times during the assay: 0.1 ml of sample was added to 0.1 ml of bovine serum albumin solution (1 mg/ml) and precipitated with 5 ml of 7% trichloroacetic acid containing 0.1 M sodium pyrophosphate. Radioactivities in the precipitates were counted as described above.

Sucrose density gradient sedimentation. [^{3}H]-RNA synthesized *in vitro* was analyzed by sucrose density gradient sedimentation on a linear gradient of 10% to 40% sucrose (w/v) in 10 mM KCl-10 mM Tris-HCl buffer, pH 7.6. Samples of 0.5 ml, each containing 0.5% sodium dodecyl sulfate, were layered on 4.5 ml of the linear sucrose gradient and centrifuged at 80,000×g for 16 hr in a Hitachi RPS-65TA rotor at 20°C. Rat liver ribosomal RNA was used as a molecular weight marker to standardize the gradient. Forty to fifty fractions were collected, and the amount of acid-precipitable radioactivity in each fraction was determined.

RESULTS

Content of DNA and histone in reconstituted DNA-histone complexes. Gel electrophoretic patterns of histone H1 and of the other four histones H2A, H2B, H3 and H4 used for reconstitution are shown in Fig. 1. The weight ratio of histone to DNA calculated for the reconstituted DNA-histone H1 complex was 0.35; for DNA-histone H2A, H2B, H3 and H4 complex, 0.71; and for DNA-whole histone complex, 0.89.

Thermal denaturation profiles of reconstituted DNA-histone complexes. The thermal denaturation profiles and their corresponding derivatives for free DNA and for the reconstituted complexes of DNA with histone H1, with four histones H2A, H2B, H3 and H4, and with whole histones are given in Figs. 2 and 3. In the derivative thermal denaturation profiles, Tm of free DNA was at 45°C under the present conditions. DNA-histone H1 complex showed a biphasic melting profile, a larger amount of melting in lower temperature range, Tm at 47°C, and a smaller amount of melting in higher temperature range, Tm at 76° and 80°–83°C. DNA-histone H2A, H2B, H3 and H4 complex showed a multiphasic

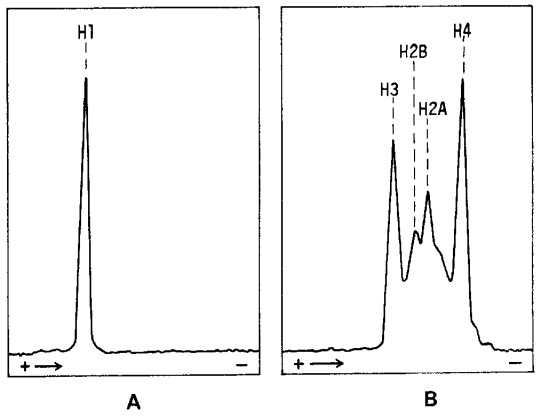

Fig. 1. Microdensitometer tracings of gel electrophoretic patterns of calf thymus histone fractions. The gel electrophoresis was carried out on 15% polyacrylamide gels containing 2.5 M urea and 0.9 M acetic acid, pH 2.7, at 1.5 mA/gel for 3 hr. (A) Histone fraction used for the reconstitution of DNA-histone H1 complex; (B) Histone fraction used for the reconstitution of DNA-histone H2A, H2B, H3 and H4 complex.

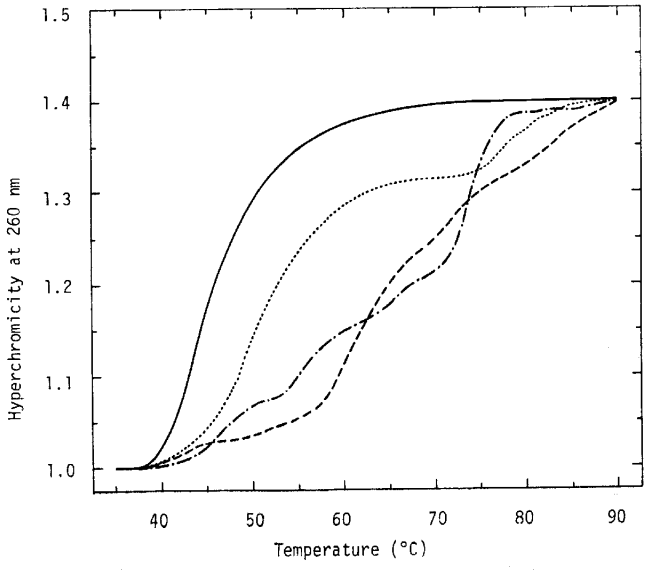

Fig. 2. Thermal denaturation spectra of free DNA and reconstituted DNA-histone complexes. The samples in 0.25 M EDTA, pH 8.0, were heated at the rate of 0.5°C per min. DNA (——), DNA-histone H1 complex (·····), DNA-histone H2A, H2B, H3 and H4 complex (- - -) and DNA-whole histone complex (-·-·-).

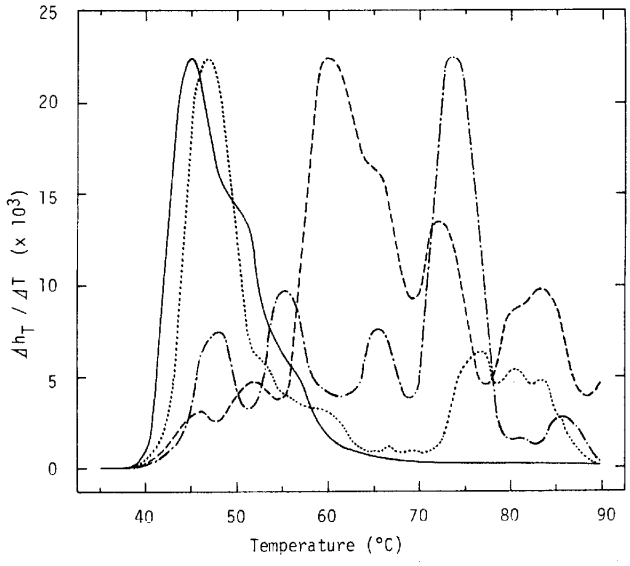

Fig. 3. Derivative melting profiles of free DNA and reconstituted DNA-histone complexes. Conditions for melting and line graphic designations are the same as those in Fig. 2. The derivative of hyperchromicity (Δh) at temperature T was calculated from the thermal denaturation spectra in Fig. 2 by the equation,

$$\Delta h_T/\Delta T = (h_{T-2} - 4h_{T-1} + 3h_T)/2,$$

where coefficients 4 and 3 are mathematical weights.

melting profile; Tm at 61°C, 65°C, 72°C and 80°-83°C, and very small amount of melting at Tm 45°C and 52°C. This profile is compatible with that of the polynucleosome structure of chromatin. DNA-whole histone complex also showed a multiphasic melting profile; a large amount of melting at Tm 74°C, a medium amount at Tm 48°C, 55°C and 65°C, and a very small amount at Tm 86°C. This profile is consistent with that of native chromatin. These data indicate significant differences in melting profiles between these complexes, suggesting that DNA-histone H1 complex and DNA-histone H2A, H2B, H3 and H4 complex greatly differ in binding characteristics.

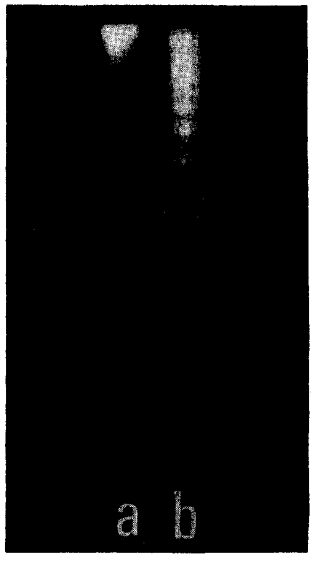

Fig. 4. Polyacrylamide gel electrophoretic patterns of DNA fragments generated by staphylococcal nuclease limit digestion of rat liver chromatin (a) and reconstituted DNA-histone H2A, H2B, H3 and H4 complex (b).

Electrophoretic analysis of DNA fragments gen-

erated by nuclease limit digestion. Polyacrylamide gel electrophoretic patterns of DNA fragments generated by limit digestion with staphylococcal nuclease of rat liver chromatin and of DNA-histone H2A, H2B, H3 and H4 complex are shown in Fig. 4. Regular periodic patterns are observed similarly in both native chromatin and reconstituted DNA-histone complex.

Transcription on reconstituted DNA-histone complexes

(a) *Template activity of DNA-histone complexes.* Template dose responsibility for transcription under the present conditions was first examined on free DNA, DNA-histone H1 complex, and DNA-histone H2A, H2B, H3 and H4 complex using 1 unit of *E. coli* RNA polymerase (Fig. 5). [^{3}H]UMP incorporated into

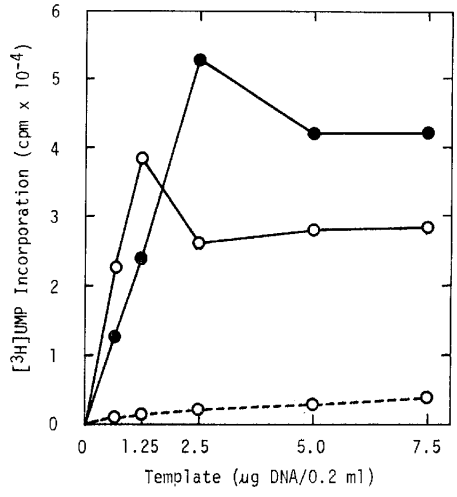

Fig. 5. Template saturation curves of free DNA and DNA-histone complexes. RNA synthesis was carried out with free DNA (○——○), DNA-histone H1 complex (○- - -○) and DNA-histone H2A, H2B, H3 and H4 complex (●——●) as templates and with one unit of RNA polymerase in 0.2 ml of reaction medium.

RNA was proportional to the amount of DNA in every template up to 1.25 μg. The template activity of free DNA was saturated with more than 1.25 μg of DNA and the template activity of DNA-histone H2A, H2B, H3 and H4 complex was saturated with more than 2.5 μg of DNA. [^{3}H]UMP incorporation on DNA-histone H2A, H2B, H3 and H4 was about 50–60% of incorporation on free DNA before saturation, but it exceeded incorporation on free DNA after saturation. Hence thereafter, less than 1 μg of template DNA was used for template activity assay with 1 unit of RNA polymerase. Template activity of DNA-histone H1 complex was less than 10% of that of free DNA.

(b) *Initiation and elongation reaction of transcription on DNA-histone complexes.* To distinguish the reaction between the initiation step of transcription and sub-

sequent chain elongation the technique of Hyman and Davidson (27) was employed. This method has already been used successfully to measure the number of initiation sites of *E. coli* and mammalian RNA polymerases on DNA and chromatin (28, 29). Initiation is carried out in low salt, using only three nucleoside triphosphates by omitting CTP. This results in the binding of RNA polymerase to the template probably accompanied by a small amount of oligonucleotide synthesis which requires no CTP. A part of the RNA synthesis may be due to contaminating concentrations of CTP, as indicated by Cedar and Felsenfeld (28, 29). The results of the initiation carried out on free DNA, DNA-histone H1 complex, and DNA-histone H2A, H2B, H3 and H4 complex are shown to the left of the arrow in Fig. 6. [^{3}H]UMP incorporation in the initiation reaction on DNA-histone H1 complex was 8–10% of that on free DNA, and [^{3}H]UMP incorporation on DNA-histone H2A, H2B, H3 and H4 complex was 50–65% of

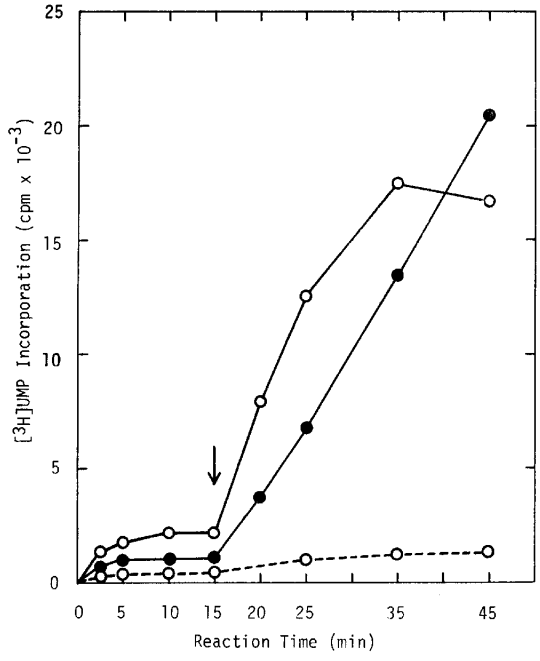

Fig. 6. Incorporation of [^{3}H]UMP in chain elongation reaction in the high salt medium after initiation reaction in the low salt medium using free DNA and DNA-histone complexes as templates. Reactions were carried out with free DNA (○——○), DNA-histone H1 complex (○- - -○), and DNA-histone H2A, H2B, H3 and H4 complex (●——●) as templates and with one unit of RNA polymerase in 1.0 ml of reaction medium. The initiation reaction was performed in the low salt medium by omitting CTP. The chain elongation reaction without reinitiation was started after 15 min, as indicated by the arrow, in the high salt medium by adding CTP.

that on free DNA. The initiation reactions were saturated within 5–10 min. As indicated by Cedar (29), when the reaction mixture is 0.4 M in $(NH_4)_2SO_4$, no further binding of RNA polymerase molecules occurs, but RNA polymerase molecules that have already initiated RNA chains remain bound and are able to make long chains when CTP is added (27–29). The results of chain elongation after 15 min of initiation reaction are shown to the right of the arrow in Fig. 6. [^{3}H]UMP incorporation in the elongation reaction on free DNA proceeded rapidly and declined gradually after 5 to 10 min and stopped after 20 min. [^{3}H]UMP incorporation on DNA-histone H2A, H2B, H3 and H4 complex was about 50–65% of that on free DNA at the initial stage but proceeded linearly for a long time (40–60 min) and exceeded that on free DNA in the later stage. [^{3}H]UMP incorporation on DNA-histone H1 was less than 10% of that on free DNA throughout the reaction. These data suggest that histone H1 may have a strong affinity to bind to the specific sites of the template and specifically inhibit the initiation, while the other four histones H2A, H2B, H3 and H4 may bind nonspecifically to the template, which results in only partial inhibition of the

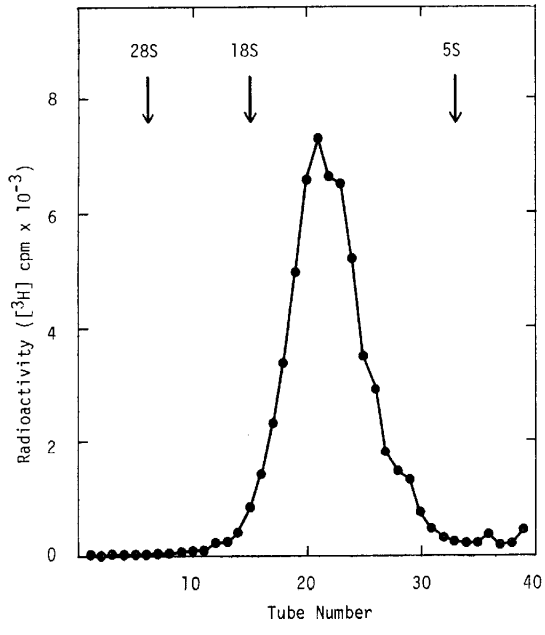

Fig. 7. Sucrose density gradient sedimentation pattern of [^{3}H]-RNA transcribed on free DNA *in vitro*. The conditions of RNA synthesis are described in the section on methods. Samples of 0.5 ml, each containing 0.5% sodium dodecyl sulfate, were layered on 4.5 ml of linear gradient of 10% to 40% sucrose (w/v) in 10 mM KCl-10 mM Tris-HCl buffer, pH 7.6, and centrifuged at 80,000×g for 16 hr at 20°C. Rat liver ribosomal RNA was used as the marker in the same tube.

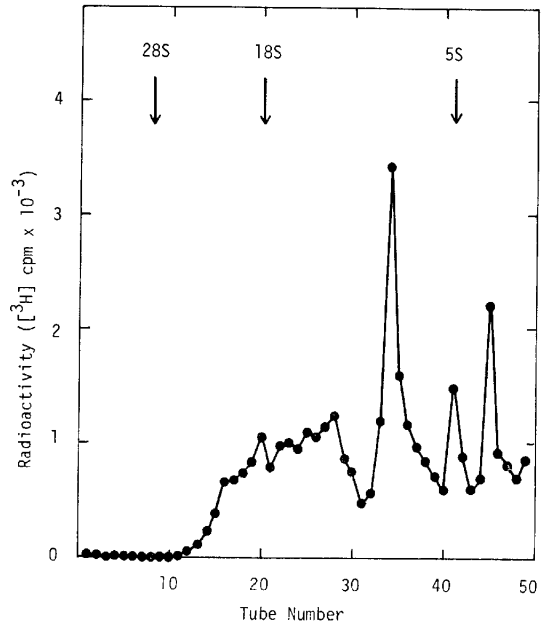

Fig. 8. Sucrose density gradient sedimentation pattern of [^{3}H]-RNA transcribed on the DNA-histone H2A, H2B, H3 and H4 complex *in vitro*. The conditions of RNA synthesis are given in the section on methods. The conditions of sucrose density gradient sedimentation are the same as those in Fig. 7.

initiation and chain elongation. The rather longer duration of chain elongation may be due to the stabilization of template structure by binding of these four histones.

Sucrose density gradient analysis of RNA transcribed on reconstituted DNA-histone complexes. Sucrose density gradient analysis was performed to examine the size of RNAs transcribed on free DNA and on DNA-histone H2A, H2B, H3 and H1 complex. Figs. 7 and 8 show their sedimentation profiles after 60 min reaction. The size of RNA synthesized from free DNA showed a broad symmetrical distribution with a peak around 10–16 S (Fig. 7), while the size of RNA synthesized from DNA-histone H2A, H2B, H3 and H4 complex showed a heterogeneous distribution with several peaks around 20–16 S, 10 S, 6–5 S and 4–2 S (Fig. 8). The contribution of larger size RNA was greater in RNA synthesized from DNA-histone H2A, H2B, H3 and H4 complex than in RNA synthesized from free DNA.

DISCUSSION

In the present communication, we have presented the profiles of thermal denaturation and template activities of the reconstituted complexes of DNA with

histone H1 and with the combinations of the other four histones H2A, H2B, H3 and H4. For the reconstitution of DNA with histones we used salt-and-urea step-gradient dialysis. The use of urea might be detrimental in reconstituting native complexes (12, 30, 31) and removing urea initially during dialysis gave better reconstitution of the subunit structure of chromatin than removing salt initially, but the presence of urea during dialysis actually served to prevent the aggregation of histones and reconstituted DNA-histone complexes. Nuclease limit digestion pattern of reconstituted DNA-histone H2A, H2B, H3, H4 complex was similar to that of native chromatin. It seemed important to keep the reconstituted complexes in a soluble or dispersed state for analyzing template activity. Furthermore, we have purified the reconstituted DNA-histone complexes by removing unbound histone through Bio-Gel column, and calculated the ratio of the amount of DNA to the amount of histones in the reconstituted complexes. The weight ratio of histone-to-DNA in the DNA-histone complexes increased by using the higher initial weight ratios of histone-to-DNA for recombination. Although analysis of DNA-histone complexes with various combinations of weight ratios is desirable, we have only analyzed the DNA-histone complexes with the initial weight ratio of one-to-one in the present study. Thermal denaturation profiles of the reconstituted DNA-histone complexes suggest that the affinity and patterns of binding of histone H1 to DNA are markedly different from those of the other four histones. In native chromatin, histone H1 has generally been considered to be bound to the DNA strand between nucleosomes and to be involved in the coiling or folding of nucleosome chains.

The analysis of the initiation reaction and chain elongation of transcription revealed that the binding of histone H1 to DNA strongly inhibited the initiation, and that the binding of the combination of the other four histones to DNA partially inhibited the initiation and chain elongation. This chain elongation proceeded linearly during prolonged periods of reaction time and exceeded the chain elongation on free DNA in the later stage. These findings on the transcription of reconstituted DNA-histone complexes are compatible with the data reported by Georgiev (10) on the transcription of histone H1-depleted chromatin and with the data reported by Barr and Butler (9) on the transcription of histone H1-DNA complex, H2A-DNA complex, H2B-DNA complex and H4-DNA complex. Histone H1 may also inhibit transcription by facilitating the association or condensation of chromatin fibers, thus preventing movement of RNA polymerase along the DNA. On the other hand, the histone H2A, H2B, H3 and H4 complex binds to DNA irrespective of base sequences and may result in nonspecific partial inhibition of transcription but does not block transcription. Recent neutron diffraction studies indicate that DNA is wound in a superhelix around the outside of the octameric protein core which consists of the four his-

tones H2A, H2B, H3 and H4 (32-34). Thus, it is conceivable that these four histones play an important role in the supercoiling of DNA and in preserving the structure and function of chromatin.

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