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In vitro core particle and nucleosome assembly at physiological ionic strength

(chromatin structure/linking number/DNases/electron microscopy/histone H1)

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ABSTRACT **Nucleosome core particles have been efficiently assembled *in vitro* by direct interaction of histones and DNA at physiological ionic strength, as assayed by digestion with DNases, supercoiling of relaxed circular DNA, and electron microscopy. Reconstitution was achieved either by the simultaneous addition of all core histones, or by the sequential binding of H3·H4 tetramer and H2A·H2B dimer to DNA. Micrococcal nuclease digestion and electron microscopy studies indicated that there is heterogeneity in the spacings at which core particles are assembled on the DNA. Length measurements of oligomeric DNA produced during the course of the digestion suggest that the core histone octamer can organize 167 (±4) rather than 145 base pairs of DNA, the extra 20 base pairs being quickly digested. Binding of histone H1 to core particles resulted in the protection of about 165 base pairs of DNA from nuclease attack. Because the core histone octamer is fully dissociated into H3·H4 tetramer and H2A·H2B dimer at physiological ionic strength, our results would suggest that *in vivo* core particle assembly may also occur by interaction of these two complexes on the nascent DNA.**

The nucleosome core particle consists of approximately 145 base pairs (bp) of DNA and an octamer of two of each core histone (H2A, H2B, H3, and H4) (reviewed in refs. 1 and 2). Although the histone octamer can exist in solution, high ionic strength is necessary for its stabilization (3–5). The high salt concentration required for its stabilization is likely to mimic the environment of the complex in the nucleosome. The assembly of the octamer in the core particle by addition, at low salt, of H2A·H2B to H3·H4–DNA complex preassembled at high ionic strength (6–8) is consistent with this view. Unlike the histone octamer, the H3·H4 tetramer and the H2A·H2B dimer are both stable at lower salt concentrations (4, 5, 9–12). Therefore, we have addressed the question of whether or not core particle assembly can spontaneously occur by mixing DNA and the octamer subunits at physiological ionic strength. In addition, we have studied the effect of H1 interaction with the reconstituted complex.

MATERIALS AND METHODS

Chicken erythrocyte histone H1s were purified by chromatography in Bio-Rex 70 (Bio-Rad) eluted with an 8–16% linear gradient of guanidine hydrochloride in 0.1 M sodium phosphate, pH 6.8/0.2 mM phenylmethylsulfonyl fluoride. Core histones were extracted with 0.25 M HCl after 5% $HClO_4$ treatment of nuclei, extensively washed with 0.35 M NaCl, renatured, and further purified as octamers in 2 M NaCl (5). H3·H4 tetramer and H2A·H2B dimer were purified by gel filtration chromatography in 0.2 M NaCl (5).

Chicken erythrocyte DNA was prepared after gentle micrococcal nuclease digestion of nuclei. Solubilized chromatin (DNA $M_r = 2.8 \times 10^6$) was fractionated in Bio-Gel A-50m (Bio-Rad) to remove size classes from mono- to pentanucleosomes (unpublished data) (see Fig. 3*A*, lane a).

Nucleohistone reconstitutions were carried out at 37°C in a rotatory shaker water bath. DNA and histones were in 10 mM triethanolamine, pH 7.3/0.2–0.4 mM phenylmethylsulfonyl fluoride/0.01% 2-mercaptoethanol/0.05–0.4 M NaCl, unless indicated otherwise. Typically, 5 ml of core histones (90 μg/ml) or 2.5 ml of each histone pair H3,H4 and H2A,H2B (90 μg/ml) were very slowly (i.e., ≈1–2 ml/hr) added to 5 ml of DNA (100 μg/ml) containing bovine serum albumin (200 μg/ml). Reconstitution experiments involving H1 (40 μg/ml) were carried out at a final H1 to DNA ratio of 0.2:1.0 (wt/wt) as described in *Results*.

The lengths of DNA fragments were determined by electrophoresis and calibration against EndoR·Bsu-restricted λdv1 (13). Reconstituted nucleohistones (5–20 μg/ml) made in the absence of bovine serum albumin were fixed in 0.1% glutaraldehyde and adsorbed onto carbon-coated Parlodion or copper grids positively charged with a monolayer of cytochrome C (14). Preparations were positively stained with 2% aqueous uranyl acetate, washed with water, air dried, and shadowed with Pt/Pd (80:20) at an angle of 8°. Other materials and methods were as described (5, 8, 15).

RESULTS

Nucleosome Core Particles Can Be Sequentially Reconstituted by Adding H3·H4 Tetramer Followed by H2A·H2B Dimer to DNA. For convenience we will refer to 0.2 M NaCl as physiological ionic strength. This salt concentration was used in most assays because it has been shown to be optimal for the nicking and closing activity (16). In addition, the results to be described were independent of the salt concentration between 0.4 and 0.05 M NaCl. We would like to emphasize that, at the histone and DNA concentrations at which the reconstitution experiments were carried out, the H3·H4 tetramer binds to DNA between 1.2 and 0.8 M NaCl, irrespective of the presence of H2A·H2B (unpublished data; see refs. 8 and 15).

Fig. 1*A* shows the result of mixing H3·H4 tetramer with relaxed pBR322 DNA (II) at 0.2 M NaCl in the presence of nicking and closing extract with further incubation at 37°C for 90 min. Complexing of histones to DNA resulted in supercoiling of the DNA, the number of superhelices introduced being dependent on the input histone (Fig. 1*A*, lanes a–c). When either nicking and closing extract or histones were omitted from the reaction, the DNA moved to the position of the relaxed form (II) (results not shown). It is known that the efficiency of H3·H4 in inducing supercoiling of DNA is about half that of core histones on a 2-fold weight basis (6, 8). Fig. 1*A* shows that addition of stoichiometric amounts of H2A·H2B at 0.2 M NaCl to the

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Abbreviation: bp, base pairs.

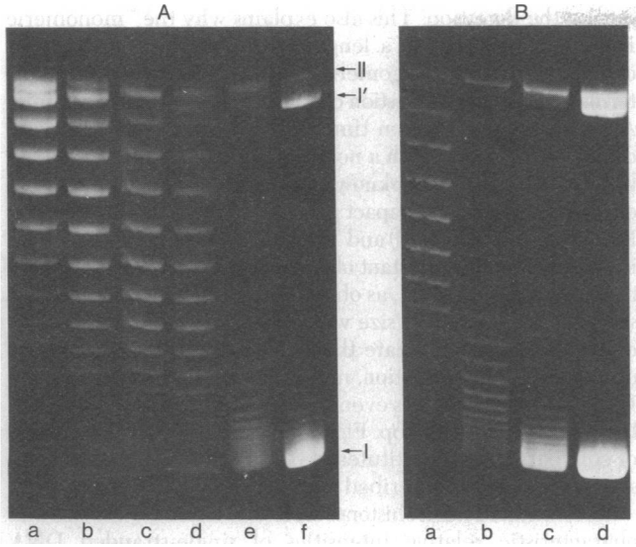

FIG. 1. Change in the linking number of relaxed pBR322 DNA (II) due to core particle assembly in 0.2 M NaCl. (*A*) Supercoiling induced by H3·H4 tetramer alone (lanes a–c) or after addition of stoichiometric amounts of H2A·H2B to the H3·H4–DNA complex (lanes d–f). Final histone to DNA ratios (wt/wt) were: 0.2 (a), 0.3 (b), 0.4 (c), 0.2 + 0.2 (d), 0.3 + 0.3 (e), and 0.4 + 0.4 (f). (*B*) Supercoiling induced by simultaneous addition of all core histones. Histone to DNA ratios (wt/wt) of 0.2 (a), 0.4 (b), 0.6 (c), and 0.8 (d). Nicking and closing extract (40 μl/μg of DNA) and bovine serum albumin (200 μg/ml) were included during the reconstitutions. After being mixed at room temperature, samples were incubated for 90 min at 37°C. DNA was analyzed by electrophoresis in a 1.6% agarose slab gel (8). (I) and (II) indicate the position of superhelical and relaxed or nicked pBR322 DNA, respectively. (I') indicates the position of a superhelical DNA from a higher molecular weight plasmid.

H3·H4 DNA complex preassembled at 0.2 M NaCl (lanes a–c) consistently shifted the distribution of superhelical turns per DNA molecule to higher values (lanes d–f). Control experiments indicated that H2A·H2B in the absence of H3·H4 produced no detectable effect (result not shown; see also refs. 6 and 8). This suggests that H3·H4 bound at physiological ionic strength to DNA has the properties of both constraining the DNA and providing the binding sites for the H2A·H2B dimer (8).

Other experiments demonstrated that H3·H4 tetramer mixed with linear DNA results in complexes that have the same characteristics as those reconstituted from 2 M NaCl when tested by DNase I (Fig. 2) and micrococcal nuclease digestion (result not shown; see ref. 8). In fact, some of the specific (17) characteristic relative intensities of the fragments obtained by the DNase I action on the nucleosome (18) are already evident in the H3·H4–DNA complex (i.e., Fig. 2 bands of about 80 and 110 bases). The extended ladder of DNA fragment multiples of about 10 bases (Fig. 2) suggests that most of the tetramers are located either at the same distances or in a 10 bp register.

Spontaneous Nucleosome Core Particle Reconstitution. At NaCl concentrations of 0.4 M or lower, the histone octamer is totally dissociated into H3·H4 tetramer and H2A·H2B dimer (4, 5). Hence, unless priming of the DNA by H3·H4 is necessary, addition of all core histones to DNA at 0.2 M NaCl should have the same effect as the sequential addition of the separate complexes. Fig. 1*B* shows a titration experiment in which core histones were added to DNA at increasing histone to DNA ratios (0.2–0.8, wt/wt) in the presence of nicking and closing extract (lanes a–d). It is clear that the distribution of supercoils is similar to that obtained in split reconstitutions of equivalent histone to DNA ratios. The core particle octamer can, therefore, be formed irrespective of the order of addition of the histone

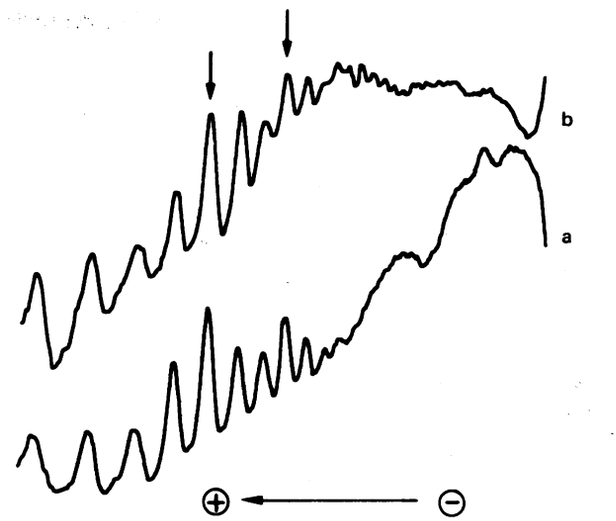

FIG. 2. DNase I digestion of an H3·H4 tetramer–DNA complex (histone/DNA = 0.45:1.00, wt/wt) assembled at 0.2 M NaCl. Densitometric scan of a 7% polyacrylamide gel containing denatured DNA fragments after DNase I (Worthington) (0.025 units/μg of DNA) digestion of the complex for 30 sec (curve b) in 50 mM NaCl at 37°C (8). Curve a is a marker from chicken erythrocyte nuclei digested until 22% of the DNA was acid soluble. The arrows point to the positions of DNA fragments about 80 and 110 bases long.

complexes. Similar results were obtained by mixing histones and DNA at 0.1 M NaCl before increasing the salt molarity to 0.2 M for the nicking and closing extract treatment (unpublished data).

Mixing core histones in equimolar amounts and linear DNA (histone/DNA = 0.9:1.0, wt/wt) at 0.2 M NaCl, under our conditions, does not result in precipitation or turbidity of the solution as determined by light scattering. The UV spectrum is typical of soluble chromatin (data not shown). These results are independent of the method of histone extraction, (e.g., acid or salt) insofar as core histones are renatured as octamers (5). In all these experiments histones were bound to DNA as determined by precipitation of the nucleohistone with 10 mM $MgCl_2$ (ref. 19; unpublished data).

The frequency of core particle assembly on DNA was analyzed by micrococcal nuclease digestion. Fig. 3 shows that early in the digestion a series of oligomeric DNA fragments with a broad size distribution was generated. At longer times, the lengths of the fragments became more homogeneous and the background disappeared (Fig. 3*A*, lanes e–g). The amount of DNA rendered acid soluble at the limit of the digestion was typically around 50%, which indicates an efficient yield of assembly. Although there may be stretches of free DNA or poorly reconstituted core particles in our preparations (i.e., see Fig. 5), this can only account for part of the background, the remainder being contributed by heterogeneity in the spacings and in the lengths of the tails (see below).

Fig. 4 shows that there is also a decrease in DNA size classes during the digestion. Extrapolation of the lengths of the oligomeric DNA gave an estimated unit repeat length of 167 bp (±4) at 0 time of digestion, whereas that of the "monomeric DNA" extrapolated at about 175 bp. The finding that the core histones alone can organize a minimum of approximately 167 bp of DNA was also supported by experiments in which H1 was added to the reconstituted core particles (see below).

The decrease in DNA length is a consequence of both exonucleolytic digestion and heterogeneity in the spacings between core particles. These two events are reflected, respectively, in a shift and in a change in the slope in plots of the oli-

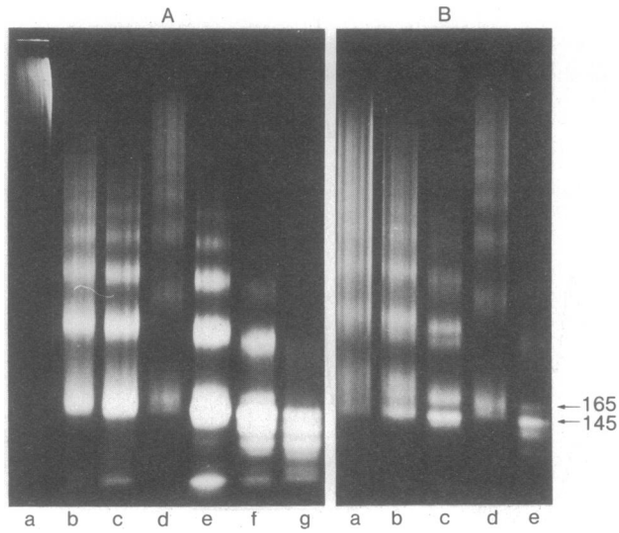

FIG. 3. Micrococcal nuclease digestion of nucleohistone reconstituted at physiological ionic strength. (*A*) Core histones were mixed with linear DNA (histone/DNA = 0.9:1.0, wt/wt) at 0.2 M NaCl, dialyzed against 0.05 M NaCl, and digested at 37°C with 0.15 units of enzyme (Worthington) per μg of DNA in the presence of 0.2 mM $CaCl_2$. DNA was deproteinized and analyzed by electrophoresis in a 2.5% polyacryalmide/0.5% agarose slab gel (8). Samples were taken at 30 sec (b), 1 min (c), 3 min (e), 10 min (f), and 30 min (g) of digestion. The DNA used for the reconstitution is shown in lane a. (*B*) H1 was added to an aliquot of reconstituted core particles as in *A* (H1/DNA = 0.2:1.0, wt/wt) at 0.01 M NaCl. The salt concentration was progressively increased up to 0.05 M and digestion was then carried out as described in *A*. Digestion times were: 30 sec (a), 1 min (b), 3 min (c), and 10 min (e). Lanes d in *A* and *B* show the DNA obtained after micrococcal nuclease digestion of hen erythrocyte nuclei (3% of the DNA rendered acid soluble).

gomeric DNA length versus oligomer number (Fig. 4*B*). Because core particles that are spaced at greater distances on the DNA should be more susceptible to the nuclease attack than those that are closely spaced, the latter should accumulate during the digestion. This also explains why the "monomeric DNA" extrapolated to a length longer than the unit repeat calculated from the oligomers. The observed repeat size at intermediate times of digestion changed to 141 bp (±4) at 45 min. At even longer digestion times the only oligomers left were dimers and trimers with a nominal periodicity of around 125 bp (Fig. 4*A*). We do not know, at present, whether the residual oligomers represent compact particles of the type described by Klevan and Crothers (20) and Tatchell and Van Holde (21). This is because nuclease-resistant oligomers are likely to be subjected to internal degradation, as observed with the monomers; consequently, their repeat size would be underestimated. In any event, our results indicate that, unless major rearrangement occurs during the digestion, not all particles are formed on the DNA at equal distances even though they have a tendency to be spaced at about 167 bp. Fig. 5 shows an electron micrograph of core particles reconstituted at 0.2 M NaCl, which illustrates some of the points described above.

Reconstituted nucleohistones were also tested for the size and characteristic relative intensities of single-stranded DNA fragments produced by digestion with DNase I and for the generation and stability of the subnucleosomal DNA fragments produced by extensive micrococcal nuclease treatment. Results not included here indicated no difference between nucleosome core particles assembled spontaneously at 0.05–0.4 M NaCl or by dialysis from 2 M NaCl (8). The pattern of DNA fragments denatured after DNase I digestion was indistinguishable from that of H3·H4–DNA complex, as reported (8). This indicates that the register of the tetramers was unaffected after H2A·H2B binding.

Nucleosome Reconstitution. Under our conditions of reconstitution, histones and DNA are mixed at ionic strengths at which H1 is bound to the DNA (22). Therefore, it was of interest to determine whether inclusion of H1 during the reconstitution could regenerate some of the characteristics of native chromatin. Several schedules of mixing were followed which produced essentially identical results. H1 was added at 0.01–0.4 M NaCl to core particles reconstituted at 0.2–0.4 M NaCl or it was mixed with the core histones during total reconstitution.

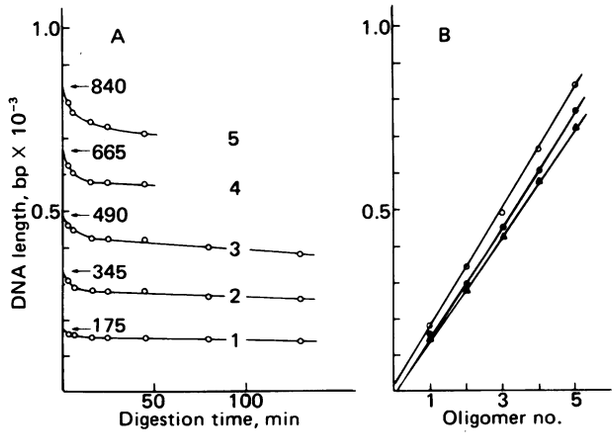

FIG. 4. Determination of the DNA repeat length of oligomeric core particles. Reconstituted nucleohistone at 0.2 M NaCl was digested at this ionic strength with 0.2 units of micrococcal nuclease per μg of DNA in the presence of 0.2 mM $CaCl_2$ at 37°C. (*A*) Shortening of oligomeric DNA during the course of digestion. The numbers given at the ordinate are the estimated DNA lengths at 0 time of digestion for the oligomers. (*B*) DNA length of the oligomers at different times of digestion (○, t = 0; ●, t = 6 min; ▲, t = 45 min) is plotted against oligomer number. In 0.2 M NaCl the rate of DNA digestion by micrococcal nuclease was about 1/13th of that at 0.05 M NaCl (unpublished results).

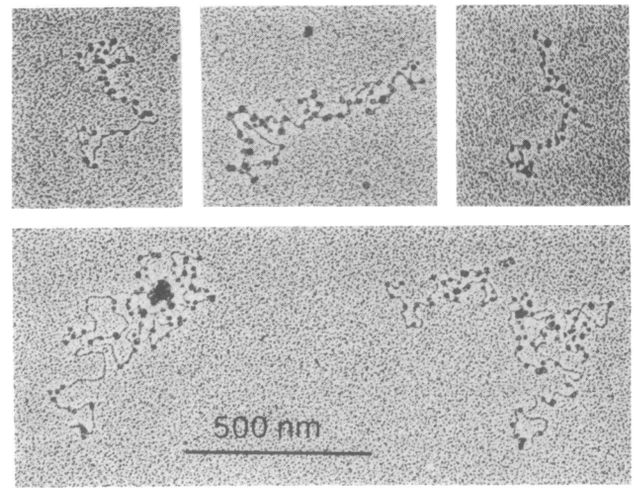

FIG. 5. Electron micrograph of reconstituted core particles. Linear DNA and chicken erythrocyte core histones were mixed at 0.2 M NaCl in the absence of bovine serum albumin. Reconstituted nucleohistone was dialyzed against 0.05 M NaCl and prepared for electron microscopy as described in the text. Average core particle diameter was 167 ± 18 Å; average diameter of free DNA was 70 ± 14 Å. The large value for both diameters is due to the coarseness of the shadowing. Similar values were obtained with native polynucleosomes.

H1 was also added after H3·H4 and before H2A·H2B in split reconstitutions at 0.2 M NaCl, or at 0.2 M NaCl before addition of core histones. Regardless of the order of mixing, the reconstitutions at 0.2–0.4 M NaCl produced nucleohistones that gave opalescent solutions and eventually precipitated. However, dialysis to 0.01–0.03 M NaCl resulted in complete solubilization of the nucleohistone. Slow dialysis up to 0.08 M NaCl (in 20 mM steps) did not result in increased turbidity. This behavior was similarly observed in polynucleosomes obtained from nuclei (unpublished data).

Micrococcal nuclease digestion of H1-containing core particles (Fig. 3*B*, lanes a,b,c, and e) showed characteristics similar to those described for the core particles but with two important differences. Firstly, the size of the "monomeric DNA" was longer, with a length average of 200 bp at 0 time. Furthermore, a fragment of approximately 165 bp was protected from digestion in a similar manner as observed in isolated H1-; or H1, H5-containing nucleosomes purified from nuclear digests (ref. 23; unpublished data) (see Fig. 3*B*, lanes b–d). Secondly, split DNA bands corresponding to dimers and trimers were observed (Fig. 3*B*, lanes a and b). Doublets of higher oligomers could not be resolved in the type of gels used. The periodicity of the DNA fragments, calculated as in Fig. 4, for the larger oligomers of the doublets, as well as for the tetramers and pentamers, was approximately 165 bp during the early course of the digestion (data not shown). Hence, binding of H1 protects approximately 20 bp of DNA from nuclease attack, presumably in core particles that are spaced at $\geq$167 bp. Control experiments confirmed that H1 is, indeed, bound in stoichiometric amounts to the oligomers and monomers of 165 bp isolated by sucrose gradient sedimentation (data not shown).

DISCUSSION

Nucleosome core particles can be reconstituted from DNA and core histones by dialysis from high ionic strength solutions (1–2 M NaCl) in the presence or absence of urea (reviewed in ref. 2). Attempts to perform reconstitution at lower ionic strengths have reportedly resulted in failure (see for instance refs. 6, 24–27). So far, core particle has been assembled *in vitro* at physiological or lower ionic strength by the addition of H2A·H2B dimer to DNA preassembled with H3·H4 at high salt concentration (6–8). At low ionic strengths, core particles (nucleosomes) have been assembled from core histones and DNA only under the influence of an "assembly activity(ies)" occurring in *Xenopus leavis* egg or oocyte extracts (24–26).

Although we can not exclude the presence of similar factors in the crude preparation of nicking and closing activity used, we believe that the results shown in Fig. 1 are not necessarily a consequence of contaminating assembly activity(ies). Faithfully reconstituted H3·H4–DNA complexes and nucleosome core particles were also efficiently obtained in the absence of nicking and closing extracts (Figs. 2–5). In addition, induction of superhelical turns on nicked pBR322 DNA by core histones was also observed after T4 ligase treatment of complexes spontaneously reconstituted at 0.2 M NaCl (unpublished observations). It is also unlikely that the histones used in the experiments reported here were contaminated by assembly activity(ies), because apart from the way in which histones were prepared (see *Materials and Methods*) the mature erythrocyte is a very inactive cell. Moreover, in heavily loaded gels, no major protein bands could be seen other than those corresponding to the histones. On the other hand, an excess of assembly activity over histones, on a weight basis, is required for efficient reconstitution (25).

We conclude therefore that, under our experimental conditions, an assembly activity of the type described (25) is not necessary for *in vitro* nucleosome core particle assembly. We would like to emphasize that this conclusion should not be taken to mean that the cell does not need any factor(s) for assembling chromatin. In a vital process like DNA packaging, the cell probably makes use of rather sophisticated mechanisms.

The biological implications of either the work of Laskey and coworkers (24, 25) or our own findings are by no means clear, but not necessarily exclusive. Assembly activity(ies) may be advantageous in cells having a large pool of DNA-free histones, as in *Xenopus* eggs or oocytes. On the other hand, our conditions of slow addition of histone complexes to DNA may reflect the mechanism of assembly during chromatin synthesis. The possibility still remains that a conjunction of both mechanisms takes place *in vivo*. Together with cytoplasmic histone modifications (28), an assembly activity (an acidic protein) (25) may, by complexing specifically with histone complexes, reduce their overall positive character. This may in turn modulate their interaction with DNA and prevent nonspecific inhibitory side reactions.

To better understand chromatin replication, it would be of great value to know which type of histone complexes are associated with the assembly activity(ies). Regarding this question, our results are strong evidence that most of the information required for the structure of the core particle resides in the sequences of the histones and in the secondary structure of the DNA. Furthermore, the H3·H4 tetramer is the key complex in the assembly process, a conclusion in agreement with previous work (5, 8). The change in the linking number (29) produced by binding of two H2A·H2B dimers to an H3·H4 tetramer–DNA complex is roughly equivalent to that produced by the binding to DNA of an H3·H4 tetramer. However, topological changes in the DNA induced by these two complexes alone may not be the same as judged by nuclease digestion of H3·H4–DNA complexes (refs. 8 and 29; this work), split reconstitution experiments (refs. 6, 8, and 15; this work), and the failure of H2A·H2B on its own to organize the DNA into a definite structure. These results are thus more consistent with the delimitation and basic organization by the H3·H4 tetramer of a length of DNA similar to that found in the fully assembled core particle as originally proposed by Camerini-Otero *et al.* (30).

Even though our findings may not be unequivocally applicable to the *in vivo* processes, it is clear that the preassembled histone octamer is not necessary for the *in vitro* assembly. Rather, the histone octamer can be assembled by interaction of the H2A·H2B dimer with the H3·H4 tetramer already bound on the DNA. Interestingly, Worcel *et al.* (31) have recently reported evidence that newly replicated chromatin is enriched in H3,H4, whereas more mature chromatin has the normal complement of all four histones.

The limiting factor for efficient spontaneous assembly appears to be the structural integrity of the H3·H4 tetramer and H2A·H2B dimer. Aggregation or precipitation of the reconstituted nucleohistone was always correlated with nonstoichiometric complexes present in the solution of histones used for reconstitution. The speed of mixing histones and DNA is also important, although mixing rates up to 4–5 ml/hr have been used without noticeable effect. Bovine serum albumin was included in most of the assays because of the higher efficiency of assembly when the amount of histones and DNA used was very small (1–2 μg); however, when larger amounts of material were handled it was sometimes omitted with no observable effect (e.g., see Fig. 5).

Another observation from our work is that the core particle is capable of organizing about 167 bp of DNA and that binding of H1 appears to be passive with no direct effect on the repeat

unit length. Because the core particle has a (pseudo) symmetrical structure (32–34), it is conceivable that the additional 20 bp represent 10-bp extensions at each end of the DNA. Simpson (35) has very recently shown that this is likely to be the case for a nucleus-derived particle containing 160 bp of DNA and the full histone complement. If 1.75 turns of DNA were to be folded around the histone octamer in the 145-bp core particle as proposed from structural studies (32–34), our results would suggest that DNA can actually make 2 complete turns in the 167-bp particle, the entry and exit sites of the DNA being located at the same position. The additional 20 bp of DNA may then be stabilized by H1. We do not know, as yet, whether this protection is effected by binding of the entire H1 molecule or of only one part of it.

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