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Size and Structure of Replicating Mitochondrial DNA in Cultured Tobacco Cells

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The BY-2 tobacco cell line was used to study the size and structure of replicating mitochondrial DNA (mtDNA). Approximately 70 to 90% of the newly synthesized mtDNA did not migrate during pulsed-field gel electrophoresis. Moving pictures of the fluorescently labeled molecules showed that most of the immobile well-bound DNA was in structures larger than the size of the BY-2 mitochondrial genome of ~270 kb. Most of the structures appeared as complex forms with multiple DNA fibers. The sizes of the circular molecules that were also observed ranged continuously from ~20 to 560 kb without prominent size classes. Pulse-chase and mung bean nuclease experiments showed that the well-bound DNA contained single-stranded regions and was converted to linear molecules of between 50 and 150 kb. MtDNA replication in plants may be initiated by recombination events that create branched structures of multigenomic concatemers that are then processed to 50- to 150-kb subgenomic fragments.

INTRODUCTION

It is likely that the structure of the mitochondrial genome profoundly influences the way it participates in its three functions: gene expression, DNA replication, and heredity. Information about the structure of this genome in vivo is therefore needed if one is to formulate accurate models for how these functions are conducted. We currently know little about the structure of most DNA molecules within mitochondria and almost nothing about their replication. This holds true not only for plants but also for most organisms other than metazoan animals (reviewed in Bendich, 1993).

The inheritance of mitochondrial (and plastid) genes appears paradoxical. Physical estimates of the ploidy obtained by dividing the mass of organellar DNA by the size of the organelle genome give a large number, whereas estimates obtained from the segregation of genetic markers give a small number (reviewed in Gillham, 1994). Thus, neither the structure nor the molecular weight of the segregating unit in mitochondria or plastids is known. DNA replication can be considered at two levels: at and near the origin of replication (a fine scale of structure) and at the level of the entire mitochondrial genome. Some research on the replication of the plant mitochondrial genome at the fine scale has been conducted (deHaas et al., 1991). Knowledge of the overall structure of replicating molecules in vivo, however, may resolve the ploidy paradox if newly replicated mitochondrial DNA (mtDNA) molecules are the vehicles of inheritance.

Although circular mtDNA molecules have been identified for some plants, these are almost always smaller than the size of the genome, always rare for intact plant tissues, and abundant only in some cultured plant cell lines (Bendich, 1993). When mtDNA is extracted by in-gel procedures and fractionated by pulsed-field gel electrophoresis (PFGE), the DNA that migrates in the gel is found in linear form at ~50 to 150 kb in size and much longer linear molecules, but much (or most) remains at the origin of electrophoresis as an immobile well-bound form of mtDNA (Bendich, 1993; Narayanan et al., 1993; André and Walbot, 1995; Muise and Hauswirth, 1995). Because restriction fragment mapping predicts a circular form for the genome and its subgenomic fractions, the immobility of mtDNA has usually been ascribed to relaxed circular molecules trapped by agarose fibers, or the well-bound mtDNA is simply ignored.

We conducted pulse–chase experiments with ^{3}H-thymidine to study mtDNA synthesis in cultured tobacco cells. We found that the newly synthesized mtDNA is primarily in the well-bound fraction and not in linear or circular forms that migrate into the gel. By using fluorescence microscopy to create moving pictures of DNA, we found that most of the well-bound mtDNA is present in complex structural forms larger than 270 kb. This is the size of the BY-2 mitochondrial genome (Satoh et al., 1993). Some of these structures of enormous molecular weight might represent segregating genetic units in mitochondria.

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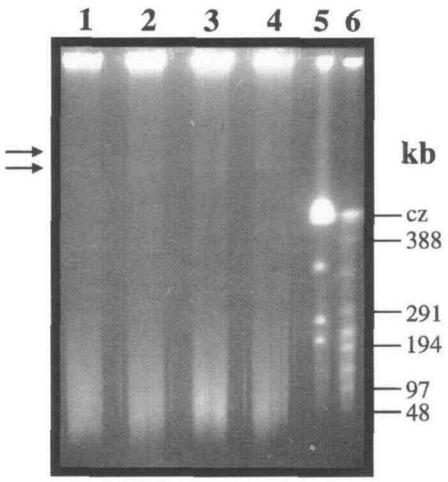

Figure 1. Size Fractionation of BY-2 MtDNA Labeled with ^{3}H-Thymidine at Varying Times after Subculture.

Shown is an EtBr-stained gel of mtDNA labeled with ^{3}H-thymidine for 4 hr before to harvesting at 12 (lane 1, sample t1), 26 (lane 2, t2), 50 (lane 3, t3), and 74 hr (lane 4, t4). Two faint bands representing circular mtDNA are indicated by arrows. The position of the compression zone (cz) is indicated. Each lane was sliced into 18 pieces, and the amount of ^{3}H incorporation is reported in Table 1. The chromosomes of yeast strain YNN295 (lane 5) and λ DNA concatemers (lane 6) served as length (in kilobases) markers.

RESULTS

^{3}H-Thymidine Labeling

MtDNA synthesis was monitored by giving a pulse of ^{3}H-thymidine for 4 hr to BY-2 tobacco cells that had been grown for 8 to 70 hr after transfer to new medium. When mtDNA from these preparations was fractionated by PFGE and analyzed by ethidium bromide (EtBr) fluorescence (Figure 1), most of the mtDNA was found as an immobile fraction at the gel origin and as a smear of linear DNA between 50 and 150 or 200 kb (henceforth termed 50 to 150 kb). The amount of DNA based on the intensity of EtBr fluorescence was estimated as between 87 and 96% for the well-bound fractions and 4 to 13% for the 50- to 150-kb fractions (see Methods). One or usually two faint bands between the well and the compression zone, representing circular DNA (see below), were present, and sometimes a faint band of DNA was observed at the compression zone, depending on how much mtDNA was loaded.

To determine which PFGE fractions contained newly synthesized mtDNA and possible replication intermediates, the PFGE-fractionated ^{3}H-mtDNA from the gel shown in Figure 1 was assayed by scintillation counting. Most of the radioactivity was found in the well-bound fraction (slice 1) of the gel (Table 1). Of the mobile mtDNA fractions, the highest percentage of label was observed in the 50- to 150-kb fraction (slices 15 and 16) for all of the cultures regardless of age. The amount of ^{3}H-thymidine incorporated in both the compression zone (slice 8) and the bands of circular DNA (slice 5) that migrated between the well and the compression zone represented <1% of the total incorporation. Therefore, the forms of mtDNA in these fractions are probably not major ones involved in mtDNA

Table 1. *Time Course of* ^{3}H-Thymidine Incorporation into BY-2 Tobacco Cell MtDNA

				Percentage of ^{3}H[a]				
Sample[b]	Time (hr)[c]	g Cells/L	Total ^{3}H[d] (cpm)	Well Slice 1	Below Well Slice 2	Circles Slice 5	cz[e] Slice 8	50 to 150 kb Slices 15 and 16
t1	12	40	4270	87	2.4	0.5	0.3	3.9
t2	26	41	5510	89	1.5	0.4	0.2	4.2
t3	50	41	5160	92	1.2	0.3	0.2	3.0
t4	74	47	2100	92	0.9	0.4	0.1	2.7

[a] The percentage of ^{3}H incorporation for each slice was determined by (cpm per slice/total cpm of all slices) × 100. The slice number, corresponding PFGE fraction, and percentage of ^{3}H incorporation for each sample are given.

[b] BY-2 cell cultures were labeled with 50 μCi of ^{3}H-thymidine at various times after transfer to new medium. Samples correspond to those in Figure 1. Each lane from the gel shown in Figure 1 was cut into 18 pieces from top (Well, slice 1) to bottom (slice 18), and the ^{3}H-labeled mtDNA in each slice was determined.

[c] The length of total growth period for each culture is given. ^{3}H-thymidine was added, and growth continued for 4 hr before harvest. For example, culture t1 was grown for 8 hr, 50 μCi of ^{3}H-thymidine was added, and cells were harvested after an additional 4 hr. Total growth time was 12 hr.

[d] Total ^{3}H incorporation into mtDNA was determined for each lane shown in Figure 1 by summing the counts per minute (cpm) from all slices (slices 1 to 18).

[e] cz, compression zone.

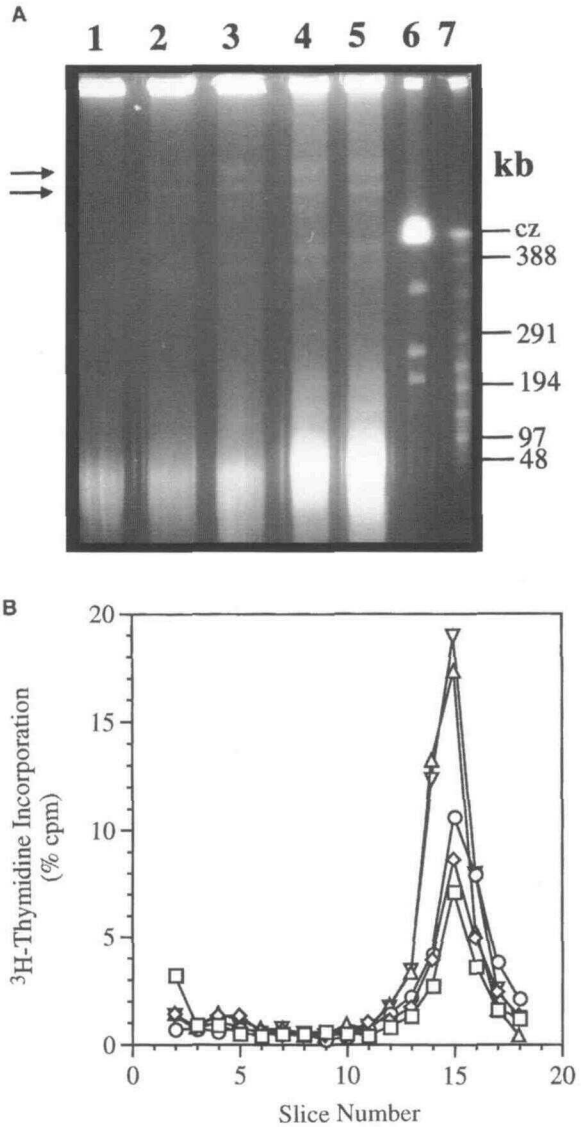

Figure 2. Size Fractionation of Pulse, Pulse–Chase, and Steady State ^{3}H-Thymidine–Labeled BY-2 MtDNA.

(A) EtBr-stained gel. Sample P3 was pulse labeled for 2 hr before harvest (lane 1). Samples PC4 (lane 2), PC5 (lane 3), and PC6 (lane 4) were labeled for 2 hr, and then unlabeled thymidine was added. Growth continued for 12, 24, and 168 hr, respectively, before harvest. Sample SS2 was grown for 170 hr with ^{3}H-thymidine (lane 5). Two faint bands representing circular mtDNA are indicated by arrows. The position of the compression zone (cz) is indicated. The chromosomes of yeast strain YNN295 (lane 6) and λ DNA concatemers (lane 7) served as length (in kilobases) markers.

(B) The percentages of the ^{3}H-labeled mtDNA that migrated into the gel for each culture are shown: P3 (□), PC4 (◇), PC5 (o), PC6 (△), and SS2 (▽). The well-bound (slice 1) fraction is omitted from this graph. Each lane was sliced into 18 pieces, and the amount of ^{3}H incorporation was determined (summarized in Table 2). The percentage of ^{3}H-thymidine incorporated is given as (cpm per slice/sum of cpm for all slices) × 100.

replication. Some ^{3}H-thymidine labeling was found in the fractions that migrated a short distance into the gel directly below the well (slice 2). This fraction may represent newly synthesized DNA that contains replication forks, because branching is known to impede DNA migration in agarose gels (Brewer and Fangman, 1987).

The ^{3}H-thymidine labeling time course showed that mtDNA synthesis had begun by 8 to 12 hr after transferring the cells to new medium and reached a maximum level by 26 hr after subculture (Table 1). Between 26 and 50 hr, the amount of synthesis per culture was similar and then decreased by 74 hr, as indicated by total ^{3}H incorporation into mtDNA. In a separate experiment, cells were pulse labeled with ^{3}H-thymidine after 120 hr of growth (mid to late log phase), and the incorporation showed that synthesis continued at a low level even in older cultures, as demonstrated previously by Suzuki et al. (1992).

Because mtDNA synthesis had begun by 12 hr after subculture, this time was chosen for pulse–chase labeling of the BY-2 mtDNA. Cells were also incubated for 170 hr with ^{3}H-thymidine to give steady state labeling. The EtBr-stained gel (Figure 2A) showed that most of the mtDNA remained in the well and that the largest amount of DNA migrating from the well was present as 50- to 150-kb linear DNA. The amount of mtDNA loaded onto the gel was greater for the older cultures due to more cells yielding more mitochondria per agarose slice. Although bands of mtDNA between the well and compression zone and in the compression zone were more prominent for the older cultures, the pattern of mtDNA migration for the different samples was similar.

The distribution of radioactivity in all of the DNA that migrated in the gel is shown in Figure 2B; that for selected gel fractions is presented in Table 2. The percentage of immobile, well-bound ^{3}H-mtDNA was highest with the pulse-labeled sample (74%) and decreased with the duration of the chase period to 50% (slice 1). Conversely, the percentage of radioactivity in the 50- to 150-kb region increased during the chase period (slices 14 to 16). Two other mobile fractions, the compression zone (slice 7) and circles (slices 4 and 5), showed no systematic change with time exposed to unlabeled thymidine. The amount of labeled mtDNA that migrated directly below the well (slice 2) was greater by 2.4- to 4.6-fold in the pulse sample than in the pulse–chase samples. These results suggest a precursor–product relationship with respect to mtDNA synthesis between the well-bound and the 50- to 150-kb fractions. They also show that neither the two discrete bands of circular molecules (in slices 4 and 5) nor the simple linear molecules of the compression zone (slice 7) are preferentially labeled forms of mtDNA. When the pulse and chase were given 24 hr after subculturing instead of 12 hr, similar results were obtained, with a decrease in the radiolabeled well-bound fraction and an increase in the radiolabeled 50- to 150-kb fraction as the chase time increased.

Our results show a rapid incorporation of ^{3}H-thymidine into tobacco cell mtDNA shortly after subculturing the cells so that 74 to 87% of the label was in the well-bound fraction, probably

Table 2. Pulse, Pulse–Chase, and Steady State Labeling of BY-2 Tobacco Cell MtDNA with ^{3}H-Thymidine

					Percentage of ^{3}H[a]				
Sample[b]	Chase[c] Time (hr)	Time[d] Harvested (hr)	g Cells/L	Total ^{3}H[e] (cpm)	Well Slice 1	Below Well Slice 2	Circles Slices 4 and 5	cz[f] Slice 7	50 to 150 kb Slices 14 to 16
P3	0	12	57	4,970	74	3.2	1.3	0.5	13
PC4	12	14	51	7,280	69	1.4	2.5	0.4	17
PC5	24	36	61	13,280	63	0.7	1.2	0.4	23
PC6	168	180	280	4,040	50	1.3	2.3	0.6	36
SS2	0	180	300	6,870	46	1.2	1.7	0.7	39

[a] The percentage of ^{3}H incorporation for each slice was determined by (cpm per slice/total cpm of all slices) × 100. The slice number, corresponding PFGE fraction, and percentage of ^{3}H incorporation for each sample are given.

[b] BY-2 cell cultures were labeled with 100 μCi of ^{3}H-thymidine. Samples correspond to those in Figure 2. Each lane from the gel shown in Figure 2A was cut into 18 pieces from top (Well, slice 1) to bottom (slice 18), and ^{3}H-labeled mtDNA in each slice was determined.

[c] The chase samples were grown for 2 hr with ^{3}H-thymidine before a 200-fold excess of unlabeled thymidine was added; growth continued for the time period shown. The pulse sample was grown for 2 hr with ^{3}H-thymidine and then harvested. The steady state sample was grown for 170 hr in ^{3}H-thymidine.

[d] Total length of time each culture was grown before harvesting, including time grown in the presence of ^{3}H-thymidine and/or unlabeled thymidine.

[e] Total ^{3}H incorporation into mtDNA was determined for each lane shown in Figure 2A by summing the counts per minute from all slices (slices 1 to 18).

[f] cz, compression zone.

due to a resumption of replication. With the steady state culture, 39% of the ^{3}H label was found in the linear 50- to 150-kb fraction but ~46% remained in the well. With the older steady state culture, some of the ^{3}H incorporation may have been due to degradation and resynthesis (turnover) of the mtDNA.

Mung Bean Nuclease Digestion to Analyze Single-Stranded DNA

There are several explanations for the immobile (well-bound) fraction after PFGE: an artifact created during preparation of the agarose slices, simple circular or rolling circular forms caught on agarose fibers, or large branched structures at recombination junctions or replication forks that may or may not contain single-stranded regions. Maleszka (1993) showed that well-bound yeast mtDNA contains single-stranded regions by digestion with the single strand–specific mung bean nuclease (MBN) and by filling in single-stranded regions with the Klenow fragment of DNA polymerase I. With the MBN treatment, a decrease in the well-bound DNA and an increase in the mobile DNA fraction, specifically the 50- to 150-kb region, were observed. We chose MBN to probe for single-stranded regions within the well-bound fraction of the BY-2 mtDNA.

BY-2 mtDNA incubated with five units of MBN for 0 to 60 min showed a gradual decrease in the amount of EtBr-stained, well-bound DNA and a concomitant increase in the 50- to 150-kb fraction with increasing incubation time (Figure 3). In addition, the size range of the DNA in this region appeared to decrease with increasing digestion time, suggesting that the mobile linear 50- to 150-kb DNA may contain single-stranded

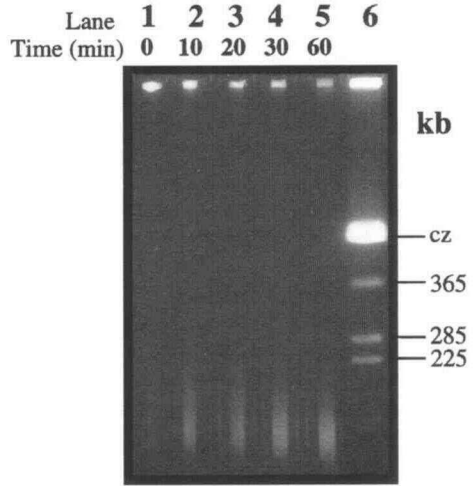

Figure 3. Size Fractionation of BY-2 MtDNA Digested with MBN.

BY-2 mtDNA–embedded agarose slices were digested with 5 units of MBN for 0 to 60 min as shown. The well-bound EtBr fluorescence decreases with increasing incubation time (from 63% to 42, 33, 30, and 27% for 0, 10, 20, 30, and 60 min of MBN digestion, respectively), and a corresponding increase in the 50- to 150-kb fluorescence is seen (from 37% to 56, 67, 70, and 73%, respectively). The chromosomes of yeast strain YNN295 (lane 6) served as length markers. cz, compression zone.

regions that retard its migration in the gel. When five different mtDNA preparations were treated with five units of MBN for 30 min, the EtBr fluorescence of the well-bound fraction decreased between 26 and 44%.

Although MBN is considered specific for single-stranded DNA (ssDNA), some double-stranded DNA (dsDNA) degradation can occur with large amounts of enzyme or with prolonged incubation time. Conditions for digestion with MBN were tested with native and denatured λ DNA and with native yeast chromosomal DNA embedded in agarose. For λ, ssDNA was completely digested, and the gel mobility of dsDNA was unaffected except at very high MBN concentrations (see Methods). The bands of linear dsDNA molecules of the three smallest yeast chromosomes appeared unaffected by the enzyme (Figure 4A). The slight increase in background fluorescent smear near these chromosome bands may have been due either to single-stranded regions in chromosomal DNA molecules trapped in the well because they were undergoing replication (Hennessy et al., 1991; Kelley et al., 1993) or to well-bound mtDNA in these total yeast cell DNA preparations. We conclude that MBN has little or no effect on simple, linear dsDNA molecules under the conditions used for digestion of BY-2 mtDNA embedded in agarose.

One of the ^{3}H-thymidine–labeled BY-2 mtDNA samples was treated with 5 units of MBN for 0, 30, or 60 min (Figure 4A). The BY-2 mtDNA lanes were sliced, and the amount of ^{3}H in each piece was determined. The amount of ^{3}H in the well decreased from 89% without MBN to 56 and 49% with a 30- or 60-min incubation with MBN, respectively. The highest increase of label following MBN treatment was in the 50- to 150-kb mobile fraction (Figure 4B). However, a general increase ranging from 0.5 to 3% was observed throughout the region between the well and the compression zone. The EtBr-stained gel showed an increase in the background fluorescence throughout the gel; again, the most intense increase was in the 50- to 150-kb region extending up to ~250 kb. Faint bands between the well and the compression zone (circular mtDNA) and in the compression zone were observed. The intensities of these bands did not decrease with MBN treatment but instead appeared to increase, again confirming that the MBN was acting only on ssDNA and not dsDNA. These results further support the idea that the 50- to 150-kb fraction is derived from the well-bound fraction and suggest that the DNA in the compression zone and the band(s) between the well and the compression zone may be derived from the well-bound material.

Moving Pictures of Fluorescently Labeled mtDNA

Fluorescence microscopy has been used to create moving pictures (Movies) of EtBr-stained DNA during electrophoresis for the purpose of investigating the structure of the mitochondrial genome (Bendich and Smith, 1990; Bendich, 1996). We used this method to determine the size and structural features of individual molecules in sample plugs taken from various regions of gels after PFGE. Measurements were made of the length

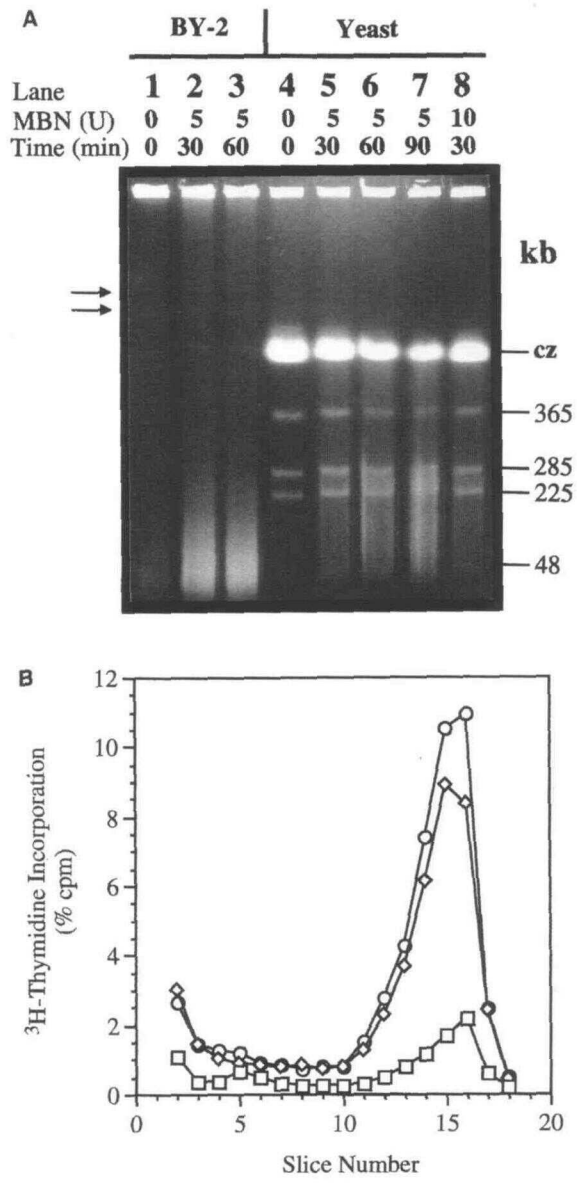

Figure 4. Size Fractionation of BY-2 MtDNA Labeled with ^{3}H-Thymidine and Yeast Chromosomal DNA Digested with MBN.

(A) EtBr-stained gel of mtDNA without MBN treatment (lane 1) and digested with 5 units (U) of MBN for 30 min (lane 2) and 60 min (lane 3), and yeast spheroplasts without MBN treatment (lane 4) and digested with 5 units of MBN for 30 min (lane 5), 60 min (lane 6), and 90 min (lane 7) and with 10 units for 30 min (lane 8). Two bands of circular mtDNA are indicated by arrows, and the position of the compression zone (cz) is shown. For the ^{3}H-labeled mtDNA, each lane was sliced into 18 pieces, and the amount of ^{3}H incorporation was determined. **(B)** The percentage of ^{3}H in the mobile fractions of mtDNA before (□) and 30 min (◇) or 60 min (o) after MBN treatment. Slice 5 corresponds to the band of circular mtDNA, slice 7 corresponds to mtDNA in the compression zone, and slices 14 to 16 correspond to the 50- to 150-kb region. The percentage of ^{3}H-thymidine incorporation is given as (cpm per slice/sum of cpm for all slices) × 100.

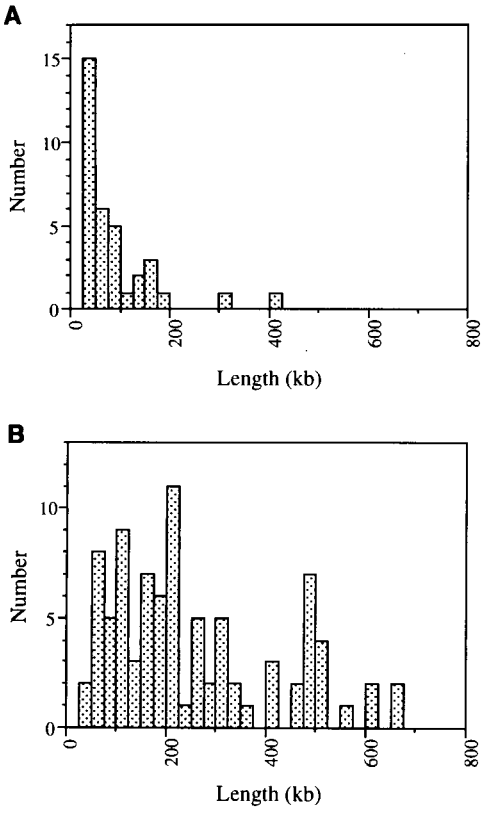

Figure 5. Size Distribution of BY-2 MtDNA Molecules from the 50- to 150-kb and Compression Zone Regions.

(A) The sizes of linear molecules from the region of the gel where linear DNA of ~50 to 150 kb migrates were determined by analyzing Movies and are grouped in intervals of 25 kb. MtDNA samples from BY-2 cultures grown for 1, 4, and 8 days were analyzed, and the data were combined. The mean length in kilobases with standard deviation, number of molecules (*n*) measured, and the size range for each sample are 87 ± 41 (*n* = 8), 28 to 150 kb for 1 day; 159 ± 137 (*n* = 8), 46 to 420 kb for 4 days; and 54 ± 38 (*n* = 18), 26 to 180 kb for 8 days.

(B) The sizes of linear molecules from the compression zone were determined by analyzing Movies and are grouped in intervals of 25 kb. MtDNA samples from BY-2 cultures grown for 1, 4, and 8 days were analyzed, and the data were combined. The mean length in kilobases with standard deviation, number of molecules (*n*) measured, and the size range for each sample are 194 ± 126 (*n* = 18), 40 to 500 kb for 1 day; 168 ± 120 (*n* = 10), 50 to 510 kb for 4 days; and 274 ± 174 (*n* = 64), 40 to 670 kb for 8 days. No visible fluorescent band of DNA was observed in the compression zone for any of the three samples.

of the fluorescent fibers on a video monitor as the DNA was stretched from impediments (agarose fibers) during its movement toward the anode. BY-2 mtDNA preparations from 1-day (lag phase), 4-day (early log phase), and 8-day (late log phase) cultures were compared by using this technique. Overall, no systematic differences were found as a function of the age of the culture. The size distributions of DNA fibers from these three mtDNA preparations are shown in Figures 5 and 6.

The Mobile DNA Fractions

Of the DNA that migrated into the gel, two classes of molecules were observed: linear molecules in and below the compres-

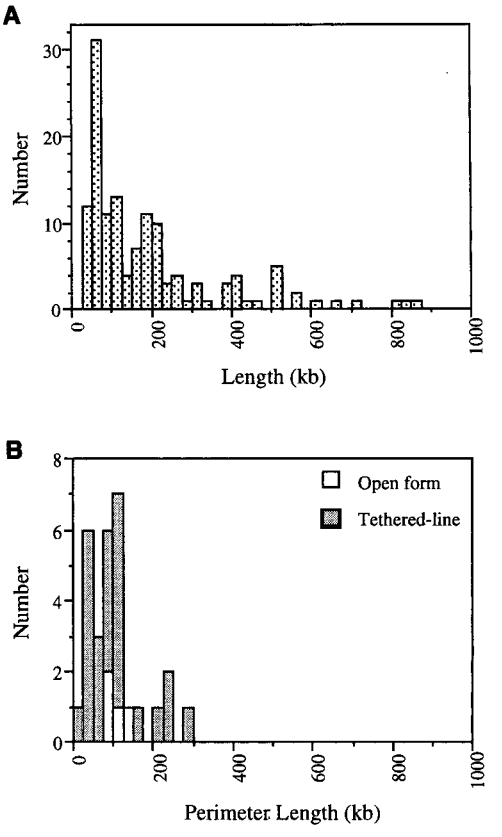

Figure 6. Size Distribution of Linear and Circular Molecules of BY-2 MtDNA from the Well-Bound Fraction.

(A) The sizes of linear molecules not associated with complex structures were measured by analyzing Movies of the well-bound DNA and are grouped in intervals of 25 kb. MtDNA samples from BY-2 cultures grown for 1, 4, and 8 days were analyzed, and the data were combined. The mean length in kilobases with standard deviation, number of molecules (*n*) measured, and the size range for each sample are 174 ± 155 (*n* = 67), 30 to 800 kb for 1 day; 246 ± 237 (*n* = 41), 34 to 870 kb for 4 days; and 154 ± 114 (*n* = 26), 50 to 500 kb for 8 days.

(B) The sizes of circular molecules in the well-bound fraction were determined by measuring the perimeter lengths in Movies and are grouped in intervals of 25 kb. Open form and tethered-line circles are represented by open and closed bars, respectively. MtDNA samples from BY-2 cultures grown for 1, 4, and 8 days were analyzed, and data were combined. The mean length in kilobases with standard deviation, number of molecules (*n*) measured, and the size range for each sample are 152 ± 94 (*n* = 9), 24 to 280 kb for 1 day; 79 ± 36 (*n* = 13), 30 to 160 kb for 4 days; and 86 ± 36 (*n* = 8), 44 to 140 kb for 8 days.

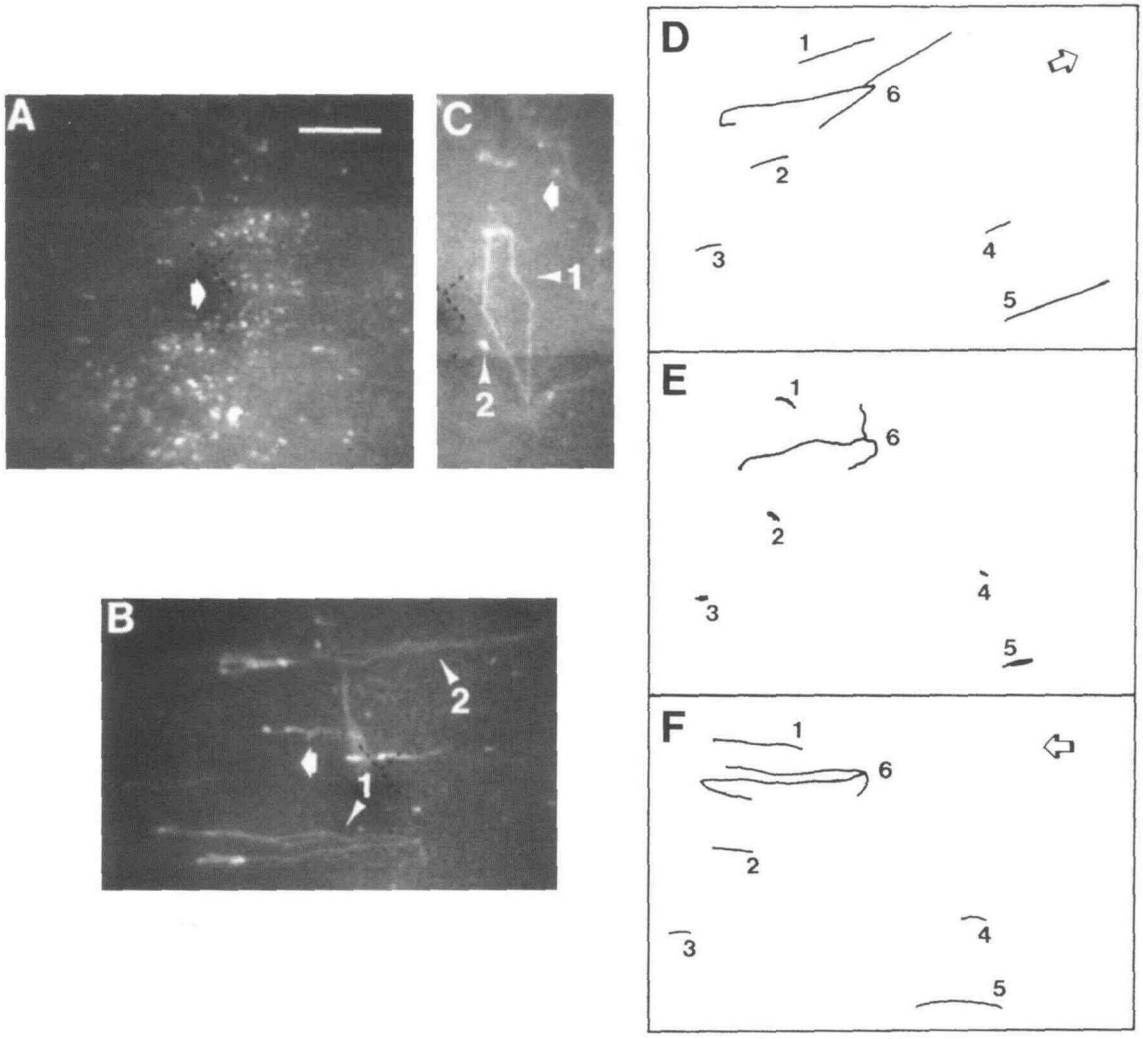

Figure 7. MtDNA Molecules from the PFGE Mobile Fractions.

(A) EtBr-stained mtDNA molecules from the 50- to 150-kb region. Many linear molecules of ~27 to 54 kb, as well as some larger molecules, are shown.
(B) EtBr-stained mtDNA molecules from the compression zone. The sizes of molecules 1 and 2 were measured as 120 μm (360 kb) and 170 μm (510 kb), respectively, although they would be longer if the molecules were fully stretched (see Methods).
(C) EtBr-stained mtDNA molecules from between the well and the compression zone. Molecule 1 is an open form circle with a perimeter length of 93 μm (280 kb). Molecule 2 was moving when the image was recorded.
(D) to **(F)** EtBr-stained mtDNA molecules from between the well and the compression zone. Drawings were made by tracing the photographs taken of the EtBr-stained DNA on the video monitor or by tracing the images directly from the monitor. Molecules 1 to 5 are tethered-line circles that pivoted around a fixed point when the polarity of the electric field was reversed. The perimeter length (twice the length of the tethered line) of these circles ranges from 13 μm (40 kb) for molecule 3 to 53 μm (160 kb) for molecule 5. Molecule 6 is a Y-form structure. The combined length of the three arms measured in **(D)** is 86 μm (260 kb).
In **(A)** to **(D)** and **(F)**, the direction of DNA migration (toward the anode) is indicated by the arrows. **(E)** is in the absence of an electric field. The bar in **(A)** = 20 μm. The photographs and tracings are all at the same magnification.

sion zone and circular molecules between the well and the compression zone. Linear molecules constantly condense and stretch as they bunch up at and move around agarose fibers during migration through the gel toward the anode under an applied electric field. Figure 7A shows a representative field of EtBr-stained DNA from the 50- to 150-kb region. The size of linear DNA molecules from this region varied from 26 to 420 kb (Figure 5A). Approximately 90% of the molecules were between ~26 and 180 kb, as expected for this region of the gel. However, on one slide a few molecules >180 kb were found.

No obvious circular molecules were observed for the 50- to 150-kb region.

The compression zone contains linear DNA molecules too large to be separated on the basis of size, and the sizes found there depend on the PFGE conditions used. Linear molecules ≥400 kb should be found in the compression zone, as shown by the DNA size markers, although some molecules were found that measured <400 kb. Figure 7B shows two large linear molecules that have been partially stretched as they migrated through the gel. DNA between 400 and 670 kb (Figure 5B) was present in this region, which is 1.5 to 2.5 times the size of the mitochondrial genome in BY-2 cells. These large linear molecules may be composed of more than one complete genome or contain multiple copies of only segments of the genome.

A faint smear of EtBr fluorescence was present in the region between the well and the compression zone that is forbidden to simple linear molecules. Approximately 50% or more of the molecules from this region were found to be circular, and the rest appeared to be linear. Circular molecules were identified as either of two types: open forms caught on more than one agarose fiber (Figure 7C), or tethered-line forms appearing to be hooked on a single agarose fiber at one end and pivoting about that anchor point when the polarity of the field was reversed (molecules 1 to 5 in Figures 7D to 7F). Occasionally, as the polarity was reversed, a tethered line would open. The circular molecules found between the well and compression zone were heterogeneous in size: 30 to 300 kb for open form circles and 20 to 560 kb for tethered lines (Figure 8). Most of the circular DNA was smaller than the BY-2 mitochondrial genome size of 270 kb, but a few molecules were seen within the range expected for a genome-size circle (250 to 300 kb), and some were greater than genome size (Figure 8). A few open circles appeared to be lariat forms and may have been rolling circle structures, although the linear segment in most cases was small relative to the size of the circle. The amount of DNA in this region usually represented <1 to 3% of the ^{3}H-thymidine–labeled mtDNA.

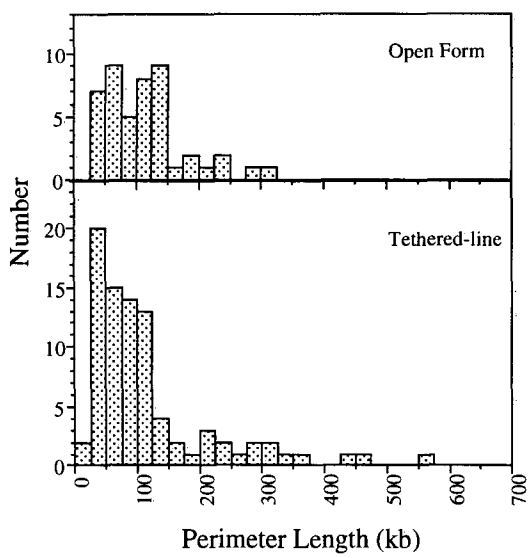

Figure 8. Size Distribution of Open Form and Tethered-Line Circles of BY-2 MtDNA from the Region between the Well and the Compression Zone.

The sizes of circular molecules that migrated between the well and the compression zone were determined by analyzing Movies and are grouped in intervals of 25 kb. Movies were made of mtDNA from an 8-day culture. The perimeter length of the circles was measured. The number of molecules measured (*n*) and the mean length and standard deviation are n = 46, 111 ± 65 kb for open form circles and n = 86, 117 ± 105 kb for tethered-line circles. Size estimates for circles <30 kb are imprecise because a tethered-line circle of this size appears as a wiggling line of only 15 mm on our video monitor.
(Top) Open form circles.
(Bottom) Tethered-line circles.

Most of the linear molecules measured in the region between the well and compression zone were small, ≤100 kb. Linear molecules were scored as DNA able to move freely through the agarose matrix or the thin liquid layer between the coverslip and the agarose. Some of the molecules scored as linear may actually have been circles not hooked on agarose fibers, because linear molecules ≤100 kb would migrate much farther into the gel. Other structures, such as Y, X, or H forms or multibranched molecules, may migrate to the region between the well and compression zone. The mobilities of these forms would be lower than those of simple linear molecules of equal molecular weight (Brewer and Fangman, 1987). In fact, a few Y-form structures were observed here (molecule 6 in Figures 7D to 7F).

The Well-Bound Fraction

Approximately 60 to 90% of the mtDNA detected by either EtBr fluorescence or ^{3}H-thymidine labeling was found in the well-bound fraction. Examination of this material by fluorescence microscopy revealed that most of the DNA was still immobile in the agarose matrix. Although mobile DNA was found, it is likely that this DNA had been released during melting and spreading of the sample on the microscope slide. We observed that most of the DNA was associated with complex structures. Some of these complexes appeared to be composed of one or more DNA fibers associated with a bright fluorescent spot of compact DNA. Both simple linear and circular molecules were also found in this fraction.

The circular DNA observed was ≤300 kb, with an average of 100 to 150 kb, so that although genome-sized circles were found, most circular DNA was less than genome size (Figure 6B; molecule 1 in Figure 9A). The linear molecules found in the well-bound DNA varied in size (Figure 6A). Molecules of 800 to 900 kb were observed, which would be equivalent in size to approximately three genome copies. Some of the lin-

ear molecules remained immobile, as shown in Figure 9A. However, possible connections with other immobilizing structures in this image cannot be ruled out.

Most of the BY-2 mtDNA was found in complex structures (Figures 9 and 10). These complexes were immobile in the gel matrix, but the associated fibers usually changed direction when the polarity of the electric field was reversed and remained attached to the central compact region. One structural type, termed a starburst (Figures 9A to 9C and 9E to 9G), had a dense core of fluorescence with several fibers of DNA emerging from this central core and was identified previously in well-bound mtDNA from other plants and fungi (Bendich, 1996). Occasionally we observed breakage of loops or other stretched fibers of DNA associated with the complexes, suggesting the presence of single-stranded regions (Figures 9C and 9E to 9G). Some of the fibers associated with these complexes were very large, 1 megabase or greater, although generally the size range was 200 to 1000 kb. When the concentration of mtDNA remaining in the well was high, few individual molecules could be distinguished. The DNA then appeared either as a tangled mass with some individual fibers of fluorescence discernible or as a clot of fluorescence without distinct fibers (molecules 3 and 4 in Figure 9A). Other complex structures were also observed, such as those appearing as whiskers (Figure 9D), a streamer (Figure 10A), and a comet (Figure 10B).

It is possible that all of the complex forms are variations of a common structural motif: duplex DNA fibers associated at foci arrayed along their length. The different appearances among the forms would depend on the density and distribution of such foci that, in turn, determine the length of fluorescent fiber emanating from each focus. Appearance may also depend on whether a complex is completely within the agarose (a comet) or at the surface near the liquid layer (a streamer) and/or the degree to which individual DNA fibers have been stretched and hooked on agarose fibers (a starburst or whiskers) during the spread of the molten agarose. The recombination-dependent replication mechanism used by bacteriophage T4 generates a many-stranded network of DNA molecules (Huberman, 1968; reviewed in Kreuzer and Morrical, 1994), and the mtDNA foci could similarly represent recombination junctions at which DNA synthesis is initiated.

Although several smaller forms of mtDNA molecules were identified among the various PFGE fractions, Movies revealed that most of the mass of total mtDNA was in molecular structures with a size >270 kb, the size of the mitochondrial genome in tobacco.

DISCUSSION

When DNA prepared by in-gel procedures is fractionated by PFGE, a substantial amount remains immobilized at the origin of the gel. Typically, this well-bound fraction is ignored when interpreting results because of the uncertainty as to the reason for its immobility. We used pulse–chase and direct visualization techniques to analyze the well-bound fraction of tobacco cell mtDNA and found that it contains most of the DNA obtained from mitochondria and most of the newly synthesized mtDNA and is structurally the most complex form of mtDNA. Furthermore, most of the mass of mtDNA is contained in molecular structures larger than the tobacco mitochondrial genome (~270 kb), leading to the conclusion that most mtDNA replication occurs on larger-than-genome-sized molecules. The linear DNA molecules found at the 50- to 150-kb region are probably the major product from these large replicating structures. The minor fraction of circular mtDNA molecules that did migrate into the gel was not rapidly labeled with ^{3}H-thymidine, suggesting that these simple circular forms (most of which are smaller than genome size) are not involved in the initiation of mtDNA replication.

The increase in ^{3}H label in the 50- to 150-kb (to 250-kb) region after MBN digestion further supports the theory that these molecules are derived from the well-bound, rapidly labeled mtDNA fraction. Gaps of ssDNA may occur between double-stranded regions of 50 to 250 kb and represent lagging strands at replication forks. MBN digestion of the ssDNA gaps would release the double-stranded regions from these replication structures, and the simple linear 50- to 150-kb (to 250-kb) molecules produced would then migrate into the gel. Although most of the dsDNA segments interspersed with single-stranded regions may be between 50 and 250 kb in the replicating structures, some double-stranded segments may be much larger (greater than genome size), and it is these segments that may be the source of the long linear molecules found in the compression zone.

MtDNA Replication from a Molecular Perspective

What do our results reveal about mtDNA replication in higher plants? It is unlikely that the replication of most of the mtDNA involves an expanding bubble (theta- or Cairns-form) mechanism as proposed for mammalian mtDNA (Clayton, 1982), because most of the DNA in the well-bound fraction was in complex structures larger than the genome size. Furthermore, the PFGE fractions that contained mainly circular mtDNA were not preferentially labeled after a pulse with ^{3}H-thymidine.

We suggest, instead, that the mechanism involves recombination events and multigenomic concatemers for the following reasons.

(1) Recombination-dependent replication is initiated by single-strand invasion of a homologous region on a duplex molecule, creating a replication fork (Kreuzer and Morrical, 1994). If mtDNA replication is a recombination-dependent process, an intermolecular recombination event occurring within a multigenomic concatemer could introduce a branch point, prevent migration in the gel, and open a single-stranded gap susceptible to MBN digestion. The complex structures, such

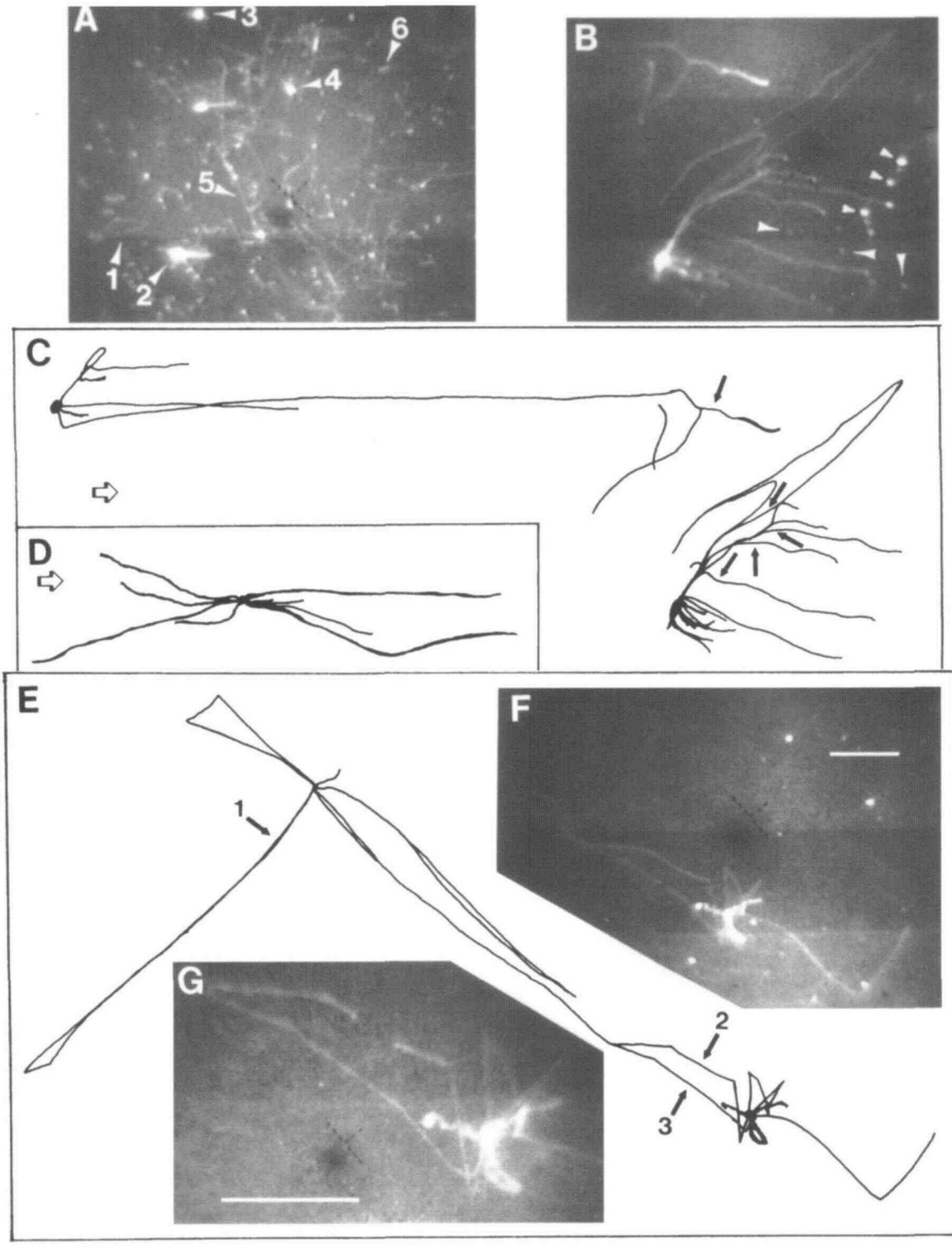

Figure 9. MtDNA Molecules from the PFGE Well-Bound Fraction.

(A) A representative field of DNA molecules in the absence of an electric field. Molecule 1 is an open form circle with a perimeter length of 30 μm (90 kb). Molecule 2 is a small starburst structure with some extended fibers emerging from the central core of fluorescence. Molecules 3 and 4 are clots of DNA with an indistinct "halo" of fibers. Molecule 5 is an immobile linear molecule. This molecule and most of the other extended linear molecules in this field did not migrate when an electric field was applied, although occasionally the ends of these immobile molecules would extend toward the anode. Arrow 6 indicates two small molecules that were fully mobile when an electric field was applied and thus are not considered to be circular. In other fields much longer, mobile molecules were common.

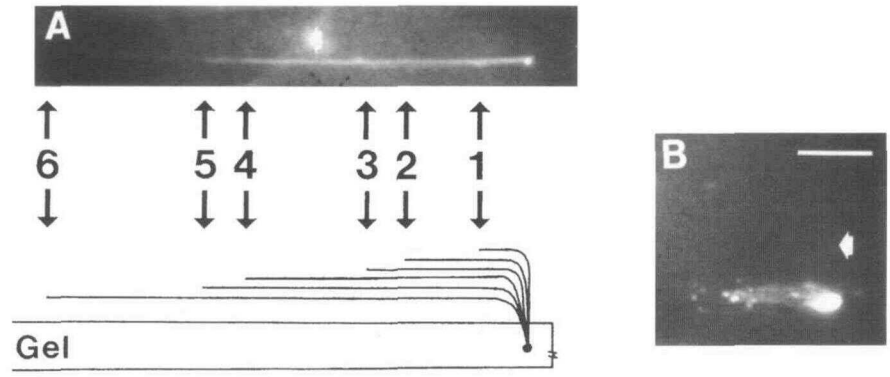

Figure 10. MtDNA Molecules from the PFGE Well-Bound Fraction.

(A) A complex molecule termed a streamer has a small, bright fluorescent head with several DNA fibers. The fibers are in the liquid layer between the surface of the agarose gel and the coverslip on the microscope slide. The head is embedded near the surface of the agarose, as depicted in the side-view drawing. This molecule contains six fibers between 13 μm (40 kb) and 130 μm (390 kb) that were observed to pivot around the head as the polarity was reversed.
(B) A complex structure called a comet is shown. DNA fibers emerged from the bright fluorescent head of the comet when an electric field was applied, and occasionally DNA fibers were observed to break from the comet's tail and move away toward the anode. The bar in **(B)** = 20 μm. The photographs are all at the same magnification. The direction of DNA migration (toward the anode) is indicated by the short, broad arrows.

as starbursts and comets, revealed in Movies of the well-bound fraction could contain branched DNA structures produced by multiple recombination events. This fraction also contained the highest amount of newly synthesized mtDNA.

(2) Rolling circle replication of the DNA from bacterial phages and plasmids is a recombination-dependent process (Viret et al., 1991). Maleszka et al. (1991) reported "lariat" structures by electron microscopy and proposed a rolling circle model for the replication of yeast mtDNA. If rolling circles participate in the replication of BY-2 mtDNA, such forms may be too large and/or complex to be mobile during electrophoresis and would be included in the rapidly labeled well-bound fraction. Although some mtDNA may replicate through a rolling circle mechanism, this may not be the only or predominant replication mechanism.

(3) We measured tethered-line circles that were between one and two times the size of the genome. These supergenomic circles have no obviously useful function, may be products of recombination events within multigenomic concatemers, and represent minor products incidental to the major replication process.

(4) The observation that circular mtDNA molecules can be obtained in appreciable yield from cell lines but not intact plant

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Figure 9. (continued).

(B) and **(C)** Photography and tracing of large starburst structures. In **(B)**, the large arrowheads indicate small DNA molecules that continued to move after this image was recorded. The DNA fibers at upper left in **(B)** extend beyond this field and are connected to a starburst structure, as shown by the tracing in **(C)**. This long DNA fiber is ~220 μm (660 kb) in length. The black arrows in **(C)** indicate points at which DNA fibers broke during continued observation of the field in **(B)**. The left-hand region of the DNA in **(C)** was irradiated for observation for a much shorter time. In **(B)**, the round objects (marked by small arrowheads) are not DNA molecules; they remained immobile upon repeated reversal of polarity of the electric field. They may be particles of EtBr that are found in some fields of view.
(D) A tracing from a photograph showing a complex structure described as whiskers. The long DNA fibers were associated with a central, bright fluorescent spot and did not reorient toward the anode when the electric field was reversed. However, some of the smaller fibers near the central spot did so move.
(E) to **(G)** Photography and tracing of a large starburst structure. **(E)** is a tracing of **(F)** that was extended to include the entire complex structure connected to the bright fluorescent core. Eighteen seconds after the image in **(F)** was taken, a single fiber was observed to break at the position indicated by arrow 1; the other fiber of this loop, however, remained intact. The upper DNA fiber (arrow 2) was observed to break 35 sec after image **(F)** was recorded. The breakage is shown in **(G)** at twice the magnification of **(F)**. The lower fiber (arrow 3) broke 11 sec later. The total amount of DNA in this single structure is ~2.8 megabases; this is equivalent to ~10 tobacco mitochondrial genomes. The bars in **(F)** and **(G)** = 20 μm. The photographs and tracings are all at the same magnification except for **(G)**, which is at twice this magnification. The direction of DNA migration (toward the anode) is indicated by the open arrows.

tissues had been attributed to two ideas (Bendich, 1985): many cells in culture divide, whereas meristematic cells are infrequent in whole-plant tissues; and circles are replicating forms of mtDNA. Currently, we believe that the increase in recombinational activity required for mtDNA replication in dividing cells explains the incidental generation of circles of any size.

The increase in EtBr fluorescence and ^{3}H-labeled DNA between the well and the compression zone following MBN treatment may have been due to release of branched structures having various mobilities due to differences in sizes and branch number and/or to small relaxed circles (<30 kb) released from rolling circle intermediates. Lockshon et al. (1995) described a yeast mutant with a number of unresolved recombination junctions (X and Y forms) in its mtDNA higher than that of wild type. They suggested that mtDNA forms multibranched recombination intermediates and that the lack of processing in the mutant strain influences the segregation of heritable units. A mechanism involving replication intermediates composed of branched multigenomic-sized concatemers that are processed to linear molecules of 50 to 150 kb could account for our results.

Backert et al. (1995) found an mtDNA hybridization signal at the compression zone when plant mtDNA in the gel was denatured by a short exposure to alkali before blotting or gel drying; however, when blotting was conducted for a long time under alkaline conditions, the signal disappeared. This result and ribonuclease experiments led them to conclude that the compression zone (but not the 40- to 200-kb) fraction was composed of "RNA molecules." Although it is possible that the long molecules in the compression zone for BY-2 cells, as well as other plants and fungi (Bendich and Smith, 1990; Bendich, 1996), contain ribonucleotides frequently distributed along their entire length, they always appear in Movies as DNA and not RNA molecules (Bendich, 1991; A.J. Bendich, unpublished data).

MtDNA Replication from a Cytological Perspective

The DNA within mitochondria can be visualized by fluorescence microscopy after staining with 4′,6-diamidino-2-phenylindole (DAPI; Kuroiwa, 1982). By comparing the fluorescence intensities of DAPI-stained mitochondrial nucleoids and T4 phage particles, the mass of DNA in individual nucleoids has been measured. For Arabidopsis, mtDNA replication is maximal in the lower part of the root tip meristem, declines in the middle and upper parts, and ceases in the more distal cells of the elongation zone (Fujie et al., 1993). The DNA content per mitochondrial nucleoid is highest in cells near the tip (~1100 and 700 kb for central cylinder and protoderm cells, respectively) and decreases rapidly to ~90 and 140 kb, respectively, for these cell types in the elongation zone within 1 mm from the tip. For geranium, the nucleoids of dividing cells contain as much as 3 and 2 to 14 megabases of DNA in root tip and ovule, respectively, decreasing to <170 and ~140 kb, respectively, in cells no longer replicating mtDNA in these two tissues (Kuroiwa et al., 1990, 1992). The size distribution of nucleoids in cultured BY-2 tobacco cells is broader (with sizes up to ~600 kb) for cells near the peak of mtDNA replication than for older cells no longer replicating mtDNA (mostly 120 to 200 kb; Satoh et al., 1993), and nucleoid size correlates with mtDNA synthesis in root tip cells of intact BY-2 plants (Suzuki et al., 1992). In nondividing epidermal cells of onion bulb leaves and internodal cells of the green alga *Nitella flexilis*, mitochondrial nucleoids contain ~70 kb of mtDNA (Nishibayashi and Kuroiwa, 1985; Fujie et al., 1993).

Because meristematic cells constitute only a small portion of bulk tissue used for extraction, most mtDNA would be derived from small-sized (~70 to 200 kb) nucleoids. If such nucleoids contained single linear DNA molecules, rather than several smaller subgenomic circular molecules (Kuroiwa et al., 1992; Satoh et al., 1993), then linear molecules should form a prominent smear at these sizes in PFGE, as we found (Figures 1, 2A, and 5A). We also found that the percentage of ^{3}H-thymidine–labeled mtDNA increased in the 50- to 150-kb fraction with increasing chase period. If the mass of mtDNA in the larger nucleoids also represented the molecular mass of individual molecules, then such replicating or newly replicated molecules might be found in the well-bound and/or compression zone fractions after PFGE. For the BY-2 cell cultures, we found linear molecules of 670 kb and fibers larger than 1 megabase associated with complex structures in the well-bound fraction (Figure 6A). In the compression zone, linear molecules >400 kb were observed, and Bendich (1996) previously reported mtDNA >1500 kb in this region for BY-2 cells.

To summarize, in plant tissues used for mtDNA extraction, most of the cells are not meristematic, are not engaged in mtDNA replication, and contain mitochondrial nucleoids with a mass of DNA similar to that for the linear molecules in the rapidly migrating PFGE fraction. The sizes of the longest individual mtDNA molecules from the compression zone and well-bound fractions are in the megabase range, similar in mass to nucleoid DNA in those cells most active in mtDNA replication. Thus, mtDNA replication in plants and fungi may involve a concatemeric array of genome-sized units that is cleaved to the ~50- to 150-kb mtDNA that we have found irrespective of genome size (Bendich, 1996).

METHODS

Organisms Referred to in Text

The common and scientific names for organisms referred to in the text are tobacco for *Nicotiana tabacum*, Arabidopsis for *Arabidopsis thaliana*, geranium for *Pelargonium zonale*, and onion for *Allium cepa*.

BY-2 Tobacco Cell Culture

The BY-2 tobacco cell line was derived from tobacco cultivar Bright Yellow 2 (Nagata et al., 1992). Suspension cell cultures were grown in Murashige and Skoog medium (Gibco BRL, Gaithersburg, MD) supplemented with 0.2 mg/L 2,4-dichlorophenoxyacetic acid. The cells

were subcultured for 10 to 14 days, using a 5% (for maintaining the cell line) or 7.5% (for cells to be harvested for isolation of the mitochondria) inoculum (v/v) of the stationary phase cells into fresh medium. The cells were incubated at 26°C on a gyratory shaker at 125 rpm and harvested by filtering through one layer of Miracloth (Calbiochem, La Jolla, CA), washed with 3% sucrose, transferred to aluminum foil packets, and stored at −80°C. For ^{3}H-thymidine–labeling experiments, cultures (400 mL) were used, and the wet weight of cells from each culture was determined by weighing the filtered cells.

Isolation of Mitochondria

The frozen BY-2 cells were pulverized to a fine powder by grinding in a coffee grinder with dry ice. The frozen powdered cells were mixed with grinding/extraction buffer (0.5 M sorbitol, 1 mM EDTA, 10 mM K_2HPO_4, 25 mM Hepes, pH 7.5, 0.5% [w/v] PVP-40 [polyvinylpyrrolidone, M_r of 40,000; Aldrich Chemical Co., Milwaukee, WI], 0.3% [w/v] BSA, 1 mM β-mercaptoethanol), which resulted in a frozen slurry. After the ice crystals had melted, the suspension was mixed by shaking vigorously and then filtered through four layers of Miracloth. The filtrate was centrifuged for 10 min at 500*g*. The pellet containing nuclei and cell debris was discarded. The supernatant was centrifuged for 8 min at 27,000*g*. The supernatant was discarded, and the pellet was gently resuspended in 3 to 5 mL of the grinding/extraction buffer without PVP-40 and transferred to a clear centrifuge tube. The tube was filled with grinding/extraction buffer and centrifuged for 5 min at 3000*g*. The supernatant was then centrifuged for 15 min at 11,400*g*. The pellet containing mitochondria was gently resuspended in 200 to 500 μL of grinding/extraction buffer without PVP-40. To remove any DNA released from broken organelles (nuclei, plastids, or mitochondria), the mitochondria were incubated with 200 μg/mL DNase I and 10 mM $MgCl_2$ for 1 hr on ice. The DNase-treated mitochondria were washed by adding grinding/extraction buffer and centrifuging for 10 min at 11,400*g*. The efficiency of the DNase treatment was assessed by examining the mitochondria under fluorescence microscopy and looking for ethidium bromide (EtBr)–stained DNA molecules, as described below. Far less than 1% of the mitochondria exhibited extra-mitochondrial DNA molecules.

Pulsed-Field Gel Electrophoresis

The mitochondrial pellet was resuspended in a small volume of buffer, 2 M sorbitol, and molten 2.5% low-melting-point agarose (LMPA) to give a final concentration of 0.35 M sorbitol and 0.5 to 0.7% LMPA. The mitochondria–LMPA mixture was pipetted into molds and allowed to harden at 4°C. Slices (70 μL) of agarose-embedded mitochondria were incubated overnight at 50°C in lysis buffer (200 μg/mL proteinase K, 1% Sarkosyl, 0.45 M EDTA, pH 9). The lysis buffer was removed, and an equal volume of 10 mM EDTA, pH 9, was added; agarose slices were stored at 4°C before pulsed-field gel electrophoresis (PFGE) analysis at 30-sec pulse time, 5 V/cm, and 24-hr run time on a 1.5% agarose gel (either SeaKem; FMC, Rockland, ME; or LMPA; Gibco BRL). The gels were stained in 0.5 μg/mL EtBr for 1 hr and destained for 2 hr with 330 mL of 0.5 × TBE (1 × TBE is 90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8) changed three times.

^{3}H-Thymidine Labeling Procedure

For the time course experiment, the BY-2 cells were transferred to fresh medium and allowed to grow for 12 to 74 hr. Four hours before the cells were harvested, 50 μCi of ^{3}H-thymidine (67 Ci/mmol; ICN, Costa Mesa, CA) was added. Cells were harvested as described above and stored at −80°C until used for preparation of mitochondria-embedded gel slices. For the pulse and pulse–chase experiments, the cells were transferred to fresh medium and grown for 10 hr, at which time 100 μCi of ^{3}H-thymidine was added to each flask. After 2 hr with the radiolabeled compound, either the cells were harvested (pulse) or a 200-fold molar excess of unlabeled thymidine was added (pulse–chase), and incubation continued for an additional 12, 24, or 168 hr before harvest. For the 168-hr chase, unlabeled thymidine was again added at 100 hr. Steady state labeling was performed by adding 100 μCi of ^{3}H-thymidine after the cells had grown for 10 hr and then harvesting the cells after another 170 hr of growth.

Mung Bean Nuclease Treatment of Agarose-Embedded DNA

Gel slices containing BY-2 mtDNA were soaked overnight in TE (10 mM Tris-HCl, pH 7.6, 1 mM EDTA). The slices were then soaked in mung bean nuclease (MBN) buffer (30 mM sodium acetate, pH 4.6, 50 mM NaCl, 1 mM $ZnCl_2$, 5% [v/v] glycerol) for 60 min. Five units of MBN (Promega, Madison, WI) was added, each sample was incubated for 30 min, unless indicated otherwise, and the reaction was stopped by the addition of 0.1% Sarkosyl and 10 mM EDTA, pH 9. Total cell DNA from spheroplasts of yeast strain YNN295 embedded in agarose was also treated with MBN as controls for single strand–specific digestion. Native and denatured (3 min at 95°C) λ DNA (50 ng) was treated with 0, 1, 5, 10, 25, or 50 units of MBN for 30 min at 37°C. As assayed by conventional gel electrophoresis, the double-stranded λ DNA remained intact except with 50 units of MBN, and the single-stranded λ DNA was completely degraded in all cases with MBN treatment.

Quantitation of Fractionated DNA

Images of the EtBr-stained gels were taken with Polaroid B&W 667 film and/or with a UVP ImageStore 7500 (UVP Inc., San Gabriel, CA) gel documentation system. Digitized images (of the original EtBr-stained gels or the Polaroid photographs) recorded with the gel documentation system were analyzed with National Institutes of Health Image software to estimate the amount of DNA in the well-bound and 50- to 150-kb fractions based on EtBr fluorescence intensities (areas under the curves). Determination of the amount of DNA was not possible for all samples or gels due to extreme differences between the intensities in the well-bound (high intensity) and the smear of DNA in the 50- to 150-kb (low intensity) regions.

For ^{3}H-thymidine–labeled mtDNA samples, after staining with EtBr and photographing, each sample lane was sliced into 18 pieces from the top (well) to the bottom of the gel. Each piece was added to 1.8 mL of H_2O and melted by steam heating for 15 to 20 min before scintillation fluid (EcoLume; ICN) was added and radioactivity assessed as counts per min in an LS7800 scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Fluorescence Microscopy and Moving Pictures of DNA

BY-2 mtDNA was fractionated by PFGE on a gel made with LMPA, and sample plugs (two or three plugs) were taken from several regions; the well (immobile fraction), the compression zone, the 50- to 150-kb region, and the region between the well and the compression zone. The sample plugs were placed in a microcentrifuge tube containing

100 μL of BET (3% β-mercaptoethanol, 0.1 μg/mL EtBr, 1 × TBE) and stored at 4°C. A 5- to 10-μL sample plug portion was melted on a glass microscope slide with 15 μL of ABET (1% LMPA in BET) at 55 to 60°C, and slight pressure was applied to the coverslip. The agarose was allowed to cool, the top and bottom ends of the coverslip were sealed with clear fingernail polish, and the left and right ends were sealed with ABET to prevent both drying of the agarose and liquid flow when buffer was added at the ends of the slide. Platinum electrodes were placed on the left and right sides of the slide, and 0.5 × TBE was added at each end to complete the electrical circuit. The direction of DNA movement (toward the anode) was changed by reversing the polarity of the electrical field.

The EtBr-stained mtDNA was visualized by fluorescence microscopy with a Nikon microphot-FX microscope (Nippon Kogaku K. K., Tokyo, Japan) equipped with an image intensifier, a CCD camera (Optronics Engineering, Goleta, CA), a video monitor, and an S-VHS video recorder. By analyzing videotape recordings, we measured individual DNA molecules. The use of λ DNA (48.5 kb) as a standard to determine mtDNA length in the 50- to 150-kb region and other length and imaging parameters were described earlier (Bendich, 1996). Measurements of long DNA molecules usually underestimate their actual length, because DNA rarely is fully stretched unless it becomes hooked on an agarose fiber, as does a large circle (Smith and Bendich, 1990; Bendich, 1996). Thus, the sizes given in Figures 5B and 6A and the legend to Figure 7B for linear molecules in the compression zone and well-bound fractions are minimal, with the underestimate greater for longer molecules. DNA sizes for the 50- to 150-kb fraction in Figure 5A are probably closer to actual length because they were estimated relative to λ DNA rather than from absolute length of fluorescent fibers. In the compression zone, DNA <400 kb could also be due to single-stranded regions retarding electrophoretic mobility and/or to shear forces generated during spreading of the melted sample plugs beneath the coverslip. Two or three sample plugs were removed from each region of the gel to ensure recovery of DNA from the area of interest; thus, a plug may be taken above or below the nominal size range. To provide a range of mtDNA concentrations for best resolution of individual molecules of the well-bound fraction, the mitochondria were diluted 1:5 or 1:10 before embedding in agarose.

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