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Ribosomal gene clusters are uniquely proportioned between open and closed chromatin structures in both tomato leaf cells and exponentially growing suspension cultures

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ABSTRACT **The accessibility of regulatory molecules to specific DNA sequences and chromatin regions in the nucleus is crucial to gene expression. In this study, we examined the chromatin structure in tomato leaf cells and in exponentially growing tomato cell suspension cultures. The structure of ribosomal chromatin was investigated by micrococcal nuclease and psoralen photocrosslinking. We showed that ribosomal genes in tomato are folded into two distinct types of chromatin: an open chromatin conformation and a closed nucleosome-containing chromatin. In contrast to previous findings in Friend cells, where half of the ribosomal genes were found to be complexed within an inactive chromatin structure, we demonstrated that the canonical nucleosome-containing chromatin is present in the majority (≈80%) of the tomato rRNA-encoding DNA clusters. The minor open chromatin population (≈20% of the ribosomal genes) could be detected only after analysis following psoralen crosslinking. The relative amounts of the two ribosomal chromatin structures are similar in stationary and exponentially growing cells. This suggests that the proportions of open and closed chromatin structures present in either stationary or exponentially growing tomato cells are not dependent on the transcriptional process.**

While the structure of inactive bulk chromatin is fairly well understood (for reviews, see refs. 1 and 2), there is still no agreement on the organization of the active chromatin in transcribing genes and replicating DNA. With DNase I, it has been found that the active chromatin is degraded more rapidly than the bulk inactive chromatin and therefore is considered to be in a less condensed, or more open, conformation (for review, see ref. 3).

Particular interest in the chromatin structure of ribosomal genes is based on the peculiar organization of the rRNA-encoding DNA (rDNA); active rRNA genes are transcribed at a maximal polymerase density [≈1 polymerase per 100 base pairs (bp) or two polymerases per nucleosomal DNA] with a high elongation rate (≈30 nucleotides per sec) (for review, see ref. 4). In spite of the high transcriptional activity, >100 rRNA gene copies are required in dividing cells to produce the needed amount of rRNA. Essentially all eukaryotic cells contain between 100 and 5000 ribosomal genes per haploid genome. The rDNA transcription rate is regulated as a function of the cellular growth rate, being down-regulated in stationary cells and up-regulated in exponentially growing cells (5, 6).

The chromatin structure of transcribing nucleolar genes is still controversial. Davis *et al.* (7) showed that the ribosomal sequences in a mouse cell line could be fractionated into a nucleosome-containing chromatin component and a component where the ribosomal genes did not show a repeating structure. Mapping the sites of topoisomerase I interaction in ribosomal chromatin of *Xenopus* oocytes, Culotta and Sollner-Webb (8) found that these sites were spaced with a nucleosomal periodicity, a result suggesting that active ribosomal chromatin is in a nucleosomal array. Ribosomal chromatin was also found in modified nucleosomes in *Physarum polycephalum* (9, 10). In contrast to these results, studies with micrococcal nuclease, DNase I, and psoralen crosslinking showed that the majority of the ribosomal genes in *Physarum* and *Dictyostelium discoideum* are not organized in the repeating structure (11–13). Using the psoralen-crosslinking technique, Conconi *et al.* (14) demonstrated the existence of open and closed rDNA chromatin populations in Friend erythroleukemia cells (a mouse cell culture line). Only the open population contained transcriptionally active ribosomal genes.

Much less is known about chromatin structure in higher plants. However, the fundamental conformational features of plant chromatin are very similar to those found in animals and in other eukaryotes (for review, see ref. 15). Thompson and Flavell (16) found that some of the rRNA genes at active, dominant loci are organized in a chromatin conformation that renders them more sensitive to DNase I than are other rRNA genes. DNase I-sensitive chromatin correlated with undermethylated CCGG sites and rDNA activity of the different loci.

Using two different approaches—micrococcal nuclease and psoralen crosslinking—we investigated the chromatin structure of ribosomal genes in tomato cells. Our results indicate that the majority of the ribosomal genes (≈80%) in tomato plant leaves and dividing cell cultures are folded in a nucleosomal structure, similar to the bulk inactive chromatin. We have used psoralen crosslinking to differentiate closed (low crosslinked) from open (highly crosslinked) ribosomal chromatin. In contrast to the mouse Friend cells where ≈50% of the chromatin is in an active conformation (14), the open chromatin structure in tomato represents only ≈20% of the total ribosomal gene clusters.

MATERIALS AND METHODS

Plant Material. Tomato suspension cultures (*Lycopersicon esculentum* × *Lycopersicon peruvianum*) were grown in Murashige and Skoog medium containing 2,4-dichlorophenoxyacetic acid (2 mg/liter) and zeatin (0.1 mg/liter), with constant shaking in the dark at 27°C.

Nuclei Isolation. Nuclei, either from 2-week-old tomato leaves [*L. esculentum* (L.) Mill., cv. Castlemart] or from tomato cell suspension culture, were basically prepared as described by Luthe and Quatrano (17).

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Abbreviations: rDNA, rRNA-encoding DNA; f and s bands, fast- and slow-migrating bands.

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Micrococcal Nuclease Digestion of Nuclei. The resuspended nuclei (≈100 μg of DNA) were adjusted to 1 mM $CaCl_2$ and incubated for 15 min at 37°C, when micrococcal nuclease (Worthington) was added to a final concentration of 23 units/ml. The reactions were terminated at different incubation times by adding EDTA to 7 mM (final concentration) and chilling the samples on ice.

DNA Extraction and Gel Electrophoresis. The micrococcal nuclease-released DNA fragments and the psoralen-photocrosslinked DNA were purified as described by Widmer *et al.* (18). Electrophoresis, blotting, and hybridizations were done as described by Conconi *et al.* (14). The length of the nucleosomal DNA ladder was determined by comparison with the fragment sizes obtained from the *Eco*RI/*Hin*dIII double digest of λ DNA and the *Hin*fI fragments of the plasmid pUC-18.

Psoralen Photocrosslinking of Nuclei. Nuclei (≈100 μg of DNA) photocrosslinking was performed in 4-ml polypropylene tubes. Psoralen (4,5',8-trimethylpsoralen; Sigma) stock solution (400 μg/ml in ethanol) was added to 1/40th of the nuclei suspension volume. After 5 min of preincubation on ice and in the dark, the nuclei were photoirradiated on ice for 20 min, with a medium-pressure Hg lamp (450 W; 320–380 nm) placed at a distance of ≈15 cm. The procedure was done a total of four times. The DNA spreading for electron microscopy analysis was performed under denaturing conditions as described by Sogo *et al.* (19).

RESULTS

Nuclei Preparation. Histone proteins were extracted on ice with 0.25 N H_2SO_4. After ethanol precipitation, the tomato chromatin proteins were analyzed by SDS/PAGE and the results were compared to standard calf thymus histones (Boehringer Mannheim). The presence of the highly protease-sensitive histone H1, and the absence of material in front of the fastest-migrating histone band, histone H4 (results not shown), suggested that degradation from contaminating proteases, if present, was very low. Since the average length of the DNA extracted from nuclei, incubated in the absence of exogenous nucleases (Fig. 1*A*, lanes 2 and 7), appeared to be at least as high as the length of genomic DNA extracted by the CTAB method (23), we assumed that the nuclear preparations were also essentially free of endogenous nuclease activities.

Micrococcal Nuclease Analysis of the Ribosomal Gene Coding Regions in Tomato Leaves and in Cell Suspension Cultures. Isolated nuclei were treated for various lengths of time with micrococcal nuclease. The digestion products were separated according to length by agarose gel electrophoresis (Fig. 1*A*). The repeat length of the nucleosomal DNA ladder was determined and the value obtained for nucleosomal DNA was 184 ± 5 bp. No difference in the bulk inactive chromatin was observed when the micrococcal patterns obtained from stationary leaf cells (Fig. 1*A*, lanes 2–6) and from dividing suspension cells (lanes 7–11) were compared.

To investigate whether the chromatin structure of the tomato leaf rRNA genes is present in an unraveled conformation, similar to what has been found in *D. discoideum* (12) and in *P. polycephalum* (11), DNA was transferred to a nylon membrane and hybridized with the radiolabeled Acc374 fragment (Fig. 1*C*) obtained from a tomato ribosomal clone (20–22) (Fig. 1*B*). An *Eco*RI fragment (schematically shown in Fig. 1*C*, E_2–E_3) that hybridized with the ribosomal probe (Fig. 1*B*, lane 1) confirms the specificity of the hybridization conditions used. The micrococcal nuclease degradation pattern found in tomato leaf rDNA and in the tomato cell line was very similar to the pattern observed for bulk inactive chromatin (compare Fig. 1 *A* and *B*, lanes 2–6 and 7–11). This well-defined micrococcal nuclease ladder (Fig. 1*B*, lanes 2–6 and 7–11) clearly demonstrates that the ribosomal genes in tomato plant leaves and in the exponentially growing tomato cell culture are mainly complexed in a nucleosomal conformation.

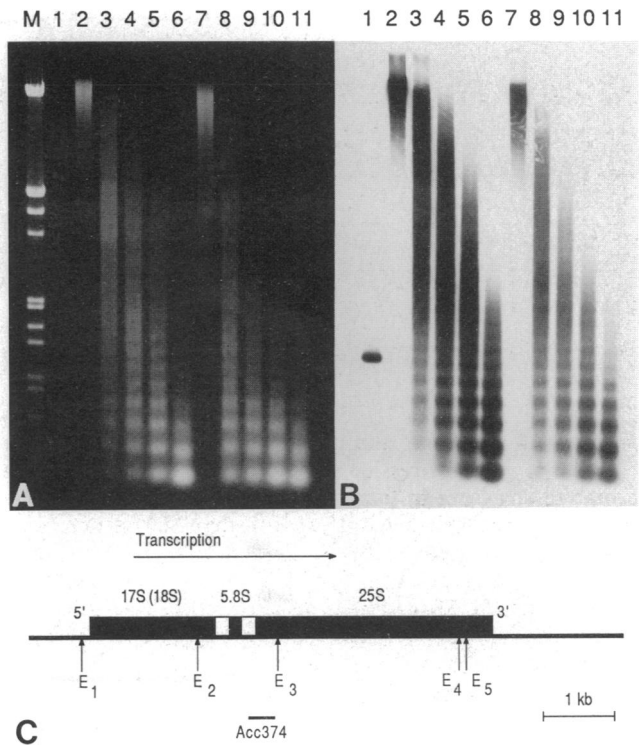

FIG. 1. Micrococcal nuclease digestion of ribosomal chromatin in the coding region. (*A*) Isolated nuclei from tomato leaves and tomato cell cultures were treated with micrococcal nuclease at 37°C for 0.5, 1, 2, and 6 min (lanes 3–6 and 8–11). As control, nuclei were incubated for 10 min under the same conditions except the micrococcal nuclease was omitted (lanes 2 and 7). DNA fragments were separated by electrophoresis in 0.8% agarose together with 1 μg of genomic DNA digested with *Eco*RI (lane 1) and the *Eco*RI/*Hin*dIII λ marker (lane M) (21.221, 5.15, 4.974, 4.271, 3.538, 2.024, 1.906, 1.584, 1.375, 0.947, 0.832, 0.564, and 0.125 kb). (*B*) DNA blotted and hybridized with the rDNA probe Acc374. Lanes 2–6 and 7–11: time course of micrococcal nuclease digestion of nuclei from tomato leaves and cell suspension cultures, respectively. Times and lanes are as described in Fig. 2*A*. (*C*) Map of transcribed region of tomato rDNA. E_{1-5}, the five *Eco*RI sites. Acc374 is an isolated fragment of the ribosomal clone (20–22) used as probe.

However, from the results obtained with the micrococcal nuclease approach, the existence of a nucleosome-depleted chromatin structure in a minor fraction of the ribosomal gene population cannot be excluded. Since we recently found that two different chromatin structures of ribosomal genes can coexist in animal cells (14), we also searched for the existence of an open chromatin conformation, possibly present in only a small portion of the tomato ribosomal gene cluster and therefore not detectable by the micrococcal nuclease assay.

Psoralen Crosslinking of DNA in Tomato Leaf Chromatin. Before using psoralen for this initial study of plant chromatin structure, we had to establish saturating conditions for photocrosslinking and determine whether plant DNA within nucleosomes is protected from crosslinking. Isolated nuclei from tomato leaves were photoreacted with 4,5',8-trimethylpsoralen. The DNA was purified, restriction enzyme digested to an optimal length average [3–10 kilobases (kb)], and spread for direct visualization of the DNA by electron microscopy under denaturing conditions (24). The results in Fig. 2*A* show that crosslinked linear tomato genomic DNA molecules are found in single-stranded bubbles connected by small duplex regions. These features are char-

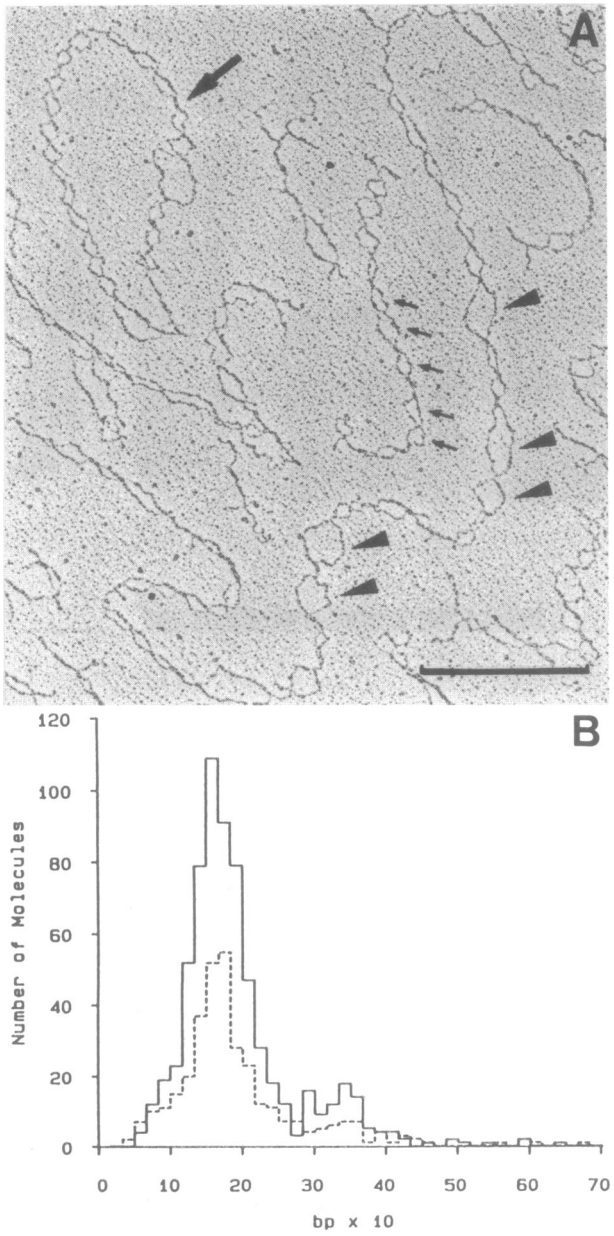

FIG. 2. Psoralen-crosslinked tomato leaf DNA. (*A*) Electron micrograph. Extracted DNA was prepared for electron microscopy under denaturing conditions. Circular simian virus DNA isolated from psoralen-crosslinked simian virus 40 minichromosomes (large arrow) were coprepared as an internal control for EM spreading. Small arrows point to single-strand DNA bubbles representing mononucleosomes and arrowheads show nucleosomal bubbles corresponding to dinucleosomal DNA. (Bar = 1 kbp.) (*B*) Direct comparison of single-stranded bubble size averages in simian virus 40 (dotted line) and tomato (solid line) DNA.

acteristic for psoralen-crosslinked DNA when complexed in nucleosomal containing chromatin (25). As an internal control, DNA from crosslinked simian virus minichromosomes (simian virus 40), previously characterized by Sogo *et al.* (26), were coprepared for electron microscopy. Tomato nucleosomal bubbles were found to be very similar in size to those obtained for simian virus 40 minichromosomes (Fig. 2*B*), which confirms the viability of the psoralen technique for plant chromatin studies. The size distribution obtained for mononucleosomal bubbles was 166 ± 29 and 324 ± 32 bp for dinucleosomal bubbles. In both simian virus 40 minichromosomes and tomato crosslinked chromatin, the major size population was found to be represented by mononucleosomal bubbles (Fig. 2), a result suggesting that the conditions used to photocrosslink tomato leaf nuclei were close to being at saturating conditions.

Accessibility of Tomato Ribosomal Gene Chromatin to Psoralen Crosslinking. Nuclei, isolated from young tomato leaves and exponentially growing tomato cell cultures, were extensively photoreacted in the presence of psoralen. The purified, crosslinked DNA was digested with the restriction enzyme *Eco*RI (Fig. 1*C*), electrophoresed, blotted, and hybridized with the Acc374 probe (Fig. 1*C*). Control, heavily crosslinked DNA was obtained by photoreacting nuclei with psoralen in the presence of SDS at 0.1% final concentration. The detergent causes nuclei to lyse, destroying the chromatin structure and allowing better intercalation of psoralen molecules into the DNA. The gel electrophoretic mobility of the photocrosslinked rDNA fragment, compared with heavily crosslinked and noncrosslinked control DNA, are shown in Fig. 3. Uncrosslinked control DNA (lanes C) migrated as a single band of 1.13 kb (Fig. 1*C*, fragment E_2–E_3) in a 1.6% agarose gel. Psoralen-crosslinked DNA from leaf cell nuclei and from exponentially growing cell culture nuclei migrated as two bands (Fig. 3, lanes 1, s and f). The mobility of the lower more intense band (f, fast-migrating band; lanes 1) was similar to the mobility of uncrosslinked control DNA (lanes C), showing only small electrophoretic retardation. This small shift (compare lanes C with f bands in lane 1) was previously found to correspond to the psoralen molecules that intercalate chromatin in the linker DNA between nucleosomes (18). The second band (s, slow-migrating band; lanes 1) is weaker and significantly retarded when compared to control uncrosslinked DNA (compare lanes C with s bands in lanes 1). When psoralen photocrosslinking was done in the presence of SDS, only one clearly retarded band could be seen (lanes 2), which migrates similarly to the s band of the doublet (compare lanes 2 with s bands in lanes 1). Lane 3 (Fig. 3*A*) shows a parallel photocrosslinking reaction of leaf nuclei, as well as the experimental variability of the psoralen photoreaction (compare lanes 1 and 3). The psoralen-crosslinked DNA is partially resistive to restriction endonuclease digestions, particularly if the crosslinks are within the restriction cutting sites. The partial digestion effect is shown in Fig. 3 (fragments E_1–E_3, E_2–E_4, and E_2–E_5, schematically represented in Fig. 1*C*). The band shift induced by psoralen crosslinking is also visible in the partial digestion fragments and the s and f bands were detected in the first partial digestion fragment (E_1–E_3; Fig. 3*A*, lanes 1 and 3 and Fig. 3*B*, lane 1). In E_2–E_4 and E_2–E_5, the resolution of gel electrophoresis was not enough to separate the s and f bands. Similar results were obtained when DNA fragments from the 17S and 25S regions of the ribosomal gene were used as probes (results not shown).

The percentage of the two different chromatin structures present in tomato ribosomal genes was calculated by scanning (Fig. 3, lanes 1). The values obtained with a densitometer were similar in both leaf cell and cell culture nuclei—≈19% for the s band and ≈82% for the f band.

As a control for psoralen photocrosslinking, purified total genomic DNA was psoralen crosslinked under the same conditions used to photoreact nuclei. Naked DNA treated with psoralen intercalates and crosslinks more psoralen molecules than the SDS-denatured chromatin structure, which crosslinked to about the same extent as the open rDNA–chromatin structure. This was detected electrophoretically by the different migration rates (13) (results not shown).

DISCUSSION

Bulk Chromatin Structure in Different Organs of Tomato Plants Has Similar Micrococcal Repeat DNA Length. In higher plants, nucleosome periodicity has been reported to be

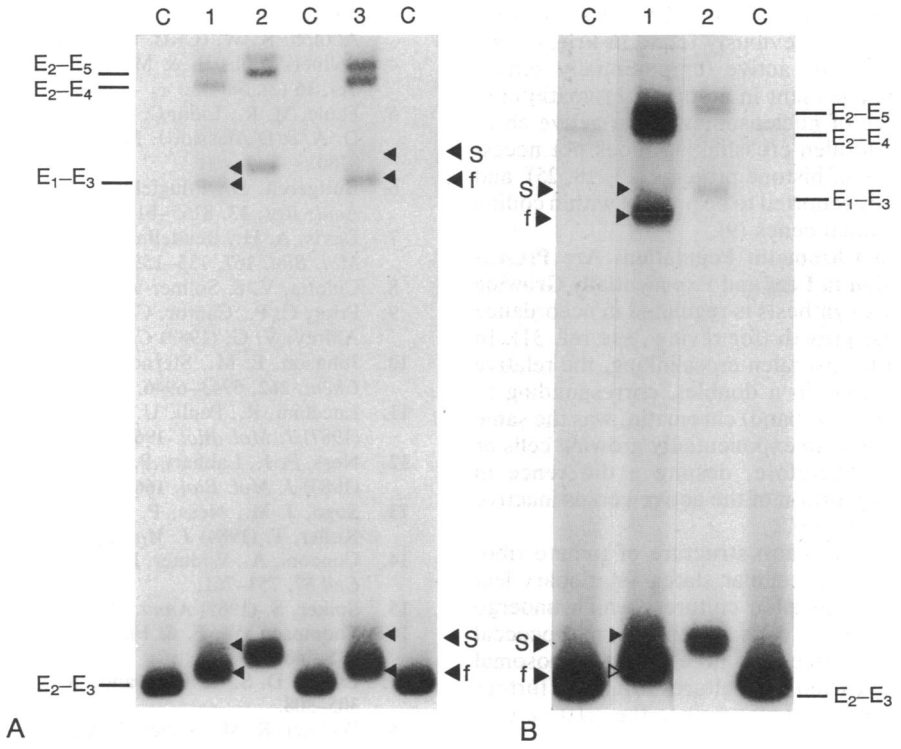

FIG. 3. Psoralen crosslinking of tomato ribosomal chromatin reveals two different chromatin structures. Isolated nuclei from tomato leaves and exponentially growing tomato cell cultures were UV irradiated in the presence of psoralen. DNA was isolated and *Eco*RI digested, electrophoresed in 1.6% agarose, blotted, and hybridized with ^{32}P-labeled Acc374 fragment (see Fig. 1*C*). (*A*) Photocrosslinking of leaf nuclei. Lanes: C, control, noncrosslinked genomic DNA; 1, psoralen crosslinking of isolated nuclei; 2, psoralen crosslinking of isolated nuclei in the presence of 0.1% SDS; 3, same as lane 1 but from a different psoralen-crosslinking experiment. (*B*) Photocrosslinking of cell suspension nuclei. Lanes C, 1, and 2, as described in *A*. s, s band; f, f band; E_1, E_2, E_3, E_4, and E_5, *Eco*RI restriction sites (see map in Fig. 1*C*).

variable; for example, the nucleosomal repeats in tobacco and barley average 194 ± 6 bp (27) and in *Brassica pekinensis* and *Matthiola incana* they average 175 ± 8 bp (28). The nucleosomal repeat in French bean (29) leaf chromatin is 191 ± 6 bp; in cotyledons, a tissue undergoing extensive DNA synthesis, it is considerably shorter (177 ± 7 bp). We found the micrococcal repeat length in tomato leaves to be 184 ± 5 bp. The leaf nucleosomal ladder was compared with the micrococcal digestion products obtained from nuclei of small green tomato fruits and young plant stems. No significant differences were found among the three different organs (results not shown). The leaf bulk chromatin repeat was further compared with the micrococcal ladder in tomato cell suspension cultures. Again, no length differences could be detected between the repeat values from the mainly stationary leaf cells and the extensive DNA-synthesizing, exponentially growing cell cultures. The results suggest that in tomato the nucleosomal repeat lengths do not change between different plant tissues.

Tomato Plant Ribosomal Genes Exist in Two Different Chromatin Structures. In the lower eukaryotes *D. discoideum* and *P. polycephalum*, the coding regions of ribosomal genes were found to be devoid of nucleosomes. In the same organisms, nucleosomes were shown to be present, similar to bulk inactive chromatin, in the nontranscribed spacers between each ribosomal gene. These conclusions were strengthened by using micrococcal nuclease and DNase I (11, 12). Therefore the results indicated that the majority of ribosomal genes in the cells of the two organisms studied were essentially depleted of canonical nucleosomes.

The micrococcal nuclease kinetic digests of ribosomal genes from Friend erythroleukemia cells showed an electrophoretic pattern that could be interpreted as representing a mixture of different chromatin structures (14). After crosslinking nuclei isolated from exponentially growing Friend cells, psoralen-crosslinked rDNA restriction fragments were found to have two different electrophoretic mobilities, called f and s bands. The f and s bands were present at roughly equal intensity, revealing the coexistence of about equal proportions of two distinct chromatin structures. With an exonuclease method, developed by Widmer *et al.* (18), the f and s bands were further characterized and found to represent nucleosome-containing and nucleosome-depleted ribosomal chromatin, respectively. Since nascent radiolabeled rRNA could be crosslinked only to the rDNA sequences present in the s band, it was shown that only the ribosomal genes depleted of canonical nucleosomes are actively transcribed (14).

Plant ribosomal genes, on the other hand, were reported to be complexed within an inactive nucleosome-like chromatin structure (28, 29). However, the expression of only a limited number of rRNA genes seems to be sufficient to produce the rRNA present in barley (30). Consequently, it is possible that the chromatin structure of a small portion of the ribosomal gene clusters, preferentially sensitive to nucleases, might be obscured from those complexed in an inactive conformation.

We have further studied the chromatin structure in cells of a higher plant, characterizing the tomato ribosomal chromatin. Southern blots of micrococcal nuclease digests hybridized with a probe from the rDNA coding region show a ladder typical for DNA folded into nucleosomes. The existence of a minor gene population within the rDNA clusters, present in an open structure, was tested by psoralen crosslinking isolated nuclei. The f band represented ≈80% of the total hybridization signal, indicating that the majority of the ribosomal genes are folded into canonical nucleosomes, similar to inactive DNA. These findings confirmed the results we previously obtained with micrococcal nuclease, but we have now also shown the existence of a minor portion of ribosomal genes (≈20%), which are complexed in a more open chro-

matin conformation. Our interpretation of the present data, by analogy with the results previously found in Friend cells (14), is that in higher plants active (or potentially active) ribosomal genes may be present in an open chromatin conformation. The absence of nucleosomes in the active chromatin, as judged by psoralen-crosslinking, does not necessarily imply the absence of histone proteins (13, 18, 25), and histones were previously reported to be present within coding regions of active ribosomal genes (9).

The Two Ribosomal Chromatin Populations Are Present with a Similar Proportion in Leaf and Exponentially Growing Suspension Cells. rRNA synthesis is regulated in accordance with the rate of cellular growth (for review, see ref. 31). In mouse Friend cells, after psoralen crosslinking, the relative intensity of the two bands in a doublet, corresponding to active (s band) and inactive (f band) chromatin, was the same whether the nuclei were from exponentially growing cells or from stationary cells. Therefore, despite a difference in rRNA synthesis, the proportion of the active versus inactive chromatin did not change (14).

We investigated the chromatin structure of tomato ribosomal genes in two different cellular states—stationary leaf cells and dividing cell suspension cultures, which undergo cell division about every 2 days. With the micrococcal nuclease assay, no difference was noted in the ribosomal chromatin between leaves and cell cultures. This was further confirmed by the psoralen technique since the relative proportional intensity of the s and f bands within the doublet did not change. As previously found in Friend cells, our data suggest that the open chromatin structure is maintained independently of transcription, perhaps by the presence of quiescent transcription machinery. Earlier work showed that the level of chromatin-bound RNA polymerases I and II was constant during the cell cycle (32) and that RNA polymerase I was found bound to the nucleolus organizer during mitosis (33).

Altogether, our results on the chromatin structure of tomato ribosomal genes suggest that while the structural organization is similar to those found in lower eukaryotes (11–13) and mouse (7, 14), the proportion of the ribosomal gene clusters in open and closed conformations is quite different. Since the proportions of the s and f bands within the doublet are unchanged among dividing and nondividing cells, our results also suggest the existence of a pre-open chromatin conformation (for review, see ref. 34). It is possible that ribosomal gene activity in plants, as in animals, is correlated with chromatin structures that may determine the maximal RNA polymerase I loading.

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