

In vitro nucleosome assembly with plant histones

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In vitro reconstitution of nucleosomes using cloned DNA sequences and animal histone proteins is straightforward and reproducible. However, there has been no report on any successful reconstitution experiment with plant histones. Here we report on the conditions under which reproducible in vitro reconstitution of different plasmids and purified plant histones takes place. Nucleosome formation is only observed with salt-extracted histones and in the presence of urea. In electron micrographs these reconstituted nucleosomes are similar to those assembled with animal histones. With the methods described prokaryotic as well as eukaryotic sequences cloned into plasmids can be packaged into nucleosomes of identical appearance. Southern transfer analyses revealed a repeat length of the reconstituted nucleosomes of 150 ± 8 bp.

Nucleosome; DNA-histone interaction; Nucleosome reconstitution; Core histone; (Plant)

1. INTRODUCTION

The nuclear DNA of eukaryotic organisms is associated with a variety of proteins to make up what is called chromatin. This nucleoprotein complex serves to package all genes and accompanying regulatory sequences at various levels. At the basic level of chromatin organization, an octamer of two of each H2A, H2B, H3 and H4 histone molecules packages 1.75 turns of superhelical DNA into so-called nucleosomes [1,2]. The interaction of core histones with one another and with DNA is reversible and salt-dependent in vitro [3] so that the assembly and dissociation can be studied in detail by, e.g. salt gradient dialysis of a mixture of purified DNA and core histones. To date, the vast majority of such reconstitution experiments have been performed either with cell extracts containing

histones or purified histones, both from animals. Usually, a repeating pattern of nucleosomes was obtained with a repeat length depending on the presence of one or more unknown nuclear factors (reviewed in [4]). Although early reports presented preliminary evidence that at least some plant and animal histones are interchangeable in reconstitution experiments [5,6], our understanding of in vitro reconstitution of nucleosomes using plant histones is scarce.

We therefore started investigating nucleosome reconstitution from linearized plasmids harbouring prokaryotic as well as eukaryotic DNA sequences and pea core histones isolated and purified by several procedures. This report presents evidence, that the efficient assembly of nucleosomes using plant histones strongly depends on the method of histone isolation and purification, and not on the origin of DNA.

2. MATERIALS AND METHODS

2.1. Plasmid material and plasmid isolation

Four different recombinant plasmids cloned in *E. coli* were used for reconstitution experiments: pBR322; pLGV0422, con-

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Abbreviations: EDTA, ethylenediaminetetraacetic acid disodium salt; β -MCE, 2-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride

taining *Hind*III – fragment 23 from the T-DNA of *Agrobacterium tumefaciens*, strain C58, cloned in pBR322 [7]; pTA250.2, spanning the 18 S, 5.8 S and 25 S rDNA of wheat, cloned in pBR322 [8] and pPSR6 2.1, harbouring the gene coding for the small subunit of ribulosebiphosphate carboxylase/oxygenase cloned in pBR327 [9].

Plasmids were isolated by the alkaline lysis method [10,11], purified by CsCl centrifugation and linearized by digestion with an appropriate restriction enzyme.

2.2. Histone isolation

Nuclei were isolated from leaves of 14-day-old pea plants according to [12]. Histones were prepared from purified nuclei either by acid extraction, ion-exchange chromatography or salt extraction.

For acid extraction, nuclei were washed twice with 0.75 M NaCl, 10 mM Tris (pH 7.5), 0.1% β -MCE and 0.5 mM PMSF, and then brought to a final concentration of 0.4 M H_2SO_4 . After stirring at 4°C for 60 min and centrifugation for 30 min at $12000 \times g$, the acid-soluble supernatants were dialysed against 95% ethanol. Resulting precipitates were spun at $12000 \times g$ for 10 min and washed twice with 100% acetone. After centrifugation at $12000 \times g$ for 10 min, the dried histone pellets were dissolved in 0.1 M NaCl and stored at $-20^\circ C$.

For ion-exchange chromatography, isolated nuclei were suspended in binding buffer (0.7 M NaCl, 50 mM phosphate-buffer, pH 7.0, 0.1% β -MCE, 0.5 mM PMSF) at 4°C using a glass homogenizer to shear DNA into smaller fragments. A column (12 cm length, 1 cm diameter) was filled with hydroxyapatite equilibrated with binding buffer. Sheared nuclei were applied to the column and washed with binding buffer with a flow rate of 30 ml/h. Core histones were then eluted with 2.5 M NaCl, 50 mM phosphate buffer, pH 7.0, 0.1% β -MCE and 0.5 mM PMSF [13] and stored in this solution at $-20^\circ C$.

Salt-extracted histones were obtained by washing purified nuclei twice in 0.7 M NaCl, 50 mM phosphate buffer (pH 7), 0.1% β -MCE and 0.5 mM PMSF at room temperature. DNA-histone complexes were then precipitated by overlaying the solution with 1.5 vols of ethanol, 'fished' with a bent glass-stick, resuspended in 5 M urea, 2 M NaCl, 50 mM phosphate buffer (pH 7), 0.1% β -MCE and 0.5 mM PMSF, and dissociated at 37°C for 30 min. After that, sucrose was added to a final concentration of 15% and the solution was centrifuged for 48 h at 35000 rpm in a Beckman SW 40 rotor in order to pellet the DNA. The supernatants containing purified histones were dialyzed against distilled water, lyophilized and stored at $-20^\circ C$.

2.3. In vitro reconstitution procedure

Nucleosome reconstitution was performed at room temperature according to [14]. Typically, 20 μg of linearized plasmid DNA were mixed with 50 μg of histones in a buffer containing 2 M NaCl and 5 M urea. After 30 min of preincubation at 37°C, the NaCl concentration was gradually lowered by overnight dialysis against distilled water. Reconstituted plasmids were stored in 10 mM Tris (pH 8.0), 1 mM EDTA at 4°C.

2.4. Electron microscopy

For electron microscopy, samples of in vitro assembled chromatin were fixed with glutaraldehyde and processed according to [15].

Deproteinized DNA was prepared for electron microscopy by the cytochrome *c* droplet diffusion method [16]. All preparations were rotary shadowed with platinum/palladium (80/20) at an angle of 8° and examined in a Zeiss EM-10 electron microscope.

2.5. Histone analysis

Reconstituted plasmids were separated from free core histones by centrifugation through a 17–51% sucrose-gradient containing 50 mM phosphate buffer (pH 7), 0.1% β -MCE, 0.5 mM PMSF (Beckman SW 40 rotor; 25000 rpm for 2–4 h) and checked for histone purity and integrity by SDS-polyacrylamide gel electrophoresis in 18% gels [17].

2.6. Micrococcal nuclease digestion and Southern analysis

Reconstituted plasmids were digested for different periods of time at 37°C with micrococcal nuclease (0.5 U/ μg plasmid DNA) in a buffer containing 10 mM $CaCl_2$. DNA from digested samples was phenol extracted, ethanol precipitated, centrifuged, dissolved in 10 mM Tris (pH 8.0), 1 mM EDTA, separated on 2% agarose gels and blotted to nitrocellulose. Blots were then hybridized to nick-translated complementary probes derived from the appropriate plasmids.

3. RESULTS

3.1. Purity of plasmid and histone preparations

Linearization and purity of plasmids was routinely checked by electron microscopy. Protein contaminations were not detected (data not shown). Purity and integrity of histones were checked by SDS-polyacrylamide gel electrophoresis (fig.2, lane A). As judged from their Coomassie brilliant blue staining patterns, the different procedures used for histone isolation resulted in similar histone preparations typical for plant core histones [18] (data not shown). The extent of contamination of histone preparations by DNA was calculated spectrophotometrically [19]. For acid-extracted and hydroxyapatite-isolated histones it was found to be less than 5%, for salt-extracted histones about 8–10%, respectively.

3.2. Reconstitution experiments

We compared the in vitro reconstitution efficiency using either hydroxyapatite-isolated histones from chicken erythrocytes or plant histones isolated by three different methods and linearized plasmids carrying DNA sequences of prokaryotic and eukaryotic origin. Reconstitution of plasmids with chicken histones usually resulted in regularly beaded nucleosomes (not shown). In contrast, the use of acid-extracted or hydroxyapatite-isolated pea histones led to the for-

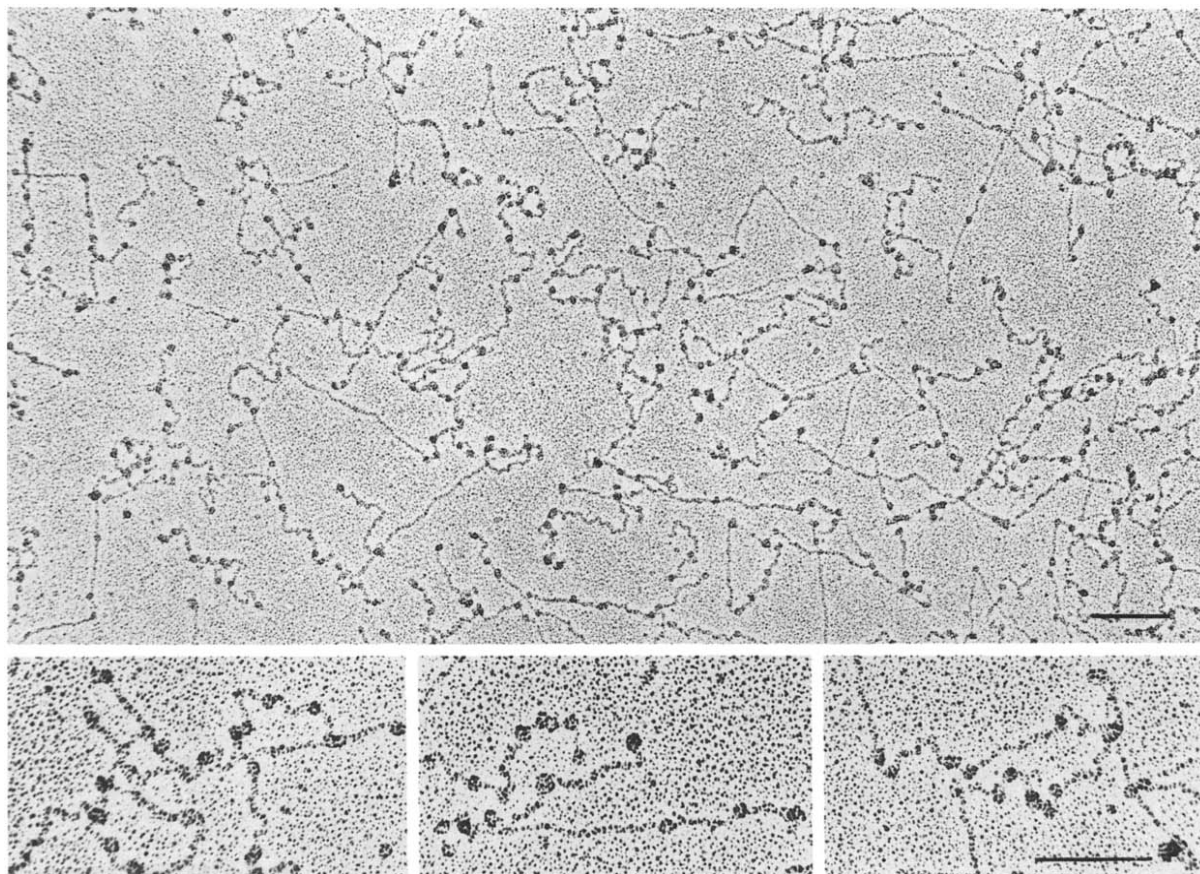


Fig.1. Electron microscopic analysis of reconstituted nucleoprotein complexes obtained with salt-extracted pea histone fractions and linearized plasmid pLGV0422 show densely beaded stretches (see insets) among other molecules associated with only a few beads. The bar represents 100 nm.

mation of bulky, irregular histone-DNA complexes as judged by electron microscopy (not shown). Although no indications for nucleosomal organization could be found, the DNA within these complexes exhibited a striking resistance towards micrococcal nuclease digestion. Incubation at 37°C for 5 min had no remarkable effect on the integrity of the DNA. Even extensive digestion for 60 min caused only slight degradation of plasmid DNA. However, typical nucleosomal ladders were not detected. On the other hand, reconstitution experiments using salt-extracted histones resulted in the formation of nucleosomes as shown in fig.1. The electron micrograph shows irregularly spaced nucleosomes on the plasmid DNA. Histone analysis by SDS-polyacrylamide gel electrophoresis indicates that the histone content

of purified reconstituates reflects the histone composition used in the reconstitution experiment (fig.2, lanes A and B). Spacing of nucleosomes was checked by micrococcal nuclease digestion and Southern analysis. Fig.3 presents evidence that chains of up to five tightly packed nucleosome cores are present in the reconstituted complexes. The repeat length was determined to be 150 ± 8 bp. In contrast, the repeat length of pea chromatin *in vivo* was found to be 185 ± 5 bp [20].

Reconstitution experiments using salt-extracted plant histones, but omitting urea in the reconstitution mixture, never resulted in nucleosome core formation as detected by electron microscopy or Southern analysis. Whereas the method of histone isolation proved to be critical for the efficiency of nucleosome assembly, the origin of DNA se-

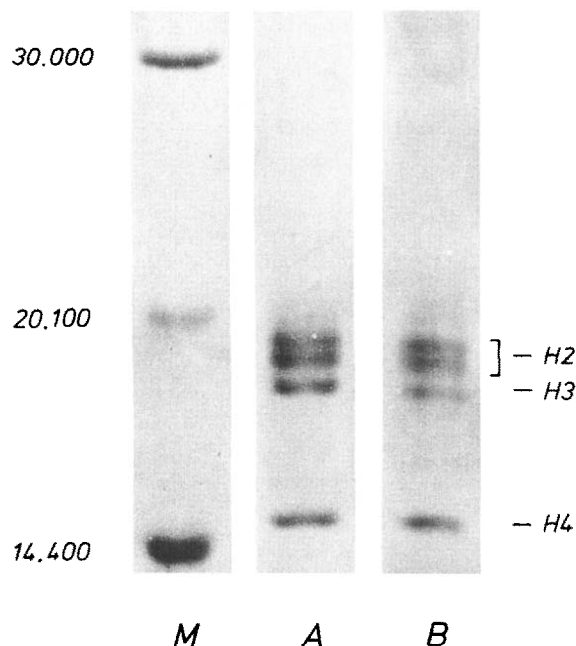


Fig.2. SDS-polyacrylamide gel electrophoresis of purified pea histones used for the reconstitution experiments (lane A) and of histones within reconstituted nucleosomes separated from a 17–51% sucrose gradient (lane B). Lane M represents molecular weight markers. For their aberrant mobilities in SDS-polyacrylamide gels [27] molecular weights of histones cannot be directly determined by comparison to protein markers.

quences had no obvious influence. Similar results were obtained for all plasmids used in the present reconstitution experiments.

4. DISCUSSION

The results of these experiments show that the efficiency of *in vitro* nucleosome assembly using plant histones strongly depends on the histone isolation procedure employed. In contrast to animal histones [14,21,22], acid-extracted or hydroxyapatite-purified histones from plant sources did not readily reconstitute with purified DNA to form nucleosomes. Reconstitution was only successful if salt-extracted core histones were used and if urea was present in the reconstitution buffer. This is in contrast to observations from animal histones, which can be most successfully reconstituted with purified DNA by simple dialysis against 2 M NaCl. Salt extraction of plant chromatin using 2 M NaCl alone did not yield

remarkable amounts of free core histones, whereas a mixture of 2 M NaCl and 5 M urea allowed the isolation of sufficient amounts of pure histones for the reconstitution experiments. The results of the present paper suggest that processes taking place during acid extraction and binding of plant histones to hydroxyapatite cause an irreversible change in their structure and reactivity which cannot be detected in an SDS-polyacrylamide gel. We hypothesize that a conformational change of one or more histones takes place. This is not true for animal histones, since even chicken erythrocyte histones eluted from SDS-polyacrylamide gels, allowing a successful reconstitution. The absolute necessity of urea for reconstitution of nucleosomes from plant histones also indicates substantial differences in DNA-histone and/or histone-histone interactions between plant and animal histones. This is not surprising, since H2A and H2B differ markedly between plants and animals [18,23,24].

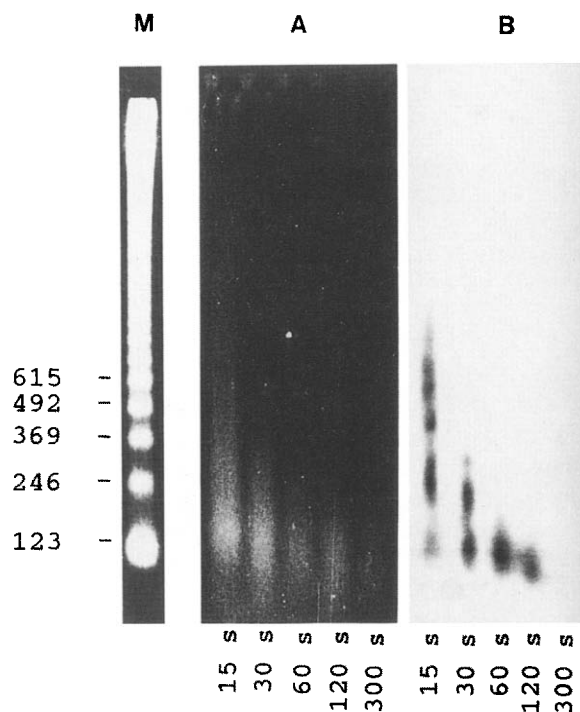


Fig.3. (A) Ethidium bromide staining pattern of purified nucleosomal DNA from micrococcal nuclease digested reconstituted plasmid pLGV0422 after separation on a 2% agarose gel. (B) Southern blot hybridization of the gel shown in A to the *Hind*III 23 fragment of pLGV0422. (M) Marker (123 bp ladder). Numbers below the figures denote digestion time in seconds (s).

Reconstitution experiments using animal cell extracts are now generally performed under conditions of physiological ionic strength and lead to nucleosomal periodicities similar or identical to the in vivo situation [25,26]. Work is in progress to create a similar system for plant nucleosome reconstitution using cellular extracts which catalyze chromatin assembly under physiological conditions.

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