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The same amount of DNA is organized in in vitro-assembled nucleosomes irrespective of the origin of the histones

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ABSTRACT

The four histones H2A, H2B, H3 and H4 from calf thymus, CHO and sea urchin gastrula cells were associated by stepwise dialysis from 2 M NaCl with SV40 DNA Form I. The *in vitro*-assembled chromatins were visualized by electron microscopy and the size of the DNA fragments generated by digestion with DNase II was determined. Irrespective of the origin of the histones, the size of the smallest DNA band generated at early times of digestion was about 190 base pairs, whereas oligomeric DNA bands were multiples of 140 bp. These results support our previous proposal that the four histones H2A, H2B, H3 and H4 are able to organize more than 140 bp of DNA, but do not provide any evidence that the variability of histones H2A and H2B plays a role in the variability of the DNA repeat length of native chromatins.

INTRODUCTION

There is a vast body of evidence that the organization of eukaryotic DNA into repeating nucleoprotein units, the nucleosomes, constitutes the first compaction step of DNA in interphase nuclei or metaphase chromosomes. The DNA repeat length of a given chromatin can be determined by nuclease digestion and corresponds to the amount of DNA contained in a nucleosome (for references, see 1-3). Remarkable variations in DNA repeat lengths have been reported, although in all cases approximately 140 bp of DNA are associated to the nucleosome core particle (references in 1-3). For instance, we have found that Chinese hamster ovary (CHO) (4) and sea urchin gastrula (5) cells have repeat lengths of 177 bp and 218 bp, respectively, whereas the DNA repeat length of rat liver cells is 196 (4), similar to that of calf thymus cells [195 bp (6)]. Changes in histone H1 (7-8) and/or changes in histones H2A and H2B (4, 9, 10) have been

invoked to explain the variability of the DNA repeat length (for additional references, see 1-3).

In vitro chromatin reconstitution experiments could be used to test these hypotheses. Previous studies have indeed shown that it is possible to generate nucleosome-like structures by associating DNA and the four histones H2A, H2B, H3 and H4 in the presence of decreasing concentrations of NaCl (11, 12). We have now prepared *in vitro*-assembled chromatin from SV40 DNA Form I and the four histones from calf thymus, CHO and sea urchin gastrula cells and we report here the results of DNase II digestion studies which indicate that the size of the smallest DNA fragment generated at early times of digestion is about 190 bp, irrespective of the origin of the histones. In all three cases, electron microscopy and DNase II digestion studies of chromatin reconstituted at a 0.9 histone/DNA ratio reveals also the presence of closely packed particles containing only 140 bp of DNA each.

MATERIALS AND METHODS

1) Sea urchin culture and preparation of nuclei.

Sea urchins, *Arbacia lixula*, were obtained from the mediterranean coast of France. Embryos were cultured as previously described (5). Nuclei from sea urchin gastrula, calf thymus tissue and CHO cells were prepared as described in (13), (11) and (4), respectively.

2) Preparation of histones H2A, H2B, H3 and H4 and in vitro assembly of chromatin.

The four histones H2A, H2B, H3 and H4 were prepared from calf thymus, CHO and gastrula cell nuclei according to Germond et al. (14), but chromatin of CHO and sea urchin gastrula was prepared by washing nuclei first in a 10 mM Tris-HCl pH 8.0 buffer containing 10 mM EDTA, 150 mM NaCl and 0.1% Triton X-100, then in the same buffer containing 80 mM NaCl, 20 mM EDTA, and finally in the above 150 mM NaCl buffer without Triton. H1 was removed from chromatin as described in (14) but a buffer containing 650 mM NaCl was used.

Associations of the four salt-extracted histones from calf thymus, CHO and gastrula cells with [^{3}H]-SV40 DNA Form I (1,500

cpm/μg) were carried out at histone/DNA ratios of 0.9 or 0.6 by stepwise disalysis at 20°C as described in (11) with the following steps: 2 M NaCl for 1 hour, 1.6 M NaCl for 15 min, 1.2 M NaCl for 15 min, 0.9 M NaCl for 1 hour, 0.8 M NaCl for 1 hour, 0.7 M NaCl for 1 hour and 0.5 M NaCl for 30 min.

3. Nuclease digestion and DNA fragment analysis.

50-70 μg (in DNA) of *in vitro*-assembled chromatin was dialyzed for 60 min against 10 mM Tris-HCl pH 6.9 at 4°C and digested at 37°C in about 750 μl of the same buffer with 1.33 u/ml of DNase II (a gift of Dr. Bernardi) (15). At different times aliquots corresponding to 5-10 μg of DNA were removed from the digestion mixture and mixed with the same volume of 50 mM Tris-HCl pH 10.0 to stop the digestion. SDS (0.5% final concentration) and proteinase K (100 μg/ml) were added and after an overnight incubation at 22°C, the samples were extracted twice with equal volumes of chloroform-isoamyl alcohol (24:1, v/v). The supernatants were combined, brought to 300 mM sodium acetate (pH 5.5) and precipitated at -20°C with two volumes of ethanol. At each time of the digestion (see legend to Fig. 3) 4-5 μg of ethanol-insoluble DNA were electrophoresed in 2% polyacrylamide-0.5% agarose gels as previously described (16). Gels were then stained with ethidium bromide and photgraphed as in (16). The size of the DNA fragments was established as previously described (4, 16) using mouse satellite and SV40 DNA restriction enzyme fragments as size markers. The percentage of acid-solubility was determined on aliquots at various times of the digestion by assaying the trichloroacetic acid-soluble counts.

4. Histone gel electrophoresis.

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate was run according to Thomas and Kornberg (17) except that the stacking gel was 6% polyacrylamide.

5. Electron microscopy.

In vitro-assembled chromatin was visualized as previously described (11, 14).

RESULTS

The four histones H2A, H2B, H3 and H4 were salt-extracted from H1-depleted calf thymus, CHO and sea urchin gastrula chromatins (Fig. 1). These histones were then associated at a histone/DNA ratio of 0.9 with SV40 DNA Form I in the presence of decreasing concentrations of NaCl (Materials and Methods). In all three cases the associations resulted in the appearance of about 18 beads (about 120 Å in diameter) on the DNA molecule with a concomitant compaction of the DNA of about 2.5 fold over its extended length. The beads were randomly distributed along the molecules and appeared either as individual beads connected by DNA filaments or as clusters of tightly packed beads with very little or no visible bridging DNA (Fig. 2a).

Fig. 3 shows the electrophoretic distribution of the DNA fragments which were obtained when the *in vitro* assembled chromatins were digested for increasing lengths of time with DNase II. It is striking that the smallest band is prominent even at early times of digestion and that for similar extents of digestion, the

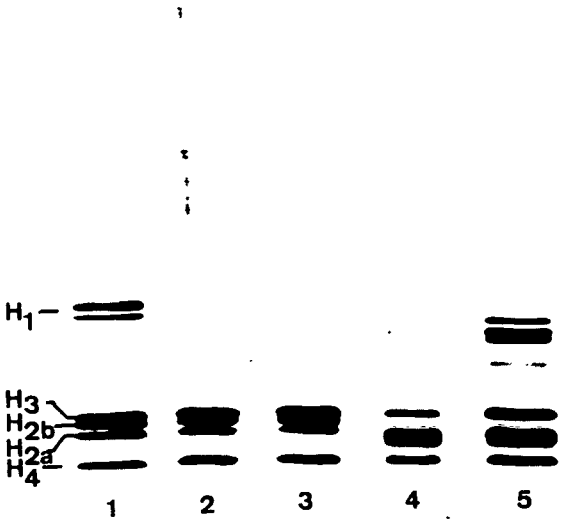

Fig. 1 : SDS-poly acrylamide gel electrophoresis of purified histones. (1) the 5 calf thymus histones, (2) the 4 calf thymus histones H2A, H2B, H3 and H4, (3) the 4 CHO histones, (4) the 4 sea urchin gastrula histones, (5) the 5 sea urchin gastrula histones. Histones were purified and electrophoresed as described under Materials and Methods.

same series of bands were obtained for all three digests. The size of the various bands after 5 min of digestion (Fig. 3, lanes 2, 6 and 10, and Table 1) was determined using a rat liver micrococcal nuclease digest (Fig. 3, lanes 1 and 14) as DNA length markers (Material and Methods). For each digest a graph of band size (in base pairs) versus band number was constructed as described by Thomas and Thompson (18) in order to determine the actual values of the DNA repeats. A value of 196 bp was found for the rat liver DNA repeat in excellent agreement with our previous determinations (16). Repeats of 140, 143 and 145 (±5) bp were calculated from the slopes of the graphs constructed with the dimer, trimer, tetramer and pentamer bands of digests of chromatins reconstituted with calf thymus, CHO and sea urchin gastrula histones, respectively. The sizes of the smallest DNA bands (Table 1, band 1) of the digests of the three *in vitro*-assembled chromatins were very similar and clearly larger (185 to 190 bp) than the repeat of the higher oligomer bands. In addition, digestion for longer times (up to 15 min, see slots 2-4, 6-8 and 10-12) indicates that the 190 bp are organized in a rather stable structure, since there was very little change in the size of

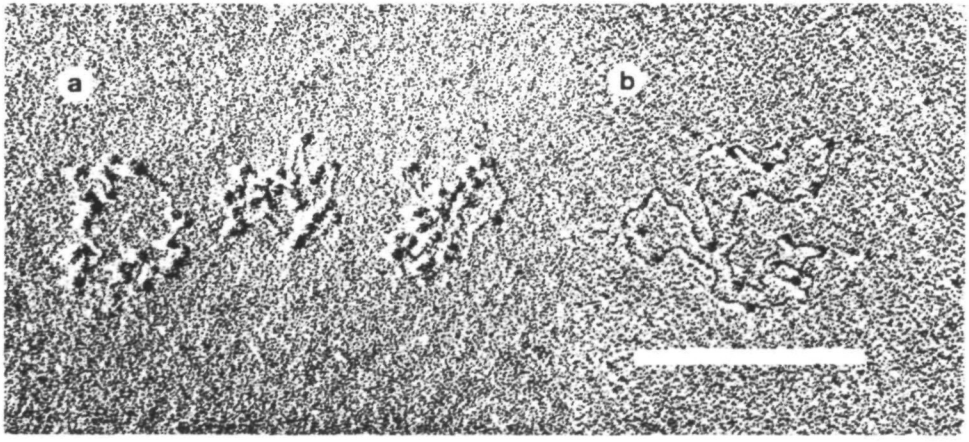

Fig. 2 : Electron microscopy of *in vitro*-assembled chromatin. (a) association at histone/DNA ratio of 0.9; (b) association at histone/DNA ratio of 0.6 (see texte and Materials and Methods). The appearance of the *in vitro*-assembled chromatins was identical, irrespective of the origin of the histones. The bar represents 0.25 µ.

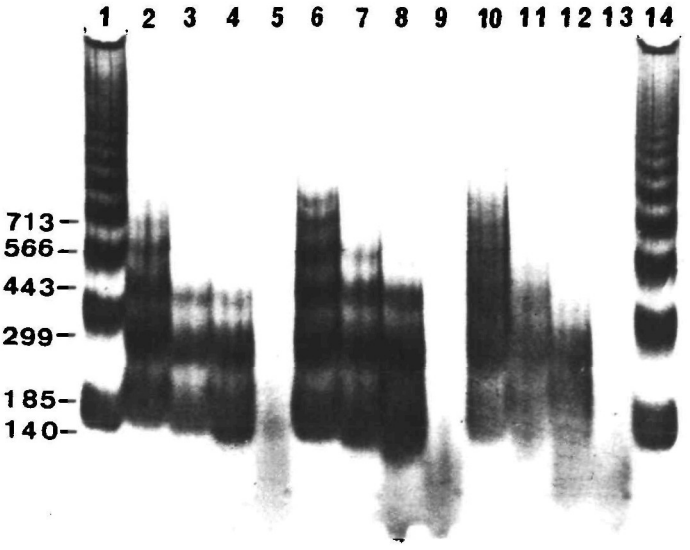

Fig. 3: Polyacrylamide gel electrophoresis of DNase II digests of *in vitro*-assembled chromatin. The four histones and SV40 DNA Form I were associated at a histone/DNA ratio of 0.9 and the reconstitutes were digested with DNase II as described under Materials and Methods. Lanes 2-5, association with the four calf thymus histones; times of digestion and percent acid-solubility were : 5 min, 7.5% (lane 2); 10 min, 8.7% (lane 3); 15 min, 12% (lane 4); 30 min, 19.5% (lane 5). Lanes 6-9, association with CHO histones : 5 min, 6.2% (lane 6); 10 min, 8% (lane 7); 15 min, 11% (lane 8), 30 min, 20.6% (lane 9). Lanes 10-13, association with sea urchin gastrula histones : 5 min, 3.4% (lane 10); 10 min, 6.6% (lane 11); 15 min, 10% (lane 12); 30 min, 16.4% (lane 13). Lanes 1 and 14, micrococcal nuclease digest of rat liver nuclei. The numbers of the left-hand side correspond to the size (in base pairs) of the center of the DNA bands shown in lane 2.

band 1 during the first 15 min of digestion. It is striking that, as DNase II digestion proceeded, there was no relative increase in the 190 bp band, nor accumulation of a 140 bp DNA fragment and of smaller discrete fragments similar to those found in micrococcal nuclease limit digests (for references, see 12). We have found a similar lack of conversion of the monomeric band to core particle and discrete subnucleosomal DNA fragments when digesting rat liver, CHO or sea urchin nuclei with DNase II, whereas the DNA repeat lengths determined by DNase II or micrococcal nuclease digestions were almost identical (unpublished results). Electron microscopy studies (not shown) during the course of the DNase II

BAND NUMBER	RAT LIVER MARKER	ORIGIN OF THE FOUR HISTONES		
		calf thymus	CHO	sea urchin gastrula
1	177	185	187	190
2	365	299	305	310
3	569	439	449	453
4	781	580	585	605
5	970	723	734	745

Table 1 : *Size of the DNA fragments generated by DNase II digestion of in vitro-assembled chromatins.* The lengths of the DNA fragments generated by DNase II digestion of the different associations were determined as described in Materials and Methods from the relative position of the center of the bands shown in Fig. 3, lanes 2 (calf thymus histones), 6 (CHO histones) and 10 (sea urchin gastrula histones). The rat liver marker corresponds to the micrococcal digest shown in lane 1 (Materials and Methods). Bands are numbered from the bottom of the gel.

digestion have shown that isolated beads were released very early in the digestion, together with clusters of very tightly packed beads. In addition there was no accumulation, at any time of the digestion, of a population of single beads which could have suggested a ready conversion of clusters to monomer particles.

When the reconstitution was performed at a lower histone/DNA ratio (0.6), about 8-12 well individualized beads were observed per molecule (Fig. 2b). Particularly noteworthy was the absence of the clusters which were found at higher histone/DNA ratio. DNase II digestion of such reconstitutes resulted in an almost complete disappearance of the oligomer DNA bands, whereas the 190 bp band was always visible, although superimposed on a high background probably corresponding to the digestion of the DNA not associated with histones (not shown).

A number of experiments were also performed varying the conditions of DNase II digestion, for example the effect of divalent cations (0.3-1 mM $CaCl_2$ or 0.3-1 mM $MgCl_2$) or different temperature (10°C or 4°C) was tested. The same patterns were obtained for DNase II digests of *in vitro*-assembled chromatins, irrespective of the origin of the histones and of the conditions of di-

gestion. However, the background was higher on the gel when the digestions were carried out at the lower temperatures.

DISCUSSION

DNase II digestion of chromatin assembled *in vitro* at high histone/DNA ratio (0.9) from DNA and the four histones H2A, H2B, H3 and H4 in the presence of decreasing concentrations of NaCl, reveals a very interesting feature of this chromatin : the smallest DNA fragment corresponds to about 190 bp, whereas the multimeric fragments are clearly multiples of 140 bp, i.e. approximately the size of the nucleosome core DNA. It is interesting to compare the results of digestion with DNase II and micrococcal nuclease. In agreement with previous reports (6, 19), we found (unpublished results) that the difference between the size of the "monomer" DNA band and that of the repeat length of the oligomeric bands was much less striking with micrococcal nuclease. The repeat length was 140 bp, whereas the size of the smallest DNA band ranged from about 180 bp, at early times of digestion carried out at 0°C, down to the DNA core particle size (140 bp) at later times. This decrease in size is most likely related to the well-known "trimming" effect of micrococcal nuclease which is very marked in the absence of histone H1 (20). Steinmetz et al. (6) have found a "monomer" band decreasing in size from about 155 bp at early times of micrococcal nuclease digestion carried out at 37°C down to 140 bp at later times. Therefore, the results obtained with DNase II which has very little or no "trimming" effect (see Results section) reflect much more accurately the actual DNA content of the released monomer particle than those obtained by micrococcal nuclease digestion.

How is one to account for the fact that the DNase II band of about 190 bp is clearly not the monomer of the oligomeric bands? Both the gel electrophoresis study of the distribution of the DNA fragments and the electron microscopy study of the distribution of single versus clustered beads during the course of the digestion indicate that the reconstituted chromatin consists in fact of two types of particles. The first type corresponds to the beads which appear well individualized in the undigested chromatin and which are readily released by DNase II digestion as single particles containing about 190 bp of DNA. The second type of par-

ticles corresponds to those which are organized in oligomeric clusters containing multiples of 140 bp of DNA. This view is also supported by the observations mentioned in the Results section, namely that chromatin assembled *in vitro* at lower histone/DNA ratio (0.6) and consisting of well-spaced beads (Fig. 2b) yields mainly the 190 bp band when digested with DNase II. All of these results are also in complete agreement with our previous electron microscopy determinations of the length of the DNA (193 ± 6 bp) associated with widely spaced nucleosomes assembled *in vitro* from the four calf thymus histones and adenovirus-2 DNA (11). Clearly, both biochemical and electron microscopy studies indicate that the four histones H2A, H2B, H3 and H4 are able to organize more than just the DNA length contained in the nucleosomal core particle provided reconstitution is carried out under adequate conditions. This finding is in agreement with our former hypothesis (2, 9, 10), namely that H2A and H2B histones could play a double role; first, they could interact with the H3-H4 subnucleosomal particle (9, 10, 12, for other references, see 1 and 2) helping to stabilize the DNA fold in the nucleosome core, and second, they could bind, perhaps together with histone H1, the linker DNA.

It is likely that, when the DNA-histone assembly is carried out at high histone/DNA ratio, the proximity of reconstituted particles allows interactions to take place between neighbouring histone cores resulting in the formation of clusters of tightly packed particles containing only 140 bp of DNA. Such an hypothesis is strongly supported by the observation (manuscript in preparation) that exposure of rat liver chromatin (depleted of histone H1 by tRNA treatment) to increasing NaCl concentrations, leads to a sliding of nucleosomes with formation of clusters of particles accompanied by a modification of the DNA repeat length from 196 bp down to 140 bp. A similar change in repeat size was found by Steinmetz et al. (6) on chromatin depleted of histone H1 by centrifugation in the presence of 0.63 M NaCl.

It is striking that the length of DNA which can by organized in well-spaced beads by the four histones is always the same (about 190 bp), irrespective of the origin of the histones used for the reconstitution. This length is very close to that of the

calf thymus chromatin DNA repeat (195 bp), but higher than that of CHO cell chromatin (177 bp) and smaller than that of sea urchin gastrula chromatin (218 bp) (see Introduction). It appears, therefore, that H2A and H2B cannot measure out specifically the linker DNA in these reconstituted chromatins. Whether this inability is due to inaccuracies in the DNA-histone interactions under the present reconstitution procedure or whether it is due to the absence of histone H1 or other chromatin or nuclear components is unknown. To answer these questions we are presently attempting to reconstitute chromatin under more physiological conditions using the Laskey system (21).

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