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Folding of the DNA Double Helix in Chromatin-Like Structures from Simian Virus 40

(chromatin structure/simian virus 40 DNA/supercoil/nucleosomes)

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ABSTRACT **Relaxed circular, covalently closed simian virus 40 DNA molecules were associated with the four histones that are present in virions. In electron micrographs the resulting complexes appear twisted, with globular structures (nucleosomes) along the DNA. Incubation with an untwisting extract converts the twisted complexes to relaxed structures. Extraction of the DNA from the relaxed complexes yields supercoiled molecules. The number of superhelical turns in these molecules corresponds to the number of nucleosomes per DNA molecule in the complexes.**

In eukaryotic nuclei, the fundamental structure of chromatin fibers appears to be a flexible chain composed of globular particles connected by DNA filaments (1, 2). In these particles, termed nucleosomes (2), about 200 base pairs of DNA are associated with the four histones $F2a_1$, $F2a_2$, F2b, and F3 (2–7). Such a repeating unit structure can be formed *in vitro* by association of the four histones and linear DNAs (2). In the nucleosomes the DNA is under constraint, since it is compacted about 5-fold compared to its length in the extended double helical form (2). The nature of this constraint can be studied by the association of histones to covalently closed circular DNA molecules, since supercoiling or unwinding of the DNA within the nucleosome during its formation would alter the supercoiling of the rest of the molecule (8). In addition, from the known thermodynamic properties of superhelical DNAs (8–10), the influence of the degree of superhelicity on the formation of nucleosome structures can provide information on whether the formation of a nucleosome is equivalent to an unwinding or winding of the double helix. Simian virus 40 (SV40) DNA is particularly attractive for such a study for two reasons. First, two circular covalently closed allomorphic forms of this DNA are available, the superhelical DNA I and the relaxed circular DNA Ir which results from the incubation of DNA I with an untwisting extract (UE) (11). This extract is thought to introduce a single-strand nick into superhelical DNA and to reseal the nick after the torsional tension in the double helix has been relieved. Second, SV40 DNA and the four histones are associated *in vivo* and the complexes can be isolated from virions (12, 13) and from infected cells. In the latter case, the complex appears as a compacted structure with about 20 nucleosomes (14).

We have used two experimental approaches to study the constraint imposed by the four histones on the double helix in DNA-histone complexes associated *in vitro* or extracted from virions. First, the number of nucleosomes per DNA molecule and the superhelicity of the complexes were monitored by electron microscopy. Second, the extranucleosomal superhelicity in the complexes was removed by treatment with UE and the DNA was then deproteinized and analyzed. Keller and Wendel (15) have recently shown that SV40 DNA molecules containing various numbers of superhelical turns can be separated by gel electrophoresis. By using this method we were able to obtain information on the constraint imposed on the DNA in the nucleosome.

Abbreviations: SV40, simian virus 40; SV40 DNA I, DNA Ir, and DNA II are superhelical, relaxed covalently closed circular and nicked (one single-strand break) circular forms of simian virus 40 double-stranded DNA, respectively; UE, untwisting extract.

MATERIALS AND METHODS

Preparation of SV40 DNA, Virus, and DNA–Protein Complexes. DNA I was extracted from African green monkey BSC-1 cells infected with freshly plaque-purified SV40 (1–5 PFU per cell) (16). DNA Ir was obtained by treating 20 μg of DNA I in 200 μl of Tris/EDTA/NaCl buffer [20 mM Tris·HCl, pH 8.0/0.2 mM EDTA/200 mM NaCl/5% (v/v) glycerol] with 20 μl of UE at 37° for 15 min. After the addition of sodium dodecyl sulfate, the DNA was purified by phenol and chloroform extraction. Linear SV40 DNA was prepared by treating DNA I with *Escherichia coli* RI restriction enzyme (17). Virus was purified from BSC-1 cells infected as above according to Crawford (18), except that treatment with receptor-destroying enzyme was replaced by ultrasonication. The DNA–histone complex was prepared by dialyzing the purified virus against an isotonic Tris-ethanolamine buffer, pH 10.5, at 4° (12) followed by sedimentation through the same buffer containing 5% sucrose onto a 60% cushion. A salt-treated complex was obtained by adding NaCl to the DNA–histone complex to a final concentration of 1 M (2 hr at 20°), and sedimentation through Tris/EDTA buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA) containing 5% sucrose and 1 M NaCl onto a 60% sucrose cushion. Both complexes were dialyzed against Tris/EDTA.

Preparation of Untwisting Extract. Krebs II ascites cells were grown in female Swiss CD/1 mice. Chromatin was prepared from 10^9 cells according to Hancock (19) with the modification that after the last washing homogenization was omitted. The chromatin was mixed with the same volume of 300 mM phosphate buffer, pH 7.5, vortexed, and centrifuged at 10,000 × *g* for 10 min at 4°. The supernatant is termed untwisting extract (UE). One μl of UE converts 10 μg of SV40 DNA I to DNA Ir in 15 min at 37°.

Conditions for Untwisting DNA and Agarose-Polyacrylamide Gel Electrophoresis. One to 3 μl of UE were added to 1 μg of DNA (either free or complexed with histones) in 20 μl of

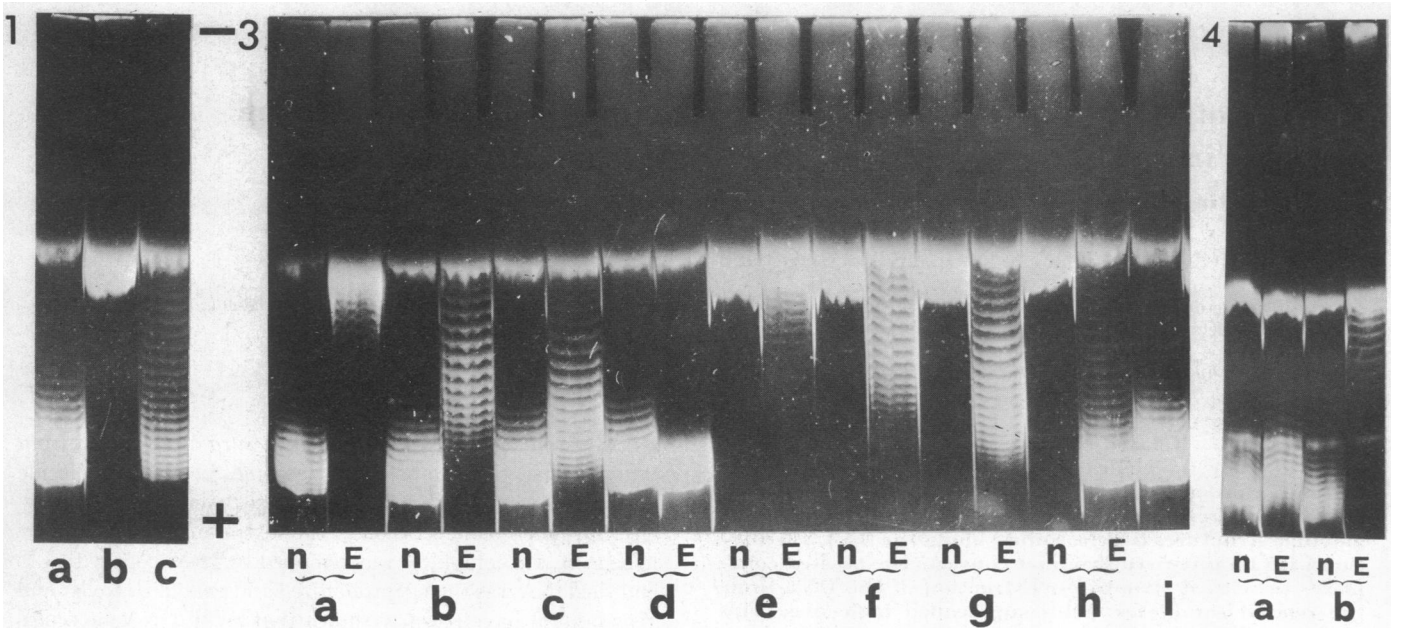

FIG. 1. Agarose-polyacrylamide gel electrophoresis of DNA I (a), DNA I treated with UE at 37° (b) or 0° (c). Each sample contains approximately 1 μg of DNA.

FIG. 3. Gel electrophoresis of DNA from untreated (n) and UE-treated (E) complexes formed *in vitro*. DNA I and the four histones were associated at histone/DNA ratios of 0.5 (a), 0.75 (b), 1.0 (c), and 2.0 (d). DNA Ir was associated with histones at ratios of 0.5 (e), 0.75 (f), 1.0 (g), and 2.0 (h). Control DNA I (i). A 5-fold increase of UE concentration did not affect the band patterns.

FIG. 4. Gel electrophoresis of DNA from untreated (n) and UE-treated (E) SV40 DNA–histone complexes extracted from virions. DNA–protein complex (a), DNA–protein complex after 1 M NaCl treatment (b). Electrophoresis time was 26 hr.

Tris/EDTA/NaCl. After 15 min at 37° the reaction was stopped by sodium dodecyl sulfate (1%). Slab gels (20), 2.5 mm thick and 100 mm long were prepared and electrophoresed in the following buffer: 36 mM Tris/30 mM NaH_2PO_4/1 mM EDTA, pH 7.7 (21). Gels contained 0.5% agarose, 1.9% acrylamide, 0.1% *N,N'*-methylene-bis-acrylamide, 0.05% ammonium persulfate, and 0.003% *N,N,N',N'*-tetramethylethylenediamine. DNA samples in Tris/EDTA/NaCl containing 10% sucrose, 1% sodium dodecyl sulfate, and 0.05% bromophenol blue were heated at 45° for 15 min, layered on the gel and electrophoresed for 22 hr at 50–60 V. Gels were stained with ethidium bromide and fluorescence was observed under ultraviolet light (22).

Association of Histone and SV40 DNA In Vitro. Purified DNA was associated with the four calf thymus histones $F2a_1$, $F2a_2$, F2b, and F3 by successive dialyses against solutions of decreasing ionic strength as previously described (2). Aliquots for electron microscopic examination were diluted with bidistilled water to 90 mM or 3.5 mM NaCl. Aliquots for treatment with UE were dialyzed for 6 hr against Tris/EDTA/NaCl buffer.

Electron Microscopy. DNA and DNA-histone complexes were visualized with a Philips 300 electron microscope as previously described (2).

EXPERIMENTS AND RESULTS

Conversion of Superhelical DNA to Relaxed, Covalently-Closed Circles. We have prepared an endonuclease-free untwisting extract (UE) from ascites cells, which converts DNA I to DNA Ir. Untwisting activities had previously been isolated from *E. coli* (23), mouse (11), and human (15) cells. The effect of UE on DNA I is illustrated in Fig. 1. DNA I has a high electrophoretic mobility, while contaminating nicked circular molecules form a slower moving band (Fig. 1a). Treatment with UE at 37° converted DNA I to DNA Ir that has the same mobility as nicked circular DNA (Fig. 1b). We, therefore, assume that DNA Ir contains no superhelical turns. When DNA I was treated with UE at 0°, the number of superhelical turns was only partially reduced. In Fig. 1c, 19 intermediate bands are visible between the most supercoiled and the fully relaxed DNA. After prolonged electrophoresis 23 intermediate bands were counted (not shown). With the assumption that all the bands were resolved, SV40 DNA isolated from infected cells contains up to 24 superhelical turns, since molecules in neighboring bands differ by one turn (15). These results confirm those obtained by Keller and Wendel (ref. 15 and private communication). Fig. 1a reveals a hetero-

TABLE 1. *Formation of nucleosomes as a function of histone to DNA ratio*

Histone/DNA (w/w)	Number of nucleosomes per SV40 DNA molecule	
	DNA I	DNA Ir
0.5	5.0 ± 1.4	4.7 ± 1.2
0.75	10.7 ± 1.4	8.9 ± 2.2
1.0	11.8 ± 3.0	9.5 ± 2.0
1.5	18.9 ± 2.0	18.0 ± 2.5
2.0	21.4 ± 2.5	18.2 ± 2.4

SV40 DNA and the four calf thymus histones ($F2a_1$, $F2a_2$, F2b, and F3) were associated as described (2), and the number of nucleosomes per DNA molecule was determined by electron microscopy after dilution to 3.5 mM NaCl. In each case the nucleosomes of more than 60 DNA molecules were counted. The numbers are mean ± standard deviation.

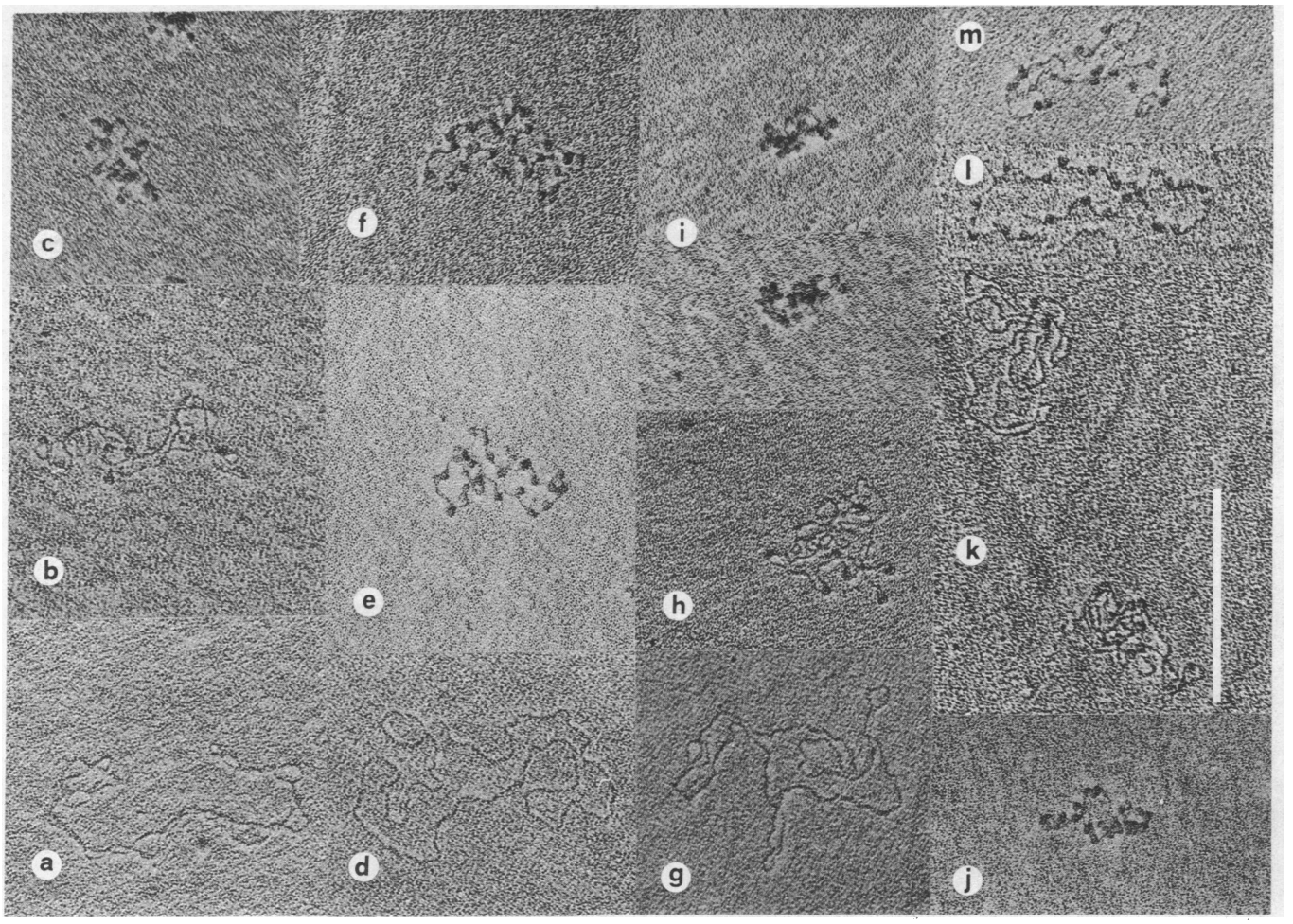

FIG. 2. Association of SV40 DNA with the four calf thymus histones $F2a_1$, $F2a_2$, F2b, and F3. (a–f) DNA I was associated with the four histones at different ratios as described in *Materials and Methods* and examined after dilution to 3.5 mM or 90 mM NaCl: (a) DNA alone, 90 mM NaCl; (b) histone/DNA 0.6, 90 mM NaCl; (c) histone/DNA 1.5, 90 mM NaCl; (d) DNA alone, 3.5 mM NaCl; (e) histone/DNA 1.5, 3.5 mM NaCl; (f) histone/DNA 2.0, 3.5 mM NaCl. (g–j) DNA Ir was associated with the four histones at different ratios as described in *Materials and Methods* and examined after dilution of 3.5 mM or 90 mM NaCl: (g) DNA Ir alone, 90 mM NaCl; (h) histone/DNA 0.6, 90 mM NaCl; (i) histone/DNA 1.5, 90 mM NaCl; (j) histone/DNA 1.5, 3.5 mM NaCl. (k) A mixture of identical amount of DNA I and II was associated with the four histones at a histone/DNA ratio of 0.6 and examined in 90 mM NaCl. (l–m) Nucleosomal complexes isolated from SV40 virions (*Materials and Methods*) were examined at 3.5 mM NaCl (l) or 90 mM NaCl (m). The bar indicates 0.25 μm.

geneity in the number of superhelical turns in DNA I extracted from infected cells. A similar heterogeneity was observed with DNA I prepared from virions (not shown).

Association of Superhelical SV40 DNA with the Four Histones: Effect of UE on the Complexes. DNA I was associated *in vitro* with the four histones $F2a_1$, $F2a_2$, F2b, and F3 at different histone to DNA ratios. Electron microscopy of the resulting complexes (Fig. 2) revealed nucleosomes (2) on the DNA. Aliquots of the samples were adsorbed to the supporting film at two different salt concentrations. At 90 mM NaCl, the complexes appeared condensed and superhelicity was visible (Fig. 2b). At 3.5 mM the complexes were extended (Fig. 2e and f), allowing an easier determination of DNA length and nucleosome number, while the supercoils appeared relaxed (compare Fig. 2a and d). In all samples tested, the number of nucleosomes per molecule was the same for both salt concentrations. The diameter of the nucleosomes was 127 ± 10 Å, as previously reported (2). Each nucleosome contains about 200 base pairs (191 ± 19), as determined from the length reduction of internucleosomal DNA as a function of the number of nucleosomes (2). The contour length of pure SV40 DNA was 1.59 ± 0.10 μm and we assume that the double helix was in the B configuration. The diameter of internucleosomal DNA appeared identical to that of naked DNA (2). The number of nucleosomes per DNA molecule increased with increasing histone to DNA ratio (Table 1). Up to 25 nucleosomes were counted per DNA molecule at the highest ratio, almost no internucleosomal DNA remaining visible. The complexes appeared more and more relaxed with increasing histone to DNA ratios, whereas their circumference decreased considerably (Fig. 2a, b, and c).

The constraint of the DNA in the nucleosome was directly estimated by treating the histone–DNA complexes with UE in order to relieve any extra-nucleosomal superhelicity. When such UE-treated complexes were deproteinized and analyzed by gel electrophoresis, we observed an increase in the number of superhelical turns per molecule when the histone to DNA ratios were increased in the association mixtures (0.5, 0.75, 1.0, and 2.0, Fig. 3, aE, bE, cE, and dE, respectively). The

TABLE 2. *Superhelicity of DNA favors the formation of nucleosomes*

SV40 DNA in the association mixture	Number of nucleosomes per SV40 DNA molecule		
	DNA I	DNA Ir	DNA II
DNA I	6.1 ± 1.8	—	—
DNA Ir	—	5.7 ± 1.8	—
DNA II	—	—	3.8 ± 2.3
DNA I + Ir	9.8 ± 2.7	2.6 ± 0.8	—
DNA I + II	8.2 ± 2.7	—	2.3 ± 0.8

The four histones and the various SV40 DNA forms were associated at a histone to DNA ratio of 0.6, as described (2). In the experiments involving two DNA forms, those were mixed in a 1:1 ratio. The number of nucleosomes was determined by electron microscopy after dilution to 90 mM NaCl. In each case the nucleosomes of more than 60 DNA molecules were counted.

controls (Fig. 3, an–dn) demonstrate that there was no change of the initial supercoiling of the DNA (Fig. 3i) when the complexes were not treated with UE before deproteinization. In the presence of the lowest amounts of histones, the DNA was almost completely relaxed after UE treatment (Fig. 3, aE). At a histone to DNA ratio of 1, the average number of superhelical turns per molecule was reduced to about 12 (Fig. 3, cE). At the highest ratio, incubation of the complexes with UE followed by deproteinization yielded DNA molecules which were highly twisted (Fig. 3, dE). It is striking that this treatment resulted in the addition of supertwists to some of the molecules (compare Fig. 3, dE and i). There is a good agreement between the average number of superhelical turns in the DNA molecule after UE treatment and deproteinization and the average number of nucleosomes per complex as determined by electron microscopy (Table 1).

Association of Relaxed Covalently Closed SV40 DNA with the Four Histones: Effect of UE on the Complexes. DNA Ir was visualized as a relaxed circle both in 90 mM (Fig. 2g) and in 3.5 mM NaCl (not shown). When DNA Ir was associated with the four histones $F2a_1$, $F2a_2$, F2b, and F3, nucleosomes similar to those obtained with DNA I were observed. With increasing histone to DNA ratio in the association mixture, the number of nucleosomes formed per complex (Table 1) as well as the number of supertwists visible per complex increased (Fig. 2h and i, compare also e and j). This indicates that the formation of nucleosomes on DNA Ir results in a supercoiled complex.

When the complexes between DNA Ir and the four histones were treated with UE and deproteinized, gel electrophoresis revealed the presence of superhelical turns in the DNA molecules. The number of these turns increased with the histone to DNA ratio (0.5, 0.75, 1.0, and 2.0, Fig. 3, eE, fE, gE, and hE, respectively), while the controls (Fig. 3, en–hn) indicate that the DNA extracted from complexes not treated with UE remained untwisted. At the highest histone to DNA ratio, the majority of the DNA molecules after the UE treatment appeared as twisted as the superhelical DNA extracted from virions (compare Fig. 3, hE and i). Again there is a correspondence between the average number of superhelical turns in the DNA molecules extracted from UE-treated complexes and the average number of nucleosomes per complex (Table 1).

The Formation of Nucleosomes Is Favored on Superhelical SV40 DNA. The fact that the binding of the four histones to DNA I reduces the number of superhelical turns in the internucleosomal DNA indicates that the free energy contained in the negative superhelical turns is favoring the formation of nucleosomes (8, 9). One would, therefore, expect that histones will bind more avidly to DNA I than to its allomorphic forms Ir and II which have a lower free energy. This is indeed the case, as demonstrated by the results presented in Table 2. Under conditions of DNA excess more nucleosomes were found on DNA I than on DNA Ir or II, when the four histones were associated with either a mixture of DNA I + Ir or a mixture of DNA I + II (Fig. 2k). Similar results were obtained when DNA I and linear SV40 DNA were mixed.

Nucleosomal Complexes Isolated from SV40 Virions. In virions the DNA is complexed with the four histones $F2a_1$, $F2a_2$, F2b, and F3 (12, 13). When these complexes were extracted from the virions and examined by electron microscopy, structures containing about 20 globular particles connected by DNA filaments were visible (Fig. 2l and m). The globular particles correspond to nucleosomes, since their diameter is 131 ± 10 Å and they contain about 200 DNA base pairs, as determined by the reduction of the length of the internucleosomal DNA (2). These complexes are very similar to the SV40 "mini-chromosomes" which were recently isolated from infected cells by Griffith (14). It is noteworthy that, although the DNA extracted from the complexes is superhelical (see below), the nucleosomal complexes appear almost relaxed (Fig. 2m) and very similar to the complexes formed *in vitro* with DNA I (Fig. 2c).

When the *in vivo* complexes were treated with UE and deproteinized, the resulting DNA molecules (Fig. 4, aE) were as twisted as those from controls without UE treatment (Fig. 4, an), indicating that the internucleosomal DNA was relaxed in the complex, whereas the nucleosomal DNA was under constraint. On the other hand, when the complexes were first partially deproteinized with 1 M NaCl and then treated with UE, there was a strong decrease of the degree of superhelicity of the DNA (Fig. 4, bE), suggesting that the salt treatment had destroyed most of the nucleosomes. In fact electron microscopy revealed that only 6.8 ± 2.1 nucleosomes were left per DNA molecule after the salt treatment.

DISCUSSION

Relaxed circular covalently closed SV40 DNA (Ir) associates *in vitro* with the four histones $F2a_1$, $F2a_2$, F2b, and F3 to form complexes. On electron micrographs these complexes appear as twisted structures consisting of a chain of nucleosomes. An untwisting extract which removes any extranucleosomal superhelical turns from the DNA converts these complexes to relaxed structures. The DNA extracted from the relaxed complexes is supercoiled, as demonstrated by gel electrophoresis. Therefore, in the relaxed complexes the DNA is under a torsional constraint which is confined to the nucleosomes. During deproteinization this constraint expands over the whole DNA molecule and induces superhelical turns. The number of these turns corresponds to the number of nucleosomes per complex prior to deproteinization. The supertwists induced in this way *in vitro* and those in DNA I have the same sense, as is indicated by the following.

When histones are complexed with a mixture of DNA Ir and DNA I, nucleosomes form preferentially on the latter.

This observation indicates that the free energy contained in the negative supercoil of the DNA is used for the formation of nucleosomes (8–10). In addition, complexes formed *in vitro* of DNA I and histones appear less twisted in electron micrographs than the original DNA. UE treatment of the complexes followed by deproteinization yields supercoiled DNA with the number of superhelical turns corresponding to the number of nucleosomes per complex.

The above results demonstrate that *in vitro* the formation of each nucleosome on a negatively supercoiled DNA molecule reduces the superhelicity of the complex by one turn. This effect is equivalent to the unwinding (denaturation) of the double helix by one turn (8).

Nucleoprotein complexes can be extracted from virions. They are relaxed and contain about 20 nucleosomes per DNA molecule. Only after deproteinization does the superhelicity of the DNA become apparent, whether the complexes were previously treated with UE or not, and also in this case the number of superhelical turns per DNA molecule corresponds to the number of nucleosomes per complex. These results indicate that the same constraint on the DNA duplex exists in nucleosomes formed *in vivo* and *in vitro*.

Replicating SV40 DNA molecules appear supercoiled after extraction (24). In the cell they are most likely complexed with proteins in the same ratio as completed circular molecules (25). These observations suggest that even during replication most of the DNA is packed in nucleosomes. However, replication of DNA and its packing in nucleosomes are not necessarily coupled. Indeed, when protein synthesis is blocked in cells infected by SV40 or polyoma virus, the subsequently synthesized DNA molecules are not associated with proteins. After extraction these molecules appear as covalently-closed circles lacking superhelical turns (26). When protein synthesis resumes, even in the absence of replication, the DNA becomes associated with proteins and appears supercoiled after deproteinization (27). This indicates that the nucleoprotein complexes are exposed to an intranuclear untwisting activity (27). Assuming that *in vivo* DNA and the four histones associate spontaneously to form nucleosomes, the above studies together with our results suggest that the intracellular pool of free histones is small and that there is no rapid exchange of the four histones between DNA molecules.

All nucleosomal structures analyzed so far appear very similar (2, 4–7, 14). We therefore assume that also in each nucleosome of chromatin fibers the torsional deformation of the DNA is equivalent to the unwinding of the double helix by one turn. This could be due to one negative superhelical turn or to an overall unwinding of the double helix by one turn. Alternatively, a combination of intranucleosomal unwinding and supercoiling could cause the torsional alteration. Current models of nucleosome structure involve wrapping of the DNA around a protein core (28–31). However, a simple supercoil loop of about 680 Å in length (200 base pairs) of DNA duplex would by far exceed the size of a nucleosome. Our results are, therefore, better accounted for by a model of nucleosome structure in which the DNA would be folded (kinked, 30), rather than by models in which the DNA would be regularly wound in a superhelical form (29, 31).

It is clear that any modification of the histone–DNA interactions (by chemical modifications of the histones for instance) will result in a modification of the constraint in the intranucleosomal DNA which would possibly lead to an unwinding or to a supercoiling of the extranucleosomal DNA. These modifications of the DNA duplex could play an important role in the binding of enzymes or regulatory factors involved in transcription and replication of the genetic material in eukaryotes (32).

Note Added in Preparation. Analogous results were obtained when histones were associated to the DNA of polyoma virus.

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