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Chromatin and core particles formed from the inner histones and synthetic polydeoxyribonucleotides of defined sequence

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ABSTRACT

Chicken erythrocyte inner histones (H2A, H2B, H3 and H4) were associated with the two complementary homopolymeric polydeoxyribonucleotides and the two alternating copolymeric polydeoxyribonucleotides. No evidence for formation of chromatin-like structures was obtained for the complexes with poly(dG)·poly(dC) or poly(dA)·poly(dT). Both poly(dGdC)·poly(dGdC) and poly(dAdT)·poly(dAdT) could be folded by histones to yield material digested by DNAase I to multiples of about 10 and by staphylococcal nuclease to 146 bp core particles. Due to the lack of sequence heterogeniety in the complex of histones with poly(dAdT)·poly(dAdT), core particles with remarkable fine structural detail are obtained. The internal organization of DNA in the AT-containing and GC-containing core particles appears not to be identical.

INTRODUCTION

Particular DNA sequences may alter the structure of the nucleic acid from the B-form structure characteristic of the bulk of DNA in solution. For example, palindromic sequences can form cruciform structures, thought by some to be of import in recognition of regulatory regions (1). Runs of a given base type or regions of high AT or GC content can also alter DNA structure; AT-rich regions melt more easily than GC-rich areas, the synthetic polymer poly(dG)·poly(dC) forms A-form structures more readily than other polymers (2), poly(dA)·poly(dT) has never been observed in an A-DNA structure (3), and RNA-DNA hybrids apparently always exist in an A-form structure (4). Structural alterations in DNA resulting from particular sequences have been proposed to play a role in both transcription and replication (5,6). Recently available DNA sequence data have provided some support for such suppositions. The 106 base pair (bp) region (5222-103) near the origin of replication of SV-40 contains 1 of the 98 runs of A or T longer than 5 residues, but 7 of the 13 runs of G or C longer than 5 resi-

dues (7), suggesting that this region may have unique structural features. In contrast to other regions of the native SV-40 minichromosome, this area near the origin is always available for cleavage by an appropriate restriction endonuclease, Bgl I (8); one plausible explanation for this result is that the DNA of this segment of the virus may not be associated with histones in the form of a normal chromatin subunit or nucleosome. We were interested to ask if some features of the sequence of DNA in this, and perhaps other, regions of eukaryotic DNAs would either preclude the formation of nucleosomes or lead to nucleosomes of distinctive structure. Such a finding would provide an attractive mechanism for a localized phasing of nucleosomes along chromosomal DNA, perhaps facilitating the reading of regulatory signals by proteins.

In studies of the effects of a particular DNA sequence on DNA structure, it is often advantageous to amplify the effect through use of synthetic polydeoxyribonucleotides. We have taken this approach to ask whether any particular DNA sequences disallow folding the nucleic acid into a chromatin particle, using the two alternating copolymers poly(dAdT)• poly(dAdT) and poly(dGdC)•poly(dGdC), and the two complementary homopolymers, poly(dG)• poly(dC) and poly(dA)•poly(dT). Each of these polymers, as well as control salmon sperm DNA, was associated with the four inner histones (H2A, H2B, H3 and H4) and the properties of the resultant complexes examined by a variety of physical and chemical methods. The results obtained demonstrate that the complementary homopolymers do not wrap around the inner histones to form nucleosomes, although failure to observe folding of poly(dA)•poly(dT) may be due to a trivial reason. The alternating copolymers form well defined chromatin-like structures, with core particles containing 146 base pairs of DNA. Certain features of core particle structure differ for the AT-containing versus the GC-containing particles. Several features of core particle structure, masked in native core particles, can be visualized in the poly(dAdT)•poly(dAdT) core particle due to its lack of DNA sequence heterogeniety.

EXPERIMENTAL SECTION

Materials Nuclei were isolated from chicken erythrocytes (PelFreez Biologicals) by washing with Triton X-100. A crude chromatin preparation was made by swelling the nuclei in 0.1 mM phenylmethane sulfonylfluoride, 1 mM EDTA, pH 8.0, for one hour, and sedimentation of the complex. The chromatin

was washed once in the same buffer containing 0.35 M NaCl and then sheared (Waring Blender, 120 v, 1 min) in a buffer containing 2.5 M NaCl. DNA was pelleted by sedimentation at 60000 rpm for 16 hr at 4°C and the crude histone preparation in the supernatant concentrated to about 20 mg/ml by pressure dialysis using an Amicon YM10 membrane. Lysine rich histones (H1 and H5) were separated from the inner histones by gel filtration. Up to 120 mg of total histone was loaded onto a 2.5 x 90 cm column of Sephacryl S200 equilibrated with 2.5 M NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.1 mM PMSF, and the column was eluted with the same buffer at 4°C. A main peak contained only the four smaller histones in nearly equal proportions, a trailing peak contained the lysine rich histones and small amounts of H2A and H2B. The inner histones were concentrated to 10 mg/ml, made 1mM in dithiothreitol and stored frozen. Histones were analyzed by discontinuous polyacrylamide gel electrophoresis as previously described (13).

Synthetic polynucleotides were obtained from Miles Laboratories (poly(dAdT)·poly(dAdT) and poly(dG)·poly(dC)) and PL Biochemicals (poly(dA)·poly(dT) and poly(dGdC)·poly(dGdC)). All had sedimentation coefficients, $S_{20,w}$ of about 10-12S, indicating degrees of polymerization in the range of 1500 base pairs. All polymers melted, in appropriate solvents, in single transitions, with an average width of less than 2°C. The polymers were dissolved in 2.5 M NaCl, 10 mM Tris-Cl, pH 8.0, 10 mM EDTA at concentrations of either 5 or 25 A_{260}/ml by stirring in the cold for 16-24 hours.

Polynucleotide kinase was purchased from Miles Laboratories and γ-^{32}P-ATP (NEG-002X) was a product of New England Nuclear Corp. ϕX174RF DNA and Hae III restriction endonuclease, used for preparation of DNA gel sizing standards, were products of Bethesda Research Labs. Micrococcal nuclease and DNAase I were purchased from Worthington Biochemicals. S1 nuclease was a product of Sigma and Proteinase K was obtained from Beckman.

Association of histones and synthetic polynucleotides The synthetic polynucleotides were mixed with histones (usual mass ratio 0.8 gm histone/gm DNA) in 2.5 M NaCl, 10 mM Tris-Cl, pH 8.0, 10 mM EDTA, and then dialyzed against a 1:1 dilution of that buffer with water for 4-8 hr at 25°C. Buffer was then changed to 0.6 M NaCl with the same concentrations of Tris-Cl and EDTA and dialysis continued for 16 hr at 25°C. Dialysis against 0.1 M NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA for 4-6 hr concluded the association protocol. Some samples were further dialyzed overnight at 4°C against two changes of 0.25 mM EDTA, pH 7.0. Performing the first three steps of the association reaction at 37°C for shorter times (2 hr at

1.25 M NaCl, 6-8 hr at 0.6 M NaCl, 3 hr at 0.1 M NaCl) was without effect on the results presented in terms of digestion by DNAase I or micrococcal nuclease or melting analysis for any of the polymers.

Nuclease digestions Synthetic chromatins were digested with DNAase I in 10 mM Tris-Cl, pH 8.0, 8 mM $MgCl_2$, 2 mM $CaCl_2$ at 37°C. Calcium and magnesium were used since Bollum (9) had noted the inability of this enzyme to degrade certain polynucleotides when only magnesium was present. Acid solubility was measured on aliquots precipitated with 1 M NaCl, 1 M $HClO_4$ for >30 min at 0°C. To determine the total amount of material present, a similar aliquot was hydrolyzed at 100°C for 15 min. Micrococcal nuclease digestions were done in 10 mM Tris-Cl, pH 8.0, 5 mM $CaCl_2$, with or without 0.1 M NaCl present, as indicated. DNA was prepared by 1) extraction with phenol after adjusting the sample to contain 1% SDS and 15 mM EDTA, 2) digestion with proteinase K (0.1-0.2 mg/ml, 37°C, 30-60 min), or 3) initial digestion with proteinase K followed by phenol extraction.

Electrophoretic analyses of DNA fragments was performed with three gel systems. Double-stranded fragments were analyzed using 5% polyacrylamide gels in the Tris-borate-EDTA buffer of Peacock and Dingman (10,11). Size standards were a Hae III digest of ϕX174 RF DNA. Single-stranded fragments were run on 12% polyacrylamide gels containing 7 M urea in the same buffer (11). Samples were dissolved in 0.2 x gel buffer in either 10 M urea or 98% formamide, boiled for two min and then applied to the gel. Single -stranded fragments of poly(dGdC)·poly(dGdC) were treated with glyoxal before electrophoresis to prevent hairpin formation; samples were incubated in 50% dimethyl sulfoxide, 1 mM potassium phosphate, pH 7, 6% glyoxal (freshly deionized by passage over a mixed bed ion exchange resin column) for 45 min at 50°C, reprecipitated and electrophoresed. All gels were stained with Stains-all (Eastman) in 50% formamide, photographed, and scanned as previously described (12).

Sucrose gradient sedimentation for isolation of core particles from the synthetic chromatins was performed as previously described (13); gradients contained 0.1 M NaCl. After dialysis against either 0.25 mM EDTA, pH 7, or the same plus 0.1 M NaCl, pooled fractions were concentrated by pressure dialysis and stored at either 4°C or frozen at -20°C. No differences in results of digestion experiments could be attributed to freezing core particle preparations.

Cutting site mapping Sites of DNAase I cutting in isolated core particles were determined by labeling core particle 5' ends with ^{32}P, digestion, gel

electrophoresis, and autoradiography, as previously described (12). In several preparations, the extent of labeling of 5' ends varied from 1.4-1.9 ^{32}P per 146 bp of DNA. Labeled core particles were digested together with a large excess of unlabeled chicken erythrocyte core particles. Digestions of poly(dAdT)·poly(dAdT) core particles were in 10 mM Tris-Cl, pH 8, 10 mM $MgCl_2$, 1 mM EGTA at 37°C, while core particles containing poly(dGdC)·poly(dGdC) were digested in 10 mM Tris-Cl, pH 8, 2 mM $MnCl_2$, 1 mM EGTA at 37°C. After electrophoresis, autoradiography was carried out using a Dupont Cronex intensifier screen at -70°C.

Quantitation of the bases at the ends of core particles modified by polynucleotide kinase was done by isolation of DNA from labeled particles, digestion with S1 nuclease after heating to 100°C (for poly(dAdT)·poly(dAdT)) or glyoxal treatment (for poly(dGdC)·poly(dGdC)), and chromatography of the digest on Whatman #3 paper with 40% ammonium sulfate in 0.1 M Tris-Cl, pH 7.5, as solvent (14). Positions of modified bases were identified by autoradiography, labeled spots were excised from the paper and counted in Aquasol.

Physical Methods Analytical ultracentrifugation was carried out in the Beckman Model E centrifuge equipped with multiplexer and automatic photoelectric scanner system. Sedimentation was performed at 20.0°C in 0.1 M NaCl, 10 mM TrisCl, pH 8, 1 mM EDTA. Data were analyzed as linear least square regression lines and all results had correlation coefficients of 0.997 or better.

Thermal denaturation measurements were made with a Cary Model 219 spectrophotometer equipped with thermostatted cell holders and temperature readout accessory. Temperature was increased by a NesLab TP-2 programmer and measured with the Cary thermal probe in a dummy cuvette. Numerical derivative melting profiles were calculated by linear least square fitting the tangent to the melting profile, using four points (at 0.5°C intervals) around each data point.

RESULTS

The complementary homopolymers do not form core particles

We have used three criteria as primary means for assessment of the fidelity of reformation of a chromatin-like structure on interaction of a DNA with the inner histones. First, DNAase I must degrade the synthetic chromatin to yield single-stranded DNA fragments which are multiples of

about 10 nucleotides in length. Second, micrococcal nuclease must degrade the reconstituted chromatin to yield a core particle length (nominally 145 bp) of DNA. Third, the synthetic chromatin must have a two phase, cooperative melting profile, like native core particles (15). All these criteria are met for complexes made with the inner histones and high molecular weight salmon sperm DNA (data not shown). Not meeting the first criterion seems the most stringent indication of failure of DNA folding since DNA digested in the presence of calcium phosphate by DNAase I yields fragments which are multiples of 10 nucleotides in length (16); absence of such digestion products provides strong evidence that a defined complex with the nucleic acid wrapped on the surface of a protein core has not been formed in the association reaction.

Complexes of chicken erythrocyte inner histones with poly(dA)·poly(dT) and poly(dG)·poly(dC) were prepared and digested with DNAase I and micrococcal nuclease, and the products analyzed as single-stranded and double-stranded fragments, respectively. In no case were sharp, distinct bands observed for digestion of either complex by either enzyme. All DNA on the gels was either of high enough molecular weight so as to only enter the gel, or degraded to fragments smaller than 15 bases (for DNAase I) or about 40 bp in length (micrococcal nuclease). These results were obtained at degrees of digestion (percent acid-soluble A_{260}) varying from 10-60%. Performing the reassociation at 37°C (versus 25°C) was without effect on the observed results.

The thermal denaturation profiles of the two samples of the complementary homopolymers associated with inner histones also suggest that defined complexes have not been formed. The complex with poly(dA)·poly(dT) melts over a range from 28°C to 85°C, as two broad transitions, comprising about 30% and 70% of the total hyperchromicity for the lower and higher transitions, respectively. Melting of the complex of histones with poly(dG)·poly(dC) is incomplete by 100°C, due to the high thermal stability of this polymer; however, the melting that is observed is a linear increase in optical density with temperature from about 50°C upwards. Broad melting profiles of this sort are characteristic of nonspecific complexes of polycationic molecules with nucleic acids and contrast to the sharply defined thermal transitions observed for native chromatin particles (15,17) and for the complex of the inner histones with poly(dAdT)·poly(dAdT) (see below). Thus, by all three primary criteria for formation of a chromatin-like structure, poly(dG)·poly(dC) and poly(dA)·poly(dT) do not fold like native

DNA on interaction with the inner histones. Additionally, electron micrographs of these complexes do not show a chromatin-like beaded structure and do not show significant length compaction of the DNA (not shown).

Poly(dAdT)•poly(dAdT) forms a well defined core particle with surprising properties

In contrast to the above polymers, poly(dAdT)•poly(dAdT) complexes with the inner histones to form a particle with all the characteristics expected for native core particles; additionally, due to the lack of heterogeneity in the DNA of this artificial complex, certain features of core particle structure are surprisingly well defined in the synthetic chromatin. Most striking of these features is the DNAase I digestion pattern of chromatin reconstituted from histones and poly(dAdT)•poly(dAdT) (Figure 1). Instead of the broad bands of approximate periodicity 10•n observed for either native chromatin or the complex of histones with native DNA (Figure 1), the synthetic complex digest has discrete bands at multiples of 2 nucleotides, clearly resolvable to over 150 bases in length in scans of longer gels (Figure 2). The envelope enclosing the highly resolved bands has periodicity of approximately ten and the variations in the intensities of the bands are akin to those observed for native or reconstituted chromatins containing sequence heterogeneous DNA. Thus, bands of about 70 and 80 bases in length are prominent while those of 60 and 90 bases length are relatively infrequently present; this latter feature is more pronounced in the DNA complex than in the poly(dAdT)•poly(dAdT) complex (Figure 1,2). Digestion of poly(dAdT)•poly(dAdT) not bound to intact histones leads to bands which are multiples of 2 but which lack this periodicity of 10 for favored cutting sites.

The single-stranded fragments in the chromatin digest occur with a periodicity of 2 bases; this arises because DNAase I cuts this synthetic polynucleotide only at the site -(pA+pT)- (18); hence all fragments will contain even numbers of bases. The high resolution shown in these gels clearly shows that DNAase I can attack chromatin DNA at any point in the core particle, not only at sites which are multiples of 10•n, although clearly preferential sites for cutting are at periodicities of about 10 nucleotides from each other. The actual periodicity for the repeat of DNA in the core particle can be calculated with great precision using these digests. Parallel digests of DNA and poly(dAdT)•poly(dAdT) reconstituted with the inner histones were electrophoresed on long polyacrylamide gels and scanned. The peak of each band in the digest of the DNA complex can be

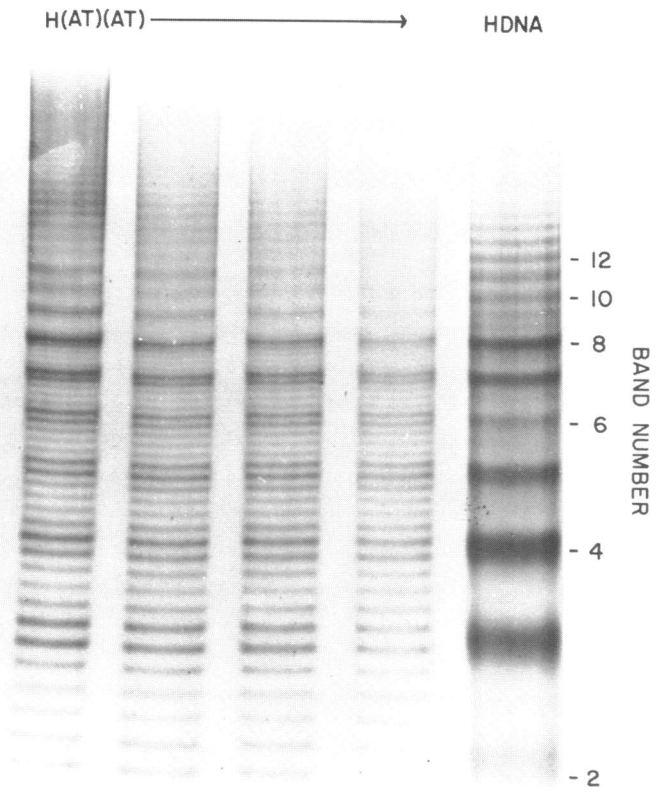

Figure 1 Single stranded DNA fragments produced by DNAase I digestion of the complex of histones with poly(dAdT)·poly(dAdT). Synthetic chromatin containing poly(dAdT)·poly(dAdT) was prepared and digested with 50 U/ml DNAase I in Ca^{++}, Mg^{++} buffer for 1, 2, 4, and 8 min at 37°C (sample order is from left to right). Electrophoresis was on a 10 cm long gel containing 7 M urea and 12% polyacrylamide. The right hand channel contains a DNAase I digest of chromatin reconstituted with salmon sperm DNA and the inner histones. Approximate DNA lengths are indicated, assuming that the DNA digest has a periodicity of 10 nucleotides.

accurately positioned using the digest of the synthetic polymer complex as standards. As shown in Table I, favored sites for DNAase I cutting of the DNA-histone complex are spaced at intervals of 10.4 - 10.5 nucleotides from one another. A similar result is obtained by calculating the repeat directly from the digest of the histone poly(dAdT)·poly(dAdT) complex. The observations that favored cutting sites are not integral multiples of 10.0 bases and that every site along the DNA in the core particle is accessible

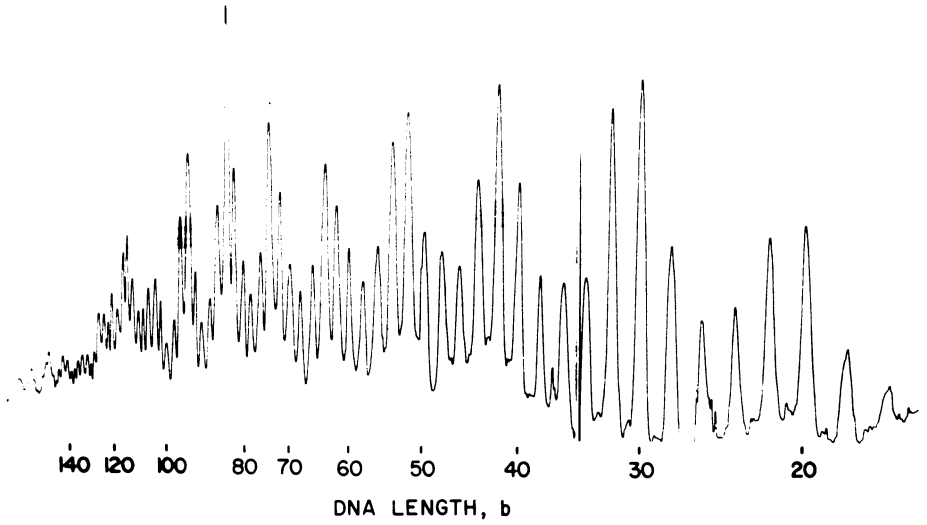

Figure 2 Scan of a DNAase I digest of the complex of histones with poly(dAdT)•poly(dAdT). A digest similar to those shown in Figure 1 was electrophoresed on a 35 cm long gel and the stained gel scanned. The ordinate is linear with optical density. DNA lengths are based on the assumption that bromphenol blue migrates with a fragment 13 nucleotides in length (11).

to DNAase I seem to preclude kinking (19,20) as the basis for the sites of nicking of DNA in chromatin by DNAase I and the "orientational" mechanism (21) for explaining the varying susceptibilities (12,22,23) of DNAase I cutting sites along the DNA of the chromatin particle.

TABLE I

Repeat Length for DNA in DNAase I Digests of Reconstituted Chromatin

DNA Band	2	3	4	5	6	7	8	9
Length, b	21.0	31.5	42.0	52.3	62.8	73.2	83.1	93.5
Length/number	10.5_0	10.5_0	10.5_0	10.4_6	10.4_7	10.4_6	10.3_9	10.3_9

The size of single-stranded DNA fragments in various bands was determined using a parallel digest of the complex of histones with poly(dAdT)•poly(dAdT) as standards.

Micrococcal nuclease digestion of the complex of histones with poly(dAdT)·poly(dAdT) yields fragments which are approximate multiples of a unit size fragment, about 146 base pairs in length (Figure 3). This observation is difficult to make when fragments are run in the gel system usually utilized for double-stranded DNA fragments; even at early stages of the digestion, a band with a mobility corresponding to length 72 bp appears. This seems to be due to core particle length fragments denaturing during phenol extraction or in the sample buffer and electrophoresing as hairpin molecules, since no bands of this size are seen when denaturing gel conditions are employed. The progression of bands from high molecular weight through a series of bands whose mobility is nearly linearly related to the logarithm of their number, to, finally, small fragments (Figure 3) is reminiscent of the digestion of chromatin lacking histone H1 and suggests further the organization of poly(dAdT)·poly(dAdT) into a chromatin-like species by the inner histones.

As the higher order bands are being degraded, a progression of intermediates is observed, with spacing approximating ten nucleotides in length (Figure 3). Thus, in addition to micrococcal nuclease cutting between particles, some of the putative dimers in this synthetic chromatin are either degraded exonucleolytically, with pauses at ten base intervals, or shortened by endonucleolytic cuts at 10 bp intervals. This observation is akin to that made for exonuclease III digestion of chromatin particles by others (24).

Core particles of remarkable homogeniety can be isolated from micrococcal nuclease digests of the complex of histones with poly(dAdT)·poly(dAdT) (Figure 4, 5). On preparative centrifugation resolution of monomer and dimer particles extends nearly to the baseline, in contrast to the case for native or reconstituted chromatins with sequence heterogeneous DNA. DNA length in the core particles was assessed by electrophoresis using a Hae III digest of ϕX174RF DNA as standards (Figure 5). The prominent band near the 72 bp standard is the core particle DNA running as hairpins. Measurements on 4 digests show that the core particle DNA length for this complex is 146±1 bp, determined from the lengths of both the double stranded and hairpin fragments.

Physical studies of both this synthetic chromatin and core particles derived from it further support the conclusion that the complex of histones with poly(dAdT)·poly(dAdT) has a chromatin-like structure. The thermal denaturation profile of the synthetic chromatin is complex, including three clearly defined transitions (Figure 6). The first transition, at 35°C,

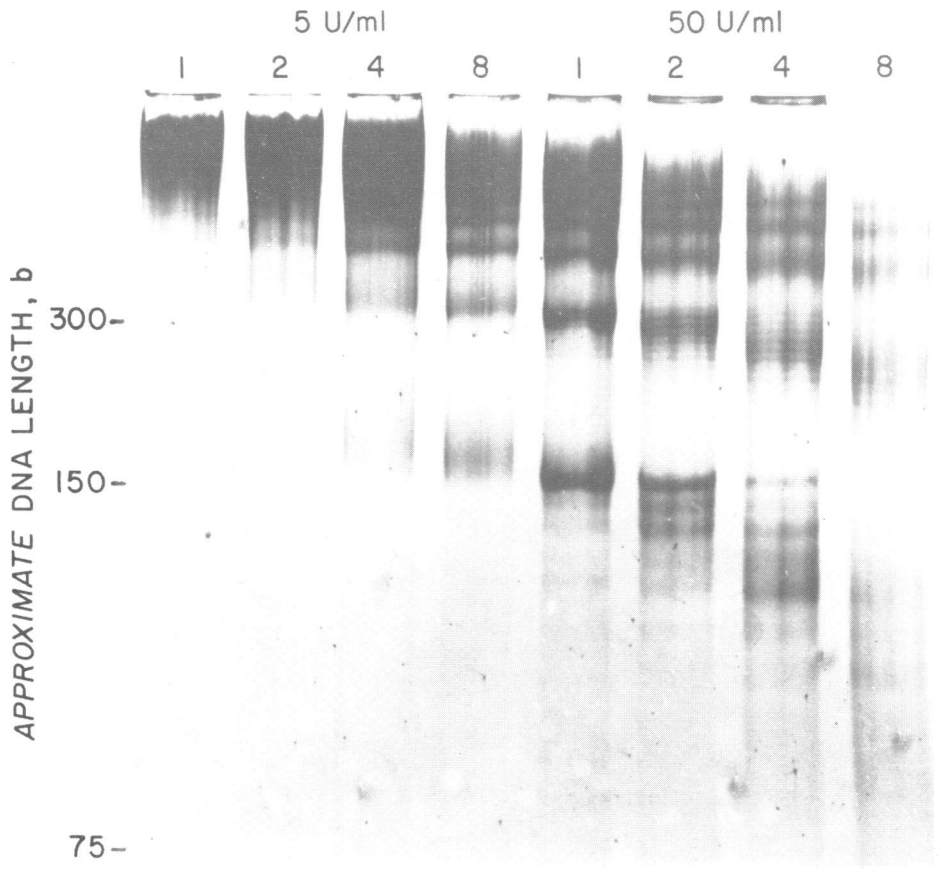

Figure 3 Fragment lengths in a staphylococcal nuclease digest of the complex of histones with poly(dAdT)·poly(dAdT). Synthetic chromatin containing poly(dAdT)·poly(dAdT) was digested at 37°C in 0.1 M NaCl with the enzyme concentrations (U/ml) and for the times in minutes indicated above the gel slots. After denaturation, samples were electrophoresed as in the legend to Figure 1, but electrophoresis was continued until xylene cyanol FF dye had migrated to the end of the gel. Approximate DNA lengths are based on the assumption that this dye migrates with a fragment 58 nucleotides in length (11).

varies in magnitude as an inverse function of the ratio of histones to polynucleotide used in the association reaction. At the "physiological" level of histones used for most studies, 0.8 gm histone/gm polynucleotide, this transition is about 40% of the total hyperchromicity (Figure 6). The melting temperature for this first transition is 20-25°C higher than that

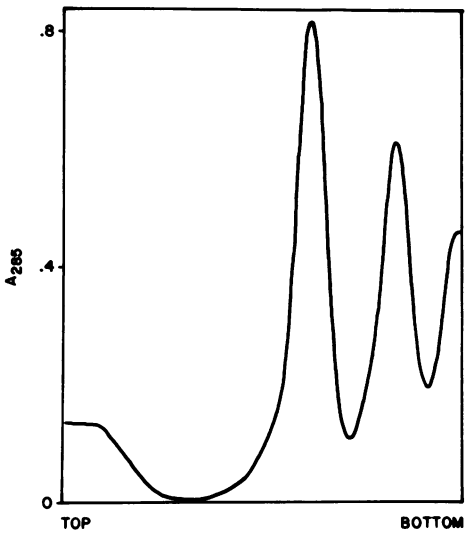

Figure 4 Sucrose gradient sedimentation of particles from staphylococcal nuclease digestion of the complex of poly(dAdT)•poly(dAdT) with the inner histones. The synthetic chromatin was digested for 1 min with 50 U/ml nuclease at 37°C, the reaction stopped by cooling and addition of EDTA to 10 mM, and samples sedimented on isokinetic sucrose gradients containing 0.1 M NaCl. Gradients were emptied through a spectrophotometer equipped with a flow cell. Sedimentation was from left to right.

for poly(dAdT)•poly(dAdT) in the same solvent, indicating stabilization of the entire polynucleotide by association with histones. By analogy with other studies of chromatin core particles (15), we feel it is likely that this melting represents polynucleotide in linker regions between core particles and probably the denaturation of some DNA at the ends of each core particle segment. The remainder of the polynucleotide melts between 60 and 70°C, a range near that for native core particles. In contrast to the single, broader transition observed for chromatin and native core particles (15), two clearly defined transitions are present for this sequence-homogeneous material (Figure 6). Melting of core particles containing poly(dAdT)•poly(dAdT) is also shown in Figure 6. All three transitions observed for chromatin are preserved in the core particle, although the magnitude of the lower transition is decreased to about 25% of the total hyperchromicity, likely due to removal of linker DNA, and the transition is sharper and occurs at a somewhat higher temperature. The two higher temperature transitions are again about equal in magnitude, although they occur at closer temperatures, 60°C and 64°C in the core particle versus 59°

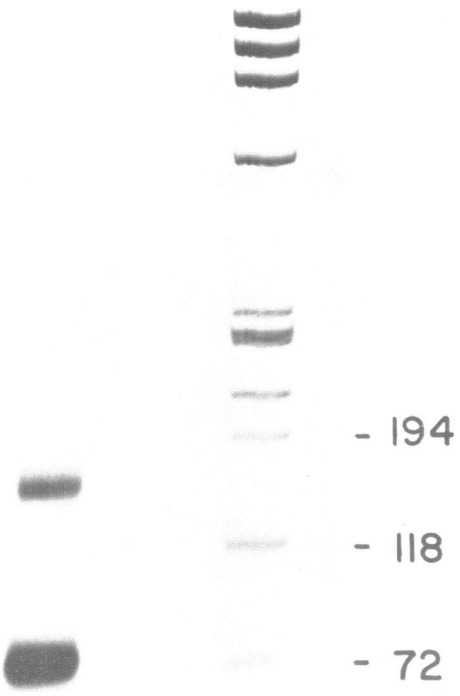

Figure 5 Size of DNA in the poly(dAdT)·poly(dAdT) core particle. DNA from the monomer fraction in Figure 4 was electrophoresed on a 5% polyacrylamide gel (left channel) together with a Hae III digest of ϕX174RF DNA (right channel). The gel shown was deliberately overloaded to show the absence of degradation products and higher order species; normally loaded gels show bands of width nearly as small as those of the restriction fragments.

and 66°C in the chromatin. Weischet et al. (15) have shown for native core particles that the 65-75°C transition accompanies disruption of histone-histone and histone-DNA interactions; the presence of two distinct transitions in this region for the synthetic core particle suggests that more information about the detailed aspects of disruption of core particle structure will be obtainable by similar studies of this complex of histones and the synthetic polynucleotide.

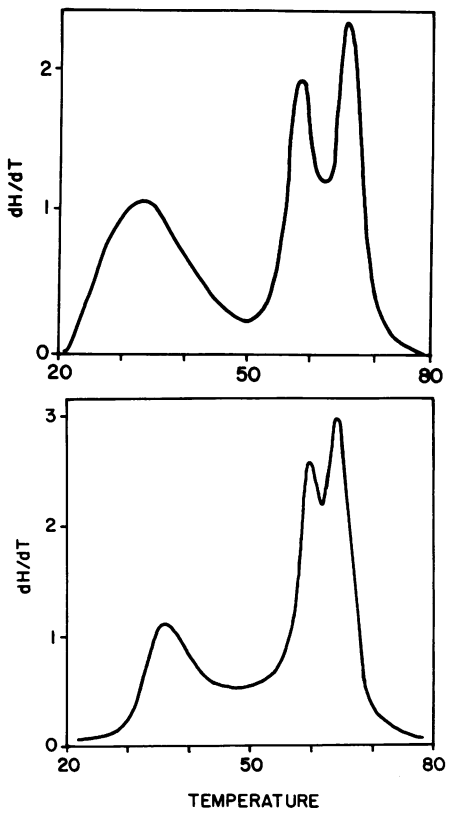

Figure 6 Thermal denaturation profiles of chromatin (top) and core particles (bottom) containing histones and poly(dAdT)•poly(dAdT). Samples were melted in 0.25 mM EDTA, pH 7.0, as described in the Experimental Section. Derivate melting profiles are shown, obtained by linear least square fitting the tangent to the melting profile over a two degree range about each point.

Hydrodynamic investigations of the core particles obtained from the synthetic chromatin further support its similarity to native chromatin core particles. The complex of histones with poly(dAdT)•poly(dAdT) of 146 bp length sediments as a highly homogeneous boundary at an ionic strength of 0.1 with a corrected sedimentation coefficient of 10.7 S.

Due to the sequence homogeniety of the core particle obtained from the complex of poly(dAdT)•poly(dAdT) with the inner histones, more detailed information about the localization of DNAase I cutting sites should be attainable with this particle than with native chromatin core particles. Both DNAase I (in the presence of Mg^{++}) and micrococcal nuclease preferen-

tially cleave DNA in AT-rich regions versus those containing high proportions of G and C (9,25). This is dramatically illustrated by the observation that optimal conditions for production of core particles from poly(dGdC)•poly(dGdC) chromatin (see below) utilize 100-times the concentration of micrococcal nuclease required for poly(dAdT)•poly(dAdT) chromatin. This difference in susceptibilities of regions of different base compositions could lead, with sequence heterogeneous DNA, to 1) lack of homogeniety in core particle DNA length, and 2) misrepresentation of the actual cutting site preferences in end label mapping experiments. For example, a normally susceptible site at 50 nucleotides from the 5' end might be cut with a lower frequency than a normally resistant site at 60 bases, if the former were GC-rich and the latter AT-rich. Accordingly, we labeled the 5' ends of the core particles containing poly(dAdT)•poly(dAdT) with ^{32}P using γ-^{32}P-ATP and polynucleotide kinase, as previously described (12), and mapped the susceptibilities for DNAase I cutting along the length of the core particle DNA.

Figure 7 shows the autoradiogram of labeled fragments obtained during a DNAase I digestion of such core particles. Scans of the autoradiogram at three times of digestion are presented in Figure 8. Up to about 60 bases from the 5' end, labeled bands are resolved at the level of individual bases. Cutting occurs at nearly every base from 10 to 60, although an apparent periodicity of 2•n is visualized as the major cutting sites. In part this arises from the fact that the particles used had about 60% of the 5' label on thymidine; the cutting mechanism of DNAase I will therefore lead to most of the fragments containing the 5' end being even numbered fragments. The major features of the cutting pattern are highly similar to those obtained by ourselves and others for native chromatin core particles in similar experiments (12,22,23,26). Major cutting sites are at bands 1, 2, 4, 5, 9, 10, 12, and 13; some nicking occurs at the 7th band from the 5' end; and the sites corresponding to bands 3, 6, 8, and 11 are very infrequently cut. The cutting pattern is remarkably symmetrical; largely due to the lack of cutting in these core particles at the 6th site from the ends. In our hands, the amount of cutting at this site has been variable from experiment to experiment with native core particles (12,26,27), in contrast to a consistent lack of cutting at the 8th site from the ends. At each of the major cutting sites for the smaller fragment sizes (less than 60 bases), two prominent bands are seen, e.g., 22 and 24, 42 and 44, and 52 and 54. This is consistent with and extends an observation made by Noll

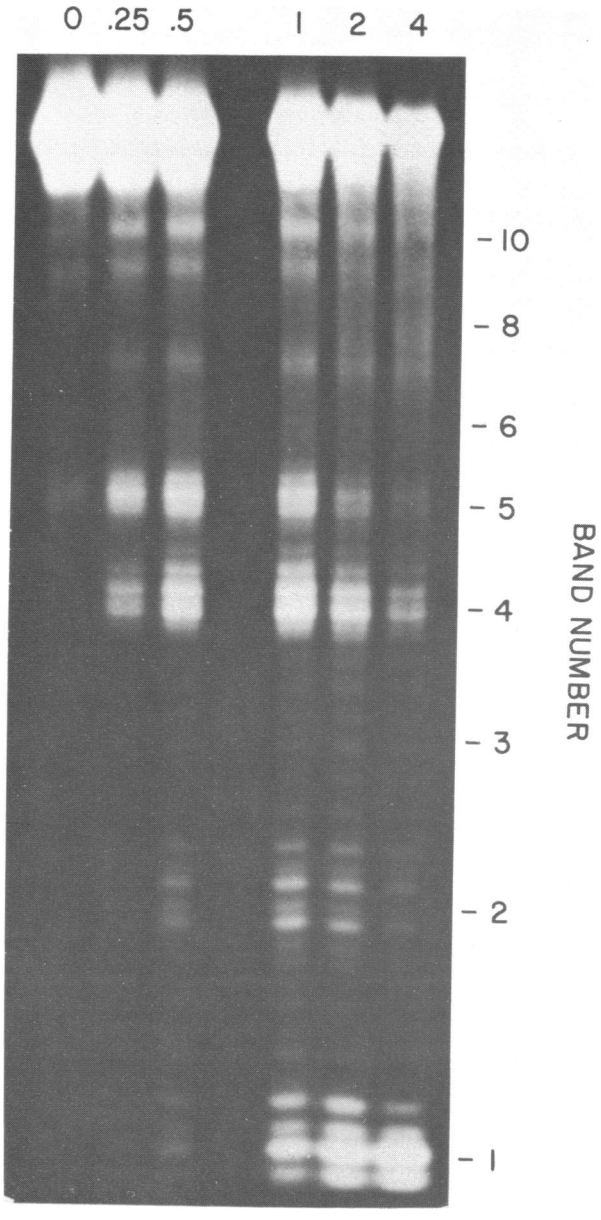

Figure 7 DNAase I cutting sites in core particles containing poly(dAdT)·poly(dAdT). Synthetic core particles labeled at the 5' end with ^{32}P were mixed with unlabeled carrier core particles and digested with 50 U/ml DNAase I for 0, .25, .5, 1, 2, and 4 min at 37°C as indicated. DNA was isolated, electrophoresed as in the legend to Figure 2, and the gel processed for autoradiography as described in the Experimental Section.

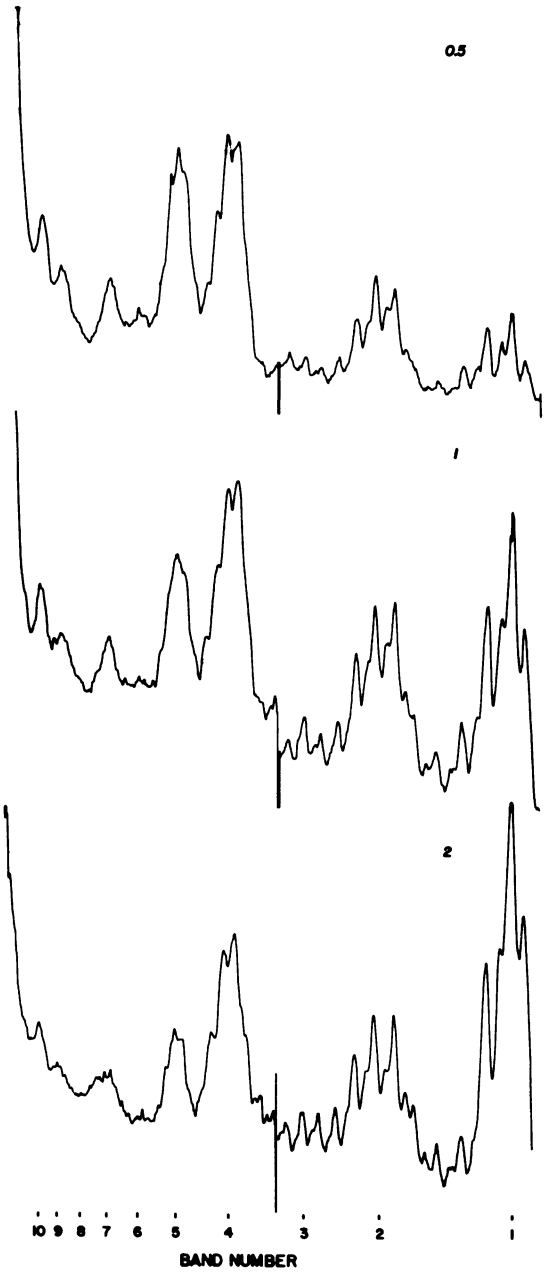

Figure 8 Scans of autoradiograms of poly(dAdT)·poly(dAdT) core particles labeled at the 5' ends and digested with DNAase I. Samples from Figure 7 at the items indicated were scanned. The figure is a direct photograph of the scans obtained. The ordinate is linear with film density.

(22) for native core particles. While the cutting sites are spaced from one another at periodicities of about 10•n, the 5' ends of the core particle are about three bases out of phase with the major cutting sites.

Poly(dGdC)•poly(dGdC) also forms a core particle

Association of the alternating copolymer of G and C with the inner histones leads to a chromatin which also satisfies the criteria employed to assess correct core particle formation, suggesting that a defined structural entity is formed on interaction with the inner histones; some differences are apparent between this complex and that formed by histones and poly(dAdT)•poly(dAdT), however. Digestion of the histone poly(dGdC)•poly(dGdC) complex by DNAase I leads to DNA fragments which appear as approximate multiples of 10 up to lengths of about 150 nucleotides (Figure 9). The GC-containing fragments form hairpins even under the denaturing conditions used for gel electrophoresis, hence prior treatment of the DNA sample with glyoxal was employed. Unfortunately, this leads to both poorer resolution of individual bands and increased molecular weight for the DNA fragments, making precise assignment of nucleotide lengths not possible. The bands do appear to have mobilities linearly related to the logarithm of their number, suggesting that the same pattern of multiples of about 10 nucleotides occurs for DNAase I digestion of this complex as for native chromatin. A scan of the stained gel shows this periodicity (Figure 10) and variations in the proportions of the various length fragments which bear similarities to that observed for native chromatin DNA. Again, bands 7 and 8 are prominent, and in this case, bands 6 and 10 are underrepresented relative to others. Differing from native chromatin digests, the GC-complex also has a low frequency of bands in the 20-40 base pair range (Figure 10).

The subunits of the complex of histones with poly(dGdC)•poly(dGdC) appear to contain the same length of the synthetic DNA that the complex of histones with native DNA contains. Discrete lengths of DNA are generated by digestion of the synthetic chromatin with micrococcal nuclease (Figure 11). The higher order bands have a periodicity of $\sim$145 + (120•n) while the monomer is again $\sim$145 base pairs in length. As digestion proceeds, discrete fragments resulting from internal cleavages in the core particles are generated, as is the case for native chromatin. The spacing of the higher order bands would be expected if the synthetic DNA formed close packed oligomers when associated with histones, as initially observed for chromatin treated with 0.6 M NaCl by Tatchell and van Holde (28). The observation that dis-

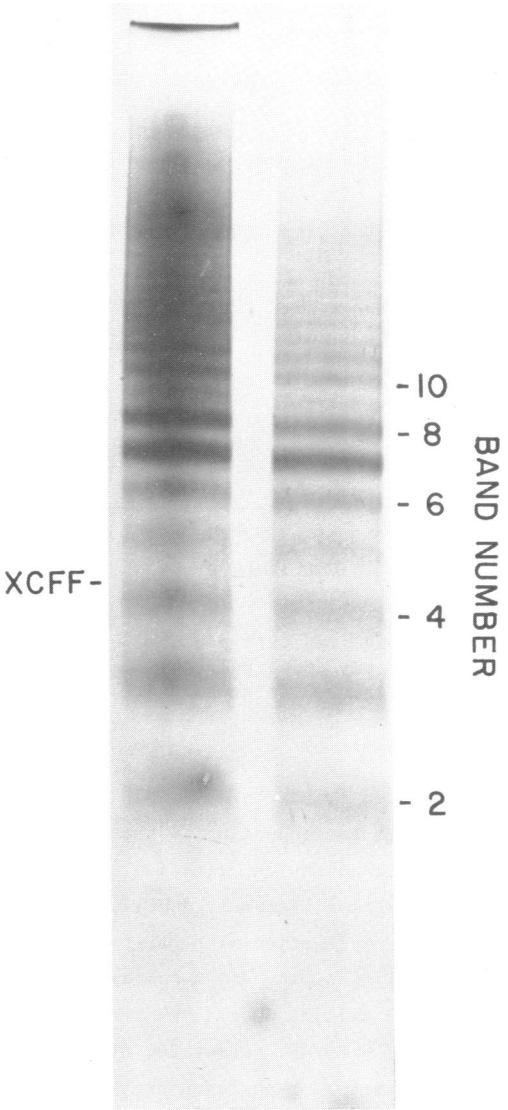

Figure 9 Single stranded DNA fragments produced by DNAase I digestion of the complex of histones with poly(dGdC)·poly(dGdC). Synthetic chromatin containing poly(dGdC)*poly(dGdC) was prepared and digested with 7 U/ml DNAase I in Mn^{++} buffer for 1 and 2 min at 37°C (left and right channels, respectively). Electrophoresis was as in Figure 1 except that samples were treated with glyoxal before electrophoresis as described in the Experimental Section. Migration position of xylene cyanol FF (XCFF) is indicated. Band numbers are based on the assumptions detailed in the text.

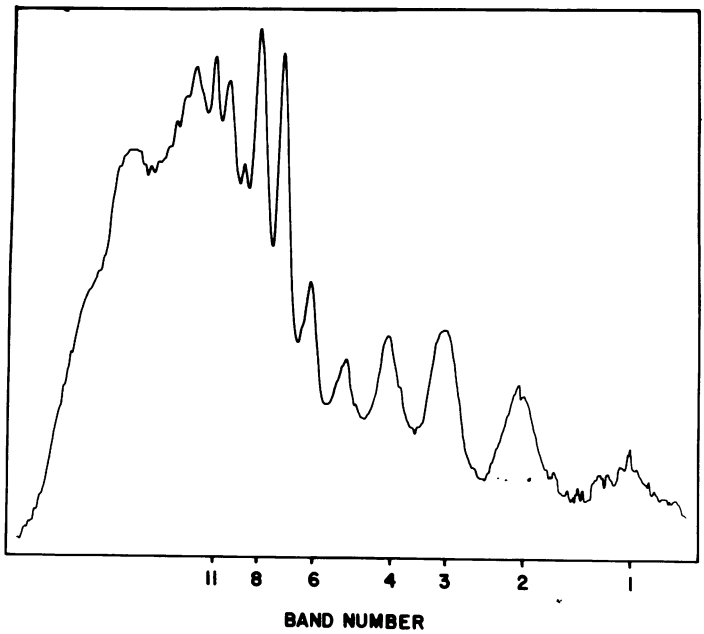

Figure 10 Single-stranded DNA fragments produced by DNAase I digestion of the complex of histones with poly(dGdC)•poly(dGdC). A scan of the right hand channel of the gel shown in Figure 9 is presented. The ordinate is linear with optical density.

crete DNA lengths are protected by histones in this complex, and that the smallest of these has a length corresponding to that in a native core particle, contrasts with observations on digestion of the complementary homopolymers and strongly suggests that poly(dGdC)•poly(dGdC) does form a chromatin-like structure on complexation with the four smaller histones.

Due to the high thermal stability of GC-containing polymers, we were not able to obtain a complete melting of this synthetic chromatin, even at very low ionic strengths (10 μM EDTA). About 30% of the expected hyperchromicity for the complex develops as a transition centered at 85°C. This likely corresponds to the low temperature transition seen for either native core particles (15) or the histone poly(dAdT)•poly(dAdT) complex (Figure 6). We can only define about half the second transition, in the range from 95-100°C, and are unable to determine if it is split as it is for the AT-complex. The melting is thus suggestive of formation of a defined complex with chromatin-like character for the inner histones and poly(dGdC)•poly(dGdC).

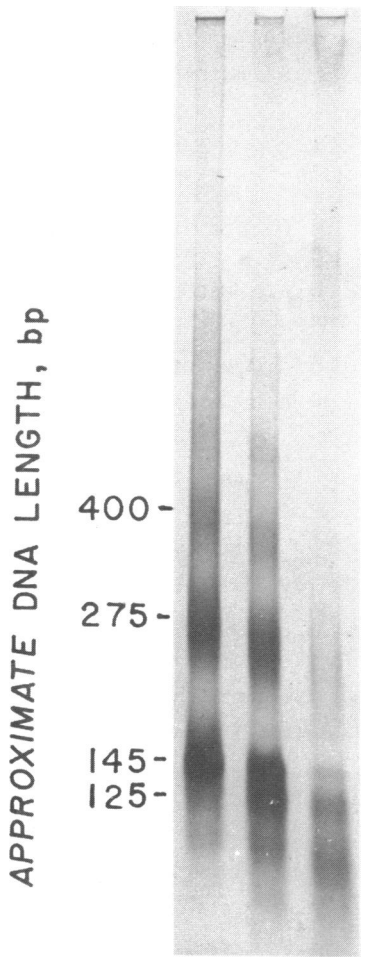

Figure 11 Fragment lengths in a staphylococcal nuclease digest of the complex of histones with poly(dGdC)•poly(dGdC). Synthetic chromatin containing poly(dGdC)•poly(dGdC) was digested at 37°C with 5000 U/ml staphylococcal nuclease without NaCl present for 1, 2 and 4 min (from left to right, respectively). Samples were electrophoresed as in the legend to Figure 5.

Core particles were isolated from this synthetic chromatin by digestion with micrococcal nuclease and sucrose gradient sedimentation. Resolution of monomer and putative dimer fractions was equivalently good to that shown above for the AT-complex (Figure 4). The DNA present in the monomer fraction was 146 bp in length (not shown); presumably any smaller fragments

are present in nucleoprotein complexes which are insoluble under the gradient conditions (13). Melting of the core particle containing poly(dGdC)•poly(dGdC) was quite similar to that of the chromatin from which it was prepared; again technical reasons precluded visualization of the full thermal denaturation profile. This core particle behaved in homogeneous fashion on sedimentation in the analytical ultracentrifuge; $S_{20,w}$, determined at ionic strength 0.1, was 11.0 S.

The distribution of DNAase I cutting sites and their relative susceptibilities in the poly(dGdC)•poly(dGdC) core particle were determined by 5' end labeling as described above. We anticipated some differences in relative susceptibilities for this particle versus the AT-containing particle in view of differences in intensities of stained bands for the two synthetic chromatins (c.f. Figures 2 and 10). Figure 12 shows the autoradiogram for several stages of digestion of 5'labeled GC-particles together with a control digest of labeled AT-particles. Scans of the autoradiogram for three times of digestion are displayed in Figure 13. Again, glyoxal treatment was employed, leading to slower mobilities and loss of resolution; the slowing is less for the AT-fragments since T will not react with the aldehyde.

Comparison of the autoradiograms and, more clearly, the scans of same, for the poly(dAdT)•poly(dAdT) core particle (Figure 8) and the poly(dGdC)•poly(dGdC) core particle (Figure 13) reveals certain clearcut differences between cutting site susceptibilities for the two synthetic core particles. Most striking of these is the high susceptibility of the 6th band site in the GC-particle, cut more frequently than the 4th or 5th band site, compared with the virtual absence of cutting at this site in the AT-containing particles. Little cutting is seen at the 8th band site in the GC-particle, an exception to the symmetry in cutting site distribution observed for the AT-particle. The second major difference is the low frequency of cutting at the 2nd band site in the GC-particle; this site is nearly as infrequently cut as the 3rd band site which is highly resistant in native core particles and the poly(dAdT)•poly(dAdT) particle. Similar patterns are observed when the poly(dGdC)•poly(dGdC) core particle is digested with higher concentrations of DNAase I (200-400 U/ml) in the presence of Mg^{++} instead of Mn^{++} (data not shown). Thus, variations in cutting site susceptibility apparently can occur due to the structure or, at least, nucleotide composition of the core particle DNA. The extent to which other differences between the polydeoxyribonucleotides, for example

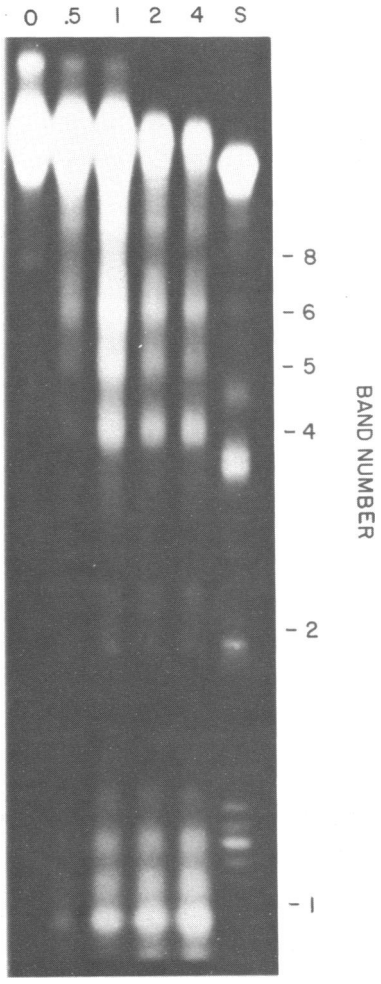

Figure 12 DNAase I cutting sites in core particles containing poly(dGdC)·poly(dGdC). Synthetic core particles labeled at the 5' end were mixed with unlabeled carrier core particles and digested with 5 U/ml DNAase I in Mn^{++} buffer for 0, .5, 1, 2, and 4 min as indicated. DNA was isolated, treated with glyoxal, and electrophoresed as the legend to Figure 2. The slot "S" contains a digest of the poly(dAdT)·poly(dAdT) core particles. Autoradiography was carried out as described in the Experimental Section.

the greater stability to strand separation of poly(dGdC)·poly(dGdC), may contribute to the obtained results is unknown.

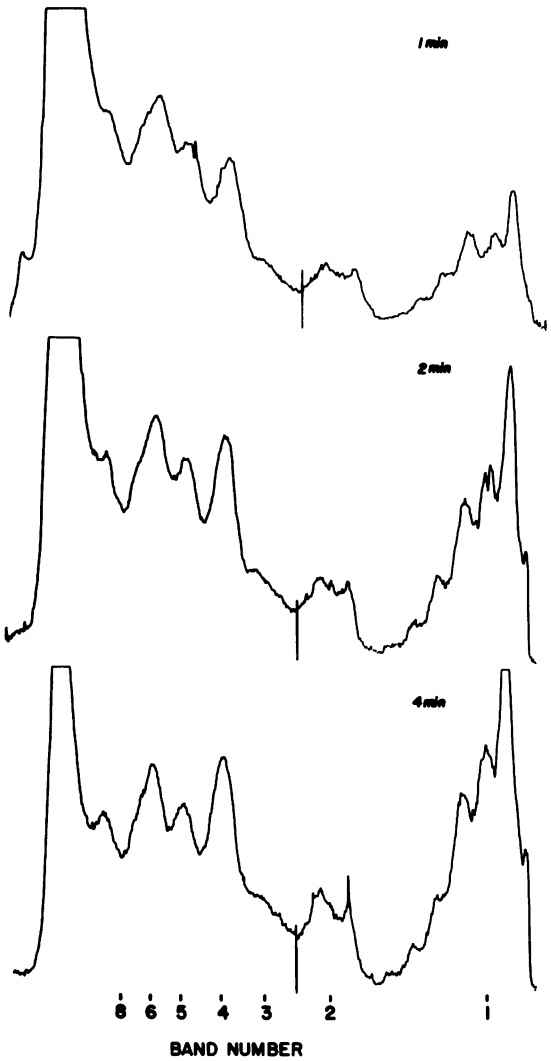

Figure 13 Scans of autoradiograms of poly(dGdC)·poly(dGdC) core particles labeled at the 5' ends and digested with DNAase I. Samples from Figure 12 at the times indicated were scanned. The figure is a direct photograph of the scans obtained. The ordinate is linear with film density.

DISCUSSION

The results obtained in this study are relevant to chromatin structure in two distinct areas: 1) the role of nucleotide sequence and DNA

structure in determining whether a particular segment of DNA will fold around a histone octamer to form a core particle and 2) the detailed information about core particle structure that should be obtainable when a simple sequence DNA is present in that particle.

Consideration of the geometries adapted by the various synthetic polynucleotides under differing conditions is relevant to the formation or lack of formation of core particles on interaction of these DNA's with the inner histones. Poly(dG)·poly(dC) prefers to adapt an A-DNA structure, taking up the B-form structure only at high salt concentrations (2). A-DNA is distinguished from B-DNA in rise per residue, residues per turn, helical repeat length, skew angle, base position relative to helix axis, helix diameter and sugar pucker (data summarized in 29). The overall effect of these differences is to make A-DNA a squatter, fatter molecule when compared to B-DNA (see reference 29 for photographs of space filling models of A- and B-DNA). Association of histones with DNA during salt gradient dialysis is a complex process and the effective local ionic strength and degree of hydration of DNA during this process are not known. Nevertheless the correlation between the proclivity of poly(dG)·poly(dC) to form A-DNA and its failure to be folded into a chromatin-like structure by the inner histones is of interest. We assume that the disposition of positive charges on the surface of the histone octamer which stabilize DNA on its path around this complex protein is rather precisely related to the geometry of the nucleic acid. The difference in disposition of phosphate negative charges between A- and B-DNA is sufficient that A-DNA may not be capable of mating with the stabilizing ionic groups on the octamer of histones, precluding formation of a stable nucleoprotein complex. The greater diameter and shorter length per residue of the A-DNA helix may also be inconsistent with the topography of the DNA-protein interactions required to fold DNA around a histone octamer.

If the reason that poly(dG)·poly(dC) does not form chromatin-like structures is related to its preference for A-form structures, an interesting biological ramification of these studies arises. RNA-DNA hybrids are A-form structures under all conditions of ionic strength (4). If A-form nucleic acids can not interact in stable fashion with a histone octamer, histone dissociation during transcription may be a simple function of a change in geometry of the nucleic acid as RNA is transcribed. Dissociated histone octamers could bind to nearby nucleosomes, as shown for native reassociating core particles by Stein (30), and rebind to naked DNA after

transcription has proceeded and the DNA has resumed a normal B-form structure.

Poly(dA)·poly(dT) also fails to form a chromatin-like structure on interaction with the inner histones. This synthetic polynucleotide has never been observed in an A-DNA structure, instead existing as B'-DNA (a minor variant of B-DNA) at most salt concentrations and humidities (3). At high salt concentrations, however, this polymer disproportionates, forming a triple-stranded helix, poly(dA)·poly(dT)$_2$, plus single-stranded poly(dA) (3). Perhaps this is the state of the polynucleotide when it encounters histones during the reassociation, leading to a trivial explanation for failure to observe folding of this polynucleotide. Such a hypothesis is supported by the observed melting of this complex; the first transition would correspond to removal of the extra poly(dT) and the second to dissociation of the poly(dA)·poly(dT)helix. While this may be the basis for our observations, it is currently only a surmise; experiments with this polynucleotide and assembly factors which allow reassociation at lower ionic strengths (31) should allow resolution of this question.

To whatever extent these observations can be extrapolated to in vivo situations, they suggest that long runs of G or C, and perhaps, A or T, may block the formation of a nucleosome on that segment of DNA. The obvious question of interest is, just how long a run is necessary?

The two alternating copolymers, poly(dAdT)·poly(dAdT) and poly(dGdC)·poly(dGdC) associate with the four smaller histones to form structures with characteristics closely like those of native chromatin lacking H1 histone. DNAase I digests the complexes to generate single-stranded DNA fragments which are multiples of approximately 10 in length. Micrococcal nuclease degrades the synthetic chromatins to produce a ladder of fragments, the smallest of which is 146 bp in length and is contained in a core particle-like structure sedimenting at about 10.7 or 11.0 S. Melting of the AT-complex is, and that of the GC-complex appears to be, biphasic, like native core particles (15). These copolymers have structural characteristics like those of sequence heterogeneous DNA under most conditions (29), suggesting their utility as reasonable synthetic polynucleotide models for studies of chromatin structure.

All previously available information concerning the structural relationships of histones and DNA in core particles has derived from structures containing complex sequence or sequence heterogeneous DNA. Such studies, using either enzymatic or physical methods for analysis, are

limited in their resolution by this sequence heterogeniety. For example, it is known that both micrococcal nuclease and DNAase I preferentially hydrolyze AT-rich DNA versus GC-rich regions (9,25). This fact would, a priori, be expected to lead to both length heterogeniety in core particle preparations and variations in cutting site frequency in DNAase I mapping experiments. By removing the heterogeniety from the DNA sequence, through the use of synthetic core particles, we should be able to amplify the features of the core particle which derive solely from DNA-protein interactions and their effects on the structure of the polynucleotide.

In this first series of experiments, this prediction has proven true. Core particles of very homogeneous length have been obtained with both synthetic alternating copolymers. Other features of core particle structure have been defined using the core particle containing poly(dAdT)·poly(dAdT). New features of the dissociation of these particles at elevated temperatures have been observed. The spreading of bands in DNAase I digests of native chromatin has been shown to arise, at least in part, from the fact that every site along the DNA can be nicked by this nuclease, even though preferential sites for nicking exist at approximate multiples of 10. This observation has made unlikely two postulated mechanisms for explaining the differential nuclease susceptibility of DNA within the core particle. The actual repeat length for DNAase I digestion of chromatin has been precisely defined. Finally, a more accurate assignment of the distances of nicking sites from the 5' end of the DNA, and their relative susceptibilities, has become possible. Further study of these sequence homogeneous synthetic core particles by other methods should lead to more detailed knowledge of histone-DNA interactions than might be obtained by study of native chromatin subunits.

The current studies of DNAase I cutting site frequencies in the two synthetic core particles suggest that two different types of interactions of DNA with histones might exist in the chromatin subunit. The DNAase I map for AT-containing particles is symmetrical, with low or nonexistent cutting at sites number 3, 6, 8, and 11. In contrast the map for GC-containing particles has low cutting frequency at sites number 2, 3, 8, and 11. Our previous studies suggested that the relatively DNAase I resistant sites in native core particles were a result of the interactions of histone amino terminal regions with DNA at these sites (26). If there are two types of core particles, exemplified by the two synthetic species discussed here, do they arise from a difference in the path of DNA around the parti-

cle, with the same structure for the octamer, or does the histone octamer have a flexibility which is affected by the structure or base composition of the DNA with which it interacts? This question should be answerable by crosslinking the histones of one type particle into an octamer (32), and then associating them with the other synthetic DNA and characterizing the product.

Whichever is the case, the observation that different DNA sequences may lead to different structures for the DNA within the same length of nucleic acid bound to an octamer of histones suggests that sequence features of DNA may effect the structure of nucleosomes, an addition to the conclusion above that sequence determined structural features of DNA may determine whether or not a particular segment of the nucleic acid is packaged with histones.

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