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Stability of nucleosomes in native and reconstituted chromatins.

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ABSTRACT

The stability of nucleosomes of SV40 minichromosomes extracted from infected cells or reconstituted by association of SV40 DNA and the four histones H2A, H2B, H3 and H4 was studied as a function of the ionic strength. As a measure of the stability of the nucleosome, we followed the disappearance of the nucleosomes from the original chromatin and their appearance on a "competing" DNA. We show here that the DNA and the histone components of the nucleosomes do not appreciably dissociate below 800 mM NaCl. At 800 mM and above, the histone moiety of the nucleosomes can dissociate from the DNA and efficiently participate to the formation of nucleosomes on a "competing" DNA.

INTRODUCTION

Evidence has been obtained over the last two years which demonstrates that the fundamental structure of the chromatin fiber is a chain of nucleosomes (for references, see Refs. 1-4). However, very little is presently known about the relationship between the structure of the chromatin fiber and its function. For example, it is unknown whether the nucleosomal structure of the chromatin fiber is conserved (totally or partially) or disappears when the DNA is replicated or transcribed. Preliminary studies in our laboratory (unpublished) have indicated that transcription by prokaryotic (E.coli holoenzyme) or eukaryotic (calf thymus class B) RNA polymerases is drastically inhibited when SV40 DNA Form I^a is associated with the four histones H2A, H2B, H3 and H4 in "natural" minichromosomes extracted from infected cells (3, 4) or in "reconstituted" chromatin made *in vitro* by association of SV40 DNA with the four histones H2A, H2B, H3 and H4 from calf thymus (5). Since these observations suggest that transcription of the DNA could

be prevented when it is folded in the nucleosomes, we have decided to study the factors which could affect the association of the DNA with the histone core in the nucleosomes. We report here the effect of ionic strength on the stability of nucleosomes of a native chromatin (SV40 minichromosome extracted from infected cells) and *in vitro* "reconstituted" chromatins.

A nucleosome can be defined as a beadlike structure of about 120 Å in diameter containing about 200 base pairs of DNA and two each of the four histones H2A, H2B, H3 and H4 (6). In addition, the DNA duplex in the nucleosome is under a constraint equivalent to one negative superhelical turn (5). As a measure of the stability of the nucleosome we have followed the disappearance of the nucleosomes from the original chromatin and their appearance on a "competing" SV40 DNA Form I as the ionic strength was increased. To do so, two complementary approaches were used. First, using electron microscopy, the number of "beads" on the DNAs was counted and their nucleosomal nature established by measuring their diameter and showing that they contain about 200 base pairs of DNA (6). Second, using gel electrophoresis, the number of nucleosomes present in a circular covalently closed nucleoprotein complex was established by counting the number of superhelical turns present in the covalently closed DNA under appropriate conditions (5).

Using these methods, we show here that the DNA and histone components of the nucleosomes do not appreciably dissociate below 800 mM NaCl. At 800 mM NaCl and above, the histone moiety of the nucleosomes can dissociate from the DNA and efficiently participate to the formation of new nucleosomes on a "competing" DNA.

MATERIAL AND METHODS

1. Preparation of DNAs, "natural" minichromosomes and "reconstituted" chromatins.

SV40 (strain 777) DNA Form I, Form Ir and Form III were prepared as previously reported (5). "Natural" SV40 minichromosomes were purified from CV1 infected cells as described else-

where (4). "Reconstituted" chromatins were prepared by associating the four calf thymus histones H2A, H2B, H3 and H4 with SV40 DNA Form I or Form III as follows. The DNA (200-300 µg/ml) and the four "salt-extracted" histones (see below) were mixed in siliconized tubes at a histone/DNA weight ratio of 1 in buffer A [20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM sodium bisulfite, 5 mM dithiothreitol (DTT) and 0.1 mM paramethylsulfonylfluoride (PMSF)] containing 2 M NaCl. After 1 h at 37°C, the NaCl concentration was progressively decreased by dilution at 37°C with buffer A : 15 min at 1.6 M NaCl, 15 min at 1.2 M NaCl, 1 h at 850 mM, 1 h at 750 mM, 1 h at 650 mM, 15 min at 500 mM and 15 min at 250 mM. The "reconstituted" chromatins were then concentrated by centrifugation in nitrocellulose tubes (SW65 Spinco rotor) through buffer A containing 5% sucrose and 250 mM NaCl onto a glycerol cushion (40,000 rpm, 14 h at 4°C). The"chromatin" pellet was dissolved in buffer A containing 250 mM NaCl at a concentration of DNA of about 500 µg/ml and stored at 4°C.

2. Preparation of the four calf thymus histones H2A, H2B, H3 and H4.

The four histones were prepared with a modification of the method previously described (6). 5 g of frozen calf thymus were homogenized in 100 ml of TMS buffer (50 mM Tris-HCl pH 7.4, 4 mM $MgCl_2$ and 0.24 M sucrose) with the help of a teflon-glass homogenizer. After filtration through 4 layers of cheese cloth the homogenate was centrifuged at 400 g for 4 min. The pellet was washed three times in TMS buffer containing 0.1% Triton X-100. Chromatin was prepared by homogenizing the nuclear pellet in 500 ml of 80 mM NaCl, 20 mM EDTA. After centrifugation (12,000 g, 10 min) the chromatin pellet was washed first in 150 ml of 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 150 mM NaCl, 0.1% Triton X-100 and then in the same buffer without Triton. The chromatin pellet was suspended in 60 ml of buffer B (25 mM sodium acetate, pH 6.6, 5 mM sodium bisulfite, 200 mM NaCl and 1 mM DTT) with the help of an Ultra-Turrax homogenizer (60 V, 1 min). 360 ml of buffer B containing 650 mM NaCl were added in order to bring the NaCl concentration to 550 mM and the chromatin was solubilized with the help of a magnetic

stirrer. After clarification by centrifugation at 12,000 g for 10 min, the supernatant was layered on 4 ml of buffer B containing 550 mM NaCl and 30% glycerol in polyallomer tubes (Spinco rotor 60 Ti). Histone H1 was quantitatively removed (as checked by polyacrylamide gel electrophoresis) by ultracentrifugation for 40 h at 245,000 g. 20 mg (expressed as DNA) of H1-depleted chromatin were homogenized in 3 ml of buffer C (1 mM sodium phosphate pH 6.6, 25 mM sodium bisulfite, 25 mM sodium acetate pH 6.6, 5 mM DTT, 0.1 mM PMSF) with the help of a conical sintered all glass homogenizer. NaCl and phosphate concentrations were raised to 2 M and 20 mM, respectively (final volume 5 ml) and the viscosity was reduced by sonication. After clarification by centrifugation (10 min at 12,000 g), the four histones were separated from the nucleic acids and the acidic proteins by chromatography on a hydroxypatite column (4 cm long, 2.5 in diameter) previously equilibrated with buffer C containing 2 M NaCl and 20 mM phosphate and eluted with the same buffer. The histones were not retained on the column and were followed by determining the absorbance at 230 nm. Fractions containing more than 1 mg of histones/ml were dialyzed overnight against 10 volumes of 10 mM sodium acetate pH 6.6, 2 M NaCl, 5 mM sodium bisulfite, 5 mM DTT, 0.1 mM PMSF and stored at -20°. The four "salt-extracted" calf thymus histones prepared in this way were pure and intact as judged by polyacrylamide gel electrophoresis (7).

The four "acid-extracted" calf thymus histones were prepared by acid extraction from H1-depleted chromatin (see above) as described previously (6). Isolated calf thymus histones H2A, H2B, H3 and H4 were provided by Dr. D. Gallwitz.

3. Conditions for studying the stability of nucleosomes.

SV40 "natural" minichromosomes or "reconstituted" chromatins (DNA concentration of about 100 µg/ml) were mixed with equal amounts of "competing" SV40 DNA Form I in 50 µl of a buffer containing 10 mM Tris-HCl pH 8.0, 0.2 mM EDTA, 1 mM DTT, 1 mM sodium bisulfite, 0.1 mM PMSF. The mixtures were incubated for 30 min at different ionic strengths and temperatures as indicated in the legends to the figures. At the end of the

incubation period the salt concentration was progressively decreased by diluting at the incubation temperature with the same buffer (100 mM steps, every 10 min) down to 500 mM and then abruptly diluting to 250 mM NaCl. The samples were separated into two fractions and the untwisting extract (UE) was added as previously described (5) in one of the two fractions. Both fractions were incubated for 15 min at 37°C. Aliquots were diluted to 0.3 µg/ml of DNA for electron microscopic examination which was carried out as previously described (5, 6). Aliquots (about 1 µg of DNA) were prepared for gel electrophoresis in order to separate the DNA molecules according to the number of superhelical turns as previously reported (5), except that the slab gel was 18 cm long and electrophoresis was for 40 h at 70 V. The gels were transilluminated with ultra-violet light, photographed with a red filter and printed as negatives.

RESULTS

I. Effect of increasing ionic strength on the stability of nucleosomes of SV40 minichromosomes extracted from infected cells ("natural" minichromosomes).

The stability of the nucleosomes of "natural" SV40 minichromosomes which contain 21 ± 2 nucleosomes (3, 4) was studied by incubating them at increasing NaCl concentrations in the presence or in the absence of an equal amount of "competing" SV40 DNA Form I. At the end of the incubation period (30 min at 37°C) the salt concentration was decreased by progressive dilution down to 250 mM (Material and Methods). All of the samples (with or without competing SV40 DNA Form I) were then separated into two fractions and the untwisting extract (UE) was added to one of the two fractions in order to remove any extra-nucleosomal superhelical turns (Material and Methods). Aliquots of the samples were then either mounted for electron microscopy for counting the number of nucleosomes or deproteinized before gel electrophoresis for counting the number of superhelical turns in the DNA molecules.

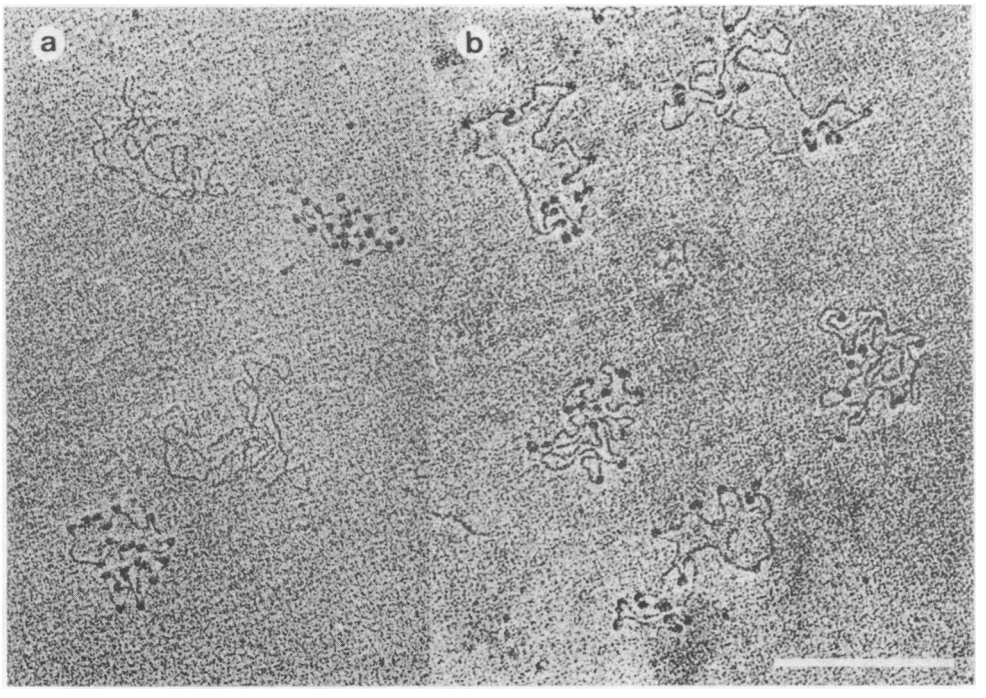

FIGURE 1 "Natural" SV40 minichromosomes were incubated (37°C, 30 min) in the presence of "competing" SV40 DNA Form I at 720 mM (a) or 880 mM (b) NaCl and processed for electron microscopy (Material and Methods). The bar indicates 0.25 µm.

A. Electron microscopic analysis.

Two populations of molecules were observed at NaCl concentrations varying between 150 mM and 720 mM. At 720 mM (Fig. 1a and 2a) one population (containing an average of 21 nucleosomes) corresponds to the original minichromosomes, whereas the other population with no or very few beads corresponds to the "competing" DNA. Below 500 mM NaCl (not shown) there were no beads at all on the competing DNA. When the NaCl concentration was increased to 800 mM (Fig. 2b) a bimodal distribution of the number of beads was observed suggesting that nucleosomes were formed on the "competing" DNA, while some of them disappeared from the original minichromosomes. At 880 mM NaCl (Fig. 1b and 2c) and above (960 mM NaCl, Fig. 2d), all of the molecules were visualized with an average of 9-14 beads suggesting that at these ionic strengths the nucleosomes were equally distributed

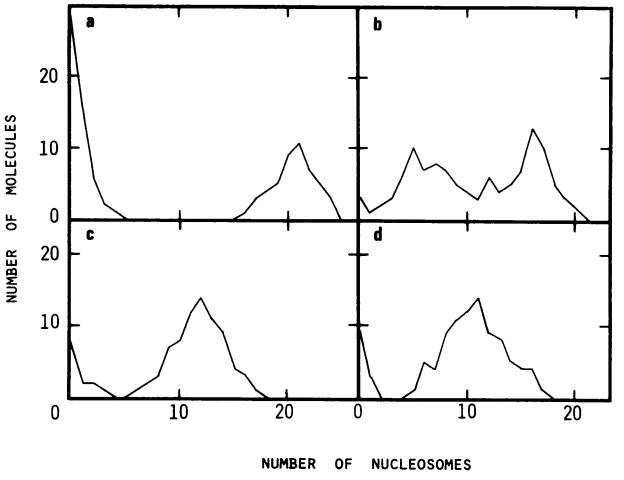

FIGURE 2 Distribution of nucleosomes on DNA molecules after incubation (37°C, 30 min) of "natural" SV40 minichromosomes in the presence of "competing" SV40 DNA Form I at 720 mM (a), 800 mM (b), 880 mM (c) and 960 mM (d) NaCl. The number of nucleosomes was determined by electron microscopic examination.

between the original minichromosomes and the "competing" DNA. This interpretation is supported by the observation (not shown) that increasing 3-fold the amount of "competing" DNA resulted in a single population of molecules with an average of 4-6 nucleosomes. The efficiency of the formation of the nucleosomes was high, since a population of complexes containing on the average 9-14 nucleosomes was formed from equal amounts of deproteinized SV40 DNA Form I and SV40 minichromosomes containing about 21 nucleosomes. As expected from the above results, when the "natural" SV40 minichromosomes were exposed to increasing salt concentrations (up to 960 mM NaCl) in the absence of "competing" DNA, no change in the number of nucleosomes was observed when the minichromosomes were observed by electron microscopy after dilution of the salt (result not shown).

In order to demonstrate that the beads which were seen on the complexes after incubations at various ionic strengths correspond to authentic nucleosomes, we have measured their diameter (125 ± 10 Å) and we have determined the average length of the DNA per bead. As described previously, this length can be determined by first measuring the total internucleosomal

DNA, then substracting it from the contour length of SV40 DNA and dividing by the number of beads (3, 4). In all cases, a value of 195 ± 15 base pairs was found in good agreement with our previous estimation (3-5) of the DNA length folded in an SV40 nucleosome.

From these electron microscopic observations we conclude that the histone moiety of a nucleosome can dissociate from DNA at 800 mM NaCl and above, and bind to another DNA to form a new nucleosome.

B. Gel electrophoresis analysis.

The constraint imposed on the DNA duplex in a nucleosome is equivalent to a negative superhelical turn (5). We have made use of this property to confirm the formation of nucleosomes on the "competing" DNA Form I, by determining the number of superhelical turns which remained in the DNA molecule after removal of all possible extra-nucleosomal constraint with an untwisting activity (UE). Under these conditions, the number of superhelical turns remaining after deproteinization corresponds to the number of nucleosomes (5). The DNA was run in an appropriate gel electrophoresis system which separates circular covalently closed DNA molecules according to the number of superhelical turns (5, 8). If no nucleosomes are formed on the "competing" SV40 DNA Form I, treatment with UE followed by deproteinization will result in relaxed DNA Form Ir molecules. The same treatment applied to a "natural" minichromosome which contains all of its nucleosomes will result in SV40 DNA Form I molecules which contain on average about 21 superhelical turns [i.e. equal to the number of nucleosomes (5)]. If nucleosomes are redistributed equally between the two DNAs after exposure to the higher ionic strength, the gel will show SV40 DNA molecules containing on average about half the number of superhelical turns present in SV40 DNA Form I. Similarly, for two populations of nucleoprotein complexes differing by their average number of nucleosomes, two populations of SV40 DNA molecules differing by their average number of superhelical turns will be obtained.

Fig. 3 shows the results which were obtained when SV40

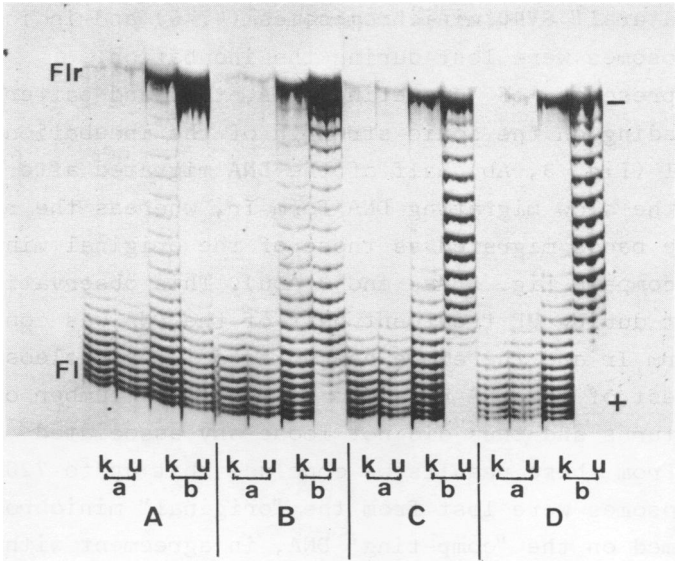

FIGURE 3 Gel electrophoresis of DNA from untreated (k) and UE-treated (u) "natural" SV40 minichromosomes which were incubated (37°C, 30 min) at various ionic strengths in the presence (b) or in the absence (a) of "competing" SV40 DNA Form I. Incubations were carried out as described in Material and Methods at 720 mM (A), 800 mM (B), 880 mM (C) and 960 mM (D) NaCl. FIr and FI indicate the position of SV40 DNA Form Ir and Form I, respectively.

minichromosomes were first incubated at increasing ionic strength (A = 720 mM, B = 800 mM, C = 880 mM, D = 960 mM) in the absence (a) or in the presence (b) of "competing" SV40 DNA Form I, and then treated (u) or not (k) with UE after dilution of the salt. As previously shown (5, 8), relaxed SV40 DNA molecules (Form Ir) have the slowest migration rate in this gel system. Molecules which contain superhelical turns are separated according to the number of superhelical turns present, neighbouring bands corresponding to molecules which differ by one turn.

The incubation of the minichromosomes at various ionic strengths, in the absence of a "competing" DNA, had no effect on the number of superhelical turns of the DNA (Fig. 3 Aa-Da). In agreement with previous results (4, 5) up to 24 bands were counted. UE treatment did not affect the band pattern (compare k and u) confirming that there is no extranucleosomal con-

straint in "natural" SV40 minichromosomes (4, 5) and indicating that no nucleosomes were lost during the incubation.

In the presence of "competing" DNA, the band pattern was modified depending on the ionic strength of the incubation. Up to 720 mM NaCl (Fig. 3, Ab) half of the DNA migrated after UE treatment as the slow migrating DNA Form Ir, whereas the remainder of the bands migrated as those of the original minichromosomes (compare Fig. 3 Aau and 3 Abu). This observation indicates that during UE treatment half of the DNA was converted to DNA Form Ir and therefore had no associated nucleosomes, whereas the rest of the DNA retained its original number of superhelical turns and thus did not loose any associated nucleosomes. From these results we conclude that up to 720 mM NaCl no nucleosomes were lost from the "original" minichromosomes nor formed on the "competing" DNA, in agreement with the electron microscopic results. When the ionic strength was increased to 800 mM, two populations of molecules differing in their average number of superhelical turns appeared after UE treatment and deproteinization (Fig. 3 Bbu). One population has a low number of superhelical turns (4 to 7), whereas the other has 13-17 superhelical turns. At a higher ionic strength (880 mM NaCl, Fig. 3 Cbu) the DNA migrated as a rather disperse population of supercoiled molecules with a mean value of 8-11 superhelical turns. The situation was not significantly modified at 960 mM NaCl (Fig. 3 Dbu). We interpret all of these results as meaning that at 800 mM NaCl, the histone moiety of some of the nucleosomes of the "original" minichromosomes start to dissociate from the DNA and can move to the "competing" DNA to form new nucleosomes, whereas at even higher ionic strengths (880 mM and 960 mM NaCl) the nucleosomes are equally distributed. These interpretations, which are in agreement with the electron microscopic observations, account for the two populations of supercoiled molecules which are seen in Fig. 3 Bbu, and for an apparent single population of supercoiled molecules which is seen in Fig. 3, Cbu and Dbu. In the control experiments run without UE treatment (Fig. 3, Abk-Dbk), almost all of the DNA molecules were superhelical irrespective of the ionic strength, as expected.

II. Effect of increasing ionic strength on the stability of "reconstituted" nucleosomes.

In order to investigate whether the stability of nucleosomes of *in vitro* "reconstituted" chromatins is identical to that of nucleosomes of a "native" chromatin, "reconstituted" chromatins were prepared by associating superhelical (Form I) or linear (Form III) SV40 DNAs with the four calf thymus histones, H2A, H2B, H3 and H4, as described in Material and Methods. Under these conditions the efficiency of the reconstitution was much higher than that previously obtained for the the same histone:DNA ratio (5). When examined by electron microscopy the circular and linear "reconstituted" chromatins contained 22 ± 2 and 20 ± 2 nucleosomes, respectively (results not shown). The stability of the nucleosomes of the "reconstituted" chromatins at increasing NaCl concentrations was studied, by gel electrophoresis and electron microscopy, as described in the preceding section for the "natural" minichromosomes.

Fig. 4 shows the results which were obtained when the

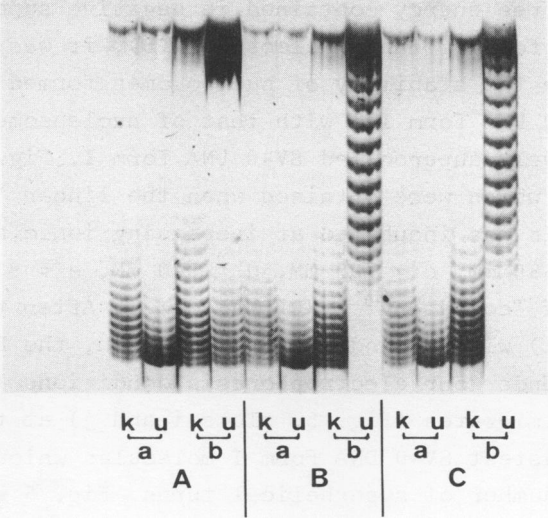

FIGURE 4 Gel electrophoresis of DNA from untreated (k) and UE-treated (u) circular "reconstituted" chromatin which was incubated (37°C, 30 min) at various ionic strengths in the presence (b) or in the absence (a) of "competing" SV40 DNA Form I. Incubations were carried out as described in Material and Methods at 720 mM (A), 880 mM (B) and 960 mM (C) NaCl.

"reconstituted" circular chromatin formed with SV40 DNA Form I was incubated at increasing ionic strengths (A = 720 mM, B = 880 mM, C = 960 mM) in the absence (a) or in the presence (b) of "competing" SV40 DNA Form I. In the presence of "competing" DNA and at similar ionic strengths, the results were almost identical to those observed for the "natural" minichromosomes (compare Fig. 3A and 4A, 3C and 4B, 3D and 4C). We interpret the results shown in Fig. 4 as meaning that below and up to 720 mM (Fig. 4A), no nucleosomes were present on the "competing" DNA molecules, whereas at 880 mM NaCl (Fig. 4B) and above (Fig. 4C) the nucleosomes were equally redistributed on the "competing" DNA molecules and on the DNA of the "reconstituted" chromatin. The interpretation of the gel electrophoresis patters was confirmed for the different ionic strengths by electron microscopic examination (results not shown). We therefore conclude that the stability of nucleosomes of a "reconstituted" chromatin formed by associating the four calf thymus histones and superhelical SV40 DNA Form I is similar to that of nucleosomes of "natural" SV40 minichromosomes.

Since the free energy contained in negative superhelical turns favours the formation of nucleosomes (5), it was interesting to compare the stability of nucleosomes formed *in vitro* on the linear SV40 DNA Form III with that of nucleosomes formed on the negatively supercoiled SV40 DNA Form I. Fig. 5 shows the results which were obtained when the linear "reconstituted" chromatin was incubated at increasing ionic strengths (a = 400 mM, b = 550 mM, c = 700 mM, d = 800 mM, e = 900 mM) in the presence of "competing" SV40 DNA Form I. After treatment (u) or not (k) with UE and deproteinization, the DNA was electrophoresed. Under our electrophoresis conditions linear SV40 DNA Form III migrates (Fig. 5, slots i and j) at the same position as the fastest SV40 DNA Form I molecules which contain the highest number of superhelical turns. Fig. 5 shows that below and up to 700 mM NaCl, no nucleosomes were present on the "competing" DNA Form I molecules, since it was quantitatively converted to DNA Form Ir by UE treatment (Fig. 5 au, bu and cu). On the contrary, at 800 mM and 900 mM NaCl, the presence of supercoiled DNA molecules was observed (Fig. 5

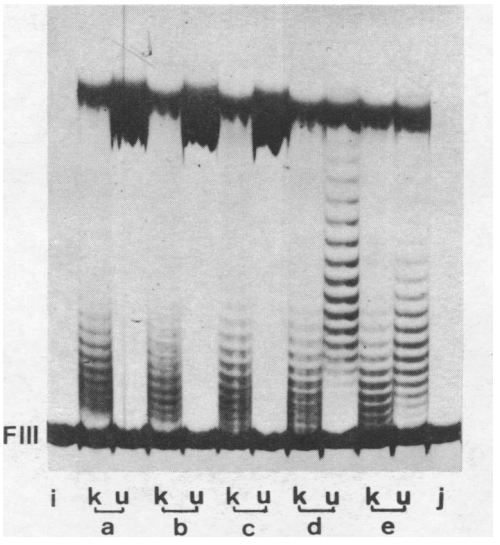

FIGURE 5 Gel electrophoresis of DNA from untreated (k) and UE-treated (u) linear "reconstituted" chromatin which was incubated (37°C, 30 min) at various ionic strengths in the presence of "competing" SV40 DNA Form I. Incubations were carried out as described in Material and Methods at 400 mM (a), 550 mM (b), 700 mM (c), 800 mM (d) and 900 mM (e) NaCl. i and j : control assays in which the linear "reconstituted" chromatin was incubated in the absence of "competing" DNA at 400 mM (i) or 900 mM (j) NaCl. FIII indicates the position of linear SV40 DNA Form III.

du and eu), indicating that nucleosomes have been formed on the "competing" DNA molecules. The presence of a population of DNA molecules with 8-13 superhelical turns at 800 mM NaCl and with 13-17 turns at 900 mM NaCl, indicate the presence on the "competing" DNA of 8-13 and 13-17 nucleosomes, respectively. These results suggest that at 900 mM NaCl almost all of the histones which were initially on the linear "reconstituted" chromatin have moved to the "competing" DNA to reform nucleosomes, since 20 ± 2 nucleosomes were originally present on the linear chromatin (see above) and the amount of "competing" DNA was equal to the amount of chromatin DNA.

This interpretation was supported by electron microscopic examination, which showed (Fig. 6) that almost no nucleosomes were left on the linear DNA molecules when the ionic

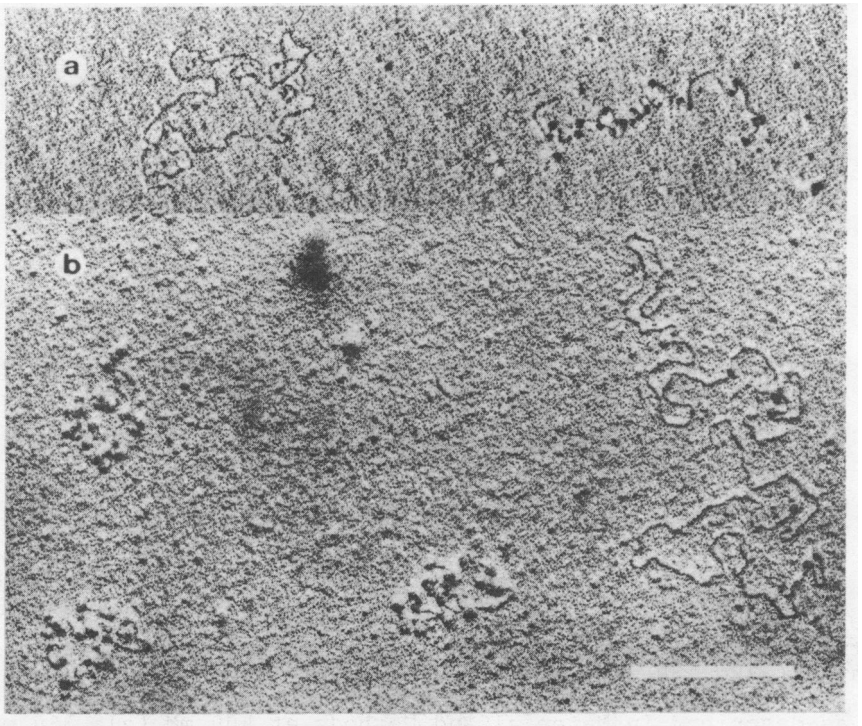

FIGURE 6 Linear "reconstituted" chromatin was incubated (37°C, 30 min) in the presence of "competing" SV40 DNA Form I at 700 mM (a) or 900 mM (b) NaCl and processed for electron microscopy (Material and Methods). The bar indicates 0.25 µm.

strength was raised to 900 mM. This preferential formation of the nucleosomes on the "competing" superhelical DNA is in keeping with our previous observations (5) showing that nucleosome formation is favoured on SV40 DNA Form I, when compared with SV40 DNA Form Ir and Form II, which have lower free energy. From these results we conclude that the stability of "reconstituted" nucleosomes at increasing ionic strength is similar, whether they were formed on a linear or on a superhelical circular DNA. Moreover, this stability is similar to that observed for nucleosomes of "natural" SV40 minichromosomes.

III. Influence of temperature and time on the stability of nucleosomes.

The physiological temperature of 37°C was chosen for the

studies described in the preceding sections and the duration of the exposure to increasing ionic strengths was uniformly 30 min. In order to get some insight into the kinetics of the process in which histones dissociate from a DNA and form new nucleosomes on another DNA, we have studied both the influence of the temperature and of the duration of the exposure to the high ionic strength on the stability of nucleosomes of "natural" SV40 minichromosomes. The studies were carried out at 880 mM, since it was known (section I) that the nucleosomes were equally distributed between the DNA of the minichromosome and the "competing" DNA after a 30 min incubation at 37°C. SV40 minichromosomes isolated from infected cells were incubated at 880 mM NaCl in the presence of an equal amount of "competing" SV40 DNA Form I for 5, 15 and 45 min (Fig. 7 a, b and c) at 37°C, 20°C or 0°C (Fig. 7 A, B and C). At the end of the incubation period the mixture was diluted and treated with UE as before for analysis by gel electrophoresis. At 37°C (Fig. 7 A), even after 5 min, a single population of superhelical DNA molecules with a mean value of 8-11 turns was observed, indicating that the nucleosomes were equally distributed between the two DNAs (see section I). On the contrary, very few nucleosomes were lost from the SV40 minichromosomes or formed on the "competing" DNA molecules after 5 min at 20°C (Fig. 7 Bau) or 0°C (Fig. 7 Cau) as indicated by the presence after UE treatment of only SV40 DNA Form I molecules (originated from the minichromosomes) and SV40 DNA Form Ir molecules (originated from the "competing" DNA). In fact, more than 15 min were required at both 20°C and 0°C in order to observe a single population of superhelical molecules corresponding to an equal distribution of nucleosomes between the two DNAs (Fig. 7 Bcu and Ccu).

We interpret these results as meaning that the process by which at 880 mM NaCl the histone moiety of a nucleosome can dissociate from its DNA and form a new nucleosome on another DNA is energetically unfavoured. Moreover, since very few nucleosomes were lost from the SV40 minichromosomes when incubated for 5 min at 20°C or 0°C (see above), we conclude that the energy-requiring step is not the formation of new nucleoso-

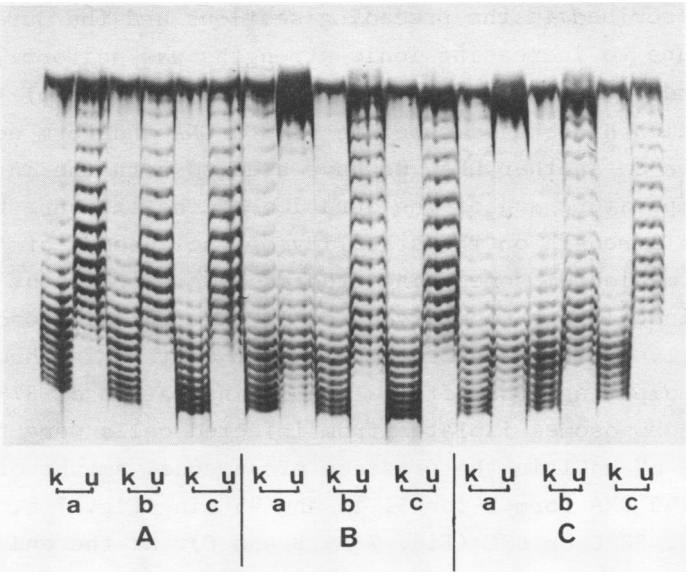

FIGURE 7 Gel electrophoresis of DNA from untreated (k) and UE-treated (u) "natural" SV40 minichromosomes which were incubated at 880 mM NaCl in the presence of "competing" SV40 DNA Form I for various times (a = 5 min, b = 15 min, c = 45 min) at different temperatures (A = 37°C, B = 20°C, C = 0°C).

mes on the "competing" DNA, but the dissociation of the components of the original nucleosomes.

IV. Analysis of the ionic strength conditions required to reconstitute nucleosomes.

Nucleosomes have been reconstituted *in vitro* by reassociating various DNAs with the four histones H2A, H2B, H3 and H4 (5, 6). The reconstitution was achieved by mixing the DNA and the four histones in 2 M NaCl and progressively dialyzing out the salt (6). Since the results described in the previous sections indicate that nucleosomes can be formed at 880 mM NaCl, the question arose whether mixing the DNA and the four histones in 2 M NaCl is actually required in order to make nucleosomes *in vitro*. Two preparations of the four calf thymus histones were used (Material and Methods). In one preparation, the histones were dissociated from the DNA in 2 M salt (salt-extracted histones), whereas in the other preparation the his-

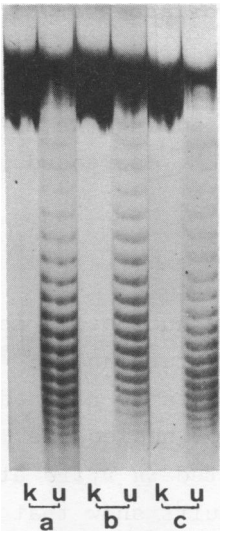

FIGURE 8 Gel electrophoresis of DNA from untreated (k) and UE-treated (u) chromatin reconstituted by associating SV40 DNA Form Ir with the four "salt-extracted" calf thymus histones. The association was carried out (a) by progressive dilution from 2 M NaCl as described in Material and Methods or (b) by adding the histones in 2 M NaCl, decreasing abruptly the salt down to 880 mM and then adding the DNA, or (c) by adding the histones in 2 M NaCl, decreasing progressively the salt down to 880 mM and then adding the DNA.

tones were acid-extracted (acid-extracted histones). The formation of nucleosomes on SV40 DNA Form I was followed by electron microscopy and gel electrophoresis after UE treatment. Only the gel electrophoresis results are shown (Fig. 8), since they were in complete agreement with the electron microscopic observations.

As shown in Fig. 8, the same distribution of supercoiled DNA molecules was observed, and therefore the same number of nucleosomes was formed, when the reconstitution, using salt-extracted histones, was carried out 1) by mixing the DNA and the histones in 2 M NaCl and progressively decreasing the ionic strength (Fig. 8a); 2) by first decreasing abruptly the salt concentration of the histone solution from 2 M NaCl down to 880 mM and then adding the DNA (Fig. 8b); 3) by first progressively decreasing the salt concentration of the histone solution from 2 M NaCl down to 880 mM and then adding the DNA (Fig. 8c). Similar results were obtained when acid-extracted histones were brought from no salt to 2 M NaCl and then used for nucleosome reconstitution. On the contrary, almost no nucleosomes were formed when the reconstitution was attempted by adding sequentially the four separated histones H2A, H2B, H3 and H4 to the DNA at 880 mM NaCl (not shown).

From these results we conclude that the presence of DNA

at NaCl concentration greater than 880 mM is not necessary in order to reconstitute nucleosomes, provided the four histones have been previously exposed to high salt, presumably in order to form the histone core octamer (9). However, adding the DNA when the NaCl concentration was less than 800 mM, decreased drastically the yield of nucleosomes (not shown).

DISCUSSION

Our present results demonstrate that the nucleosome is a very stable structure at physiological temperature and ionic strength. A similar conclusion has been recently reached by Cremisi et al. (10), who found no evidence of nucleosome movement when SV40 minichromosomes were incubated *in vitro* at physiological ionic strength. However, our results show that there is a sharp transition in the nucleosome stability around 800 mM NaCl. At this ionic strength the histones can dissociate from the DNA and efficiently participate to the formation of nucleosomes on another DNA molecule. Since all four histones H2A, H2B, H3 and H4 are actually required in order to reconstitute a nucleosome (P. Oudet et al., in preparation), we conclude that the four histones are in fact moving from one to another DNA molecule. Inasmuch as the sequential addition of the four separated histones H2A, H2B, H3 and H4 to DNA at 880 mM NaCl (see section IV) does not allow the formation of nucleosomes, we also conclude that the histone core of the nucleosome, presumably in the form of an octamer (9), is not dissociated when the histones move at 880 mM NaCl from one to another DNA molecule to reform a nucleosome. However, our results indicate that, even at 880 mM NaCl, this concerted movement of the four histones is energetically unfavoured, the equilibrium being strongly in favour of formation versus dissociation of the nucleosomes. There is therefore no discreapancy between our present results and the previous reports (for references, see Refs. 11-12), which have shown that a much higher ionic strength is required to quantitatively remove the four histones from the DNA by ultracentrifugation. In fact, we have observed that there was no loss of nucleosomes when "native" or reconstituted minichromosomes were centri-

fuged for 2 hours at 10°C and 240,000 g in a 5-20% sucrose gradient containing 800 mM NaCl (Germond, unpublished result). However, it should be recalled that Clark and Felsenfeld (13) have previously reported that it is possible to remove the histones from chromatin by running it through a DNA-cellulose column in the presence of 800 mM NaCl.

Our results demonstrate that the stability of nucleosomes at increasing ionic strength is similar whether the nucleosomes belong to a "natural" SV40 minichromosome extracted from infected cells or to a chromatin reconstituted *in vitro* by associating the four calf thymus histones to SV40 DNA. It therefore appears that the degree of acetylation of the histones has no marked effect on the stability of the nucleosomes at increasing ionic strength, since the histones of SV40 minichromosomes are highly acetylated when compared to those of calf thymus chromatin (14, and M. Bellard, unpublished results). In addition, it does not appear that there are marked variations in the stability of nucleosomes of the bulk of the SV40 minichromosomes extracted from infected cells, since the histones of all of them appear to move to another DNA molecule to reform nucleosomes within a small increment of the ionic strength. Studies are now in progress in our laboratory to investigate whether the stability of the nucleosomes could be affected at physiological ionic strength by histone modification and (or) the presence of other components of the chromatin and of the cell nucleus, in order to allow transcription to occur.

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[a]ABBREVIATIONS

DTT, dithiothreitol; PMSF, paramethylsulfonylfluoride; SV40 DNA Form I, Form Ir and Form III, are superhelical, relaxed covalently closed circular and linear forms of simian virus 40 double-stranded DNA, respectively. UE, untwisting extract.

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