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A nucleosome-like structure containing DNA and the arginine-rich histones H3 and H4

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ABSTRACT

The low-angle X-ray diffraction pattern from fibres of reconstituted H3/H4/DNA complexes is very similar to that of chromatin and has well defined maxima at 10.6, 5.4, 3.4 and 2.6 nm. Staphylococcal nuclease digestion of reconstituted H3/H4/DNA yields DNA fragments of length 49, 69, 100, 128, 193 and 255 b.p. as principal components. Comparison of the relative amounts of DNA fragments shows that the larger components (100 and 128 b.p.) increase with respect to the smaller (49 and 69 b.p.) as the histone to DNA ratio increases. A structural unit containing $\sim$ 65 b.p. of DNA and tetrameric $(H3/H4)_2$ is proposed such that longer DNA fragments result from multiples of this unit. The principal nucleo-protein particle resulting from nuclease digestion contains 128/139 b.p. of DNA and has electrophoretic mobility very close to that of 'core' nucleosome. It probably represents a dimer of the basic structural unit.

INTRODUCTION

The critical importance of H3 and H4 for the maintenance of nucleosome structure has been demonstrated by two approaches. Firstly, using X-rays, only histone/DNA complexes containing this histone pair were able to generate some of the diffraction rings characteristic of chromatin[1]. Secondly, the use of biochemical structural probes such as nucleases and proteases has been extended to complexes reconstituted from DNA and different histone combinations[2,3] and the primary conclusion reached was that the arginine-rich histones H3 and H4 play a central role in nucleosome formation and are able to order at least 70 base pairs (b.p.) of DNA and possibly more. The importance of H3 and H4 in generating the supercoiling of DNA in the nucleosome has been demonstrated by the finding that the H3/H4 pair is able to induce as much supercoiling in a closed circular DNA as the combination of all 4 'core' histones H2A, H2B, H3 and H4[4].

It is shown here that the H3/H4 pair when recombined with DNA can form a nucleoprotein complex having the complete X-ray fibre diffraction pattern of chromatin. When treated with staphylococcal nuclease this material yields discrete DNA fragments of up to 255 b.p. in length and discrete particles, the most abundant of which has electrophoretic mobility almost identical to that of the nucleosomal 'core' particle and contains the same length of DNA (∿ 128-139 b.p.).

EXPERIMENTAL PROCEDURES

Calf thymus DNA (Sigma type 1) was complexed with all four histones H2A, H2B, H3 and H4 and with the histone pairs (H2A, H2B) and (H3, H4). The histones were prepared by the procedure of van der Westhuyzen and von Holt[5] but were recovered by 0.5 M perchloric acid precipitation. The equimolar histone pairs (H2A, H2B) and (H3, H4) were found to be electrophoretically pure as judged by gel electrophoresis in 2.5 M urea-acetic acid[6] and in 0.1% SDS at neutral pH (Ref.7 and legend to Fig. 1). The histone mixtures were dissolved in 10 mM Tris, 0.1 M β Mercaptoethanol and left overnight at 4°C. DNA was dissolved at 2 mg/ml in 2 M NaCl, 20 mM Tris-HCl, 10 mM sodium bisulphite, 0.6 mM $CaCl_2$, pH 7.5 at 4°C. Equal volumes of the histone and DNA solutions were rapidly mixed and dialysed at 4°C through the following NaCl molarity steps; 0.8 M, 0.6 M, 0.4 M and 0.2 M NaCl all in 10 mM Tris-HCl, 5 mM sodium bisulphite, 0.3 mM $CaCl_2$, pH 7.5 and finally to 0 M NaCl, 10 mM Tris-HCl, 5 mM sodium bisulphite, 1 mM EDTA, pH 7.5. All complexes gave clear gels except when the histone:DNA (w/w) ratio, r, was greater than 1, when the complexes were increasingly more particulate. For X-ray diffraction gels were pelleted by centrifugation at 300,000 g for 3-4 hours at 4°C. Fibres were pulled on pointed-end forceps and left to dry at room temperature. A control sample of calf thymus chromatin was prepared by the method of Panyim et al.[8] and a fibre drawn.

RESULTS AND DISCUSSION

Fig. 1 shows X-rays diffraction patterns from 3 reconstituted complexes and from chromatin. The diffraction from H2A/H2B/H3/H4/DNA and from H3/H4/DNA are strikingly similar to that from chromatin. The densitometer scans in Fig. 1 show that the definition of the pattern from H3/H4/DNA is as good as that from chromatin. In contrast the H2A/H2B/DNA complex shows no discrete low-angle diffraction except for the equatorial arcs at ∿ 3.7 nm probably due to the packing of free DNA. It is particularly noteworthy that the pattern from H3/H4/DNA shows a meridional arc at 10.6 nm (which was not observed in previous studies of this complex[1]) and which has been assigned in chromatin to the pitch of a 'coil'[9] or 'solenoid' of nucleosomes[10]. If this ring has the same origin as in chromatin, it implies that H3/H4 alone are able to generate the nucleosomal coil. Since however the diameter of the nucleosome is about 11.0 nm other arrangements of nucleosome-like particles could give rise to a similar reflection from the H3/H4/DNA complex e.g. the absence of H2A/H2B could result in the interparticle spacing of a chain of H3/H4/DNA particles being observed. The other striking feature of the H3/H4/DNA diffraction pattern is the meridional orientation of the 5.4 nm reflection.

The histone (H3,H4):DNA ratio (r) of the fibre used in Fig. 1 was 1:1 (w/w). Diffraction patterns were also obtained for fibres having r=0.3, 0.5, 0.8 and 1.5. The composition of the fibres having r=0.3, 0.5, 0.8 and 1.0 (input ratios 0.25, 0.5, 0.75 and 1.0 respectively) was checked using infra red spectroscopy[11]. Since the fibre compositions agreed within the

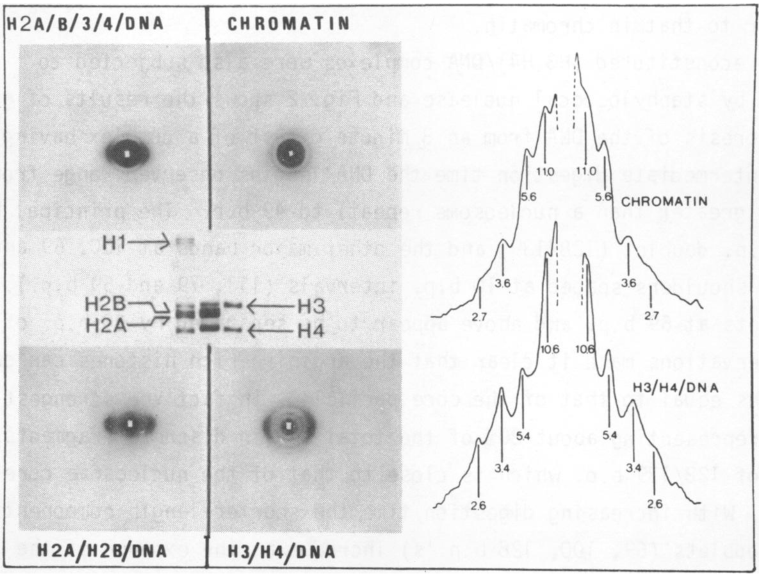

FIGURE 1 Low-angle X-ray diffraction patterns from chromatin and reconstituted nucleoprotein. Right hand side shows meridional densitometer scans with reciprocal spacings in nm. A Franks camera was used having a sample to film distance of 9.3 cm together with an Elliott GX20 rotating anode X-ray generator. Fibre samples were held at 98% r.h. Gel electrophoresis is of histones extracted from the X-ray fibres with 0.4 N H_2SO_4. The samples are: left hand side, whole histone from chromatin; centre, H2A/H2B/H3/H4 from 'core' histone reconstitute; right hand side, H3/H4 from arginine-rich histone reconstitute. Electrophoresis in 0.1% SDS, 16% acrylamide, essentially as in ref. 7.

experimental error with the input composition, the protein was fully bound to the DNA. At r=1.5 and 2.0 (input ratios) the samples were too opaque for infra red spectroscopy. No diffraction pattern was observed at r=0.25 but between r=0.5 and 1.0 the pattern was observed with increasing intensity. At r=1.5 a fibre was difficult to obtain because of the particulate nature of the complex and the 10-11 nm ring was absent, perhaps due to the large excess of histone present. From the close similarity of the (H3,H4)/DNA diffraction pattern to that of chromatin we conclude that H3 and H4 are necessary and sufficient to generate the structural features of chromatin which give the characteristic low angle X-ray pattern.

Digestion of chromatin by pancreatic DNase I gives rise to DNA fragments spaced by 10 b.p.[12] and a similar set of fragments has been reported for H3/H4/DNA reconstitutes[3]. We also observe that DNase I digestion of an H3/H4/DNA reconstitute having r=1 generates a well-defined ladder of fragments spaced by 10 b.p. This suggests that the DNA fold in the complex is similar to that in chromatin.

The reconstituted (H3,H4)/DNA complexes were also subjected to digestion by staphylococcal nuclease and Fig. 2 shows the results of gel electrophoresis of the DNA from an 8 minute digest of a complex having r=1. At this intermediate digestion time the DNA lengths observed range from 255 (somewhat greater than a nucleosome repeat) to 49 b.p. The principal band is a 10 b.p. doublet (128/139) and the other major bands at 100, 69 and 49 b.p. also have shoulders spaced at 10 b.p. intervals (111, 79 and 59 b.p.). The DNA doublets at 69 b.p. and above appear to be separated by 30 b.p. of DNA. These observations make it clear that the arginine-rich histones can order DNA lengths equal to that of the core particle. In fact the strongest doublet (representing about 30% of the total DNA in discrete fragments) has a length of 128/139 b.p. which is close to that of the nucleosome core particle. With increasing digestion time the shorter-length component of the DNA doublets (69, 100, 128 b.p.'s) increases at the expense of the longer component. Finally, at long digestion times, as noted in reference 2, the 49 b.p. peak becomes the strongest component and the longer lengths disappear from the digest.

As a more detailed probe of the structural elements giving rise to discrete DNA fragments a further set of digestion experiments was conducted on complexes of H3/H4 to DNA where the w/w ratio, r, was varied between 0.25 and 2.0. It was observed that as this ratio increased up to 1.0 the summed intensity of all the discrete DNA bands also increased for a constant

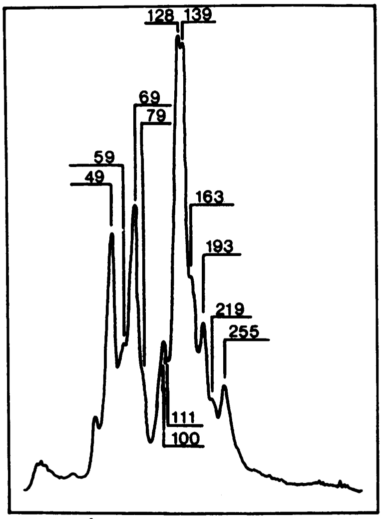

FIGURE 2 The DNA of the H3/H4/DNA complex after 8 minutes staphylococcal nuclease digestion, separated on a 6% polyacrylamide tris-borate-EDTA slab gel prepared and run as described by Miniatis et al. (1975)[13] but with 0.1% SDS added to the gel and electrode reservoirs. The total digest was made up to 0.1% SDS, 5% glycerine and applied directly to the gel. Gel stained by ethidium bromide and photographed under UV light. The densitometer scan is of the photograph. Digestion was performed at 37°C in 10 mM tris-HCl, 5 mM sodium bisulphite, 1 mM EDTA with 150 units[14] per ml of enzyme (M.R.E. Porton) plus 1.3 mM added Ca^{++}. DNA fragments in the range 120 to 260 b.p. were sized by comparison with λ dv-1/Bsu restriction fragments[15] (a gift from Drs M Steinmetz and H Zachau) as recalibrated by Dr M Steinmetz. Fragments below 120 b.p. were also calibrated against DNase I fragments of chromatin[12] by the following procedure: DNA was extracted with chloroform-isoamyl alcohol (24:1) in 1 M NaCl, 0.1% SDS, precipitated with 2 vols of ethanol at -16°C, dissolved in H_2O, lyophilised and redissolved in 98% formamide. Electrophoresis was on 12% polyacrylamide, 7 M urea gels[13].

8 minute digestion time. At a weight ratio of 1.5 the sample digested more slowly and at a ratio of 2.0 there was no digestion at all even after 32 minutes. This was presumably a consequence of the physical state of the samples as described previously. With increasing r although the sizes of the DNA fragments produced did not alter, there were changes in the relative amounts of the main fragments. Now if an increasing histone/DNA ratio, r, resulted simply in the generation of an increasing amount of one type of structural unit then for constant time of digestion the distribution of DNA fragments would be expected to remain unchanged. This is the case (Fig. 3) for the two smallest DNA fragments which maintain a constant intensity ratio I_{49}/I_{69},

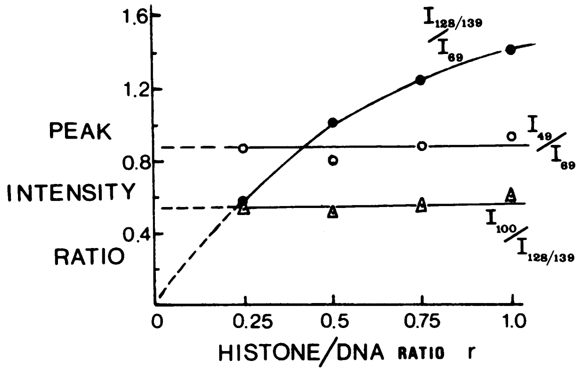

FIGURE 3 Variation in relative fragment yields with H3/H4:DNA ratio in 8 minute staphylococcal nuclease digests. Data obtained as described in Fig. 2.

and also for the ratio of the larger fragments 100 and 128/139 b.p. In contrast however, the amounts of the larger class DNA fragments 100 and 128/139 b.p. were found to increase with respect to the smaller class fragments (49 and 69 b.p.) as illustrated in Fig. 3 by the ratio $I_{128/139}/I_{69}$. Thus the 49 and 69 b.p. DNA fragments appear to come from one structural unit while the larger DNA fragments come from a second and larger structural unit, the amount of which increases with increasing r. The simplest explanation is that the smaller unit is composed of 69 b.p. DNA and the $(H3/H4)_2$ tetramer and two of these units adjacent to one another form the larger unit. Fragments greater than 139 b.p. would result from runs of more than two of the smallest unit. Fig. 2 shows that the bands at 69, 128, 193 and 255 b.p. are more intense than the intermediate bands at 100, 163 and 219 b.p. This supports the proposal that a basic structural unit occurs at about every 65 b.p. with the intermediate bands resulting from internal cleavage of this unit.

The staphylococcal nuclease digestion products of the H3/H4/DNA complexes can also be separated by electrophoresis. Fig. 4 shows the densitometer scan of an 8 minute digest of a sample having r=1.0. The most intense particle band has mobility *very* close to that of a mono-nucleosome reconstituted with all four 'core' histones. A slice was therefore taken from the centre of this band (as indicated in Fig. 4) and the DNA from it electrophoresed in a borate/SDS system. The DNA fragment distribution is shown as an inset to Fig. 4. It can be seen that the most abundant particle contains DNA lengths of about 135 and 70 b.p.'s in roughly equal proportions

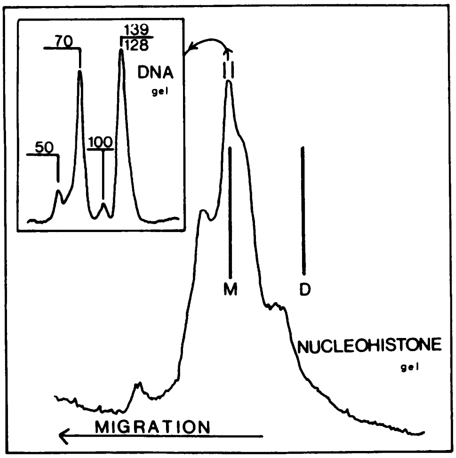

FIGURE 4 Gel electrophoresis of nucleohistone complexes from the 8 minute staphylococcal nuclease digest of Fig. 2. 4% polyacrylamide slab gel prepared as in reference 15 with the modification that the tris-borate-EDTA system is replaced by 20 mM tris, 2 mM EDTA, pH 7.5. Electrophoresis for 3 hours at 10V/cm. Densitometer trace of photograph of ethidium bromide stained gel. Coomasie blue staining carried out directly following ethidium bromide staining, using 0.1% dye, 50% TCA for 6 hours. Destaining in 20% ethanol, 7% acetic acid. A cut from a parallel gel was taken at the position indicated and placed in a slot of a 6% tris-borate-EDTA gel[13] containing 0.1% SDS, and the DNA electrophoresed and visualised in this gel as described in Fig. 2. Resolution was somewhat poorer than in Fig. 2 due to the finite size of the gel cut taken. M=mono- and D=di-nucleosome mobility.

and much lower amounts of 100 and 50 b.p.'s. The DNA length distribution in the particle is similar to the overall length distribution of Fig. 2, with the exception that DNA fragments longer than 139 b.p. are absent and that the 50 b.p. fragment is reduced in intensity. We conclude that the principal particle of the H3/H4/DNA reconstitute contains 139 b.p.'s of DNA (see Fig. 2) and has a highly accessible internal cleavage site about half way along and a less accessible site about 30 b.p. in from either or both ends.

CONCLUSIONS

Since the principal particle in the 8 minute digest contains 128/139 b.p. of DNA, it probably consists of two basic structural units, each of ∿ 65 b.p. of DNA and one $(H3/H4)_2$ tetramer, as argued above. This represents a histone/DNA ratio close to that of the nucleosomal 'core' particle and this composition is supported by their virtually identical mobilities (Fig. 4). It must however be emphasised that there is no *direct* evidence here for the histone/DNA ratio in the complex that gives rise to the defined DNA fragments and X-ray diffraction. If the basic structural unit readily dimerises to form the 139 b.p. particle this must be brought about by protein interactions. This accords with the aggregating capacity of the $(H3/H4)_2$ tetramer[16].

If both a unit and dimeric structure are formed in the H3/H4/DNA reconstitutes, the question arises as to which structure gives rise to the characteristic X-ray diffraction pattern. Since the pattern is not observed at a histone/DNA ratio r of 0.25 and increases in intensity as r goes from 0.5 to 1.0, it is probable that the diffraction is generated by dimers (and multiples) of the 65 b.p./$(H3/H4)_2$ unit structure. Such a view accords with the suggestion[17] that the 5.5, 3.7, 2.7 and 2.2 nm peaks are in large measure part of the transform of the nucleosomal 'core' particle containing $\sim$ 140 b.p. of DNA and octameric histone.

If the principal structural features of the nucleosome can be generated by the arginine-rich histone pair alone, the possibility arises that the function of the moderately-lysine rich pair H2A/H2B is by no means only a passive structural one. Such a view accords with the considerably greater variability noted in the sequences of H2A and H2B molecules[18], when compared to the H3, H4 molecules.

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