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DNA associated with nucleosomes in plants

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ABSTRACT

50 to 55 % of tobacco and barley nuclear DNA is accessible to micrococcal endonuclease digestion. The DNA fragments resulting from a mild endonuclease treatment are multiples of a basic unit of 194 ± 6 base pairs in tobacco and 195 ± 6 base pairs in barley. After extensive digestion, a DNA fragment of approximately 140 base pairs is predominant. Hence the "extra-core" or "linker"-DNA is 55 base pairs long. Other fragments having 158 and less than 140 base pairs are present as well. Treatment with DNase I results in multiples of 10 bases when analysed under denaturating conditions.

These results show that the general organization of the DNA within the nucleosomes is about the same in higher plants as in other higher eukaryotes.

INTRODUCTION

It has been demonstrated by biochemical and morphological evidence that the chromatin of eukaryotes consists of repeating nucleohistone subunits (for reviews see 1 and 2). Mild endonuclease treatment of either intact nuclei or chromatin has shown that a repeating unit of DNA of approximately 200 base pairs is associated with each subunit (nucleosome)[3,4] and thus partially protected against endonuclease digestion. Recent data suggests that the DNA repeat length is variable, ranging from 154 in *Aspergillus nidulans*[5] to 241 base pairs in sea urchin sperm cells[6]. However, whatever the length of the DNA repeating unit may be after a mild treatment, the fragment remaining after a semi-extensive digestion is approximately 140 base pairs long in all the chromatins studied so far[4-7]. This means that the DNA associated with the histone core is about the same length in all the eukaryotes, the variability mentioned above being due to differences in the "extra-core" or "linker"-DNA[6]. Whether or not this variability is a reflection of differences between species of core histones or of histone H1 is still controversial. Within the nucleosome it was shown from DNase I-treatment that the DNA was wound around the core of histones[8].

We have recently shown that the chromatin of higher plants is also composed of nucleosomes[9,10]. On the other hand it was demonstrated that even if histones H2A and H2B have higher molecular weights[11,12] and differ slightly in their amino acid composition, the cross-complexing pattern of the histones is the same in plants and in animals[13,14]. Up to now, no systematic study had been done on the size of the DNA associated with each nucleosome in higher plants.

Here we report on the action of micrococcal endonuclease and DNase I on nuclei extracted from tobacco and barley leaves and protoplasts. The products of endonuclease digestion were compared with restriction enzyme Hae III fragments of PM2 and SV40 DNAs. Fragments resulting from DNase I treatment were calibrated against sequenced t-RNA.

EXPERIMENTAL PROCEDURES

1. Isolation of nuclei

The techniques for the isolation of tobacco protoplasts and of nuclei from either protoplasts or leaves have been published previously[9,10]. The established procedures were followed except that the sucrose concentration was increased to 0.25 M in grinding buffer. When nuclei were prepared from barley leaves, the concentration of Triton X-100 was reduced to 0.5 % in the grinding medium. On the contrary attempts to reduce the concentration of detergent during the isolation of nuclei from tobacco (leaves as well as protoplasts) were unsuccessful.

2. Treatment of nuclei with micrococcal endonuclease and DNase I

The isolated nuclei were suspended in 10 mM Tris-HCl, 1 mM $CaCl_2$, 0.25 M sucrose buffer pH 7.5, and washed once with this buffer by a 450 g centrifugation. The treatment with micrococcal endonuclease (Sigma) was performed at 37°C. After digestion for the desired length of time, the reaction was terminated by addition of 20 µl of 0.1 M Na_2 EDTA pH 7.0 per ml and chilling in ice. To determine the amount of DNA digested by the endonuclease, intact nucleoprotein was precipitated for 1 h by addition of 1 volume of 10 % perchloric acid (PCA). The precipitate was sedimented by centrifugation at 3000 g for 10 min, and the amount of free nucleotides present in the supernatant fluid was determined from the UV absorption at 260 nm. The hyperchromicity of tobacco DNA was calculated after an extensive digestion with micrococcal endonuclease of deproteinized tobacco DNA. The amount of DNA present in the pellet (i.e. undegraded by the endonuclease treatment) was estimated by either the colorimetric technique of

Giles and Myers[15] or UV absorption at 260 nm after 1 h hydrolysis in 10 % PCA at 70°C.

10^7 nuclei were suspended in 0.1 ml of the following buffer : 10 mM MES, pH.7.0, 10 mM $MgCl_2$, 10 mM Natrium bisulphite, 5 mM mercaptoethanol and 0.25 M sucrose. 25 units of DNase I (Worthington) were added and the mixture was incubated for 30 seconds at 37°C. The incubation was stopped by addition of EDTA and SDS up to 20 mM and 1 % respectively.

3. Deproteinization and analysis of DNA fragments

The DNA was extracted from endonuclease-treated nuclei and deproteinized by an overnight incubation with proteinase K (100 µg/ml) in the presence of 1 % SDS. The incubation was followed by two treatments with 1 volume of phenol and two treatments with chloroform-isoamyl alcohol (9:1). The DNA contained in the aqueous phase was then precipitated with 2 volumes of distilled ethanol at -20°C. The precipitate was washed twice with ethanol, quickly dried under vacuum and finally resuspended in 10 mM Tris-HCl, 20 % glycerol, 1 mM Na_2 EDTA, pH 8.0, containing bromophenol blue as a marker.

The DNA fragments resulting from micrococcal endonuclease treatment were separated by slab-gel electrophoresis as described by Peacock and Dingman[16] except that the running buffer was Tris-HCl 0.04 M, sodium acetate 0.02 M, Na_2EDTA 1 mM, at pH 7.8. The concentration of acrylamide in the gel was 2 % after a mild treatment and 6 % for the products of an extensive digestion. Migration was for 2.5 h at 80 volts for 2 % and 7 h at 40 volts for 6 % gels.

The fragments resulting from the DNase I digestion were analysed on 12 % acrylamide gels, containing 7 M urea as described by Maniatis et al[17]. Running buffer was 0.09 M Tris-Borate, pH 8.3 containing 2.5 mM EDTA. The DNA samples were suspended in 10 mM Tris pH 8.3, 1 mM EDTA and treated with 0.1 M NaOH just before application on the gel. Migration was for 5 h at 80 V at room temperature. After migration, the gels were stained for 30 min with 1 µg/ml of ethidium bromide dissolved in 10 mM Tris-HCl pH 8.0, Na_2EDTA 1 mM. After staining, the gel was quickly rinsed with the same buffer and photographed under UV illumination, using an orange filter.

RESULTS

1. Accessibility of nuclear DNA to micrococcal endonuclease

Nuclei from tobacco leaves or protoplasts were digested with 6 units of micrococcal endonuclease (Sigma) per 10^6 nuclei for various lengths of time. Figure 1 shows the percentage of total DNA which became acid-soluble after

digestion. After 60 min incubation a plateau was attained where more than 50 % of the DNA had been digested. The susceptibility of the DNA to endonuclease degradation was the same whether the nuclei were isolated from leaves or from protoplasts. This indicates that the organization of the chromatin with respect to accessibility to endonuclease is not significantly modified by the protoplast isolation procedure. Some differences found between tobacco and barley leaves nuclei will be reported elsewhere.

By electron microscopic studies, we have shown previously that when ten to a hundred times less endonuclease is used, the digestion is more gradual and filaments consisting of several nucleosomes can be isolated[18]. Figure 2 shows the pattern obtained with DNA extracted from nuclei subjected to such mild endonuclease digestion. Digestion was for 10, 30 and 60 min and the digest was run on a 2 % acrylamide gel. After 10 min digestion, no DNA corresponding to the monomer was visible but after 60 min this class of DNA was the only one present. The 30 min digest shows a very typical pattern of a mixture of DNA fragments whose lengths are multiples of a basic repeating unit.

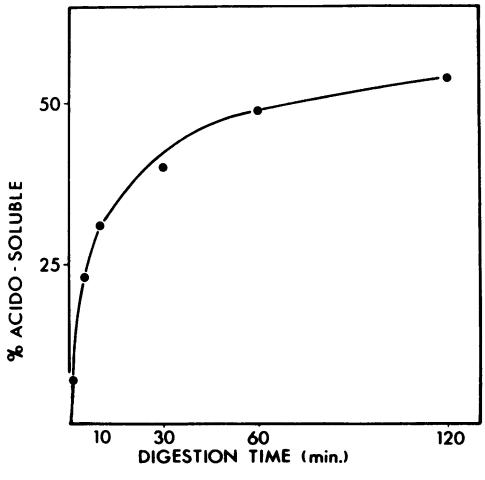

Figure 1. Kinetics of the "in situ" digestion of tobacco nuclear chromatin DNA by micrococcal endonuclease (6 units/10^6 nuclei).

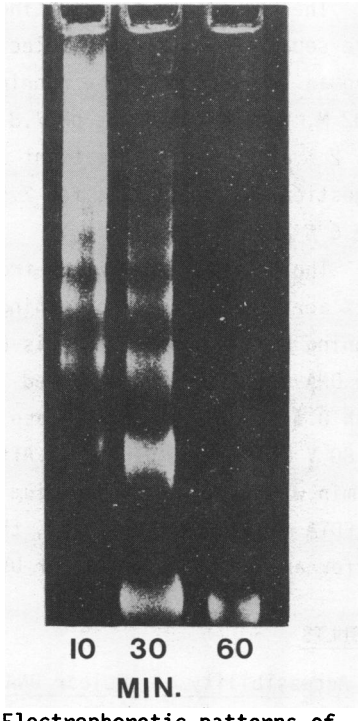

Figure 2. Electrophoretic patterns of DNA fragments after 10, 30 and 60 min digestion of tobacco nuclei with endonuclease (0.2 units/10^6 nuclei).

2. Size of the DNA repeating unit

The lengths of the DNA fragments isolated after a mild treatment with the micrococcal endonuclease were determined by comparison with the calibrated Hae III fragments of PM2 and SV40 DNAs. The sizes of the Hae III fragments of PM2 DNA were those determined by Noll[7] and of SV40 DNA by Subramanian et al[19]. The figure 3 compares the electrophoretic patterns of the DNA fragments resulting from a mild treatment of nuclei from tobacco and barley leaves. The fragments from tobacco migrate slightly faster than those of barley nuclei. The difference is due to the fact that the tobacco nuclei were slightly more extensively digested (10 % acido soluble DNA) than barley nuclei (5 % acido soluble DNA) as judged by the higher proportion of monomers. The sizes of the different fragments were determined using the calibration curve shown in figure 4 and the found values are reported in table I.

It was shown that during the micrococcal nuclease treatment at 37°C, degradation of the cleaved fragments occured by release of DNA from the ends[19]. To overcome this problem which may introduce uncertainty in the

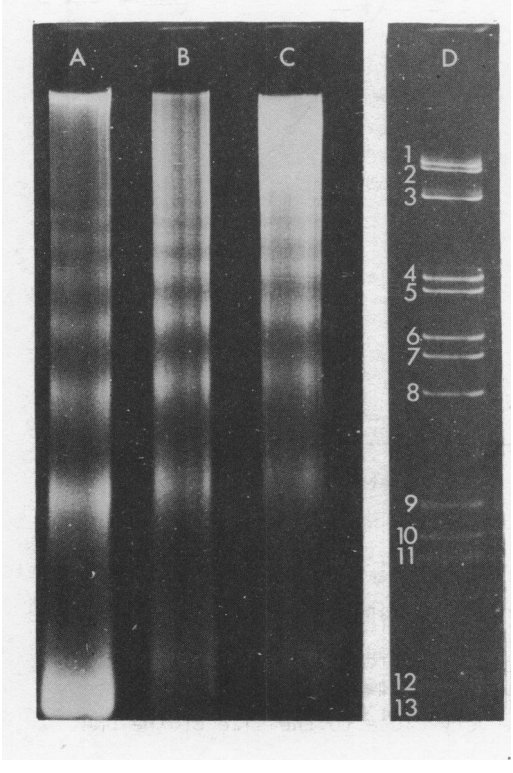

Figure 3. Polyacrylamide gel electrophoresis of micrococcal nuclease digests of nuclei from tobacco (A), barley (B), rat liver (C). The Hae III fragments of PM2 DNA (D) migrated with the other samples but the picture was printed separately.

Table I. Lengths of DNA fragments isolated from tobacco and barley nuclei, after a mild treatment with micrococcal endonuclease.

PM2 DNA by Hae III[7]		SV40 DNA by Hae III[19]		TOBACCO		BARLEY	
Band	Base pairs	Band	Base pairs	Band	Base pairs	Band	Base pairs
1	1860	1	1550	1	162	1	172
2	1720	2	775	2	341	2	366
3	1410	3	525	3	531	3	562
4	890	4	350	4	728	4	741
5	845	5	325	5	917	5	929
6	672	6	310	6	1122	6	1142
7	615	7	300	7	1310	7	1340
8	525	8	225				
9	333	9	170				
10	295						
11	272			Repeat length :			
12	167						
13	152			194 ± 6		195 ± 6	

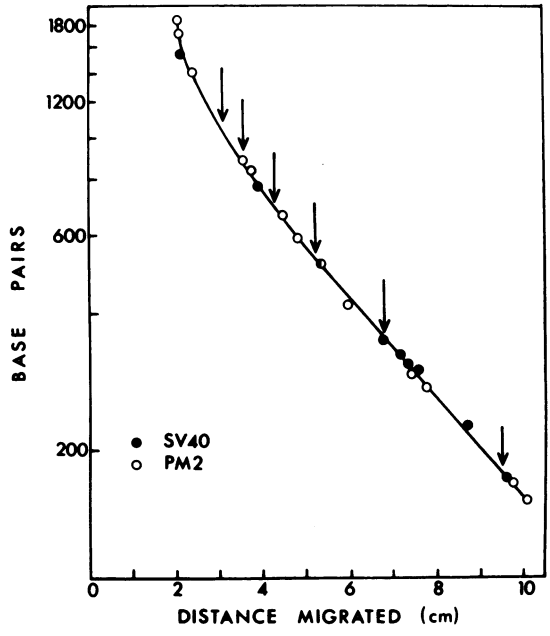

Figure 4. Calibration curve for sizing of the DNA fragments of the gel shown in figure 3. Hae III fragments of PM2 and SV40 DNAs were used as markers. The arrows indicate the position of DNA fragments from barley.

determination of the size of the repeating unit, we have measured the differences between the second and the following multiples, as described by Noll and Kornberg[21]. The values found by this method are 194 ± 6 for tobacco and 195 ± 6 for barley. These values were the same whether the nuclei came from leaves or from protoplasts. They are very close to the size of the DNA

repeating subunit determined in a majority of chromatins from higher eukaryotes[22], but significantly higher than the values found in fungi[5,7] and yeast[23].

The electrophoretic pattern of an extensive endonuclease digest run on a 6 % acrylamide gel is seen in Figure 5. In this figure we compare the DNA extracted from extensively digested rat liver, tobacco leaf and protoplast nuclei. A major band is present which has a size of 135 base pairs in both the tobacco and rat liver digests. This DNA fragment corresponds to the 140 base pairs which have been universally found to be associated with the core of histones. Minor bands were also found, having sizes of 110, 84 and 59 base pairs and which are also present in both rat liver and tobacco digests. However, a component of 158 base pairs is visible in tobacco which does not appear clearly in the rat liver digest.

3. Digestion with DNase I

It had been shown that the digestion of chromatin with DNase I revealed under denaturating conditions a series of DNA fragments whose lengths are multiples of 10 bases[8]. Otherwise, the digestion pattern was believed to reflect the arrangement of the DNA with the core of histones within the

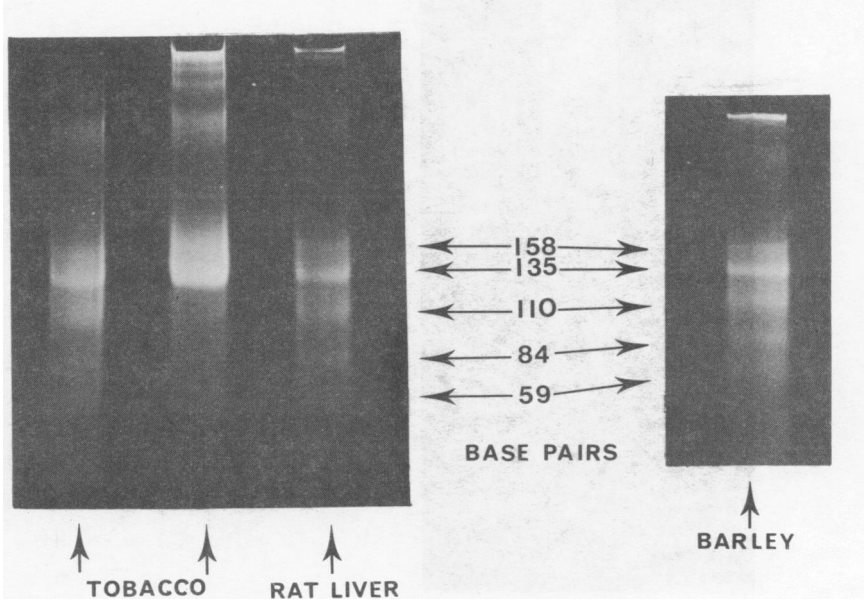

Figure 5. Electrophoretic pattern of DNA fragments isolated from extensively digested tobacco, barley and rat liver nuclei.

nucleosome. Intact nuclei from either tobacco or barley leaves were treated with DNase I under the conditions described above, and the DNA fragments analysed on 12 % polyacrylamide gels under denaturating conditions. As it was demonstrated that between 10 and 150 nucleotides the mobilities of denatured DNA and RNA were the same[17], we used sequenced t-RNAs and fragments of t-RNAs to establish the calibration curve. The markers were yeast t-RNA^{Ser} (85 nucleotides), yeast t-RNA^{Phe} (76 nucleotides), sequenced fragments of specifically cleaved yeast t-RNA^{Phe} (39 and 36 nucleotides) and a fragment of t-RNA^{Asp} (19 nucleotides).

The figure 6 shows the electrophoretic pattern of DNA fragments isolated from tobacco nuclei digested with DNase I. A series of bands are visible which are multiples of 10 nucleotides. No fragment can be clearly detected below 30 and above 130 nucleotides. It was mentioned in DNase I digests from several other chromatins that differences existed in the intensities of some frag-

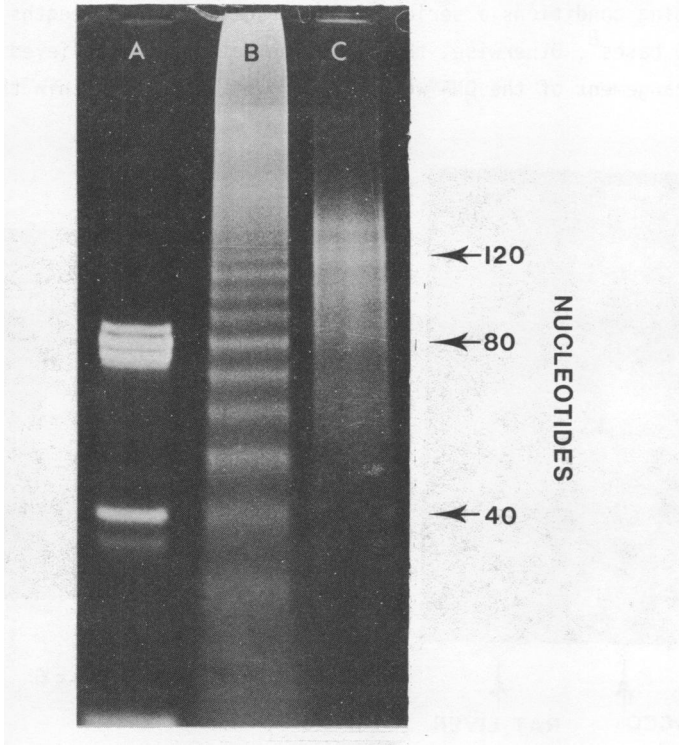

Figure 6. DNA fragments of tobacco nuclei digested with DNase I (B) as compared with marker RNAs (A) and DNA fragments resulting from extensive digestion of nuclei with micrococcal endonuclease (C).

ments. The 80 nucleotide fragment is generally broader whereas the 60 nucleotide fragment is weaker[7,8]. These results were generally interpreted as a proof of preferential cutting sites in the DNA within the nucleosomes. Such differences do not appear clearly in our material.

DISCUSSION

Our results show that in nuclei isolated from higher plants, approximately 50 to 55 % of the DNA is accessible to added micrococcal endonuclease. Similar values have been found in a number of other eukaryotes. Analysis on polyacrylamide gels of the DNA extracted from nuclei treated under mild conditions showed a typical pattern of DNA fragments in which the larger fragments have lengths which are integral multiples of the smallest. The resolution of the different DNA fragments demonstrates that little, if any, degradation of the nucleosomes occurs during preparation of nuclei.

The DNA associated with a nucleosome was found to be 194 ± 6 base pairs long for tobacco and 195 ± 6 for barley. These values are of the same order as reported for several other higher eukaryotes[22]. It is higher however than the length of the nucleosomal DNA in lower plants such as Neurospora crassa (170 base pairs[5]) and Aspergillus nidulans (154 base pairs[7]). A previous estimate of the size of the DNA repeating length in a higher plant was 170 base pairs for pea seedlings[24]. But, in this case, the endonuclease treatment was performed on extracted chromatin in rather uncontrolled conditions and such treatment generally leads to a less clear electrophoretic pattern than when intact nuclei are treated.

When the nuclei were extensively treated with the endonuclease a DNA fragment of 135 base pairs was predominant. It corresponds to the DNA length (140 base pairs) which was found to be associated with the octamer of histones in all the chromatins studied so far. Another fragment exists, having 158 base pairs, which could correspond to the 160 base pairs DNA fragment mentioned by Noll in rat chromatin when H1 was not depleted[17]. The difference between the length of the DNA associated with the nucleosome (194 b.p.) and that of the DNA associated with the octamer (135 b.p.) represents the "extra-core" or "linker"-DNA. It is about 60 base pairs long.

These results show that the size of the DNA associated with the nucleosome as well as that of the "linker" DNA is of the same order in higher plants that in the majority of other higher eukaryotes. On the other hand, the DNase I digestion pattern which is very similar in higher as well as lower eukaryotes indicates that the general organization of the DNA within the nucleosomes is the same. It thus appears that the nucleosomes of orga-

nisms which are evolutionarily very distant and in which several histones differ by their molecular weights as well as their chemical composition have apparently the same architecture.

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