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The presence of RNA in a double helix inhibits its interaction with histone protein

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ABSTRACT

The binding of core histones (H2A, H2B, H3, H4) to a circular plasmid DNA and to a circular DNA-RNA hybrid molecule of similar size has been compared. Circular hybrid molecules were formed from single stranded fd DNA by synthesis of the complimentary strand with ribonucleotides using wheat germ RNA polymerase II. Upon reconstitution of plasmid DNA circles with histone, the sedimentation profiles of the DNA remained sharp by increased several fold in rate. Material from the peak fractions of these sedimentations appeared to be condensed circular loops of nucleosomes when examined by electron microscopy (EM), and the mass ratio of DNA to histone (at the histone concentrations which produced the fastest sedimentations) was typical of native chromatin. In contrast, the sedimentation behavior of DNA-RNA hybrid circles after addition of histone remained unchanged except for a minor fraction which exhibited a broad and faster sedimentation rate. Examination by EM revealed that most of the molecules appeared identical to protein free hybrid circles while the minor, faster sedimenting fraction appeared to be two or more circles bound together by protein aggregates. Finally, a linear molecule consisting of about 3000 base pairs of duplex DNA covalently joined on both ends to 1500 base pairs of RNA-DNA hybrid helix was constructed. Reconstitution of this molecule with core histone showed nucleosome formation only on the central DNA duplex region. Isopycnic banding of fixed hybrid-histone mixtures showed that little or no histone had bound to the bulk of the full hybrid molecules. We suggest that the presence of RNA in a nucleic acid duplex inhibits the condensation of the duplex into a nucleosomal structure by histone.

INTRODUCTION

RNA base paired to DNA occurs *in vivo* near replication forks where RNA primers are formed (1) and is thought to exist at transcription sites if nascent RNA remains transiently in an RNA-DNA hybrid (2). With many questions to be answered concerning the arrangement of nucleosomes near points of replication and transcription, a comparison of the interaction of histone protein with DNA-RNA-hybrid and duplex DNA molecules should prove valuable. One reason to believe that histones might interact differently with these two double helical forms is that the helix parameters of hybrid molecules are of the A form and are significantly different from

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that of B form DNA (3,4). If nucleosome formation does depend on the precise alignment of certain elements of structure on the histone core and the DNA helix, as current models suggest (5,6), and if DNA in nucleosomes is in a B family structure, DNA-RNA hybrids might not be able to form these contacts. To investigate this we have used the in vitro chromatin reconstitution system of Germond et. al. (7) in which histones are mixed with DNA in 2M NaCl and the salt concentration lowered step-wise to 250mM. The association of purified core histones with 6400 base pair DNA-RNA hybrid molecules and with a similar sized duplex DNA was thus examined. Whereas nucleosome formation was observed on duplex DNA by EM and sedimentation analysis, no such interaction of histones with the hybrid molecules was detected. When a linear duplex consisting of roughly 3000 base pairs of DNA-DNA helix flanked on both ends by 1500 base pairs of RNA-DNA helix was reconstituted with core histone, nucleosome formation was observed only on the central duplex DNA portion. The implications of these findings are discussed.

MATERIALS AND METHODS

Preparation of enzymes, plasmid DNA, and DNA-RNA hybrid substrates

Supertwisted plasmid DNA (^{3}H thymidine labeled) was prepared from Escherichia coli (E. coli) JC411 carrying the col.E_1 plasmid by the method of Modrich and Zabel (8) and relaxed with pancreatic DNase in a solution of ethidium bromide as described by Greenfield et. al. (9). Phage fd (the gift of Dr. R. Webster) were grown in E. coli K37 and purified by the procedure of Makino et. al. (10). Exo_{III} was prepared by the procedure of Roger and Weiss (11). RNA $polymerase_{II}$ from wheat germ was prepared according to Jendrisak et. al. (12).

fd DNA-RNA hybrids were prepared by incubating fd single stranded DNA (10 μg/ml) with wheat germ RNA $polymerase_{II}$ (3.4 units/ml, from Worthington Biochemicals) and all 4 ribonucleotide triphosphates (1mM each) in the following buffer: 0.2M Tris HCl pH 7.9, 0.02M $MgCl_2$, 1mM $MnCl_2$, 0.01M 2-mercaptoethanol, and 0.05M NaCl. Following incubation at 37° for 6 hrs the volume was increased to 6 ml with 0.01M Tris HCl pH 8.0, 1mM ethylenediamine-tetraacetic acid (EDTA) and 0.5% sarcosyl. Solid Cs_2SO_4 was added to a final density of 1.51 and the sample centrifuged 48 hrs, 34,000 rpm at 20° in a type 40 rotor. Peak fractions were dialyzed against 0.01M Tris HCl pH 8.0, 1mM EDTA overnight, precipitated with ethanol and resuspended in a small volume.

Col. E_1 heterohelices were prepared by digestion of 10 μg of linear

DNA (prepared by Eco R_1 treatment) with 420 units of Exo_{111} at a DNA concentration of 40 μg/ml using the digestion buffer described by Roger and Weiss (11). Following incubation for 55 min at 28°, phenol extraction and ethanol precipitation, the single stranded ends were filled in with RNA using the conditions described above.

Preparation of core histones

Core histones were prepared from calf thymus or chicken erythrocyte cells by modification of the method of Germond et. al. (7). Briefly, purified nuclei were lysed and the chromatin pellet washed with 0.8M NaCl to remove histones H1 and H5. After centrifugation into a cushion of 2.2M sucrose the chromatin depleted of histone H1-H5 was resuspended in a buffer containing 2M NaCl, sonicated and passed over a hydroxyapetite column equilibrated with the same buffer. Peak histone fractions which showed an absence of histone H1 and H5 but the normal patterns for the 4 core histones on polyacrylamide stacking gels (13) were used in the reconstitution experiments.

Sedimentation and cesium salt banding of reconstituted complexes

Aliquots (200 μl) of the reconstituted complexes were layered over 5%-20% sucrose gradients containing 0.01M Tris HCl pH 8.0, 1mM EDTA 0.01M NaCl and centrifuged 90 min, 4° at 50,000 rpm in an SW 60 rotor. For the purpose of isopycnic banding, reconstituted complexes were concentrated by centrifugation into 30% glycerol, diluted 2-fold and fixed with formaldehyde and glutaraldehyde as described previously (14). Following fixation the complexes were layered over preformed CsCl (ρ = 1.35 to 1.55) or Cs_2SO_4 (ρ = 1.30 to 1.60) gradients and centrifuged 15 hrs at 35,000 rpm in a type 40 rotor.

RESULTS

Preparation and characterization of fd DNA-RNA hybrid molecules

fd DNA-RNA hybrid molecules were prepared as described above. Following synthesis of the complimentary RNA strand, the density of the DNA in Cs_2SO_4 equilibrium density bandings shifted as a discrete peak from 1.44 to 1.51, the established density of full DNA-RNA hybrid molecules under these conditions (15), (Fig. 1). When examined by EM, these molecules appeared as relaxed duplex circles with no visible discontinuities, similar to that reported earlier (Fig. 1C in ref. [16]. Following treatment of the hybrids with endonuclease S_1, a population of relaxed circles and full length linear molecules was seen suggesting that the complimentary RNA

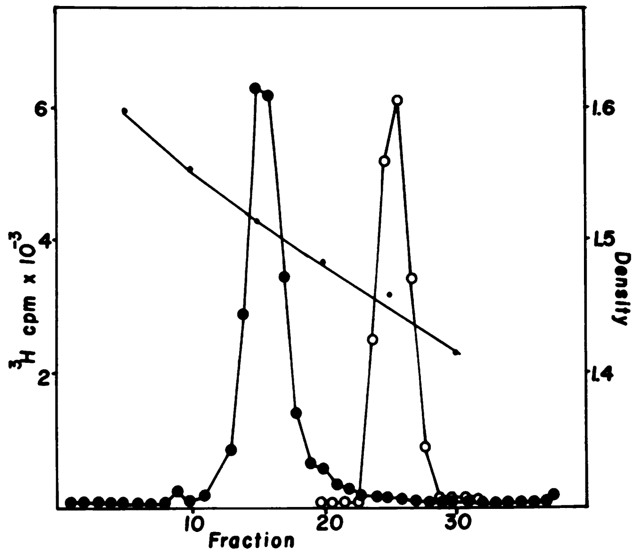

Figure 1. Equilibrium density Cs_2SO_4 banding of single stranded fd DNA (o) or fd DNA-RNA hybrids following synthesis of the complimentary RNA strand (•). The DNA component was labeled with ^{3}H thymidine (see above). Samples were run in separate but parallel gradients and the profiles superimposed for the plot above. The line indicates density as determined by refractive index.

strands contained few S_1 sensitive gaps or nicks. Indeed, RNA polymerase$_{II}$ is known to synthesize long RNA transcripts on single stranded DNA unlike the E. coli RNA polymerase which generates much shorter RNAs (17).

Single stranded fd DNA was chosen for these experiments because it showed a much more efficient conversion to full hybrid molecules than ϕX174 or G4 single stranded DNAs. Plasmid col.E_1 DNA was used for comparison due to its similar size and ease of isolation.

Sedimentation analysis of reconstituted histone-DNA and histone-hybrid complexes

The addition of histone to DNA results in the formation of nucleosomes and a compact fast sedimenting complex (7). To study the interaction of histone with DNA-RNA hybrid molecules it was necessary for us to demonstrate that our preparations of core histones would form nucleosomes on a similar sized, relaxed duplex DNA molecule. Using the *in vitro* reconstitution system of Germond *et. al.* (7), relaxed col.E_1 DNA was mixed with varying amounts of purified core histones in 2M NaCl and the salt lowered by a

series of step-wise dilutions to a final concentration of 250mM. Aliquots of the reconstitution mixture were layered over 5% to 20% sucrose gradients and sedimented as described above. As shown in Fig. 2 A-D, upon addition of increasing amounts of core histone the col.E_1 DNA was found in single discrete peaks having progressively greater sedimentation rates. At high concentration of histone the DNA was found pelleted to the bottom of the

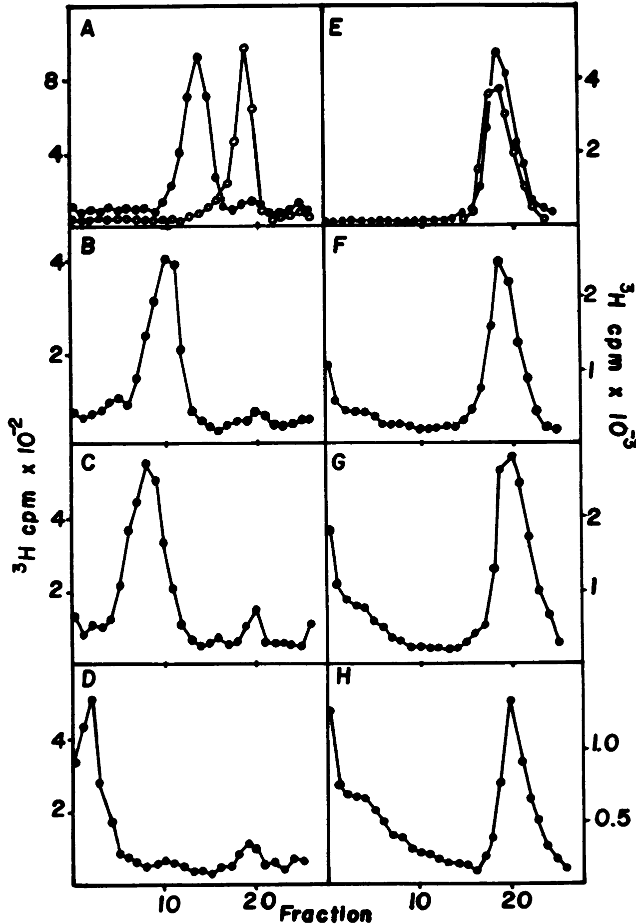

Figure 2. Sucrose velocity sedimentation of reconstituted histone-DNA (A-D) or histone-hybrid (E-H) complexes at core histone to DNA or hybrid ratios of 1:1 (A,E), 1.5:1 (B,F), 2:1 (C,G) or 2.5:1 (D,H). Following step-wise dilution of histone-nucleic acid-salt mixtures, aliquots were layered directly over sucrose gradients and centrifuged as described above. (•) Sedimentation was right to left as shown. DNA was labeled with ^{3}H thymidine. (o) denotes sedimentation of naked DNA or hybrid molecules.

gradient. These results are consistent with the formation of nucleosomes, and buttressed by further evidence cited below, show that these histone preparations were capable of nucleosome formation in vitro.

Reconstitution of core histones with fd DNA-RNA hybrid molecules under otherwise identical conditions yielded different results. In numerous experiments addition of even high concentrations of histone failed to shift the sedimentation rate of much of the hybrid molecules from that of the control (Fig. 2 E-G). The remaining hybrid was found in a continuum of faster sedimenting structures whose non-condensed nature was to be elucidated by EM. Thus, no evidence was found for the formation of a regularly compacted structure after reconstitution of histone and DNA-RNA hybrid molecules under conditions where such structures routinely formed with duplex DNA. Direct EM was applied to further characterize the hybrid molecules in these reconstitution experiments.

Electron microscopy of reconstituted histone-DNA and histone-hybrid complexes

Aliquots of reconstituted histone-DNA or histone-hybrid complexes were taken from sucrose gradients as in Fig. 2, fixed, and prepared for EM by methods we have described elsewhere (18). As shown in Fig. 3 a-d peak material from the sedimentations of reconstituted histone-DNA appeared as open circular chains of nucleosomes. The number of nucleosomes per loop appeared to be greater at higher histone concentrations and the contour length of the loops, coordinately shorter. The highest histone concentrations (2.5:1, histone to DNA) yielded compact structures much like native SV40 minichromosomes in appearance (19).

In contrast, histone-hybrid complexes taken from the sedimentation peaks (fractions 20-25, Fig. 2 E-G) were indistinguishable by EM from hybrid circles examined prior to reconstitution (Fig. 4a). Hybrid complexes taken from the faster sedimenting regions (fractions 1-10, Fig. 2 E-G) appeared as several non-compacted circular molecules bound together by large protein-like aggregates (Fig. 4b). Together with the sedimentation data these results argue that nucleosomes will not form on RNA-DNA helices alone. However it is possible that nucleosomes present on regions of duplex DNA could migrate to adjacent regions of RNA-DNA hybrid helix and remain stably associated. To test this possibility a heterohelix was constructed.

Linear col.E_1 DNA was treated with Exo.$_{111}$ under conditions established in this laboratory for synchronous digestion. Contour length measurements [Griffith (19)] of the digested col.E_1 DNA showed that 50 ± 9% remained as

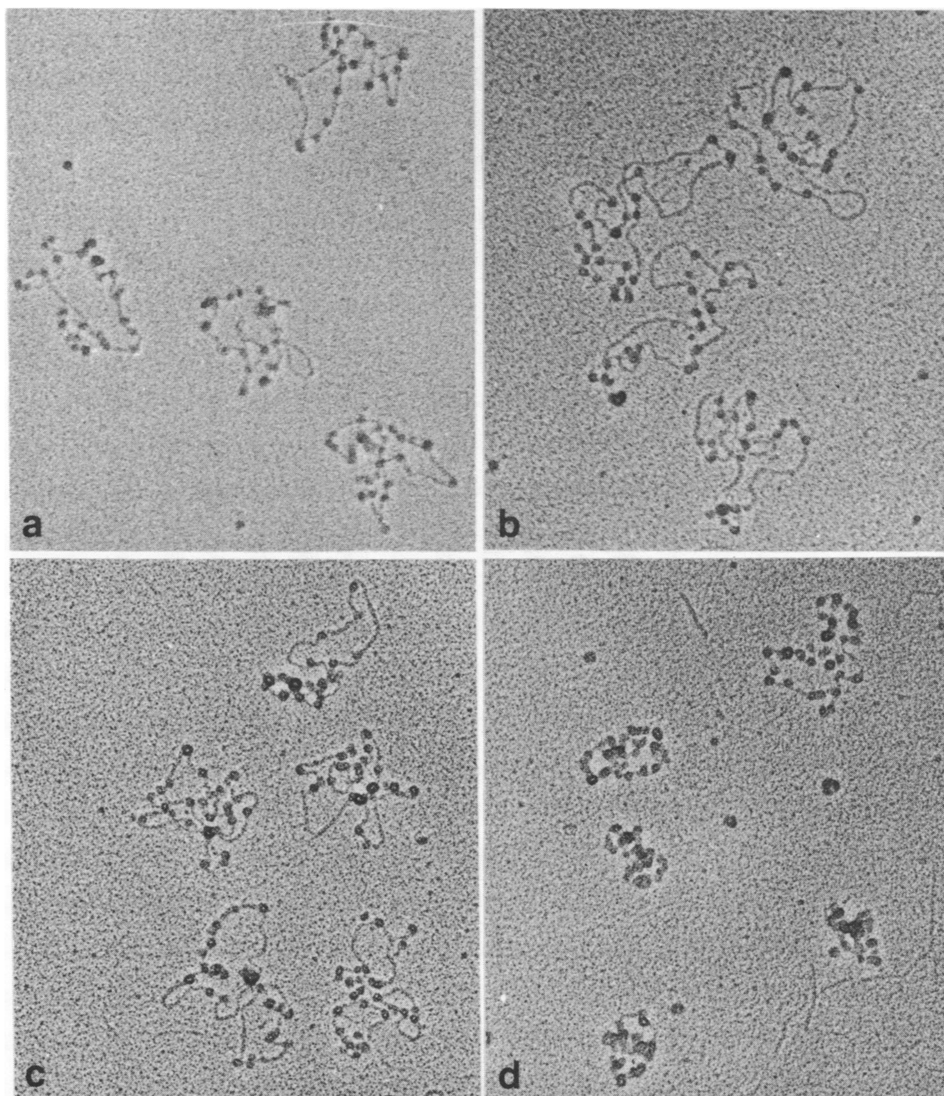

Figure 3. EM visualization of reconstituted histone-DNA complexes. Core histones were reconstituted with col.E_1 DNA circles at histone:DNA ratios of (a) 1:1, (b) 1.5:1, (c) 2:1, (d) 2.5:1 and sedimented in sucrose gradients as in Fig. 2. Peak fractions were fixed and examined by direct mounting with spermidine (18) on thin carbon supports by tungsten rotary shadowing. Bar equals 1 micron.

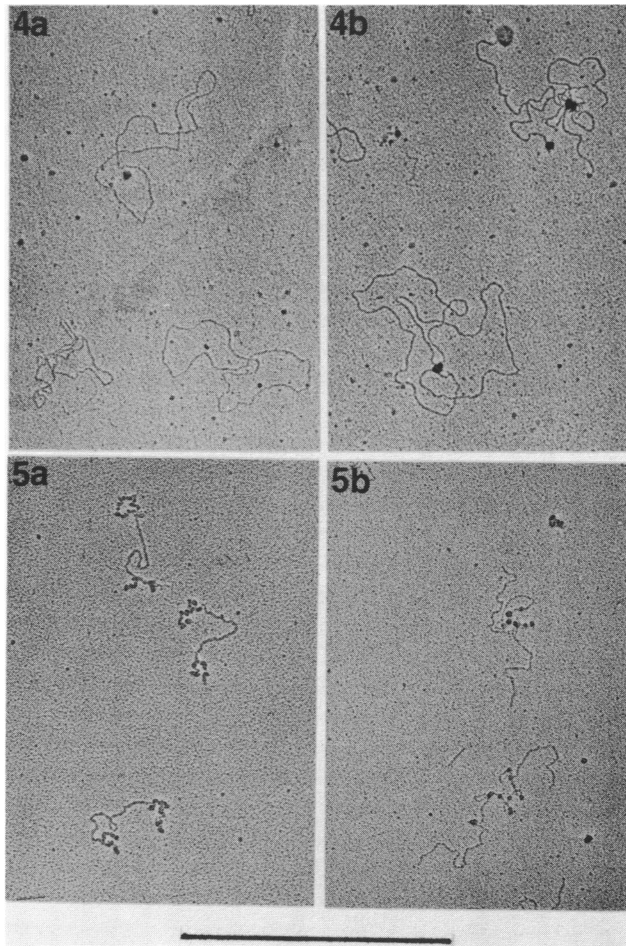

Figure 4. EM visualization of reconstituted histone-hybrid complexes. Fractions from sucrose gradients as in Fig. 2 E-H were fixed and prepared for EM as in Fig. 3. (a) corresponds to material about fraction 20, Fig. 2 (b) corresponds to material typical of fractions 1-10, Fig. 2. Bar equals 1 micron.

Figure 5. Preparation of col.E_1 heterohelix and its reconstitution with histone. Linear col.E_1 DNA molecules were digested with Exo.$_{III}$ such that 25 ± 5% of each end was single stranded. Visualized here following addition of single strand binding protein, the single stranded ends appear thicker (and foreshortened 2 to 3X) relative to the central duplex region (a). Following synthesis of an RNA compliment on the single stranded ends, reconstitution with histone (see above) yielded complexes with nucleosomes only on the central DNA duplex regions (b). In 19 molecules as shown here the mean length from each end to the first nucleosome was 0.40 ± 0.06 μ (with 9 ± 2 nucleosomes), somewhat greater than the mean length of each hybrid arm (0.38±0.02μ).

duplex DNA, the other 50% being single stranded DNA equally divided between both ends. This is illustrated in Figure 5a where the single stranded ends are stained with single stranded DNA binding protein (the gift of Dr. Arthur Kornberg). Following incubation of these molecules with RNA polymerase[11] the length was restored to within 99% of that expected for such a heterohelix. These heterohelices and linear col.E_1 DNA were reconstituted with core histone under conditions described above. Whereas linear col.E_1 DNA showed nucleosomes packed end-to-end, (data not shown) nucleosomes were found only on the central 50% of the heterohelices, the hybrid arms remaining nucleosome-free (Fig. 5b).

Isopycnic density banding of histone-DNA and histone-hybrid complexes

Although our results argue against nucleosome formation on DNA-RNA hybrid structures (under the reconstitution conditions we have used) we cannot rule out the possibility that histone bound to the hybrid molecules in some other manner, as for example, in a smooth thin sheath about the DNA-RNA duplex. To investigate this possibility we examined the composition of reconstituted histone-DNA and histone-hybrid complexes.

Reconstituted histone-DNA and fd histone-hybrid complexes were separated from any free histone by sedimentation into glycerol, fixed with formaldehyde and glutaraldehyde and banded in CsCl or Cs_2SO_4 as described above. As shown in Fig. 6a, histone-DNA complexes formed at histone to DNA ratios of 2:1 were found (after fixation and banding) in a single sharp peak at a density of 1.44 in CsCl, a density typical of native chromatin (20). In contrast after reconstitution of histone with full (fd) hybrid molecules at a ratio of 2:1, fixation, and banding, the bulk of the molecules remained at the density of protein-free fd hybrid; the remainder was spread broadly across lighter densities (Fig. 6b). The ratio of the two species was very similar to the ratio of the fractions which ran either slowly or faster in the sedimentation studies.

DISCUSSION

In this study we compared the ability of DNA-RNA hybrid with the ability of duplex DNA molecules to interact with core histones, using a common chromatin reconstitution system. Upon addition of histone to duplex DNA circles, discrete physical changes typical of nucleosome formation were detected. The sedimentation profiles of the DNA remained sharp but increased several fold in rate with added histone. When examined by EM the DNA-histone complexes from these sedimentations appeared as condensed loops of

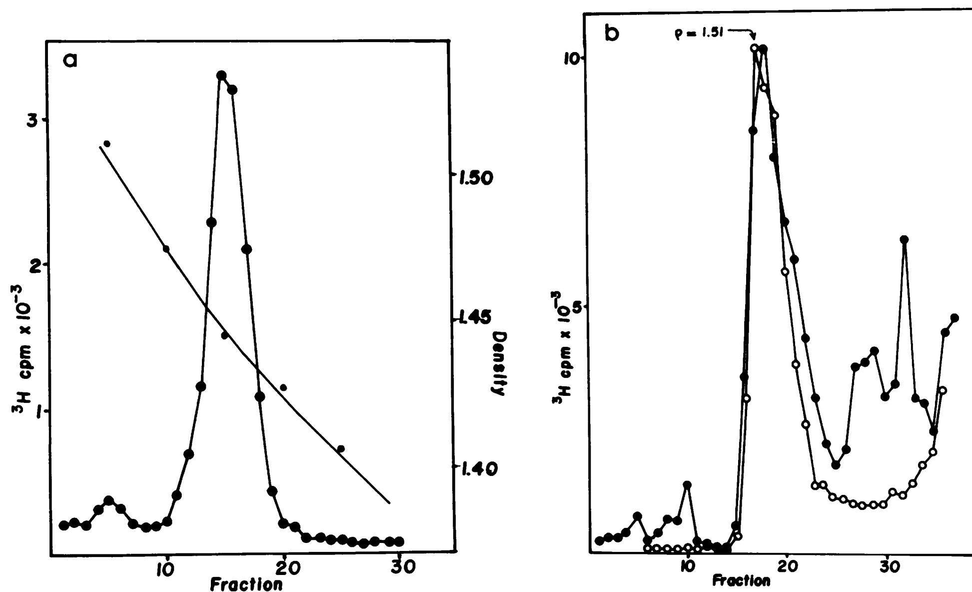

Figure 6. Equilibrium density banding of fixed histone-DNA (a) or histone-hybrid (b) complexes after reconstitution, sedimentation into 30% glycerol, and fixation. Histone to DNA or hybrid ratio used was 2.0:1. Histone-DNA complexes were banded in CsCl and histone-hybrids in Cs_2SO_4 solutions as described above. In (b) the banding pattern of hybrid alone (not reconstituted with histone) from a parallel gradient is shown superimposed (o). DNA was labelled with ^{3}H thymidine.

nucleosomes, and the mass ratio of DNA to protein (at the histone concentrations which produced the fastest sedimentations) was typical of native chromatin as determined by density banding. In contrast, no such changes were observed when histone was added to similar sized circular DNA-RNA hybrid molecules under identical reconstitution conditions. The sedimentation rate of the hybrid molecules remained slow and unchanged with added histone except for a minor fraction which exhibited a broad and faster sedimentation. Examination by EM showed the slow sedimenting material to appear as fully extended duplex loops, identical in appearance to protein-free hybrid circles. The faster sedimenting minor fraction was composed of two or more hybrid circles bound together by large protein aggregates. Finally, when a linear duplex consisting of about 3000 base pairs of duplex DNA flanked by 1500 base pairs of RNA-DNA hybrid helix on both ends was reconstituted with core histone, nucleosomes were seen only on the central duplex region. Isopycnic banding of fixed histone-hybrid mixtures showed that little

or no histone had bound to the bulk of the hybrid molecules. A portion did show a dispersed shift to lighter densities and this fraction appeared to correspond to the fraction of hybrid molecules in multimolecular aggregates.

RNA-DNA hybrid helices, like duplex DNA, carry a large negative charge density while histone cores carry a strong positive charge. We were surprised, therefore, at the absence of any stable complex formation between hybrids and histones at salt concentrations (250mM) far below the range (0.8-1.0M) where nucleosomes form on duplex DNA. This might suggest that the stability of the nucleosome depends on interactions between certain elements of structure on DNA and on histones over an extended distance on the octamer surface and that these interactions are much less favored with the hybrids due to their different helical structure. Indeed, Simpson and Kunzler (21) have shown that the complimentary homopolymers poly(dA)(dT) and poly(dG)(dC) will not reconstitute nucleosomes with added histone and they noted that the latter homopolymer forms A-like structures more readily than other polymers they studied. Alternatively, the primary structure of the hybrid may also influence the formation of stable nucleosomes. Possibly too, we might have found evidence for complex formation under other ionic conditions or isolation procedures, or if we had employed chromatin assembly factors such as those described by Laskey et. al. (22) and Nelson et. al. (23). Even so, our results argue that any nucleosome-like structure that might form between DNA-RNA hybrids and histone would be much less stable than the usual nucleosome formed on duplex DNA. Furthermore, our reconstitutions of histone with the heterohelices argued that nucleosomes which have formed on a DNA duplex region will not move easily into adjacent segments of RNA-DNA hybrid nature. This experiment also served to buttress the conclusions derived from reconstitutions of histone with col.E_1 DNA and fd hybrid circles where the reconstitutions were carried out in separate reaction mixtures.

We are interested in the manner in which RNA might influence the structure of chromatin. Because RNA is covalently joined to DNA at replication forks and possibly base paired with DNA during transcription, we have studied the in vitro interaction of DNA-RNA hybrids with histone. Until the precise architecture of replication forks and transcription complexes are elucidated, we cannot conclude what role if any DNA-RNA hybrid structures play in these dynamic processes. RNA primers at replication forks would seem to be spaced too far apart from the fork itself to be able to play a direct role in opening up the nucleosomal structure ahead of the moving fork. However, if DNA-

RNA hybrid structures do exist at transcription sites due to transiently base paired RNA (2), this might provide a mechanism for destabilizing the nucleosomal structure about the moving RNA polymerase and thus facilitate the movement of the transcription complex.

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