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H3·H4 Tetramer Directs DNA and Core Histone Octamer Assembly in the Nucleosome Core Particle†

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ABSTRACT: The way in which histones interact with DNA during in vitro assembly of nucleohistone has been examined. Chicken erythrocyte core histones H2A, H2B, H3, and H4 and λDNA in 2 M NaCl were allowed to interact by stepwise decrease in the salt concentration. Binding, although weak, was first observed at 1.4 M NaCl and was essentially completed at 0.6 M NaCl. Analysis of the DNA-bound histones revealed that each of the histones in the pairs H2A,H2B and H3,H4 was always present in equimolar amounts and that the relative proportion of each pair was constant between 1.4 and 0.8 M NaCl. Evidence is presented suggesting that binding occurred via complexes of the four histones, the nature of which is likely to reflect the equilibrium among the octamer and its products of dissociation (Ruiz-Carrillo, A., & Jorcano, J. L. (1979) *Biochemistry* (preceding paper in this issue)). The presence of complexes of the four core histones is, however, not required for the correct assembly of the nucleosome core particle. Nucleohistones obtained by adding at progressively lower ionic strengths the dimer H2A·H2B to the H3·H4–DNA complex (split reconstitutions) had the same characteristics as those assembled with the core histone complexes.

Recent work has demonstrated that core histones (H2A, H2B, H3, and H4) can form an octameric complex in solutions of high ionic strength and neutral pH (Thomas & Butler, 1977; Ruiz-Carrillo & Jorcano, 1979) and that the H3·H4 tetramer plays a central role in its organization (Ruiz-Carrillo & Jorcano, 1979). The existence of a histone octamer in the absence of DNA strongly suggests that the information for the nucleosome core particle assembly may reside in the structure of the histone complex. Therefore, if the structure of the histone octamer in solution and in the nucleosome is similar (Thomas et al., 1977), it could be expected that the octamer would bind to DNA and thus generate nucleosome-like structures. Alternatively, the preassembled histone octamer may not be necessary if the H3·H4 tetramer bound to DNA can also direct the assembly of the octamer and, as a consequence, the nucleosome core particle.

In the present report we examine this question and show that a complex of the four core histones does, indeed, bind to DNA and generates nucleosome core particle-like structures. However, evidence is also presented indicating that this can also be achieved by the sequential binding of H3·H4 tetramer and H2A·H2B dimer.

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Experimental Procedures

Cells, Isolation of Nuclei, and Histone Preparation. Nuclei from chicken erythrocytes and salt- or acid-extracted core histones and histone pairs were prepared as described previously (Ruiz-Carrillo & Jorcano, 1979). Histone concentrations were estimated by their absorbance at 275 nm (Ruiz-Carrillo & Jorcano, 1979).

Warburg ascites cells (a kind gift of Dr. E. Liss) were propagated in female NMRI mice.

Radioactive Labeling of Histones. Reductive methylation of chicken erythrocyte core histones in 2 M NaCl, 0.2 mM PMSF,[1] 10 mM sodium borate, pH 9.0, was carried out by a modification of the procedure of Rice & Means (1971) as described by Ruiz-Carrillo et al. (1975). Methylated histones

[1] Abbreviations used: b.p., DNA base pair; nucleosome core particle, 145 b.p. of DNA complexed with two of each of the core histones; core histones, H2A, H2B, H3, and H4; DNA (I), superhelical form of covalently closed circular DNA; DNA (II), relaxed form of covalently closed circular DNA; NCE, nicking and closing extract; buffer A, 5 mM Tris-HCl, 0.01% 2-mercaptoethanol, 0.1–0.3 mM PMSF, pH 7.2; buffer B, 10 mM Tris-HCl, 0.2 mM EDTA, 5% glycerol, pH 7.9; buffer C, 5 mM Tris-HCl, 0.1 mM EDTA, 0.01% 2-mercaptoethanol, 0.1 mM PMSF, pH 7.2; PMSF, phenylmethylsulfonyl fluoride; SSC, 0.15 M NaCl, 0.015 M sodium citrate; EDTA, sodium salt of ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; TBE, 90 mM Tris-boric acid, 2.5 mM EDTA, pH 8.3.

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were purified from nonreacted formaldehyde by Sephadex G-25 (fine) chromatography in 2 M NaCl in buffer A. The specific activity of histones was 11 000 cpm/μg. This means that, on the average, 0.33 lysyl residue per histone molecule was methylated. This labeling method produced no observable cross-links of the core histones which were labeled in the following ratios: H4 (1.0), H3 (1.4), H2A (1.8), and H2B (3.3). The labeling ratios were determined by scintillation counting of slices of the corresponding electrophoretic bands previously digested with Protosol (New England Nuclear) for 16 h at 60 °C. These ratios were confirmed by densitometry of nonsaturated fluorographs of the radioactively methylated histones. Histone pairs H3,H4 and H2A,H2B were labeled by reductive methylation as described for core histones except that the NaCl concentration was reduced to 0.2 M NaCl.

Binding experiments were carried out with both in vivo and in vitro labeled histones. The use of methylated histones was preferred because of their identical binding characteristics but much higher specific activities than in vivo labeled histones. The validity of using methylated histones was tested by a variety of criteria. First, methylated histones behaved as octamers in the same way as nontreated core histones as determined by analysis of the complex in Bio-Gel P-200 chromatography in 2 M NaCl, buffer A (Ruiz-Carrillo & Jorcano, 1979). Second, nucleohistones reconstituted with methylated and control histones had the same characteristics as tested by micrococcal nuclease and DNase I digestion. Third, methylated histones induce similar changes in the linking number of circular DNA (II) after nicking and closing extract (NCE) treatment as do control histones at the same histone/DNA ratio.

It has to be realized, however, that due to the disproportionate labeling of the different core histones the electrophoretic bands of H2A and H2B appear more intense than those of H3 and H4 by fluorography. Acid- and salt-extracted methylated core histones behaved indistinguishably by the above criteria (see also Ruiz-Carrillo & Jorcano, 1979).

DNA Isolation and Purification. Highly polymerized calf thymus DNA (Sigma) was further purified by digestion with RNase A (Sigma), deproteinized with 0.05 M Tris-HCl (pH 7.9)–saturated phenol, and ethanol-precipitated. DNA was dissolved in SSC at 2 mg/mL and sheared in a Virtis 60 homogenizer at 30 000 rpm for 30 min. The weight average molecular weight of the sheared DNA, determined by velocity sedimentation, was 6.5×10^6.

Plasmid pBR322 (Bolivar et al., 1977) was prepared from *Escherichia coli* K-12 C600 (kindly provided by Dr. K. Timmis). Plasmid DNA was purified according to Timmis et al. (in press). DNA was finally dialyzed against 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.9, and stored at 4 °C. Supercoiled pBR322 DNA (I) was converted into the relaxed form (II) by incubation with NCE (Keller & Wendel, 1974). The reaction was stopped with buffered phenol and the DNA purified as described above.

λDNA was prepared from λCI857S7 (kindly provided by Dr. R. Thompson) grown in *E. coli* M65 (Allet et al., 1973). The DNA was homogeneous in length as determined by both velocity sedimentation and agarose gel electrophoresis.

Nicking and Closing Extract (NCE) Preparation. NCE was extracted from chromatin of Warburg ascites cells prepared according to Hancock (1974), essentially as described by Germond et al. (1975). The relaxing activity was precipitated with ammonium sulfate (Keller & Wendel, 1974), dissolved in 0.2 M NaCl in buffer B, dialyzed extensively against the same buffer, and stored at −70 °C.

Binding Experiments. Several aliquots of λDNA and radioactively methylated core histones were mixed at a histone/DNA ratio of 0.8:1.0 (w/w) in 2 M NaCl in buffer C at 0 °C. Subsequently, buffer C was added sequentially to the mixtures at 10–20-min intervals to decrease the salt molarity until a final concentration of either 1.6, 1.4, 1.2, 1.0, 0.8, or 0.6 M NaCl was reached. The concentration of histones after the last dilution was in all cases 400 μg/mL. The reconstitutes were allowed to interact for an additional period of 90 min in the final salt concentration. DNA-bound and free histones were separated by 5–20% sucrose gradient centrifugation for 130 min at 58 000 rpm in a Spinco SW 60Ti at 1 °C. The radioactivity of each fraction was measured by counting 10-μL aliquots, diluted with 1 mL of water, in a toluene–Triton X-100 based scintillation mixture.

Individual or pooled fractions from the gradients were precipitated with 18% (w/v) trichloroacetic acid after addition of 20 μg of nonlabeled core histones and 20–60 μg of bovine serum albumin (Pentax, Miles) as carriers. The precipitates were electrophorized in 15–19% $NaDodSO_4$ polyacrylamide gradient slab gels as described by Ruiz-Carrillo & Jorcano (1979). Fluorography was carried out according to Bonner & Laskey (1974) and Laskey & Mills (1975) using Kodak X-Omat R film. Different exposure times were used in order to perform a semiquantitative analysis of the data. The amount of each pair of histones (H3 + H4 and H2A + H2B) in a given fraction was estimated from the percentage of the total radioactivity recovered in the fraction and from the areas of tracings of fluorographs, using an Ortec 4310 densitometer equipped with an automatic integrator. The areas were corrected according to the differential labeling of each histone. Binding of radioactively methylated histone pairs H2A,H2B and H3,H4 to DNA was carried out essentially as described for the core histones except that the histone/DNA ratio was 0.4:1.0 (w/w) for each pair.

Reconstitution Experiments. Control nucleohistones were made by mixing sheared calf thymus DNA and acid- or salt-extracted core histones in 2 M NaCl in buffer A at 4 °C at a histone/DNA ratio of 0.4:1.0 or 0.8:1.0 (w/w). The reconstitution regime included 90-min dialysis (Spectrapor 3) at 4 °C, in a rotatory shaker, against 2, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2, and 0.05 M NaCl in buffer A. Reconstituted samples were left overnight in the last step buffer. Complexes of DNA with the pairs of histones H2A,H2B and H3,H4 were made as outlined above except that the ratio of each histone pair to DNA was 0.4:1.0 (w/w). Split-reconstituted nucleohistones were made by mixing H3,H4 and sheared DNA in 2 M NaCl in buffer A at a histone to DNA ratio of (0.2 or 0.4):1.0 (w/w). Histones H2A,H2B in 2 M NaCl in buffer A were dialyzed stepwise parallel to the H3·H4–DNA complex. H2A,H2B was then added slowly to the H3·H4–DNA complex at a H2A·H2B/DNA ratio of (0.2 or 0.4):1.0 (w/w), at the end of the indicated ionic strength step. The reconstitution was then carried on through the remaining steps. Relaxed pBR322 DNA (II)–histone complexes were made at the indicated histone/DNA ratios as described above, except that buffer B was used throughout. Reconstitution was achieved by stepwise dilution with buffer B down to 0.2 M NaCl. Bovine serum albumin at 200 μg/mL was included during the reconstitution. The reconstituted complexes were treated with 20–40 μL of NCE/μg of DNA at 37 °C for 60 min. The reaction was stopped with buffered phenol and the DNA purified as described above.

Nuclease Digestions. Micrococcal nuclease (Worthington) digestions were carried out at 37 °C with 0.05–0.2 unit of

enzyme/μg of DNA in the presence of 0.1 mM $CaCl_2$. The kinetics of the digestion was followed by determining the percentage of DNA rendered acid soluble in 1 M NaCl, 1 N perchloric acid in duplicate aliquots. The absorbance of the supernatants was measured at 260 nm and corrected for the hypochromic effect of DNA digested with the nuclease, estimated to be 1.68. The DNA concentration of the starting sample was determined by measuring the absorbance at 260 nm of triplicate aliquots made 1% in $NaDodSO_4$. At different times during the course of the digestion, samples were taken for DNA electrophoresis. The reaction was stopped by the addition of EDTA to 10 mM.

DNase I (Worthington) digestions were carried out at 37 °C with 0.024 unit of enzyme/μg of DNA in the presence of 2 mM $MgCl_2$. The kinetics of the digestion and sampling of aliquots for DNA electrophoresis was carried out as described above, except that the hypochromicity of DNA digested with DNase I was estimated to be 1.4.

DNA Electrophoresis. Micrococcal nuclease limit digests were analyzed in 5–8% polyacrylamide gradient slab gels (acrylamide/bis(acrylamide), 20:1). Gel and running buffer was TBE (Peacock & Dingman, 1968). Micrococcal nuclease limited digests were analyzed by electrophoresis in 2.5% polyacrylamide–0.5% agarose composite slab gels according to Peacock & Dingman (1968).

Changes in the linking number of relaxed pBR322 DNA inducted by histone complexing were determined by electrophoresis (Keller, 1975) in 1.6% agarose slab gels in 35 mM Tris, 30 mM KH_2PO_4, 1 mM EDTA, pH 7.9. Gels were run at 3.5 V/cm for 18–22 h at 22 °C.

DNA was denatured and analyzed in 8% polyacrylamide slab gels [acrylamide/bis(acrylamide), 11:1] containing 7 M urea (ultrapure grade, Schwarz/Mann) according to Maniatis et al. (1975).

DNA gels were stained with 0.5–1.0 μg/mL of ethidium bromide and photographed under short-wave UV light with a Kodak wratten filter no. 16. The lengths of the DNA fragments produced by micrococcal nuclease digestion were estimated by comparison with EndoR·*Hin*cII and EndoR·*Hin*dIII (Miles) doubly restricted λDNA (Maniatis et al., 1975).

Results

Binding of Histones to DNA. The way in which core histones bind to DNA was examined by allowing histones and DNA, at a histone/DNA ratio of 0.8:1.0 (w/w), in 2 M NaCl, buffer C, to interact by a stepwise decrease in the salt concentration (Figure 1). At each ionic strength studied DNA-bound and free histones were separated by sucrose gradient sedimentation.

Control experiments indicated that under the conditions used the DNA pelleted totally. At 1.6 M NaCl, an ionic strength at which binding was not observed, unspecific trapping of histones in the DNA pellet or smearing of the counts along the gradient did not occur (Figure 1A). The pattern of histones after centrifugation of a core histone–DNA mixture in 1.6 M NaCl was indistinguishable from that of the histones in the absence of DNA in the same conditions. Moreover, sucrose gradient centrifugation of core histones in the absence of DNA at ionic strengths ranging between 1.4 and 0.6 M NaCl produced essentially identical profiles of radioactive histones as that shown in Figure 1A. In no case was radioative material ever found in the bottom of the tubes (J. L. Jorcano, & A. Ruiz-Carrillo, unpublished observations). Interaction, although very weak, began at 1.4 M NaCl (Figure 1B). This can be seen as a change in the shape of the radioactive histone

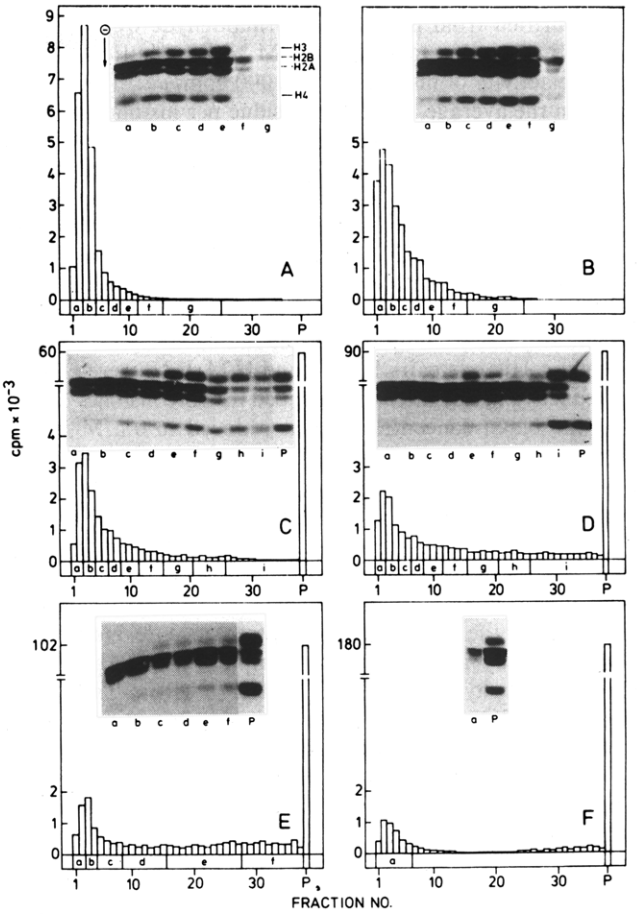

FIGURE 1: Binding of histones to DNA. λDNA and radioactively methylated core histones [histone/DNA ratio, 0.8:1.0 (w/w)] in 2 M NaCl (buffer C) were allowed to interact by stepwise dilution with buffer C. DNA and nucleohistones were pelleted through 5–20% sucrose gradients containing NaCl at the same concentration of the loaded samples. The radioactivity of 10-μL aliquots is shown (bars). P stands for the radioactivity recovered in the pelleted nucleohistone. Panels A–F: DNA-bound and free histones in 1.6, 1.4, 1.2, 1.0, 0.8, and 0.6 M NaCl, respectively. Insets: fluorography of the histones in the pooled fractions as indicated alphabetically.

Table I: Binding of Histones to DNA at High Concentrations of Histones and DNA[a]

NaCl (M)	% bound histone[b]	(H3 + H4)/(H2A + H2B) in bound complex[c]
1.4	5	1.50
1.2	36	1.57
1.0	61	1.44
0.8	75	1.50
0.6	90	1.33

[a] The conditions of the binding experiment were as described in Figure 1. See Experimental Procedures. [b] Estimated as the difference between the total radioactivity loaded in each gradient and that recovered in the top of the gradients between fraction 1 and 15. [c] Calculated from densitometric analysis of fluorographs of the histones along each gradient. See Experimental Procedures.

profile along the gradient. At lower salt concentrations clear binding was observed (Figure 1C–F), and at 0.6 M NaCl most of the histones were already bound to DNA (Table I). Similar types of experiments in which the ionic strength was decreased to lower salt molarities indicated that at 0.4 M NaCl more than 98% of the histones were bound to DNA (J. L. Jorcano, & A. Ruiz-Carrillo, unpublished observations). Analysis of the histones along the gradients and in the pelleted nucleohistones indicated that the four core histones were recovered

in the pellets at all salt concentrations as well as in the lower half of the gradients at the higher ionic strengths (Figure 4C, D). We interpret the presence of histones in the lower half of the gradients as resulting from their dissociation from the DNA–histone complex as it sediments.

In all cases each histone in the pairs H3,H4 and H2A,H2B was always present in equimolar proportions in the bound, dissociated, and free pools, already suggesting that histones may bind to DNA as complexes. The histone stoichiometry in the orginal DNA–histone complex before sedimentation, estimated taking into account pellet-bound and dissociated histones along the gradient, was rather constant during the first steps of binding (i.e., 1.4–0.8 M NaCl) (Table I). The value of the (H3 + H4)/(H2A + H2B) ratio in the DNA-bound histones was around 1.5 (Table I). We argue that these data can be interpreted as being the result of the binding of histone octamer as well as its products of dissociation for the following reasons. Firstly, experiments similar to the ones described but carried out with several concentrations of the isolated pairs H3,H4 or H2A,H2B showed that H3,H4 interacted with DNA at 1.0 M NaCl and was almost totally bound at 0.8 M NaCl. On the other hand, binding of H2A,H2B was observed to start between 0.8 and 0.6 M NaCl (Ruiz-Carrillo & Jorcano, 1977, and unpublished observations). Binding of H3,H4 and H2A,H2B to DNA is likely to occur via tetramer and dimer, respectively, since these are the stable complexes these pairs make in solution in this range of salt concentration in the absence of DNA (Ruiz-Carrillo & Jorcano, 1979). Hence, the presence of H2A,H2B in the histone–DNA complex at 1.2 and 1.0 M NaCl (Figure 4C,D), salt concentrations at which the dimer has no affinity for DNA (see above) or for the H3·H4–DNA complex (Ruiz-Carrillo & Jorcano, 1977), strongly suggests that H2A,H2B was not bound to DNA but rather to the H3·H4 tetramer. Secondly, the stoichiometry (H3 + H4)/(H2A + H2B) of the histones bound to DNA (Table I) is consistent with the binding of complexes of the four histones. At the salt and protein concentration at which binding took place the octamer is more dissociated than in 2 M NaCl, as determined by a fall in its sedimentation coefficient (Ruiz-Carrillo & Jorcano, 1979, and unpublished observations). The net effect of the change in the equilibrium caused by lowering the ionic strength is the release of H2A·H2B dimer from the octameric structures (Ruiz-Carrillo & Jorcano, 1979). As would be expected, this phenomenon is reflected in the stoichiometry of the bound complex. Finally, the pattern in which histones dissociated from the complex interacting with DNA is in keeping with our interpretation. In Figure 1 (B–D) it is shown that between 1.4 and 1.0 M NaCl the histones dissociated from the DNA-bound complex were progressively depleted of H3,H4, which was enriched in the pelleted nucleohistone. As opposed to this, the H2A·H2B dimer continued to dissociate from the DNA-bound complex in the same ionic strength interval. This is in agreement with the behavior of the isolated pairs (see above). It was not until the salt molarity was decreased to 0.8 M that the H2A·H2B dimer per se showed affinity for DNA and as a result its relative proportion in the pelleted nucleohistone increased (Figure 1E,F).

Split Reconstitution. In order to determine whether the H3·H4 tetramer bound to DNA can also organize both the octamer of histones and the nucleosome core particle, nucleohistone was reconstituted first in the presence of H3·H4 tetramer alone. At progressively lower ionic strengths, at which the H3·H4 tetramer was known to be bound to DNA, the H2A·H2B dimer was added in stoichiometric amounts to

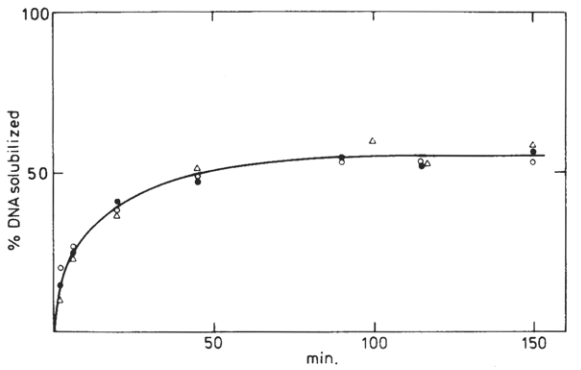

FIGURE 2: Kinetics of DNA digestion of split-reconstituted nucleohistone. Control reconstituted nucleohistone (●), 0.8 M (○), and 0.2 M (△) split-reconstituted nucleohistones with a final histone/DNA ratio of 0.8:1.0 (w/w) were digested with micrococcal DNase (0.2 units/μg of DNA) at 37 °C in the presence of 0.1 mM $CaCl_2$. The percentage of DNA rendered acid soluble was determined in aliquots made 1.0 M NaCl and 1.0 M perchloric acid. Split-reconstituted nucleohistones at 0.6 and 0.4 M NaCl gave similar kinetics (not shown).

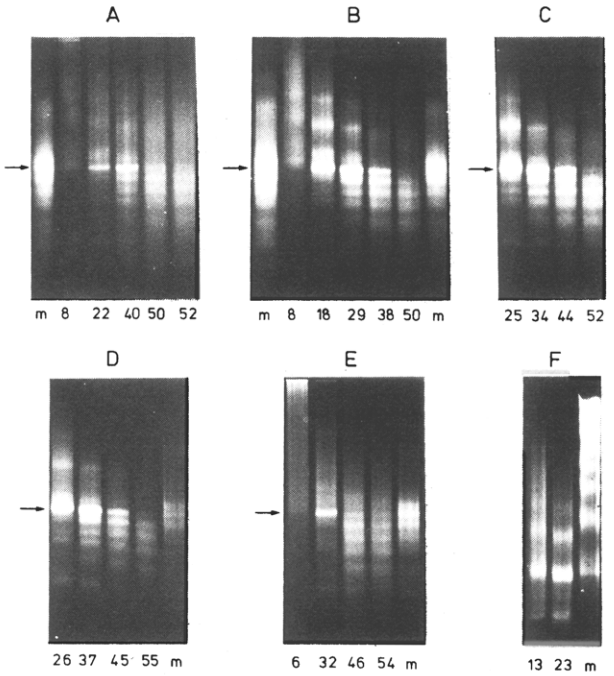

FIGURE 3: Micrococcal nuclease digestion of split-reconstituted nucleohistones. (A–E) 5–8% polyacrylamide gradient and (F) 2.5% polyacrylamide–0.5% agarose gel electrophoresis of DNA fragments produced by micrococcal DNase digestion of reconstituted nucleohistones. The final ratio of histone/DNA was 0.8:1.0 (w/w) in all cases. Direction of migration is from top to bottom. (A) Control reconstitution; (B–E) 0.8, 0.6, 0.4, and 0.2 M NaCl split reconstitutions; (F) 0.8 M NaCl split reconstitution. The numbers at the bottom of each lane refer to the percentage of the DNA rendered acid soluble, and m is a chicken erythrocyte nuclei limit digest marker. The arrow in A–E points to the position of a 145 b.p. DNA fragment. For further details, see the legend to Figure 2.

those of H3,H4 (split reconstitutions). The characteristics of these nucleohistones were examined using a control nucleohistone reconstituted at a protein concentration at which binding of the core histones as complexes was known to take place (see above). The rate of micrococcal nuclease digestion (Figure 2) and the lengths and rate of appearance of the protected DNA fragments (Figure 3) of split-reconstituted (0.8, 0.6, 0.4, and 0.2 M NaCl) and control nucleohistones were very similar. In all cases a very prominent DNA fragment with a narrow size distribution around 145 b.p. (the

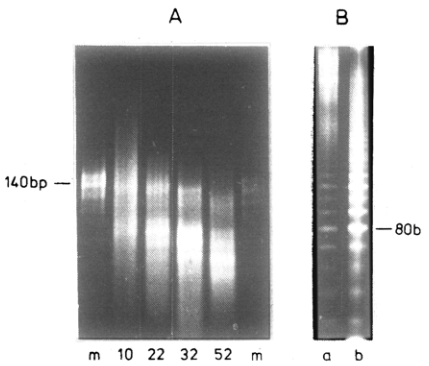

FIGURE 4: DNase digestion of H3·H4–DNA complex. Reconstituted H3·H4–DNA complex at a histone/DNA ratio of 0.4:1.0 (w/w) was digested with micrococcal nuclease (A) or with DNase I (B). (A) Electrophoresis of double-stranded DNA fragments. The numbers at the bottom of each lane refer to the percentage of the DNA rendered acid soluble and m is a chicken erythrocyte nuclei limit digest marker. (B) 8% polyacrylamide–7 M urea electrophoresis of denatured DNA fragments after digestion of the nucleohistone to 40% of the DNA acid soluble (b); chicken erythrocyte nuclei marker, 12% of DNA acid soluble (a). For further details, see the legend to Figure 3.

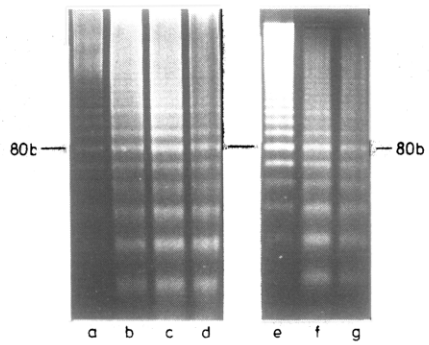

FIGURE 5: DNase I digestion of split-reconstituted nucleohistone. Electrophoresis of denatured DNA fragments after DNase I digestion of control and split-reconsitituted nucleohistones (histone/DNA, 0.8:1.0, w/w). (a and e) Chicken erythrocyte nuclei marker, 12% of the DNA acid soluble; (b) control nucleohistone 25% of the DNA acid soluble; (c, d, f, and g) 0.8, 0.6, 0.4, and 0.2 M NaCl split-reconstituted nucleohistones digested to 35% (c), 37% (d), 42% (f), and 42% (g) of the DNA acid soluble, respectively. See the legend to Figure 3 for further details.

size of the nucleosome core particle) was generated early in the digestion. In addition to this, larger DNA fragments with a length multiple of 145 b.p. were also observed (Figure 3A–F), in agreement with previous reports (Yaneva et al., 1976; Steinmetz et al., 1978). The lengths, rate of appearance, and stability of the DNA fragments produced by the nuclease action on nucleohistone reconstituted only with H3·H4 were clearly different (Figure 4A), whereas nucleohistone reconstituted only with H2A·H2B failed to show protection of DNA (results not shown) (see also Camerini-Otero et al., 1976). The results obtained with the split-reconstituted nucleohistone, therefore, evidenced that at least a great proportion of the H2A·H2B dimers were able to find the correct binding sites in the H3·H4 tetramer. These findings also suggest that the H2A·H2B may slide along the DNA till it interacts with the tetramer. This possibility was further supported by experiments, similar to the ones shown, in which the final ratio of histones to DNA was 0.4:1.0 (w/w). The nucleohistones thus prepared presented the characteristic DNA banding patterns of the nucleosome core particle as in Figure 3 (results not shown).

In the case of DNase I, again no significant difference was found between the pattern of digestion of DNA in control and split-reconstituted nucleohistones (Figure 5). In both cases

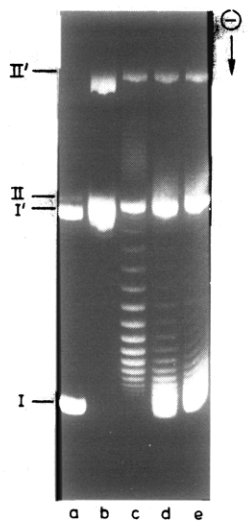

FIGURE 6: Change in the linking number of circular DNA (II) in split-reconstituted nucleohistone. 1.6% agarose slab gel electrophoresis of pBR322 DNA from control and split-reconstituted nucleohistones. (a) Superhelical pBR322 DNA marker; (c) H3·H4–DNA nucleohistone (histone/DNA, 0.35:1.0, w/w); (b and d) 0.2 M NaCl split-reconstituted nucleohistone (histone/DNA, 0.7:1.0, w/w), before (b) and after (d) treatment with NCE; (e) control reconstituted nucleohistone (histone/DNA; 0.7:1.0, w/w) after treatment with NCE. I refers to the position of superhelical DNA and II to that of the relaxed and/or nicked DNA. I′ and II′ correspond to the positions of superhelical and relaxed DNA from a higher molecular weight contaminating plasmid.

the characteristic ten base pattern was observed in the denatured DNA (Noll, 1974), and the intensity of the DNA fragments of 80 and 110 bases long was also higher, suggesting that the differential accessibility of the DNA in the nucleosome core particle was also preserved (Simpson & Whitlock, 1976). However, DNase I digestion is not a good criterion for core particle assembly since the pattern of digestion of DNA of nucleohistone containing only H3·H4 was undistinguishable from that containing the four core histones (Figure 4B). It is worthwhile to point out that the relative frequency of nicking by DNase I in the H3·H4–DNA complex is also similar to that in the core particle as reflected in the higher intensity of the bands of around 110 and 80 bases. In addition, the ten base periodicity extends up to at least 270 bases, which is the limit of resolution of the gels used. These observations are at variance with those reported by other workers (Sollner-Webb et al., 1976).

Finally, it is known that core histones induce, by complexing to covalently closed relaxed circular DNA (II) and treatment with NCE, a change in the linking number (Fuller, 1971; Crick, 1976) of the DNA molecule (Germond et al., 1975). This change can be observed after the removal of histones (Germond et al., 1975) as an increase in the electrophoretic mobility of the DNA due to supercoiling. H3,H4 alone at a histone/DNA ratio of 0.35:1.0 (w/w) produced a change in the linking number which was smaller than that produced by the core histones at a histone/DNA ratio of 0.7:1.0 (w/w) (Figure 6C,E) (see also Camerini-Otero & Felsenfeld, 1977). H2A,H2B alone, on the other hand, produced no visible effect (Camerini-Otero & Felsenfeld, 1977; J. L. Jorcano and A. Ruiz-Carrillo, unpublished observations). Figure 6 shows, however, that addition of H2A,H2B at 0.2 M NaCl to the H3·H4–DNA (II) complex increased the number of supercoils per DNA molecule to an extent comparable to that of control nucleohistone assembled with the same histone/DNA ratio (Figure 6D,E).

The degree of supercoiling of circular DNA, the percentage of DNA rendered acid soluble in micrococcal nuclease limit digestions, and the relative intensities of the DNA fragments over background after micrococcal nuclease or DNase I digestion clearly indicated that a large proportion of the nucleohistone had in all cases been faithfully reconstituted.

Discussion

The data presented in the first part of this report indicate that the octamer of core histones and its intermediate products of dissociation can bind to DNA in an in vitro assembly of nucleohistone. Evidence is presented suggesting that the histone stoichiometry of the complexes which bind to DNA at the higher ionic strengths reflects the equilibrium among the histone complexes existing under identical conditions but in the absence of DNA. These results bring together two sets of apparently conflicting observations. Namely, the report that the nucleosome core particle can be reconstituted with chemically cross-linked histone octamer (Stein et al., 1977) and that interaction of non-cross-linked core histones to DNA takes place by the sequential binding of H3·H4 tetramer and H2A·H2B dimer (Ruiz-Carrillo & Jorcano, 1977; Wilhelm et al., 1978). It is worthwhile to emphasize here that, in addition to the ionic strength, the stability of the complexes of the core histones is heavily influenced by the concentration of protein (Ruiz-Carrillo & Jorcano, 1979), which in turn influences the type of complexes interacting with DNA. Concerning this, it is relevant that at the protein concentration at which our previous binding studies were performed (Ruiz-Carrillo & Jorcano, 1977) the octamer was totally dissociated into its structural subunits, the H3·H4 tetramer and the H2A·H2B dimer (J. L. Jorcano and A. Ruiz-Carrillo, unpublished observations). Another conclusion from this work is that even in the conditions in which binding of complexes of the core histones is observed, interaction with DNA occurs through the H3·H4 tetramer subunit.

The importance of the role played by the H3·H4 tetramer in the assembly of the nucleosome core particle is also highlighted by the split reconstitution studied. These experiments showed that the H3·H4 bound to DNA is able to direct the organization of the core particle octamer similarly to the way it does in solution and therefore that the preassembled octamer is not required for the correct reconstitution of nucleohistone. The properties displayed by the H3·H4 tetramer suggest that it may have two separate structural domains. One would be involved in the constraint of DNA into prenucleosomal structures, while the other would provide the interacting sites for the H2A·H2B dimer.

From the above discussion and from the work of Finch et al. (1977), Hjelm et al. (1977), and Richards et al. (1977), we suggest that in vitro the H3·H4 tetramer organizes the path of DNA through its DNA binding domain in structures which have many of the characteristics of the nucleosome core particle (Richards & Pardon, 1970; Kornberg & Thomas, 1974; Boseley et al., 1976; Camerini-Otero et al., 1976; Sollner-Webb et al., 1976; Ruiz-Carrillo & Jorcano, 1977; Camerini-Otero & Felsenfeld, 1977; Bina-Stein & Simpson, 1977; and this work). The pre-core particle thus formed still has two equivalent binding sites, presumably on opposite surfaces, for the H2A·H2B dimers. The H2A·H2B bound to the DNA through the N termini can then slide along the DNA phosphate backbone until it is within interacting distance of the H3·H4 tetramer. The binding of two H2A·H2B dimers per H3·H4 tetramer results in the assembly of the core protein octamer and in the additional constraint of the DNA as observed in the nucleosome core particle.

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^{1}H NMR and ESR Studies of Oxidized Cytochrome c_{551} from *Pseudomonas aeruginosa*†

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ABSTRACT: Near neutral pH, Fe(III) cytochrome c_{551} exhibits an ESR absorption due primarily to a single species with g values of 3.24, 2.06, and 1.48. These g values are somewhat different from those of horse heart cytochrome c and can be interpreted by the generalizations of Brautigan et al. [(1977) *J. Biol. Chem. 252*, 574] to be due to Fe binding by the imidazole anion of histidine rather than by neutral imidazole. The NMR spectrum of Fe(III) cytochrome c_{551} exhibits a number of hyperfine-shifted peaks whose pattern shows similarities to but many differences from that of horse heart cytochrome c. Variation in shifts of some of the peaks in the pH range 5–9 is ascribed to ionization of a somewhat buried propionic acid side chain (pK = 5.8) and to ionization of the N-terminal NH_3^+ group (pK = 7.7). At alkaline pH >9.4, as shown by a variety of optical and ESR spectral changes, the Met-61 S ligand is replaced by other ligands.

The cytochrome c_{551} of the bacterium *Pseudomonas aeruginosa* (Ambler, 1963) is a primitive version of the "mammalian" cytochrome c of eukaryotes (Dickerson et al., 1976). For example, both horse heart cyt c and cyt c_{551}[1] have a single heme group covalently bound through two thioether linkages to cysteines of the amino acid chain, and both have a histidine and a methionine as fifth and sixth ligands, respectively (Almassy and Dickerson, 1978). However, cyt c_{551} has only 82 amino acids in contrast to horse heart cyt c which has 104 (cf. Table I for a comparison of properties of these two cytochromes).

Antonini and co-workers (1970) discovered a remarkable electron-transfer reaction between cyt c_{551} and a companion copper protein, azurin from *Pseudomonas*, which proceeds in each direction with rate constants $\sim 10^6$ M^{-1} s^{-1}. The redox potentials of horse heart cyt c and cyt c_{551} are both 286 mV, and that of azurin is 314 mV. We expect, therefore, that the biological role of both cyt c_{551} and azurin is as a soluble electron carrier. In order to understand the electron-transfer kinetics of these two proteins (Rosen & Pecht, 1976; Coyle & Gray, 1976), we have studied azurin by emission spectroscopy (Ugurbil & Bersohn, 1977; Ugurbil et al., 1977a), by ^{1}H NMR (Ugurbil & Bersohn, 1977), and by ^{13}C NMR (Ugurbil et al., 1977b). Toward the same goal we have now studied cyt c_{551} by ^{1}H NMR and by ESR.

Experimental Procedures

There were two cyt c_{551} preparations. One was made by Ugurbil whose isolation procedure, previously described (Ugurbil & Bersohn, 1977), is a modification of Ambler's procedure (Ambler, 1963); the other was purchased from Microbiological Research Establishment, Porton, Salisbury, U.K. The NMR sample usually had a concentration of ~3 mM with 0.1 M NaCl and 0.05 M phosphate buffer. A severalfold excess of $K_3Fe(CN)_6$ was added to ensure that cyt c was completely oxidized. NMR measurements were made on a Varian 220-MHz spectrometer. ESR measurements were made on a Varian E-9 X-band spectrometer. In the low-temperature range (<50 K) a carbon resistor was used as a thermometer. Before each electrophoresis on polyacrylamide gel the protein was boiled with 1% $NaDodSO_4$ for 3 min.

ESR Spectra and Assignments. In order to slow down the rapid spin–lattice relaxation rates, the ESR spectra were taken at ~11 K. The spectrum at pH 4.5 (Figure 1) contains contributions from four paramagnetic species. The smallest peak at g = 4.3 is due to a rhombic high-spin Fe(III) complex which has been found in a number of cytochromes (Brautigan et al., 1977) as a small impurity. The sharp peak at g = 2.07 is the envelope of the perpendicular hyperfine components of a Cu impurity whose concentration, as measured by atomic absorption spectrophotometry, is ~1% of the total protein concentration; this is probably azurin (Brill et al., 1968) which, in any case, remains with cyt c_{551} until the last stage of purification. The small peak at g = 5.74 most likely belongs to an irreversibly denatured high-spin cyt c_{551} (see the next section for details). The absorption-like peak at g = 3.24, the broad derivative peak spread over ~700 G at g = 2.06, and the small, barely visible and often undetectable shoulder at g = 1.48 are all assigned to the dominant low-spin species of cyt c_{551}. The minor high-spin components probably have greater amplitude in the observable spectrum because of their narrow line width.

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[1] Abbreviations used: cyt c_{551}, *Pseudomonas aeruginosa* ferricytochrome c_{551}; NMR, nuclear magnetic resonance; ESR, electron spin resonance; TSP, sodium 3-(trimethylsilyl)propionate.

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