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Nucleosomes will not form on double-stranded RNA or over poly(dA)·poly(dT) tracts in recombinant DNA

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ABSTRACT

We have been unable to "force" double-stranded RNA to fold into nucleosome-like structures using several different histone-RNA "reconstitution" procedures. Even if the histones are first stabilized in octameric form by dimethylsuberimidate cross-linking they are still unable to form specific complexes with the RNA. Moreover double-stranded RNA is unable to induce histones to assemble into octamers although we confirm that the non-nucleic acid homopolymer, polyglutamic acid, has this ability. We have also determined, using pyrimidine tract analysis, that nucleosomes will not form over a sufficiently long segment of poly(dA)·poly(dT) in a recombinant DNA molecule. Thus nucleosomes cannot fold DNA containing an 80 base pair poly(dA)·poly(dT) segment but a 20 base pair segment can be accommodated in nucleosomes fairly well. Segments of intermediate length can be accommodated but are clearly selected against. Poly(dA)·poly(dT) differs only slightly from natural DNA in helix structure. Therefore either this homopolymer resists folding, or nucleosomes are very exacting in the nucleic acid steric parameters they will tolerate. Such constraints may be relevant to nucleosome positioning in chromatin.

INTRODUCTION

Recently there has been an increasing awareness of the non-uniformity of double-helical structure in nucleic acids of different sequences.[1-8] The possible physiological significance of sequence dependent variations in structure along a DNA molecule in vivo has attracted considerable attention.[9-13] We have been interested in the nature of DNA-histone interactions in nucleosomes[14] and the possible role of DNA sequence in specifying nucleosome position.[15] In this report we show that nucleic acids with double-helical parameters different from those of "average" DNA cannot form nucleosome-like complexes with histones. Moreover, one of these, poly(dA)·poly(dT), prevents nucleosome formation even in cloned form as part of a natural DNA.

The basic features of nucleosome structure are now well established.[16,17] A central histone core constrains 168 base pairs of DNA[18] into two superhelical turns[17] wound over the protein surface. The DNA appears to be fixed firmly

to the protein core at specific positions along its length which act as temporary barriers to nuclease digestion of nucleosomes.[17,19] The familiar "core particle", which contains 146 base pairs of DNA, is generated from nucleosomes by digestion of the DNA to the first of these symmetrically disposed binding locations.

In order to determine whether stable histone-DNA interactions may depend upon specific aspects of DNA double-helical structure several attempts have been made to assemble histones with various "non-typical" nucleic acids. Three, DNA-RNA hybrid, poly(dA)·poly(dT) and poly(dG)·poly(dC), were found not to support nucleosome formation.[20-23] These results have raised important practical and theoretical questions. For example, the attempts to assemble histone with DNA-RNA hybrid[21] were carried out using a simple step-wise dilution protocol. Conceivably the optimal experimental parameters for histone-hybrid assembly differ from those for DNA, thus formally leaving open the basic question of whether DNA-RNA hybrids can form nucleosomes. A similar kind of experimental uncertainty was raised concerning the attempts to assemble poly(dA)·poly(dT) with histones.[22,23] In this case the potential for disproportionation into triple helix structures during reconstitution has precluded any strong inferences concerning the ability of poly(dA)·poly(dT) to be assembled into nucleosomes. On the other hand, if indeed it is the duplex form of poly(dA)·poly(dT) which does not support nucleosome formation, then the important question of what minimum length can be included in a nucleosome arises because short homopolymer stretches are known to occur within or near regions of potential regulatory significance in natural DNA.[24-32]

In the studies reported here we show, using several different reconstitution strategies, that RNA-RNA duplexes cannot be incorporated into nucleosomes. We also show that the inability of synthetic poly(dA)·poly(dT) homopolymers to form nucleosomes is related fundamentally to the *duplex* structure and that as little as 20 base pairs inserted into natural DNA are detectably disfavored during nucleosome formation while an 80 base pair segment is virtually excluded.

MATERIALS AND METHODS

dsRNA isolation. P1 double-stranded RNA (dsRNA) was isolated from *Saccharomyces cerevisiae* S7, a strain containing elevated amounts of P1 kindly provided us by C.S. McLaughlin (see Ref.33). dsRNA was prepared from unbroken cells as described by Fried and Fink[34] through the LiCl precipita-

tion step. The ethanol pellet was then dissoved in 2% $NaDodSO_4$ 10 mM Tris, 1 mM EDTA, pH 7.5 and run over a Sepharose CL-4B (Pharmacia) column (2.3 cm x 95 cm) equilibrated in the same buffer. Fractions from the early-eluting peak were pooled, precipitated twice with 2 volumes of 95% ethanol, and dissolved in 10 mM Tris, pH 8.0. After addition of $MgCl_2$ to 1 mM the sample was treated with 2 µg/ml DNase (Worthington, chromatographically purified, lyophilized powder) at 20°C for 30 min. Digestion was stopped by the addition of EDTA to 10 mM, after which nucleic acids were precipitated by adding 1/20 volume 3M NaCl and 2 volumes of 95% ethanol. Figure 1 shows that the dsRNA obtained after DNase I treatment migrates as a single band on a 2.6% polyacrylamide gel. As a final purification step the sample was centrifuged into a 5-20% sucrose gradient in 0.15 M NaCl, 1 mM Tris, 1 mM EDTA, pH 7.2 (31,000 rpm, 14 h, 4°C in an SW 41 rotor). Pooled dsRNA fractions were dialyzed into 0.15 M NaCl, 10 mM Tris, pH 8.0 and stored at -20°C.

Preparation of recombinant Ml3 replicative form (RF) DNA. E. coli JM 101 from an overnight culture were used to inoculate 1 liter flasks of M9 medium containing 0.4% glucose, 0.5% casamino acids and 2 µg/ml vitamin B_1. The cells were grown with vigorous shaking to an A_{595} of 0.8-1.0 and infected with the M13 recombinant, mH2 (a gift of Dr. Bryant Villeponteau), at 10^{10} PFU/ml. After 4 h additional growth, cells were harvested by centrifugation at 4000 g for 5 min. Cleared lysates were prepared using a standard procedure[35], extracted twice with phenol/$CHCl_3$ and three times with ether. Nucleic acids were precipitated with ethanol and dissolved in 10 mM Tris, 1 mM EDTA, pH 7.4 (TE). The preparation was treated with 50 µg/ml of RNase A (Sigma,

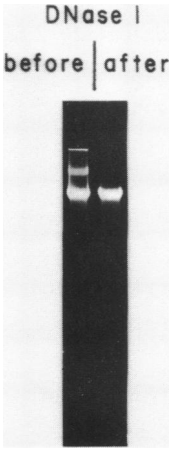

Figure 1. Characterization of dsRNA on a polyacrylamide gel before and after treatment with DNase I. Approximately 10 µg of RNA were loaded per lane.

Type II-A; pre-heated at 90°C for 10 min) for 30 min at 37°C, followed by phenol/$CHCl_3$ extraction and ethanol precipitation. The pellet was dissolved in 4 ml TE and fractionated on a 2.3 cm x 95 cm column of Sepharose CL-4B (Pharmacia) equilibrated with 0.1 M NaCl, 10 mM Tris, 0.1 mM EDTA, pH 7.8. Column fractions were analyzed by absorbance at 260 nm, and DNA in the early-eluting peak was precipitated with 3 volumes of ethanol. The pellet was dissolved in TE and stored at -20°C. Analysis of the RF DNA preparation on a 0.7% agarose gel containing 0.5 μg/ml ethidium bromide indicated approximately equal amounts of supercoiled and nicked forms (as well as some contamination with host DNA). The principal results to be described are therefore characteristic of both torsionally constrained and unconstrained DNA.

Core histone isolation. Core histones for reconstitution experiments were prepared by several different methods.

Salt-extracted histones for dsRNA reconstitution were prepared from calf thymus nuclei which were isolated as described previously[36]. The nuclei were lysed by suspension in 2 M NaCl, 10 mM Tris, 0.7 mM EDTA, pH 7.5 (phenyl-methylsulfonyl fluoride was also added to the buffer at 1 mM final concentration just before use), and the DNA was pelleted by centrifugation at 50,000 rpm for 24 h at 4°C in a Ti 75 rotor. After final purification of the supernatant over a Sephadex G100 (Pharmacia) column the core histones were concentrated using a Millipore Immersible Separator. The concentration of core histones in 2 M NaCl was determined spectrophotometrically assuming an A_{278} of 0.45 for 1 mg/ml of core histone.[20]

Salt-extracted histones for reconstitutions with the mH2 recombinant RF DNA were prepared by a modification of the hydroxylapatite procedure of Simon and Felsenfeld.[37] This method avoids ultracentrifugation which Philip et al.[38] have suggested may destabilize the histone octamer. Approximately 2 mg of salt-washed nucleosomes which lacked H1[36] were loaded on a Biogel HTP (Bio Rad) column (0.9 cm x 18 cm) equilibrated with 0.35 M NaCl, 50 mM potassium phosphate, pH 6.7. After washing with about 25 ml of the 0.35 M NaCl buffer, core histones were eluted with 2 M NaCl, 25 mM potassium phosphate pH 7.2. Fractions were monitored by absorbance at 278 nm and histone containing fractions were pooled and dialysed against 2 M NaCl, 10 mM Tris, 0.7 mM EDTA, pH 7.5. Electrophoresis on 18% polyacrylamide/ $NaDodSO_4$ gels indicated that the histones were undegraded and present in roughly stoichiometric amounts with respect to each other.

Acid-extracted histones were prepared using 0.4 N H_2SO_4 extraction of calf thymus nuclei suspended in distilled water. H1 was removed by three

extractions with 5% perchloric acid. These histones were dissolved in distilled water.

To obtain cross-linked histone octamers dimethylsuberimidate cross-linked monomer particles were prepared[39] and the histone octamer was then separated from the monomer DNA by virtue of its insolubility in 3 M NaCl.[40]

Reconstitution Protocols. Method 1: Salt-extracted histones in 2 M NaCl, 10 mM Tris, 0.7 mM EDTA, pH 7.5 were mixed with an equal mass of nucleic acid which was first adjusted to 2 M NaCl. The mixture, at a DNA concentration of 0.2-0.45 mg/ml, was dialyzed in Spectrapor 1 dialysis tubing. For volumes of $\leq$ 250 µl a microdialysis procedure was used.[41] Gradient salt dialysis was carried out as described by Tatchell and Van Holde[42] except that the final dialysis was with 3 changes of 50 mM NaCl, 10 mM Tris, pH 8.0.

Method 2: Salt-extracted histones were mixed with DNA as described for Method 1. The mixture, with both DNA and histone at a concentration of 0.2 mg/ml, was then dialyzed against a buffer containing 2 M NaCl and 5 M urea, followed by salt/urea gradient dialysis as for Method 3.

Method 3: Acid-extracted core histones (at 11 mg/ml in H_2O) were adjusted to pH 7-8 using 0.25 M Na_3PO_4, incubated in 10% β-mercaptoethanol at 37°C for 24 h, adjusted to approximately 5 M urea and 2 M NaCl by adding solid urea crystals and 5 M NaCl, respectively, and then incubated for an additional hour at 37°C. The histones were then added to an equal mass of nucleic acid which had been adjusted to 2 M NaCl, and the mixture (at a DNA concentration of about 0.25 mg/ml) was dialyzed using Spectrapor 1 membrane. Salt/urea gradient dialysis was carried out by the protocol of Camerini-Otero et al.[43] except that all buffers contained 10 mM Tris, 0.1 mM EDTA, pH 8.0 in addition to appropriate NaCl and/or urea concentrations. The final dialysis was against 50 mM NaCl, 10 mM Tris, pH 8.0.

Method 4: Reconstitution of cross-linked histone octamers followed essentially the protocol of Stein et al.[40] The nucleic acid was adjusted to 2 M NaCl and was added dropwise to an equal mass and volume of cross-linked histones dissolved in TE buffer. The mixture was dialyzed first against 1 M NaCl, 10 mM Tris, 0.7 mM EDTA, pH 7.5, then against 0.6 M NaCl, 10 mM Tris, 0.1 mM EDTA, pH 8.0 and finally against 50 mM NaCl, 10 mM Tris, pH 8.0.

Polypyrimidine tract analysis. RF DNA-histone reconstitution mixtures were adjusted to 1 mM $CaCl_2$, digested with 0.024 units/ml of staphylococcal nuclease (Sigma, Grade VI) at 37°C for 10 min, and then quenched with 1/50 volume of 0.1 M EDTA, pH 7.0. Samples were centrifuged into 5-20% sucrose gradients containing 50 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5 (SW 60; 59,000

rpm, 4 h, 4°C). Nucleosome monomer and dimer fractions were precipitated with 3 volumes of ethanol and dissolved in TE buffer.

The nucleosome samples were extracted once with phenol/$CHCl_3$, twice with $CHCl_3$, and then precipitated with ethanol. The pellet was dissolved in 50 µl distilled water. DNA was hydrolyzed to liberate polypyrimidine tracts by a modification of an established procedure.[44] All steps up to the phenol extraction were carried out in a single Eppendorf bullet. 100 µl of 3% (w/v) diphenylamine/98% formic acid were added to 50 µl DNA samples, and the mixture was incubated for 18 h at 30°C. After adding 75 µl of 8 M urea, 1 M sodium acetate and 1/4 µg poly(U) as carrier, the diphenylamine and formic acid were removed by two extractions with 0.5 ml ether. Nucleic acids were precipitated by adding 1 µl 1 M $MgCl_2$ and 250 µl ethanol and incubating for 30 min in a dry ice-ethanol bath. They were reprecipitated once and dissolved in 10 µl distilled water.

The next step involved labeling of 5'-ends by exchange with γ-^{32}P-ATP mediated by polynucleotide kinase in order to detect the polypyrimidine tracts on a gel. γ-^{32}P-ATP was prepared using $^{32}P_i$(ICN) to phosphorylate ADP.[45] The mixture of polypyrimidine tracts and carrier poly(U) was adjusted to a total volume of 24 µl in the presence of 100 µCi γ-^{32}P-ATP and 50 mM Tris, 10 mM $MgCl_2$, 10 mM dithiothreitol, pH 7.6. The unreacted ADP from the γ-^{32}P-ATP preparation provided enough excess nucleotide to promote exchange labeling of the 5'-phosphorylated polypyrimidine tracts. The reaction proceeded with incubation at 37°C for 30 min in the presence of 2 units T4 polynucleotide kinase (P & L Biochemicals). Nucleic acids were precipitated with the addition of 25 µl 4 M ammonium acetate and 150 µl ethanol. The ^{32}P-labeled poly(U) produced by the reaction was hydrolyzed by treatment with 50 µg/ml heat-treated RNase A at 37°C for 1 h in 0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4. Then $NaDodSO_4$ was added to 0.3% (w/v), along with 1 µg of calf thymus monosome DNA as carrier, and the sample was extracted with phenol/$CHCl_3$ followed by $CHCl_3$ alone. Nucleic acids in the final aqueous layer were precipitated with 3 volumes of ethanol, washed once with cold 70% ethanol and dissolved in 6 µl of 0.5% $NaDodSO_4$. Just prior to loading on a gel the samples were adjusted to 80% (v/v) with deionized formamide, placed in a boiling water bath for 2 min and cooled immediately in ice.

Polyacrylamide gel electrophoresis. High molecular weight double-stranded RNA preparations were analyzed on 2.6% polyacrylamide slab gels using the recipe described by Loening.[46] Samples were dissolved in 2% $NaDodSO_4$, 15% sucrose, 18 mM Tris, 15 mM sodium phosphate, 0.5 mM EDTA for electrophoresis.

Nondenaturing 4% polyacrylamide gels containing $NaDodSO_4$[47,36] were used to separate nucleic acid fragments after staphylococcal nuclease digestion. Dimethylsuberimidate-cross-linked histones were electrophoresed on 12% polyacrylamide/$NaDodSO_4$ gels prepared according to Laemmli.[48] Polypyrimidine tracts were visualized after autoradiography of 12% polyacrylamide gels containing 7 M urea and 0.1% $NaDodSO_4$. These gels are similar to standard urea denaturing gels[49] except that we have added $NaDodSO_4$ in order to reduce nonspecific aggregation and allow the direct electrophoresis of protein-nucleic acid complexes.

RESULTS

Double-Stranded RNA Cannot Form Nucleosomes. We have isolated a 5000 base pair dsRNA from yeast[33] and attempted to complex it with core histones using salt gradient dialysis procedures. Most of the experiments described below were carried out using at least two different reconstitution protocols and with histones prepared by various methods. In no case did the dsRNA yield any reconstituted material resembling nucleosomes.

When a dsRNA-histone reconstitution mixture was sedimented in a sucrose gradient the results in Figure 2 were obtained. There was no discrete shift of the dsRNA peak to a heavier position in the gradient, but instead about 50% pelleted under these conditions. (The broadened leading edges of the peaks apparently reflect aggregation of naked dsRNA, since dsRNA purified from fractions at the leading edge and at the peak itself migrate identically when resedimented. This apparent slight aggregation occurs at salt concentrations ranging from 1 mM EDTA to 0.15 M NaCl.) The absence of a discrete shift for the dsRNA-histone mixture is in contrast to the greatly increased sedimentation rates of plasmid DNA-histone complexes over that of free DNA.[21]

In order to assay by a different method whether dsRNA can be folded by histones, the dsRNA-histone reconstitution mixture was subjected to staphylococcal nuclease digestion and the RNA was analyzed on a non-denaturing polyacrylamide gel. The results (Fig. 3A) do not indicate protection of any specific-sized band(s) for the reconstitution mixture when compared to that for the purified dsRNA. In both cases nuclease digestion generated a very similar complicated banding pattern on the gel, presumably reflecting specific sequence preferences of this enzyme. The only apparent differences between the reconstituted and the naked RNA digests is a minor decrease in digestion kinetics and a slightly enhanced background for the dsRNA reconsti-

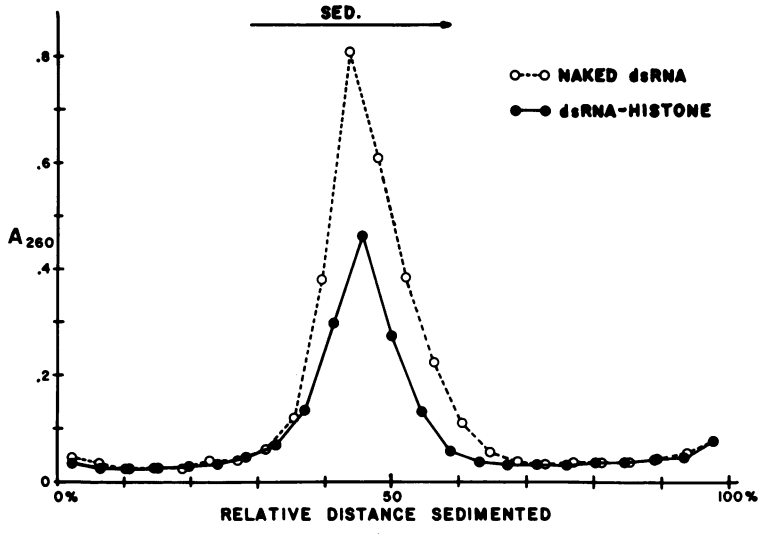

Figure 2. Sedimentation of dsRNA-histone "reconstitution" mixture. Acid-extracted histones and dsRNA were reconstituted by Method 3. 0.6 ml samples of the dsRNA-histone mixture as well as a "naked" dsRNA control were separately adjusted to an A_{260} of 2 in 50 mM NaCl, 10 mM Tris, pH 8.0. They were centrifuged in parallel on 5-20% sucrose gradients containing the same buffer (14 h, 4 °C, 30,000 RPM, SW 41). 10-drop fractions were collected from the bottom.

tution mixture. In contrast, staphylococcal nuclease digestion of a parallel DNA-histone reconstitute yielded discrete core-sized DNA bands on the DNA gel (Fig. 3B).

The inability of the dsRNA-histone reconstitution mixture to yield either a discrete, faster-sedimenting peak in a sucrose gradient or a protected fragment(s) after staphylococcal nuclease digestion provides evidence that dsRNA does not fold around histones to form stable nucleosome-like complexes. We next asked whether dsRNA, like other non-nucleosome forming polyanions (see Discussion), could nevertheless induce core histones to associate into nucleosomal octameric complexes. The $NaDodSO_4$ gel in Figure 4A shows that no octamer histone species is formed after cross-linking with dimethylsuberimidate under conditions which give rise to an intense octamer band from a DNA-histone reconstitution mixture. In contrast a similar experiment carried out with the polyanion, polyglutamic acid, did yield a cross-linked histone octamer (Fig. 4B) as first reported by Stein et al.[50] It should be noted that the faint band just inside the resolving gel of

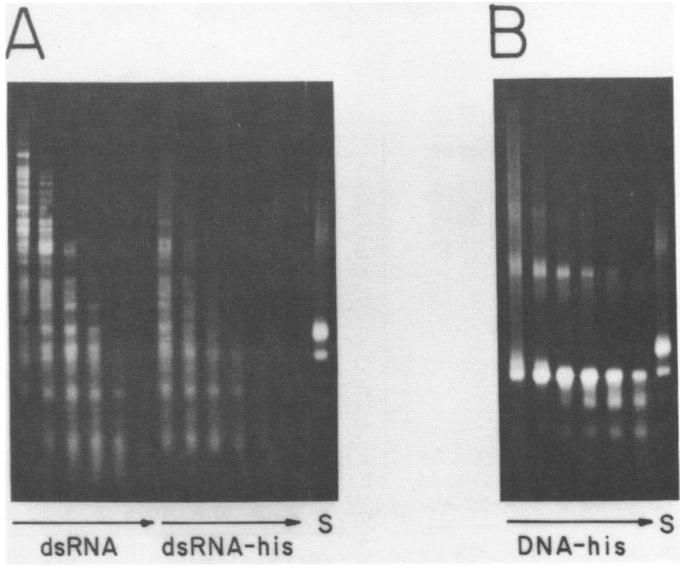

Figure 3. Staphylococcal nuclease digestions of (A) a dsRNA-histone "reconstitute" and (B) a DNA-histone reconstitute. Reconstitution was by Method 1. After the final dialysis step the mixtures were adjusted to 0.1 mM Ca^{++} using 10 mM $CaCl_2$. Staphylococcal nuclease digestions were carried out at 37°C using 5 units/ml for dsRNA samples and 0.2 units/ml for the DNA. At desired times (naked dsRNA: 1, 2, 5, 10, 30, 60 min; dsRNA-histone: 5, 10, 15, 30, 60, 120 min; DNA-histone: 2, 5, 10, 15, 30, 60 min) samples were removed, added to 1/10 volume of 0.1M EDTA, pH 7.0, placed on ice and then analyzed by gel electrophoresis as described. Panel A shows the digestion of naked dsRNA (left lanes) and the dsRNA-histone mixture (right lanes). Panel B illustrates nuclease digestion of a DNA-histone reconstitute. Lane S is a standard mixture of chromatosome and core particle DNA obtained from the staphylococcal nuclease digestion of calf thymus nuclei.

the RNA lanes in Figure 4A is due to staining of dsRNA by Coomassie Blue. The persistence of this band as well as a more careful analysis on a 2.6% polyacrylamide gel (results not shown) indicated that the pH 10 condition used in the cross-linking experiment does not degrade dsRNA. Therefore we conclude that dsRNA cannot induce the core histones to become organized into an octamer complex.

dsRNA may be unable to form nucleosomes simply because of its inability to induce the formation of histone octamers. In order to uncouple the ability to assemble histones into octamers from the ability of the nucleic acid to be folded, dsRNA was mixed with intact octamers which had been cross-linked by dimethylsuberimidate and purified by precipitation in 3 M

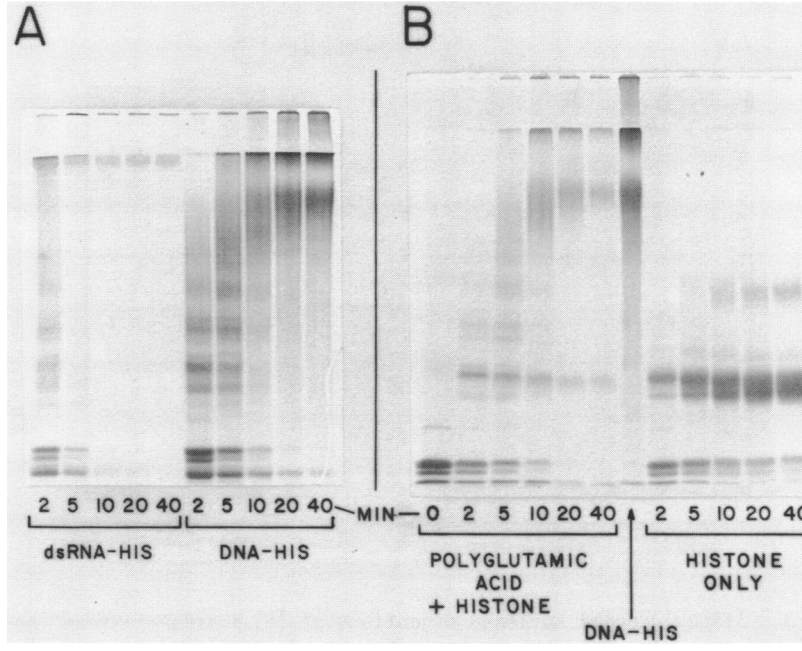

Figure 4. Dimethylsuberimidate cross-linking of (A) dsRNA-histone and DNA-histone reconstitute, and (B) a polyglutamic acid-histone mixture. For (A), reconstitution was by method 1 except that the final dialysis was against 0.1 M NaCl, 1 mM Tris, pH 8.0. One ml of each mixture, at an A_{260} of 7-8, was added dropwise with constant vortexing at room temperature to 4 ml of a freshly made solution of dimethylsuberimidate (Pierce) at 6.25 mg/ml in 0.1 M sodium borate, pH 10.0. Aliquots were removed at time intervals and added to an equal volume of 1 M sodium acetate, pH 5.0 to stop the reaction. Then they were dialyzed against distilled water, lyophilized, and dissolved in 2% $NaDodSO_4$, 10% glycerol, 5% β-mercaptoethanol, 0.125 M Tris (pH 6.8) for analysis on a 12% polyacrylamide/$NaDodSO_4$ gel. For (B), acid-extracted histones were first treated with urea and β-mercaptoethanol as described previously[36] and then adjusted to 0.15 M NaCl and 0.69 mg/ml of histone with a final addition of an equal mass of sodium polyglutamate (5 mg/ml in H_2O, Sigma, type III-B). For the "Histone Only" control, water was added in place of the polyglutamate solution. Cross-linking with dimethylsuberimidate was carried out exactly as described above except that the concentration of dimethylsuberimidate in the reaction mixture was 2.5 mg/ml. The DNA-histone lane in panel (B) is the same sample as used for the 40 min lane in panel (A). The cross-linking experiment using polyglutamate has also been repeated using salt-extracted histones with essentially identical results.

NaCl.[40] Such octamers are known to be competent in nucleosome formation.[40] When the dsRNA-histone octamer reconstitution mixture was sedimented in a sucrose gradient or digested with staphylococcal nuclease, virtually

identical results were obtained as in Figures 2 and 3 (results not shown). Thus we conclude that dsRNA is not folded into nucleosome-like complexes even when mixed with preformed histone octamers.

Nucleosomes avoid poly(dA)-poly(dT) segments in double-stranded DNA. For our studies we have utilized the double-stranded replicative form (RF) DNA of a recombinant M13 bacteriophage, mH2, which contains long and short homopolymer stretches of poly(dA)·poly(dT) separated by natural DNA (see Fig. 5). This phage was a gift from Dr. Bryant Villeponteau who used the dA-dT tailing procedure to introduce a piece of foreign DNA into the cloning

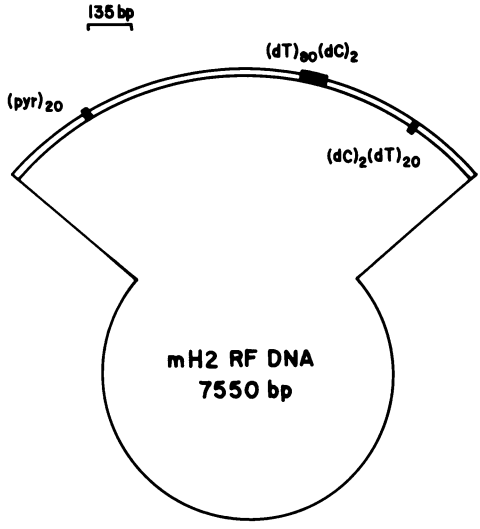

Figure 5. Diagram of the recombinant mH2 RF DNA used for reconstitution. Orientation is 5'→3' for the top strand and the diagram is drawn roughly to scale. mH2 was generously provided by Dr. B. Villeponteau and is a derivative of M13 mp2[61]. It was constructed by insertion of a poly(dA) tailed 258 bp chicken globin DNA Hae III restriction fragment from pHbl001[62,63] (provided by Dr. Winston Salser) into the poly(dT) tailed Eco RI site of M13 mp2. Each poly(dT) tract in the mH2 DNA is terminated by two dC residues which originate from the Hae III termini of the globin fragments. The 20 base polypyrimidine tract is a natural feature of the M13 sequence.[64] The poly(dT) tracts were shown by pyrimidine tract analysis of the adult M13 single-stranded DNA to be in the orientations illustrated. The base compositions of the three polypyrimidine tracts were verified by pyrimidine tract analysis of RF DNA which had been nick translated with α-^{32}P-dCTP or α-^{32}P-dTTP. The "mystery" pyrimidine tracts, x and y (see Fig. 7 and Text), were also confirmed to be poly(dT) by this analysis. We note parenthetically that mH2 contains the lac operator sequence (carried over from M13 mp2) centered at about 40-45 bp to the left of the $(dT)_{80}$ tract. However we do not believe that the role of this region in nucleosome positioning[15] affects the interpretation of the results in the present study.

vehicle, M13 mp2 (provided by J. Messing).

The DNA of mH2 contains three well separated segments relevant to the present work; an approximately 80 base pair (bp) poly(dA)·poly(dT) segment, a 20 bp poly(dA)·poly(dT) segment and a 20 bp polypurine·polypyrimidine segment. A complete description of this DNA is given in Figure 5.

In order to determine the extent to which the homopolymer segments can be accomodated in nucleosomal structures, the mH2 DNA was reconstituted with histones by Method 1 (salt extracted histones, salt dialysis), digested briefly with staphylococcal nuclease, and the resulting nucleosome monomers and dimers isolated from sucrose gradients, as shown in Figure 6, for analysis of their DNA. The DNA was hydrolyzed with formic acid/diphenylamine to liberate polypyrimidine tracts, and these were kinased with γ-^{32}P-ATP in

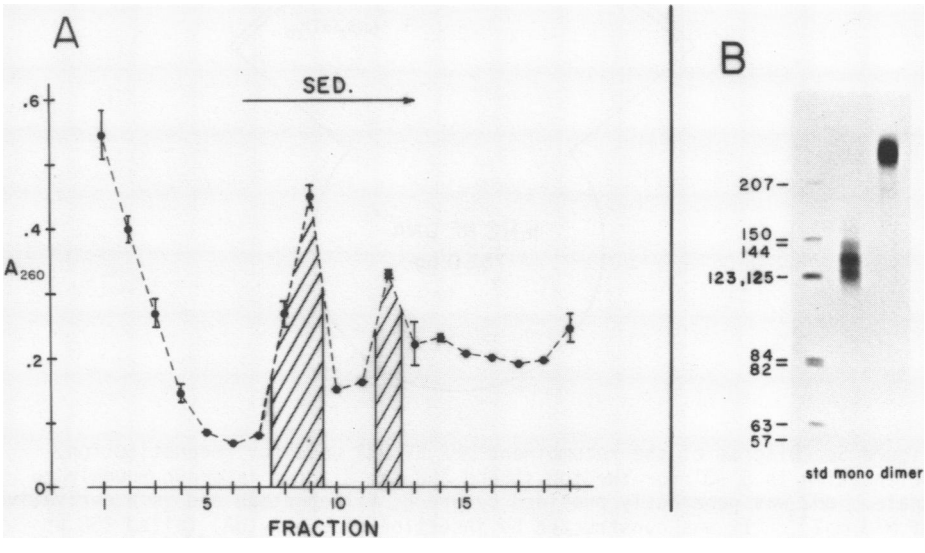

Figure 6. Isolation and characterization of reconstituted mH2 monomer and dimer nucleosomes. Part (A) shows the averaged profile of two preparative sucrose gradients. Fractions were collected from the bottom and pooled as shown. More than 50% of the total A_{260} in the gradient sedimented at the mononucleosome position or beyond (without adjusting for hyperchromicity). For part (B) the monomer and dimer nucleosomes were labeled with γ-^{32}P-ATP and T4 polynucleotide kinase, followed by electrophoresis on a denaturing gel (6% polyacrylamide, 7 M urea, 0.1% $NaDodSO_4$) and autoradiography. The calibration lane on the left is a mixture of kinased Hpa II and Hha I restriction fragments produced from a piece of *lac* UV5 DNA.[15] From this gel we estimate the single-strand DNA sizes of our monomer and dimer preparations to be 135 bases ± 10% and 270 bases ± 10% respectively (>90% of the band intensity within the 10% limits).

order to detect them after electrophoresis on a 12% polyacrylamide gel. The autoradiograph of such an experiment is shown in Figure 7A and scans of each lane are presented in Figure 7B.

From Figures 7A & B it is immediately apparent that the 80 base poly(dT) tract is scarcely present in either the monomer or dimer nucleosome samples (lanes 2 and 3) although it is, of course, well represented in the original DNA sample (lane 1). In contrast, the 20 base poly(dT) and polypyrimidine tracts are almost equally represented in all samples. Essentially the same results are obtained using reconstitution by Method 2 (salt extracted histones, salt-urea dialysis; see Fig. 7C, lanes 1-3) or by Method 3 (acid extracted histones, salt-urea dialysis; not shown). As a control, naked mH2 DNA also was digested with nuclease and analyzed for polypyrimidine tracts. Figure 7C shows that this procedure does not result in detectably diminished recovery of the long, relative to the short, poly(dT) tract. The lack of any obvious preference of staphylococcal nuclease for poly(dA).poly(dT) is in agreement with the recent study of Dingwall et al[51] on this enzyme. Thus preferential digestion of poly(dA).poly(dT) is not the cause of its depletion in nuclease prepared nucleosomes. We therefore conclude that some intrinsic property of the double helical structure of poly(dA).poly(dT) prevents inclusion of long segments of this homopolymer in nucleosome cores.

The exclusion of poly(dA)·poly(dT) from nucleosomes is clearly length dependent. The 80 bp poly(dA)·poly(dT) segment appears to be completely excluded from core particles whereas the 20 bp segment is represented at a substantial level (Fig. 7). Also nucleosome *dimers* can accommodate the presence of the 80 bp poly(dA)·poly(dT) segment (see Fig. 7C, lane 2) although it is strongly disfavored compared to the 20 bp segment. Since our nucleosome dimers are "closely spaced" (ca.270±30 bp; see Ref. 52) this shows that poly(dA)·poly(dT) can be present in nucleosomes at least to the extent of 40 bp per nucleosome although it is strongly selected against.

DISCUSSION

Nucleic Acid Helix Structure and the Ability to Form Nucleosomes. We have demonstrated that two different species of double-stranded nucleic acid are preferentially excluded from nucleosome-like structures during assembly with core histones. The first, double-stranded RNA, is incapable of being folded around histones into specific structures, even when mixed with stably cross-linked, intact histone octamers. The other, an 80 bp segment of poly(dA)·poly(dT) which is part of a double-stranded recombinant DNA, is

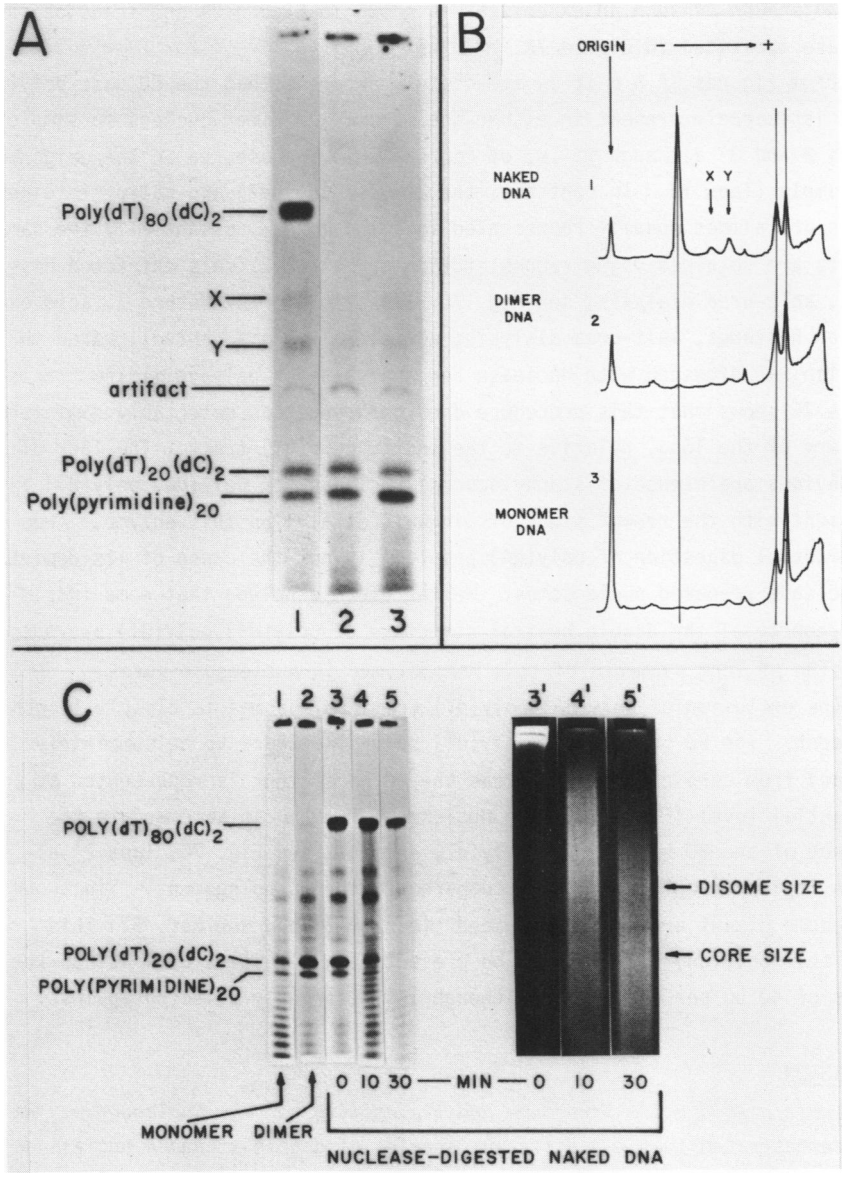

Figure 7. Polypyrimidine tract analysis of reconstituted nucleosomes. A. Analysis of naked mH2 DNA (lane 1), dimer DNA (lane 2) and monomer DNA (lane 3). A 6½ h exposure of a 12% polyacrylamide gel is shown. Note that the pyrimidine tracts are end-labeled so autoradiographic intensity should be proportional to the number of tracts recovered. From lane 1 it can be seen that, using our procedure, either the initial labeling and/or the

subsequent recovery is more efficient for the long compared to the short, poly(dT) tract. This effect is fairly constant throughout our experiments and serves to dramatize the absence of poly(dT)$_{80}$ in lanes 2 and 3. Bands x and y are also poly(dT) tracts which probably reflect contaminating phage in our preparation (see Text). The "artifact" band has appeared only in this particular experiment.
B. Densitometric scans of the lanes in panel A. For comparison, the statistically expected representation of 22 and 82 base tracts in DNA of dimer size is 92% [(270-22)÷(270)] and 70% [(270-82)÷(270)] respectively of that for high molecular weight DNA. The corresponding numbers for monomer size DNA (135 bases) are 84% and 39%. Thus in the case of the long poly(dT) tract, for example, its representation in monomer DNA should be at least half of that for the dimer DNA (39÷70=0.56). Yet long autoradiographic exposures show that the poly(dT)$_{80}$ tract, if present at all in monomer DNA, is present at only negligible levels compared to the dimer DNA.
C. Polypyrimidine tract analysis of nuclease digested naked mH2 DNA. The DNA was adjusted to 0.2 mg/ml in 50 mM NaCl, 10 mM Tris (pH 8.0), 1 mM $CaCl_2$ and digested at 37°C with 0.025 units of staphylococcal nuclease/ml. At intervals aliquots were removed. About 1.5 µg of DNA from each aliquot was electrophoresed on a non-denaturing gel and stained with ethidium bromide to determine the overall extent of digestion (lanes 3', 4', 5'). The mobilities of core particle and closely-spaced dinucleosome DNAs are indicated by the arrows. The rest of each aliquot was subjected to polypyrimidine tract analysis with the results shown in lanes 3-5. The monomer and dimer lanes (1 and 2) show, for comparison, the results of pyrimidine tract analyses of mH2 DNA reconstitutes (prepared by Method 2). Autoradiographic exposure was 75 h except for lane 2 which was 17 h. Note that the probability of containing an 80 base tract rapidly approaches zero for DNA of less than core particle size. Most of the submonomer length DNA of the size visible in lane 5' therefore does not contribute poly(dT)$_{80}$ tracts to lane 5. The ladder pattern at the bottom of each autoradiographic lane is a consequence of incomplete digestion of the carrier poly(U) in this experiment. It is based on such ladders that we have estimated the pyrimidine tract lengths for the DNA of mH2.

absent from nucleosome cores prepared by nuclease digestion following reconstitution of the DNA with histones.

The helical structure of dsRNA is of the A family[53] which differs markedly from the usual form of DNA in solution. The structure of poly(dA)·poly(dT), however, differs only slightly from that of DNA (Ref. 2). Nevertheless the double helical structures of both dsRNA and poly(dA)·poly(dT) are characteristically inert to environmental influences.[2,6,11,53] Consequently they may resist adoption of the helical parameters necessary for nucleosome formation. We do not know what aspects of helix structure might be important in this regard. Perhaps dsRNA and poly(dA)·poly(dT) are unable to accommodate certain specific contacts with the histones that may be required in nucleosome formation. Alternatively, perhaps these nucleic acids resist bending, a property which would clearly disfavor nucleosome formation (see Refs. 12, 54).

We have found that double-stranded RNA is incapable even of organizing histones into octamer complexes. Single-stranded RNA, in contrast, apparently can organize the histones as shown by its ability to promote octamer-like trypsin resistance in histones[55] and to facilitate chromatin assembly.[56] Also, in one out of three preliminary experiments (unpublished), we obtained histone octamers by dimethylsuberimidate cross-linking in the presence of partially degraded single-stranded RNA. [The lack of reproducibility is probably related to the subsequent observation of Nelson et al.[56] that the histone-RNA interaction depends critically on a sufficient single-stranded RNA length.] The ionic environment provided by 2M NaCl or by flexible acidic polypeptides also can stabilize the octamer complex[57,50] (Fig. 4) as can single-stranded DNA.[55] All of these observations are consistent with the proposal that the principal barrier to octamer organization and nucleosome formation by dsRNA is its inert secondary structure and lack of ability to assume appropriate helical parameters.

Birnboim et al.[58] have shown that natural nucleosomes display no detectable selection against polypyrimidine tracts of mixed sequence regardless of length. Therefore the failure of long poly(dA)·poly(dT) tracts to be incorporated into nucleosomes is apparently a property of the homopolymer sequence specifically and not more generally of the overall purine·pyrimidine asymmetry inherent in the sequence. We also have not noticed any obvious selection in our reconstitutes against the natural 20 bp mixed polypyrimidine sequence contained within the mH2 phage DNA. We do however notice that the ratio of $poly(dT)_{20}$/$poly(pyrimidine)_{20}$ is slightly diminished in our reconstitutes compared to the control (e.g. Fig. 7B), consistent with the notion that these two types of sequence differ in their ability to be folded into nucleosomes.

Length Dependence of poly(dA)·poly(dT) Incorporation into Nucleosomes. From Figure 7 it is evident that the ability of a nucleosome to constrain poly(dA)·poly(dT) depends on the length of the homopolymer tract. The 20 bp tract is fairly efficiently incorporated within core particles whereas the 80 bp tract is not.

We cannot tell from our data whether there is a well defined "cut-off length" between 20 and 80 bp or whether the tendency to exclude the homopolymer is proportional to homopolymer length. However, the following considerations seem to favor the latter possibility. First there is the observation, mentioned above, that even the short $poly(dT)_{20}$ sequence appears to be selected against slightly in the reconstitutes. Second there

is the observation that the reconstituted closely-spaced dinucleosomes, in contrast to the core particles, do contain low but significant levels of the poly(dT)$_{80}$ tract. Since core particles are essentially devoid of poly(dT)$_{80}$ this implies that the opportunity to share this long sequence between two cores (i.e. reducing to 40 bp the average length incorporated per core) correspondingly increases its structural acceptibility. Also consistent with there being a gradual rather than a discrete length dependence is the behavior of the "mystery" tracts, x and y, of intermediate length in Figure 7B, which are recovered, but in clearly reduced amounts, from the core particles. We have confirmed that x and y are poly(dT) tracts (see legend to Fig. 5) and suspect that they arise from minor rearranged populations of phage DNA in our preparation (the mH2 phage was not plaque purified for our experiments).

DNA Sequence Aversion and the Positioning of Nucleosomes in Chromatin. A DNA sequence with strong aversion to being included within nucleosomes has the obvious potential to specify the positions of nucleosomes with respect to DNA sequence in chromatin. Clearly any sequence with such properties would occur preferentially in spacer regions and could thus specify the positions of the flanking nucleosomes. Longer stretches could specify nucleosome free regions. However even segments as short as 20 bp could also play a role in nucleosome positioning. Since a 20 bp segment of poly(dA)·poly(dT) maintains its characteristic structure in solution even when flanked by natural DNA of differing pitch,[5,6] it seems unlikely that its inclusion within a nucleosome would be without consequence. For example such a segment may exert a strong preference toward being placed *between* the sites of tightest histone-DNA contact[17,19] in the nucleosome core and additionally may prefer the "wing" domains which are more conformationally flexible.[59]

Thus even a 20 bp segment of poly(dA)·poly(dT) could serve to position nucleosomes. It is interesting that such segments are found in the 5' flanking domains of many genes at locations presumed to have regulatory significance.[25,27,30,31] Perhaps segments less obviously recognizable in sequence but similar in function reside near other eukaryotic genes. We are currently testing these possibilities using cloned genomic β-globin genes from chicken.[60] One of these has already been shown to contain a 16 bp poly(dG)·poly(dC) segment in its 5' flanking domain.[26] Poly(dG)·poly(dC), like poly(dA)·poly(dT), does not support nucleosome formation.[22,23]

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