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Self-assembly of single and closely spaced nucleosome core particles

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ABSTRACT

Self-assembly of DNA with the four core histones but in the absence of H1 generates nucleosome core particles which are spaced randomly over large distances. Closely spaced core particles, however, exhibit a preferred short linkage which is not a multiple of 10 base pairs. They bind about 140 base pairs whereas apparently shorter DNA lengths per nucleosome observed after digestion with micrococcal nuclease are the result of degradation from the ends. The DNA length of one superhelical turn in the core particle is 83 ± 4 base pairs. Single core particles may bind more DNA than closely spaced core particles but probably less than two full turns or 168 base pairs. The internal structures of single and of native core particles are very similar as judged by their amount of DNA, sedimentation coefficient, appearance in the electron microscope, and digestion with DNase I. In addition to core particles, a particle is described which sediments at 9 S and consists of 108 base pairs of DNA bound to the histone octamer. It appears to be the smallest stable "core particle" but it is not a degradation product of the 146-base-pair core particle. Digestion of end-labeled 9 S and nucleosome core particles with DNase I shows distinct differences.

INTRODUCTION

Numerous efforts have been made to reconstitute chromatin from histones and DNA (1-5). From these experiments it emerged that at least one assembly factor is required (2,3) to obtain the same DNA length per nucleosome in reconstituted as in native chromatin. Although this finding indicates that the assembly of chromatin *in vivo* is not a self-assembly process, reconstitution without an assembly factor is possible in the absence of histone H1 (1). However, this type of reconstituted chromatin exhibits a considerably shorter DNA length per nucleosome (4). Such a reduced DNA size per nucleosome has been reported to exist transiently in newly replicated chromatin *in vivo* (6) and may occur also in other regions of native chromatin devoid of H1. In this study, the self-assembly of DNA and the four histones of the nucleosome core is used to examine the possible arrangements of nucleosomes in such regions and to analyze the structure of the nucleosome core in greater detail.

MATERIALS AND METHODS

Preparation of histones. So called native chromatin was prepared from rat liver nuclei as described (7), and histones were obtained by stepwise washings of the chromatin in NaCl (8): To native chromatin ($A_{260} \simeq 30$) in 1 mM EDTA, pH 7, 0.5 mM PMSF (PMSF was always diluted from a 50 mM stock solution in isopropanol) an equal volume of 20 mM Tris-HCl, pH 7.5, 0.6 M NaCl, 2 mM DTT, 0.5 mM PMSF was added slowly with mixing on a Vortex. The chromatin was centrifuged in a Beckman SW 50.1 rotor through 0.5 ml of 1 M sucrose, 10 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1 mM DTT, 0.5 mM PMSF for 12.5 hr at 50,000 rpm and 2°C. The pellet was rinsed, dissolved in 4.5 ml of 10 mM Tris-HCl, pH 7.5, 0.6 M NaCl, 1 mM DTT, 0.5 mM PMSF, and spun through a 0.5 ml 1 M sucrose cushion containing 10 mM Tris-HCl, pH 7.5, 0.6 M NaCl, 1 mM DTT, 0.5 mM PMSF for 16 hr as before. The supernatant contained histone H1 and none of the core histones (Fig. 1). The H1-depleted chromatin pellet was rinsed and dissolved in 20 mM Tris-HCl, pH 9.0, 2 M NaCl, 1 mM DTT, 0.5 mM PMSF. Insoluble matter was removed by a quick spin for 10 min at 10,000 rpm. The supernatant ($A_{260} \simeq 20$-25) was layered over 0.5 ml of 1 M sucrose, 20 mM Tris-HCl, pH 9.0, 2 M NaCl, 1 mM DTT, 0.5 mM PMSF and centrifuged for 19 hr at 50,000 rpm. About 1 ml of the supernatant slightly above the pellet (to avoid contamination with DNA) showed the highest concentration of histones H2A, H2B, H3, and H4, typically 2-3 mg/ml. Less than 4% DNA (by weight) was present, and no contamination with histone H1 was evident (Fig. 1). Histone concentra-

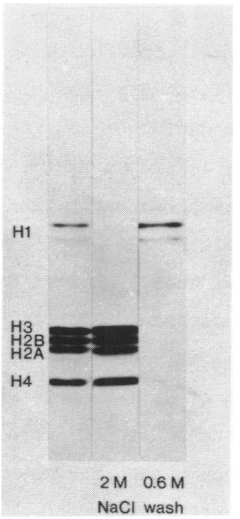

Figure 1: Polyacrylamide gel analysis of fractionated histones. Histone H1 and the core histones H2A, H2B, H3, and H4 are analyzed in 18% polyacrylamide gels as described (9). The left lane shows the histones of native chromatin for reference.

tions measured either by absorbance at 230 nm (4.25 mg/A_{230}, ref. 8) or by amino acid analysis varied not more than 5-10%. No proteolytic degradation of the histones was detectable before or after reconstitution of chromatin (Fig. 1). The 0.3 M NaCl wash of the procedure removing most nonhistone proteins may be omitted if no purification of histone H1 is required.

Preparation of DNA. High molecular weight DNA was purified from salmon sperm DNA (Serva) according to Marmur (10). Circular DNA of the plasmid pSF 2124 (11) containing a 3 kb deletion (P. Schedl and S. Artavanis-Tsakonas, unpublished) was linearized with Eco RI.

Reconstitution of chromatin. For chromatin assembly from DNA and histones H2A, H2B, H3, and H4 a modified procedure of Germond et al. (12) was used. Salmon sperm DNA (0.2-1 mg/ml; Serva) and histones H2A, H2B, H3, H4 were mixed in 2 M NaCl, buffer R (20 mM Tris-HCl, pH 8.0, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.1 mM PMSF) at the ratios indicated. The mixture was dialyzed at 37°C against buffer R and stepwise reduced concentrations of NaCl: 1.6 M (15 min), 1.2 M (15 min), 0.85 M (1 hr), 0.75 M (1 hr), 0.65 M (1 hr), 0.5 M (15 min), 0.25 M (15 min). In a final step the reconstituted chromatin was dialyzed against 0.2 mM EDTA, pH 7 at 4°C overnight.

Enzymes. Nucleases were purchased from Sigma Chemical Co. (micrococcal nuclease and DNase I) or from Worthington Biochemical Corporation (DNase II and micrococcal nuclease).

Electron microscopy of reconstituted chromatin. For electron microscopy, samples were adsorbed onto positively charged carbon films, stained with an aqueous solution of 2% uranyl acetate, and rinsed with water (13). They were observed in dark-field CTEM for length measurements or in a STEM for high resolution analysis (14). Length measurements were calibrated by adding DNA molecules of a known length to the preparation.

Analysis of distribution of internucleosomal DNA lengths. For simplicity we consider the problem of combining randomly N_o histone octamers with a single DNA molecule of the length L to form N_o nucleosome core particles. With a DNA length L_o bound in each particle the average length $\bar{l}$ of the internucleosomal DNA is $(L-N_oL_o)/N_o$. In analogy to the radioactive decay, the number N(l) of intervals of internucleosomal DNA larger than l is obtained from

$$\frac{dN(l)}{dl} = - kN(l). \qquad (1)$$

Employing the boundary conditions $N(0) = N_o$ and $\int_0^\infty N(l)dl = L - N_o L_o$, the solution of this equation becomes

$$N(l) = N_o \exp(-l/\bar{l}). \qquad (2)$$

In Figure 6, however, we plot the number $\Delta N(l)$ of intervals of internucleosomal DNA between l and $l + \Delta l$, i.e.

$$|\Delta N(l)| = \frac{N_o \Delta l}{\bar{l}} \exp(-l/\bar{l}). \qquad (3)$$

From this equation it is easily verified that the tangent to the curve at $l = 0$ intersects the l-axis at $l = \bar{l}$.

In Figure 6 the average lengths of internucleosomal DNA were 359 Å, 698 Å, and 1090 Å whereas the values for $N_o \Delta l/\bar{l}$ were 56.3, 67.0, and 52.3 for $R_{nom} = 0.8$, 0.6, and 0.4, respectively. From $\bar{l}$, L_o, the DNA mass per unit length μ, and the molecular weight M of the histone octamer the histone/DNA ratio of the reconstituted chromatin, R_{real}, is calculated to be equal to $M/(\bar{l} + L_o)\mu$.

RESULTS

Self-assembly of string of nucleosome core particles

Chromatin was reconstituted by combining equimolar amounts of the four core histones H2A, H2B, H3, and H4 (Fig. 1) with high molecular weight DNA at 2 M NaCl and subsequent reduction of the salt concentration by stepwise dialysis at 37°C (see Materials and Methods). Such H1-deficient chromatin reconstituted at histone/DNA ratios between 0.4 and 1.7 was partially digested with micrococcal nuclease, and the DNA fragments were analyzed in a polyacrylamide gel as shown in Figure 2. Two features are evident from Figure 2. The DNA size per nucleosome of reconstituted chromatin is considerably smaller than that of native chromatin, and it is the same at all histone/DNA ratios tested except for ratios above 1.3. Clearly, the discrete bands originate from a regular close spacing of nucleosome core particles.

Two views of the assembly process are compatible with the experiment shown in Figure 2. It is possible that nucleosome core particles assemble at random intervals. In this case, more distant core particles linked by free DNA would contribute in Figure 2 only to a continuous size distribution of DNA fragments above the DNA size of a single nucleosome. Moreover, this contribution to a continuous background is expected to be reduced with increasing

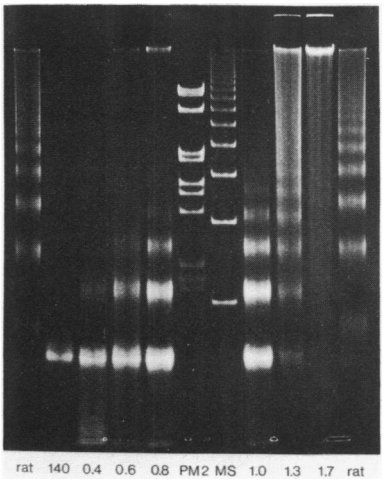

Figure 2: Micrococcal nuclease digestion of reconstituted chromatin. Chromatin reconstituted at the histone/DNA ratios indicated was digested with micrococcal nuclease (30 units per 100 µg of DNA; 75 units/ml) for 30 s at 37°C as described in the legend of Table 1. Analysis of the DNA in a 2.5% polyacrylamide slab gel (15) was carried out as described (16). The lanes at the left and right contain DNA of a 0°C micrococcal digest of rat liver nuclei (16), the lanes labeled PM2 and MS exhibit a Hae III digest of PM2 DNA and a partial Eco RII digest of mouse satellite DNA, respectively, and the lane labeled "140" shows DNA of the "140-base-pair" core particle.

digestion as free DNA is degraded more rapidly than the DNA of closely spaced core particles. Alternatively, the results could be explained by a preferentially close spacing of core particles due to a cooperative assembly. Both views are consistent with the observation of DNA bands corresponding to higher multiples of closely spaced core particles with increasing histone/DNA ratio.

Although the DNA size of single core particles in reconstituted chromatin is slightly larger than 140 base pairs (17, 18) and the same as in native chromatin (Fig. 2), the DNA size per nucleosome of closely spaced core particles appears to be shorter. Averaging the size differences between successive multimers (16) which have been calibrated with DNA fragments of known size shows that there are only 127 ± 4 base pairs bound per nucleosome (Fig. 3).

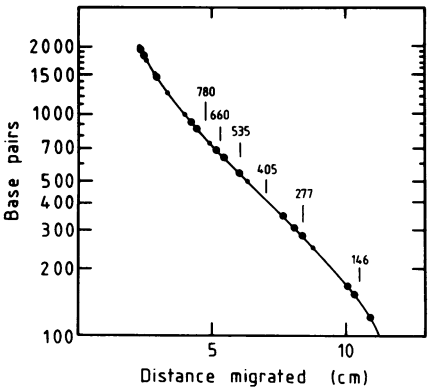

Figure 3: Calibration of DNA length per nucleosome in reconstituted chromatin. The DNA sizes obtained after micrococcal nuclease digestion of reconstituted chromatin were determined from Figure 2. Hae III restriction fragments of PM2 DNA (⊛) and an Eco RII digest of mouse satellite DNA (•) served as markers. The mouse satellite DNA represents multiples of 248 base pairs (16), the Hae III restriction fragments of PM2 DNA are slightly larger than in a previous determination (19): 1930, 1820, 1485, 915, 860, 695, 630, 540, 340, 300, 277, 167, 152, 120, 95, 59, and 54 base pairs.

The effect is probably the result of DNA degradation from the ends (7,16) for the following reasons. As evident from Figure 4, the DNA size of oligonucleosomes is reduced with increasing digestion. At very mild digestions, a much larger DNA size per nucleosome of about 140 base pairs is observed (Table 1). In addition, digestion with DNase II which results in little degradation from the ends (refs. 4, 20, and Fig. 4) demonstrates a similar DNA length of the closely spaced core particles (Table 1). Thus under conditions that suppress degradation from the ends, a DNA size per nucleosome is obtained which within limits of the error of measurement does not differ from the 146 base pairs of single core particles. The monomeric DNA size of 168 base pairs obtained after DNase II digestion is about 20 base pairs larger than that of a micrococcal nuclease digestion. This does not reflect a change in chromatin structure induced by Ca^{2+} because the same difference is detected if Ca^{2+} is present during DNase II digestion.

The question of cooperative or random assembly of the nucleosome core particles was examined by electron microscopy of the reconstituted chromatin (Fig. 5). At low histone/DNA ratios the core particles appear randomly spaced. With increasing histone/DNA ratio the average spacing is reduced until at a ratio of 1.3 all DNA is packed into tightly spaced core particles. A quantitative analysis of the spacing of the core particles does not exhibit any significant deviation of the measured distribution from that expected for random assembly (Fig. 6) and hence argues in favor of a random assembly pro-

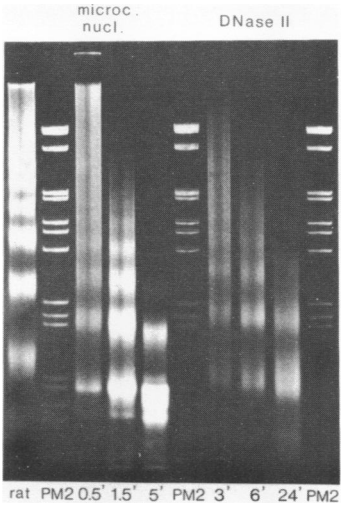

Figure 4: Time course of micrococcal nuclease and DNase II digestion. Reconstituted chromatin was digested with micrococcal nuclease or DNase II for the times indicated as described in the legend of Table 1. The DNA was analyzed in a 2.5% polyacrylamide slab gel as in Figure 2. At the left a micrococcal nuclease digest of rat liver nuclei is shown for comparison. A Hae III digest of PM2 DNA was used for calibration. For sizes see Figure 2.

Table 1: DNA size of mono- and oligonucleosomes (base pairs) after digestion with micrococcal nuclease or DNase II

	micrococcal nuclease				DNase II			
time of digestion (min)	0.5	1.5	5	15	3	6	24	54
monosome	146	140	130	120	168	163	160	160
disome	292	280	258	245	305	300	295	295
trisome	430	415	380		450	445	440	
tetrasome	565	555			590	590		
pentasome	700	695						
average repeat	139	139	125		141	142	140	

Reconstituted chromatin (0.3 mg/ml; histone/DNA = 0.9) was digested with micrococcal nuclease (45 units/ml) in 5 mM Tris-Ac, pH 7.8, 0.6 mM $CaCl_2$, 0.2 mM EDTA or with DNase II (90 units/ml) in 10 mM Tris-HCl, pH 6.9, 0.2 mM EDTA at 37°C for the times indicated. No change in DNase II digestion was observed in the presence of 0.6 mM $CaCl_2$.

cess. The resolution is not sufficient, however, to allow us to rule out nonrandomness for very short spacings of the core particles (< 100 Å). Furthermore, such an effect could be obscured by shearing of the chromatin when applied to the grid (21).

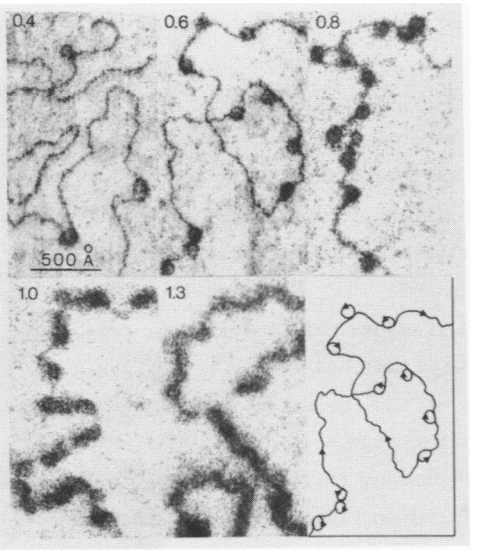

Figure 5: Electron microscopy of reconstituted chromatin. Electron micrographs of reconstituted chromatin were recorded by a STEM. The various histone to DNA ratios of the reconstituted chromatin are indicated. The right panel of the bottom line illustrates how the DNA length between nucleosomes was determined, using the electron micrograph of chromatin reconstituted at a histone/DNA ratio of 0.6 as an example. The lengths were measured along the DNA between corresponding sites of the core particles located at half the distance between entry and leaving points of the DNA at the periphery of the nucleosomes.

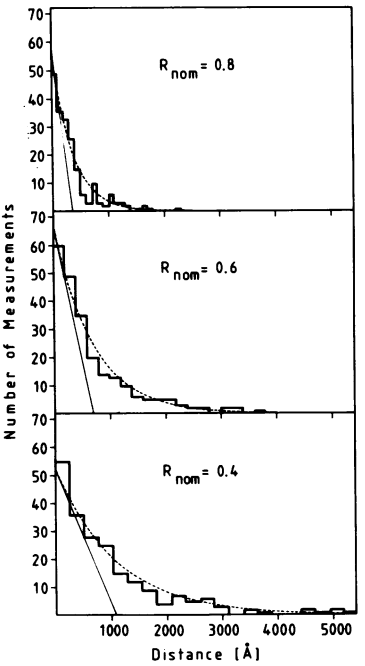

Figure 6: Histograms of internucleosomal DNA in reconstituted chromatin. The distribution of the DNA lengths between nucleosomes was measured in the way indicated in Figure 4 for chromatin reconstituted at histone/DNA ratios of R_{nom} = 0.4, 0.6, and 0.8 (R_{real} = 0.34, 0.47, and 0.69, respectively). Since the procedure of measuring the internucleosomal DNA (Fig. 5) does not result in distances shorter than about 100 Å, all measurements were reduced by 100 Å for the histogram. The dashed line indicates the expected distribution for random assembly, the intersection of the thin line with the abscissa represents the average length of internucleosomal DNA (see Materials and Methods).

From DNA and histone content and the average spacing of the core particles the histone/DNA ratio in the reconstituted chromatin may be calculated (see Materials and Methods). Comparing these values with the input ratios we find that at least 80% of the histones have been assembled into chromatin.

Length of DNA per supercoil in reconstituted nucleosomes

Because the nucleosomes are spaced randomly in the reconstituted chromatin, the bands in Figure 2 reflect the DNA length in closely spaced core particles. The amount of DNA bound to the histone octamers in more widely spaced nucleosomes could be larger. Particularly, it is conceivable that more than 146 base pairs found to be protected in single core particles (Fig. 2) are associated with the histone core. We have examined this question by reconstituting chromatin from core histones and DNA of defined length at various histone/DNA ratios and measuring the length of the chromatin as a function of the number of core particles in the electron microscope (Fig. 7).

The length of the chromatin was measured by following the DNA in the nucleosomes along their periphery as described in Figure 5, neglecting the ad-

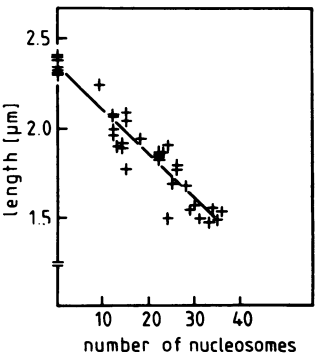

Figure 7: Intranucleosomal DNA per superhelical turn in reconstituted chromatin. Chromatin was reconstituted at histone/DNA ratios of 0.5 and 0.8. Linearized DNA of the plasmid pSF 2124 (11) reduced by a deletion of about 3 kb (P. Schedl, personal communication) was used. Its size determined in the electron microscope by comparison with PM2 DNA (Fig. 3) is 7983 ± 340 base pairs. The DNA length of the reconstituted chromatin was measured as indicated in Figure 5 and plotted as a function of the number of nucleosomes. The reduction in length per nucleosome of 83 ± 4 base pairs obtained by the slope of the linear regression line represents the intranucleosomal DNA per superhelical turn.

ditional DNA bound in one or several complete turns on the outside of the histone cores. Hence the slope in Figure 7 represents the amount of DNA bound by the histone octamer in an integer number of superhelical turns. As Figure 7 demonstrates, this slope is 83 ± 4 base pairs and thus represents only one complete superhelical turn of the DNA in the nucleosome (22,23). Therefore, the total amount of DNA associated with the histone cores is found to be less than two complete turns or less than 166 base pairs.

These measurements do not argue for models in which considerably more DNA is bound to the core histones than the 168 base pairs found to be protected against the action of DNase II in single core particles. Although this result may need correction for a shearing effect (21) and does not rule out the possibility that more DNA interacts with the histones in solution, it appears to be in conflict with earlier measurements obtained by a similar technique in which 194 ± 7 base pairs were reported to be bound to the core histones (1). To substantiate this claim, it was reported that digestion with DNase II resulted in monomer DNA of a size close to 190 base pairs (20). In a similar experiment, we found that the kinetics of both DNase II and micrococcal nuclease digestion did not reveal any resistant core particles with a DNA size of 190 base pairs. On the contrary, during the entire time course the DNA size of mononucleosomes was always shorter than 170 base pairs (Fig. 4 and Table 1).

Closely spaced core particles

It has been demonstrated previously that removal of histone H1 from na-

tive chromatin reduces its sedimentation velocity considerably (16). This effect has been attributed to a stabilization of the interaction of neighboring nucleosomes mediated by histone H1. Depletion of H1 causes a destabilization and results in an increase of the axial ratio of oligonucleosomes reflected in the reduction of the sedimentation coefficients (16,24). Therefore, for further characterization, micrococcal nuclease digests of chromatin reconstituted without H1 were analyzed in isokinetic sucrose gradients (Fig. 8). Brief digestion of chromatin reconstituted at a high histone/DNA ratio (Fig. 8a) or long digestion of chromatin reconstituted at a low histone/DNA ratio (Fig. 8c) reveals peaks at sedimentation values of 13.7 S (Fig. 8a,c) and 15.4 S (Fig. 8a) which are close to those of di- and trinucleosomes of H1-depleted chromatin (16). These peaks represent closely spaced di- and trinucleosomes as ex-

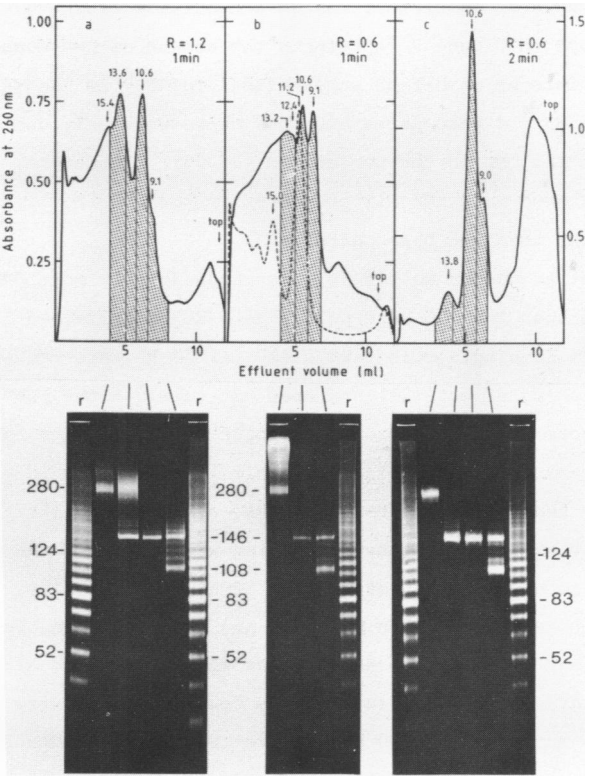

Figure 8: Sedimentation analysis of reconstituted chromatin digested with micrococcal nuclease. Chromatin reconstituted at the histone/DNA ratios R indicated was digested with micrococcal nuclease (20 units per 100 µg of DNA; 180 units/ml) at 37°C for 1 min (a,b) and 2 min (c). Sedimentation of 5 (a,b) and 10 A_{260} units occurred in isokinetic sucrose gradients (25) at 40,000 rpm and 4°C in a Beckman SW41 rotor for 14 hr (a), 15.75 hr (b), and 16.5 hr (c) as described (16). The dahed line in panel b shows the sedimentation profile of a micrococcal digest of native chromatin. The DNA of the gradient fractions indicated was extracted and analyzed in 8% polyacrylamide slab gels in 7M urea (26). For size reference (r) DNA of a DNase I digest of chromatin is shown (27). Analysis of the DNA under nondenaturing conditions revealed an identical size distribution (not shown).

pected from the conditions of digestion and verified by analysis of their DNA size in polyacrylamide gels (lower part of Fig. 8). The lower molecular weight of the closely spaced nucleosomes may account for their diminuished S-values compared to native chromatin consisting of the same number of nucleosomes (13.6 S vs. 15.9 S and 15.4 S vs. 19.8 S). However, because no comparable effect is observed for mononucleosomes (10.6 S vs. 11.2 S), we consider this explanation less probable. It appears more likely that the interaction of closely spaced nucleosomes is as weak in chromatin reconstituted without H1 as in H1-depleted chromatin. After brief digestion of chromatin reconstituted at a low histone/DNA ratio, on the other hand, no pronounced peaks of di- or trinucleosomes are obtained after sedimentation analysis (Fig. 8b). This is expected because under these conditions not only closely spaced core particles resist digestion and hence the spacing of nucleosomes remains heterogeneous.

It is conceivable that even closely spaced core particles exhibit heterogeneity of spacing. To test this possibility, we digested the closely spaced dinucleosomes shown in Figure 8c with DNase I. If the spacing is defined, the pattern of discrete single-stranded DNA fragments will extend beyond the DNA size of a single core particle. Alternatively, should the spacing be heterogeneous, a smear or high background is expected. As evident from Figure 9a,

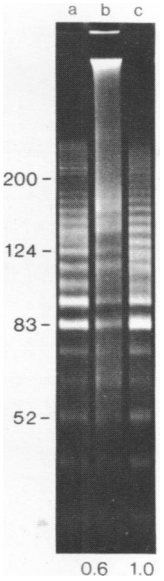

Figure 9: DNase I digestion of closely spaced dinucleosomes. Dinucleosomes (0.2 A_{260}/ml) of the gradient fraction indicated in Figure 8c were digested with DNase I (10 units/ml) in 5 mM Tris-Ac, pH 7.8, 0.2 mM EDTA, 0.6 mM $MgCl_2$, 0.6 mM $CaCl_2$ for 30 s at 0°C (a). For comparison chromatin reconstituted at histone/DNA ratios of 0.6 (b) and 1.0 (c) was digested with DNase I (6.7 units/100 µg of DNA; 60 units/ml) for 5 min at 0°C. The DNA was analyzed as in Figure 8.

a clear pattern of bands is visible up to the DNA size of the dinucleosomes, indicating that the length of the DNA linking close dinucleosomes is defined. In contrast, in a brief DNase I digest of chromatin reconstituted at a histone/DNA ratio of 0.6, bands appear only above a high background (Fig. 9b). The background disappears again if the histone/DNA ratio is raised to 1.0 (Fig. 9c). A similar effect of diminuishing background was observed when dinucleosomes obtained after increasing exposure of rat liver nuclei to micrococcal nuclease were digested with DNase I (not shown), suggesting that closely spaced dinucleosomes exist also in native chromatin and are linked by a defined length of DNA.

Interestingly, there is a region in the gels shown in Figure 9a and c between 90 and 140 nucleotides (more precisely, on the average the bands are multiples of 10.3 to 10.4 nucleotides as pointed out by Prunell et al. (17)) in which the bands are broader. This effect could be explained if the length of the DNA linking close dinucleosomes was different from the spacing of the DNase I cleavage sites within the core particles. This explanation is supported by the observation that the DNA bands above 12x10.3 nucleotides are about 3 nucleotides shorter than nx10.3 nucleotides (where n is an integer and larger than 12). Thus the size of the DNA linking close dinucleosomes is not a multiple of 10 nucleotides but rather about 7 (plus possibly a multiple of 10) nucleotides. Discrete DNA fragments between 146 and 290 base pairs that are multiples of about 10 base pairs are found also in mono- and dinucleosomes obtained after digestion of closely (Fig. 8a and c) but not of widely spaced core particles (Fig. 8b) with micrococcal nuclease. This observation is in accord with our conclusion that the DNA size of closely spaced core particles is defined.

9 S particles

Surprisingly, in addition to nucleosome cores sedimenting at 10.6 S, a peak or shoulder is detected in Figure 8 that sediments more slowly at about 9 S. Whereas these 9 S particles contain the same amount of histones as the core particles as demonstrated by analysis in an 18% polyacrylamide gel (ref. 9 and Fig. 1), analysis of their DNA reveals that it is reduced to about 108 base pairs (Fig. 8). The 9 S fraction in Figure 8 is contaminated with 146-base-pair core particles and with particles of discrete DNA sizes of about 136, 127, and 117 base pairs. However, upon further purification their DNA

appears to consist mainly of 108 base pairs (Fig. 12). Although the intermediate bands between 146 and 108 base pairs might suggest a precursor-product relationship between the 146-base-pair core particle and the 9 S particle, digestion of purified core particles with micrococcal nuclease did not indicate any 9 S intermediates upon analysis in sucrose gradients but resulted only in smaller fragments remaining at the top of the gradient (not shown).

Evidently, 9 S particles are generated at both low and high histone/DNA ratios (Fig. 8a,b) and after brief as well as extensive micrococcal digestions (Fig. 8b,c). Indeed, when 146-base-pair core particles accumulate upon further digestion, the concentration of 9 S particles remains nearly constant (note the different scales in Fig. 8b and c). These observations lend further support to the notion that 9 S and core particles are produced independently. In addition, they indicate that 9 S particles are not readily degraded by micrococcal nuclease. Reducing the histone/DNA ratio increases the yield of 9 S particles relative to that of 146-base-pair core particles at a given degree of digestion (Fig. 8a,b and Fig. 10). This is consistent with relatively stable 9 S particles that may be even more resistant towards degradation by micrococcal nuclease than 146-base-pair core particles. Although the origin of 9 S particles remains unclear at the moment, it is interesting that after micrococcal nuclease digestion of H1-depleted chromatin a similar sedimentation pattern and the same corresponding DNA sizes were observed as in Fig. 8a (M. Noll and R.D. Kornberg, unpublished observation).

In addition to the unusual 9 S particles, a more slowly sedimenting peak shows up at an apparent S-value of 5 to 6 S in Figures 8 and 10. However,

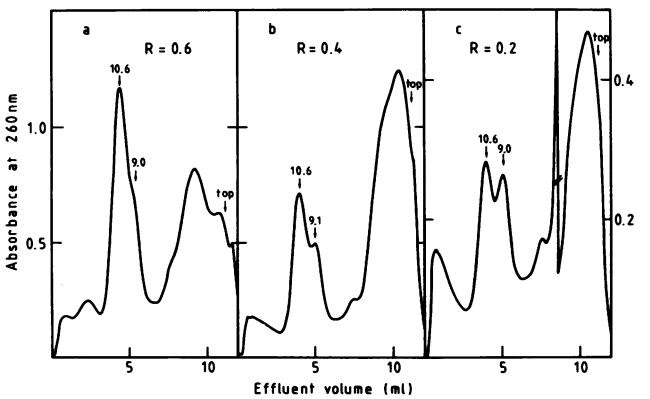

Figure 10: Sucrose gradient analysis of 9 S particles. Chromatin (5 A_{260} units) reconstituted at the histone/DNA ratios R indicated was digested with micrococcal nuclease and sedimented in isokinetic sucrose gradients for 20.5 hr as in Figure 8c.

this material does not represent histone-associated but rather free DNA of heterogeneous size shorter than 108 base pairs (not shown). Thus no stable particles that consist of less than 108 base pairs bound to the histone octamer are found.

The mass of the 9 S particles is only about 12% smaller than that of the 146-base-pair nucleosomes which sediment at 10.6 S. This reduction in mass cannot account entirely for the relatively large difference of the sedimentation coefficients. The remaining difference could be explained by a relatively large axial ratio of the 108- compared to the 146-base-pair core particles, i.e. the shape of the 9 S particles may appear less compact than that of the nucleosome core particles. This is indeed observed in the electron micrographs shown in Figure 11. The 9 S particles seem partially unfolded and reveal frequently an open C-like structure. Although the 9 S particles were highly purified containing mostly 108 base pairs of DNA as shown in Figure 12, no uniform shape is evident in the electron microscope (Fig. 11). For reasons explained below, we prefer to think that this reflects a deformation originating from the preparation of the 9 S particles for electron microscopy rather than a true heterogeneity of the 9 S particles.

Internal structure of 9 S and core particles

Whereas the spacing of nucleosomes in chromatin reconstituted without H1 differs from that in native chromatin, it is still possible that the internal structures of the core particles are similar in native and reconstituted chro-

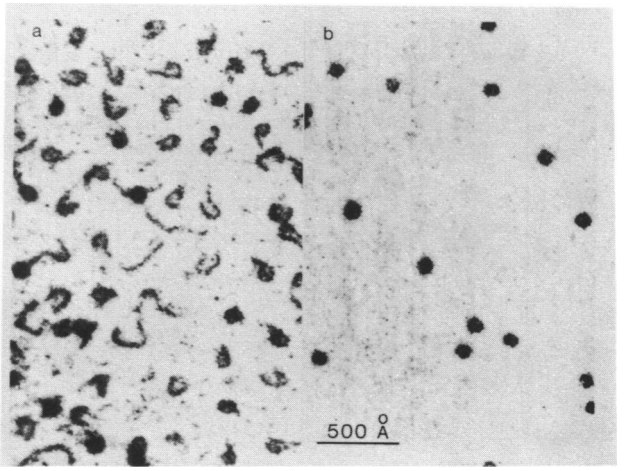

Figure 11: Electron microscopy of 9 S and core particles. The appropriate gradient fraction in Figure 8c was centrifuged in an identical second isokinetic sucrose gradient for further purification of the 9 S particles. The purified 9 S particles were dialyzed against 1 mM EDTA and observed in a STEM (a). For comparison 146-base-pair core particles are shown (b).

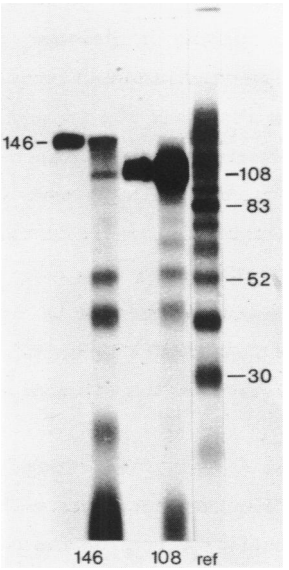

Figure 12: DNase I digestion of end-labeled 146-base-pair nucleosomes and 9 S particles derived from reconstituted chromatin. Purified 146-base-pair core particles and 9 S particles (prepared as described in the legend of Figure 11) were labeled at their 5'-ends by γ-^{32}P-ATP and kinase (22) and digested with DNase I (1 unit/µg of DNA; 50 units/ml) for 30 s at 0°C. After extraction, the DNA was analyzed in 12% polyacrylamide-7 M urea slab gels (26). The autoradiogram shows the DNA of undigested and digested 146-base-pair nucleosomes and 9 S particles on the left and the right, respectively. For size reference, a labeled DNase I digest of chromatin (22) run in the same gel is shown.

matin. Indeed, the same DNA lengths, sedimentation coefficients, and patterns of DNase I digestion of core particles derived from native or reconstituted chromatin indicate a structural similarity. However, the most stringent test for a properly reconstituted internal structure represents a partial DNase I digestion of the core particles labeled at the 5'-ends of their DNA. As demonstrated previously, the single-stranded DNA fragments obtained after a mild DNase I digestion exhibit a characteristic distribution of intensities which reflect the relative frequencies of cutting at the internal cleavage sites (22,28,29). An experiment of this type is illustrated in Figure 12 (on the left). The pattern is very similar to that obtained from native core particles in two aspects. First, it shows the characteristic relative intensities of bands with lows at about 30, 60, 70, 80, 90, and 110 and highs at approximately 10, 20, 40, 50, 100, 120, and 130 nucleotides. Second, the distances of the cleavage sites from the 5'-ends are the same as in core particles derived from native chromatin and about 2 to 3 nucleotides larger than the normal series of multiples of about 10 nucleotides. Therefore, even with respect to this stringent test the internal structure of the reconstituted core particles is very similar to that in native or H1-depleted chromatin.

In an analogous experiment, the DNase I cleavage pattern of 9 S particles was determined (left hand side of Figure 12). After DNase I digestion of the

108-base-pair nucleosomes labeled at their 5'-ends, a characteristic pattern of bands appears. Although it exhibits also a regular spacing of cleavage sites at 10 nucleotide intervals, it differs clearly from the pattern obtained for the 146-base-pair core particles. Cleavage occurs at a very low frequency not only 30 but also 20 nucleotides from the 5'-ends whereas no other pronounced extremes are evident. In addition, sites at 60, 70, and 80 nucleotides from the 5'-ends are cut relatively frequently compared to those in core particles. The low probabilities of cleavage at 20 and 30 nucleotides from the 5'-ends suggest that the 108-base-pair nucleosomes are homogeneous in structure although a mixture of particles of which the DNAs differ by multiples of 10 base pairs at the ends and which are not cleaved at the sites proximal to the 5'-ends is not strictly ruled out.

The spacing of the cleavage sites in 9 S particles at multiples of about 10 nucleotides probably reflects the pitch of the DNA regularly arranged on the outside of the histone octamer as suggested previously for nucleosomes (27). It is difficult, however, to interpret the asymmetry of cleavage frequencies, consisting of low probabilities near the 5'-ends and relatively high frequencies near the 3'-ends, in terms of the handedness of a DNA superhelix as has been argued for core particles (29). Since the pitch of the superhelix would consist of about 80 base pairs (Fig. 7 and refs. 22 and 23; a much shorter pitch is ruled out by energetic considerations (30,31)), the argument would be compatible only with an asymmetry between sites proximal and distal to 80 nucleotides from the 5'- or 3'-ends depending on the handedness of the superhelix. Hence such an argument would predict a right-handed superhelix in 9 S particles as opposed to the left-handed superhelix known to be present in the nucleosome (32). Contrary to the argument of Lutter (29), it follows that the asymmetry of the cleavage pattern has its origin in features which are not directly related to a superhelical structure of the DNA.

DISCUSSION

Reconstitution of chromatin from DNA and the four histones H2A, H2B, H3, and H4 generates nucleosome core particles which are spaced randomly except for very close spacings (Figs. 5 and 6). Over short distances, core particles are spaced considerably closer than in native chromatin (Figs. 2-4, Table 1) and in a nonrandom manner (Fig. 9). The observation of closely spaced core particles in reconstituted chromatin confirms earlier reports in which a re-

peat length of 140 to 150 base pairs was measured (4,20,33). A similar reduction of nucleosome spacing occurs when native chromatin is exposed to NaCl concentrations that release histone H1, an effect which has been attributed to a sliding of the histone octamers along the DNA (18,34-36). Taken together, these results support the idea that histone H1 plays a decisive role in the spacing of the nucleosomes in the chromatin fiber (19, 37-39).

One might expect that the closely spaced core particles of reconstituted chromatin are the same as the "compact oligomers" found in chromatin depleted of H1 in 0.6 M NaCl (18) because similar transient states probably exist during reconstitution and H1-depletion of chromatin at the same NaCl concentrations. However, the results reported in the literature are not consistent with such an expectation. Thus a DNA size of 140 to 150 base pairs is found in the closely spaced core particles (Fig. 4 and Table 1, refs. 4,20,33,34,36) whereas a significantly shorter repeat length is associated with compact oligomers (18,35). In addition, the sedimentation coefficients of compact oligomers (18) appear to be larger than those of the corresponding closely spaced core particles reported here (Fig. 8) and similar to those of native chromatin fragments (16) although a smaller S-value was measured for compact dimers (35).

Our results show that caution is indicated when the repeat length is determined after partial digestion with micrococcal nuclease. Thus after brief digestion a repeat of about 140 base pairs is obtained (Fig. 4, Table 1). However, after prolonged digestion significantly shorter repeat lengths are observed (Figs. 2-4, Table 1). These results may be explained in two ways. Either the shorter repeats are an artifact of degradation from the ends (7,16) or they exist and appear only later during digestion due to a higher resistance of compact oligomers to micrococcal nuclease digestion. Because digestion with DNase II does not reveal shorter repeats (Fig. 4, Table 1) and since micrococcal nuclease clearly degrades the DNA of single core particles from the ends (Figs. 4,8, Table 1), only the first explanation is consistent with our experiments. It is noteworthy that for higher oligomers degradation from the ends appears to be larger, resulting in a size reduction of about 70 base pairs for hexamers (Fig. 3). The reduced ability of DNase II to degrade the DNA from the ends has been noticed previously (4,20). The effect cannot be explained by the absence of divalent cations during digestion as the same results were obtained in the presence of Ca^{2+} (Table 1 and ref. 4).

From the previous considerations it appears that detailed models account-

ing for repeats shorter than 140 base pairs are premature (18,40) unless it is demonstrated that compact oligomers do not arise by degradation from the ends. The association of closely spaced core particles of 140 base pairs reported here and in other studies (4,20,33,34,36) remains unclear. Allowing only left-handed DNA superhelices, two extreme ways of linking core particles seem possible. Either the core particles interact between top and bottom planes of one another (Fig. 13a) or they are assembled laterally (Fig. 13c). Both types of interaction have been observed with native core particles (23,41). Also intermediate relative positions may have to be considered (Fig. 13b). One type of arrangement is transformed to the other by a rotation as indicated in Figure 13c and requires a change in the length of the DNA link to accomodate the change of the twist on the DNA. Whatever the arrangement, it depends on the exact length of the DNA associated with each core particle. The fact that closely spaced disomes contain DNA of discrete sizes (Fig. 8a and c; ref. 18) and generate a pattern of discrete bands up to their full length of DNA upon digestion with DNase I (Fig. 9; ref. 18) suggests that the DNA size associated with closley spaced core particles is not arbitrary but defined. Although arrangements of the type shown in Figure 13a explain a defined spacing best and may prevail in solution, in the electron microscope (Fig. 5) chiefly arrangements of the type shown in Figure 13b and c are observed. Such arrangements exhibiting large axial ratios are probably also favored at high hydrostatic pressures in the ultracentrifuge (42) which would explain the relatively small sedimentation coefficients of the closely spaced core particles (Fig. 8).

The DNA of 140 base pairs in closley spaced core particles is considerably shorter than the 194 ± 7 base pairs reported to be bound in more widely and randomly spaced core particles of reconstituted chromatin (1). Our diges-

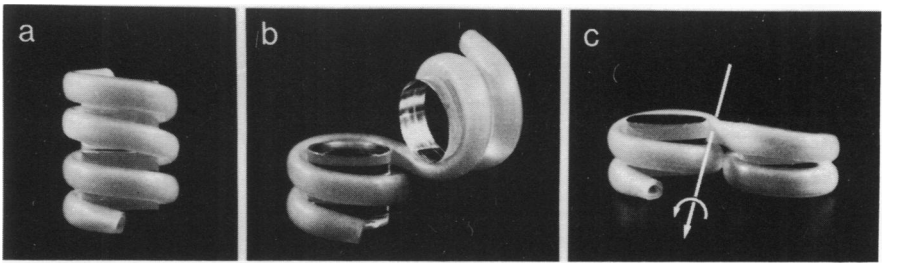

Figure 13: Linkage of closley spaced nucleosome core particles. For explanation see text.

tions with DNase II show that indeed about 170 base pairs may be associated with single core particles, a value which is still lower than the 190 base pairs published previously (20). In an attempt to measure the amount of DNA in core particles in the electron microscope, we found that always less than two full superhelical turns were bound (Fig. 5). One turn was measured to consist of 83 ± 4 base pairs (Fig. 7) which is in accord with models proposed previously (22,23,43,44). Hence, these results are consistent with those obtained by DNase II digestion. Although it is possible that more than 170 base pairs of DNA can bind to the histone octamer, we were unable to detect it. The discrepancy between our and previous results (1,20) might be explained at least partially by the difference in the origin of the core histones.

Although the spacing of nucleosome core particles is not properly reconstituted in the absence of histone H1, it appears that their internal structures closely resemble those of native core particles. This is suggested by the appearance of their shape in the electron microscope (Fig. 5), the sedimentation coefficient of 10.6 S, and the DNA size of 146 base pairs (Fig. 8). According to these criteria, reconstitution of core particles from its constituents (i.e. using 146 base pair DNA) has been demonstrated previously (45).

The most stringent test for proper reconstitution of core particles represents digestion of core particles labeled at their 5'-ends with DNase I (22,28,29). Minor quantitative discrepancies between such DNase I cleavage patterns of core particles derived from native (22) and H1-depleted chromatin (29) have been reported. The differences consist mainly in more pronounced low frequencies of cutting at sites 30 and 110 nucleotides from the 5'-ends in core particles obtained from H1-depleted chromatin. In this respect the cleavage pattern of reconstituted core particles (Fig. 12) resembles that of core particles derived from H1-depleted chromatin more closely. This is not surprising since during reconstitution probably a state is reached which is similar to the one produced when chromatin is depleted of H1 by washing in 0.45 M NaCl (29). The question now arises whether the quantitative differences observed in the cleavage patterns are based on structural deviations of the "reconstituted" core particle of Lutter (29) from the "native" core particle studied earlier (22,28) or whether they are an artifact due to a slight heterogeneity of the DNA size in the preparation of core particles as has been discussed previously (22).

In addition to 146-base-pair core particles, a novel type of 9 S parti-

cles consisting of an intact histone octamer and only 108 base pairs of DNA was detected in reconstituted chromatin (Figs. 8,10,12). As suggested by DNase I digestion of end-labeled 9 S particles (Fig. 12), they probably represent a homogeneous structure. The observation that their shape exhibits an increased axial ratio in comparison to that of core particles (Fig. 11) is supported by the relatively large decrease of the sedimentation coefficient from 10.6 S to 9.0 S. It appears that 108 base pairs represent the smallest DNA size which is stably associated with the histone octamer. The significance of the 9 S particles remains unclear. They are not a degradation product of 146-base-pair core particles and represent at a histone/DNA ratio of 1:1 a small fraction of the reconstituted chromatin. They probably exist also in H1-depleted chromatin (M. Noll and R.D. Kornberg, unpublished observation) and correspond to the subnucleosome SN_7 found by Bakayev et al. (46) in native chromatin.

It is much easier to think of a possible physiological role of the closely spaced core particles. Their similarity to structures observed at the replication fork is striking (6). In both cases dinucleosomes of about 300 base pairs that are relatively resistant to cleavage by micrococcal nuclease are observed. Thus it seems likely that assembly of chromatin during replication involves a transient state of closely spaced core particles followed by a spacing step which inserts histone H1 (6).

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