Structural and functional characterizations of mung bean mitochondrial nucleoids

Hwa Dai*, Yih-Shan Lo, Alexandra Litvinchuk, Yuh-Tai Wang, Wann-Neng Jane, Lin-June Hsiao and Kwen-Sheng Chiang[1]

Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan 11509, ROC and
[1]Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637, USA

ABSTRACT

Mitochondrial nucleoids isolated from mung bean seedlings exhibited a chromatin-like structure associated with a membrane component. A similar structure, which underwent discrete changes during cotyledon development, was identified *in situ*. Isolated nucleoids consisted of essentially the same phospholipids, including cardiolipin, as whole mitochondria and proteins of inner- and outer-mitochondrial-membrane origin. Actin was consistently found with mitochondrial nucleoids prepared with different detergent concentrations. Formaldehyde cross-linking of cytochalasin B- and proteinase K-treated mitochondria further revealed that actin was associated with DNA in nucleoids. Mitochondrial nucleoids were self-sufficient in directing DNA synthesis *in vitro* in a pattern mimicking mtDNA synthesis in isolated mitochondria. In pulse-field gel electrophoresis, newly synthesized mtDNA separated into two major components, well-bound and fast-moving forms. Nucleoids DNA synthesis was resistant to aphidicolin but sensitive to N-ethylmaleimide, which indicates that a γ-type DNA polymerase was responsible for this activity. Mitochondrial nucleoids were capable of self-directed RNA transcription in a non-random fashion *in vitro*. Consistent with and complementary to results from fungi and human cells done mostly *in situ*, our present work helps to establish the important paradigm that mitochondrial nucleoids in eukaryotes are more than mere mtDNA compaction and segregation entities but are centers of mtDNA maintenance and expression.

INTRODUCTION

Electron microscopy (EM) revealed that DNA in each mitochondrion of a mouse cell appeared as clumped or thickened fibers in 2–6 centers termed nucleoids by Nass (1). Yeast mtDNA was organized into 10–20 nucleoprotein complexes as observed on EM in 1981 (2). Recently, Spelbrink *et al.* (3) delineated the nucleoids structure in human mitochondria by colocalization of a Twinkle-green fluorescent protein fusion protein with mtDNA, and for the first time showed that nucleoids in mammalian cells are stable assemblies of multiple mitochondrial proteins with mtDNA. Combining immunocytochemistry, mitochondrial-specific dye staining and fluorescent protein tagging analysis, several hundreds of nucleoids were shown to be organized in each human cell (4–6). In higher plants, it is generally believed that only one nucleoid exists per mitochondrion (7). Mitochondrial nucleoids have been isolated as entities with defined density from several organisms and these entities appear to retain their structure *in vitro* (8,9).

Traditionally, *in vitro* studies of nucleoids have focused mainly on their characteristic as a DNA–protein complex for mtDNA compaction, maintenance and segregation. More recently, the functional significance for several DNA-binding proteins have been shown to be involved in *in vivo* nucleoids DNA replication and RNA transcription in yeast and human cells (4–6,10–15). Regardless of the genome size and topology of restriction-endonuclease-generated genome map (circular or linear), a fraction of plant mtDNA migrates as a smear zone to where 50–200 kb linear DNA molecules do, while the remainder remains in the well at the origin of electrophoresis (16,17). The intermediate of replicating mtDNA in yeast and plants has been suggested to be a rolling circle (18,19) and/or a multifiber network similar to bacteriophage-T4-replicating molecules (17,20,21). Both forms of these replicating molecules have been considered

as the probable cause for their immobility of a fraction of plant mtDNA in pulsed-field gel electrophoresis (PFGE) (17,21). Most plant mtDNA replication studies have been carried out with cultured cells or isolated organelles; however, little is known about DNA replication in plant mitochondrial nucleoids *in vivo* and *in vitro* (7,22). Submitochondrial fractions of membrane-bound DNA with nucleic acid synthetic activity but undelineated structure have been also reported in plants (23,24).

In general, multiple nucleoids exist in a mitochondrion, but the number of mitochondrial nucleoids in each organelle after propagation varies. It is generally assumed that nucleoids are involved in mitochondrial DNA segregation, inheritance and mitochondrial propagation (4–6,14,15, 25–27). Recently, the relation between mitochondrial nucleoids division and mitochondrion propagation became better understood. The nucleoids in mammalian cells were shown to be stable and dynamic assemblies that could move or divide in concert with the dynamics of the mitochondrial network and could also diffuse efficiently into mitochondria devoid of mtDNA. These phenomena may imply that the transmission of mtDNA to daughter mitochondria occurs during mitochondrial development and propagation (4–6). The movement and segregation of mitochondrial nucleoids may be managed by the association of mitochondrial nucleoids to actin or tubulin directly or indirectly (6,14).

In the course of our investigation, we found that isolated functionally competent mitochondrial nucleoids were chromatin-like structures with an intriguing membrane system consisting of phospholipids including cardiolipin with relative content essentially the same as that of the whole organelle and of proteins constituents originating from both the inner and the outer mitochondrial membrane. While the membrane protein contents of isolated nucleoids varied qualitatively according to the non-ionic detergent concentration used for their preparation, actin was consistently associated with isolated nucleoids despite a 4-fold variation in the detergent concentration used. Furthermore, we showed the association of actin with mtDNA after CsCl gradient fractionation on homogenized mitochondrial nucleoids that were isolated from formaldehyde cross-linked mitochondria pre-treated with an actin-depolymerizing agent and proteinase K.

In addition, we demonstrated that mung bean mitochondrial nucleoids were self-sufficient in directing DNA replication *in vitro*. This DNA synthesis was resistant to aphidicolin and sensitive to N-ethylmaleimide (NEM), which indicates that mung bean mitochondrial nucleoids contain a γ-type DNA polymerase. In PFGE, newly synthesized mitochondrial-nucleoid DNA separated into a well-bound and a fast-moving component, which appeared to have different labeling kinetics and stability. This pattern of mtDNA synthesis was also observed *in vivo* and in isolated mung bean mitochondria. We also showed that mung bean mitochondrial nucleoids were capable of self-directed RNA transcription. Thus, our present work indicates that higher-plant mitochondrial nucleoids, like their counterparts in fungi and mammalian cells, are capable of self-directed DNA replication and RNA transcription and play a more active role in mitochondrial biogenesis than was previously believed.

MATERIALS AND METHODS

Preparation of mitochondrial nucleoids

Mitochondria were prepared from 3-day-old etiolated mung bean seedlings (*Vigna radiata* L. [Wilzed] cv. *Tainan* No. 5) as described previously (28). Nucleoids were isolated essentially as described previously (8,9). Briefly, the sucrose-gradient-purified mitochondria were pelleted, suspended at a concentration of 5 mg protein equivalent per milliliter in isolation buffer [15 mM Tris–HCl, pH 7.6, 1.5 mM EDTA, 0.38 M sucrose, 0.6 mM spermidine, 5.3 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by 0.5% NP-40 or as specified. After centrifuging at 14 000 g for 20 min, the clear supernatant was loaded on 5-layer sucrose gradients (15, 30, 40, 50 and 60% w/v) in gradient buffer (20 mM Tris–HCl, pH 7.6, 1 mM EDTA, 1 mM spermidine, 7 mM 2-mercaptoethanol, 0.4 mM PMSF) and centrifuged at 46 000 g for 1 h. Nucleoids were recovered from the 15 to 30% sucrose boundary, diluted with 2 vol of gradient buffer and pelleted at 46 000 g for 1 h.

Electron microscopy

Isolated mitochondrial nucleoids and nucleoids in plant tissue were examined under an electron microscope (Philips CM100). Thin-section transmission electron microscopy was as described previously (29).

Phospholipid analysis

Phospholipids of mitochondrial nucleoids and mitochondria were extracted and analyzed by thin layer (Kieselgel 60 F_{254}, Merck) chromatography with the use of molybdenum blue (1.3% molybdenum oxide in 4.2 M sulfuric acid) spray reagent (30).

Southern blot analysis

Southern blot analysis was performed by the conventional method (31) with CsCl-gradient purified mtDNA used as a probe. The absence of chloroplast and nuclear DNA in this probe was verified by PCR with primers specifying for chloroplast or nuclear gene products (see below). PCR involving pure mtDNA and the primer pair: cx3-34U, gtagatccaagtccatg-gcct and cx3-458L, gcatgatgggcccaagttacggc produced a mitochondrion-encoded *cox 3* DNA fragment, which was also used as a probe. The same results were obtained with both types of probe.

Immunoblot and DNA blot analysis

Methods for immunoblot and DNA blot analysis were described previously (29). For dot hybridization, newly synthesized ^{32}P-labeled RNA isolated from mitochondrial nucleoids or mitochondria was used as the probe. Total mtDNA isolated from DNase I-treated mitochondria (1 μg, 500 ng, 40 ng, 8 ng/spot) and gene fragments of *cox 2* (20 ng, 4 ng, 0.8 ng, 0.16 ng/spot), *cox 3* (20 ng, 4 ng, 0.8 ng, 0.16 ng/spot) and *rbc*L (20 ng, 4 ng, 0.8 ng, 0.16 ng/spot) were used as the template. Mitochondrial, chloroplast and nuclear DNA used as template for hybridization and/or PCR were all purified through CsCl gradient fractionation. Conditions for dot hybridization followed the manufacturer's instructions (Promega).

Cross-linking protein with mtDNA in mitochondrial nucleoids

Formaldehyde cross-linking was performed as described previously (12). Briefly, highly purified mitochondrial samples with or without cytochalasin B (CB, Sigma) treatment (30 min incubated at 30°C in 50 μM CB in 0.4 M mannitol and 10 mM Tricine-KOH, pH 7.2, followed by re-isolation of mitochondria through a sucrose cushion) were treated with DNase I and proteinase K. Samples were then incubated with 1% paraformaldehyde for 8 h and quenched with glycine. Mitochondrial nucleoids were isolated by sucrose gradient centrifugation after 0.5% NP-40 treatment as described above. The purified nucleoids were homogenized and treated with 1% sarkosyl and 50 μg/ml RNase A, and the total mixture was then subjected to CsCl gradient fractionation. The distribution of mtDNA and proteins in each fraction was determined by dot hybridization using *cox* 3 fragment as a probe and by immunoblotting, respectively.

Pulsed-field gel electrophoresis

Mitochondria or mitochondrial nucleoids were resuspended in M1 buffer (28), mixed with an equal volume of 1.5 % low-melting-point agarose (LGT) and kept at 42°C. This mixture was pipetted into molds and allowed to set on ice for 10 min. The plugs were treated with ESP (1 mg/ml proteinase K, 1% sarkosyl, 0.5 M EDTA, pH 9.0) at 50°C overnight. Fresh ESP was replaced twice during incubation. The plugs were then washed with ES buffer (1% sarkosyl, 0.5 M EDTA, pH 9.0) at 50°C for 2 h. After replacement with fresh ES buffer, PFGE was performed at a 30–60 s pulse time (at the ratio A:B = 1), 150 V (11.8 V/cm) on a 1.2% agarose gel for 24 h in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA) at 13°C. Gels were then stained with ethidium bromide, de-stained by washing and underwent conventional Southern blotting analysis. For DNA synthesis analysis, the gels were dried first before being exposed to X-ray films. For counting of α-[^{32}P]dCTP incorporation into newly synthesized wb (well-bound) and fm (fast-moving) DNA, the dried gel was sliced into wb and fm zones guided by autoradiography and then counted in an scintillation counter (Hewlett Packard).

Mitochondrial-nucleoid DNA synthesis *in vitro*

The method for mtDNA synthesis in wheat mitochondrial lysate (23) was adapted with modification. Briefly, mitochondrial nucleoids isolated from 2 mg protein equivalent of sucrose-gradient-purified mitochondria were incubated at 30°C in 400 μl of reaction mixture containing 50 mM Tris–HCl, pH 7.5, 20 mM MgCl$_2$, 40 mM KCl, 2 mM DTT, 1 mM ATP, 50 μM each of dATP, dGTP and dTTP and 100 μCi of [α-^{32}P]dCTP (3000 Ci/mmol). For pulse experiment, samples were withdrawn at each time point, stopped by adding unlabeled dCTP and EDTA at a final concentration of 40 μM and 10 mM, respectively. Samples were pelleted and imbedded in an agarose plug for proteinase K digestion and PFGE. For pulse-and-chase experiment, after a 30 min [α-^{32}P]dCTP pulse-labeling period, 1000-fold molar excess of unlabeled dCTP was added, and the incubation continued for various chase times. For DNA polymerase inhibitor assay, different concentrations of aphidicolin (Sigma, stock solution dissolved in DMSO), NEM (Sigma, stock solution dissolved in ethanol)

or ddCTP (Roche) was supplemented to a DNA synthesis reaction mixture at the onset (also see Table 2).

In organello DNA synthesis of mitochondria

mtDNA synthesis *in organello* was carried out as described as above for mitochondrial-nucleoid DNA synthesis, except with a different reaction buffer (20 mM MgCl$_2$, 40 mM KCl, 2 mM DTT, 1 mM ATP, 50 mM Tris–HCl, pH 7.5, 50 μM each of dATP, dGTP and dTTP and 50 μCi of [α-^{32}P]dCTP [3000 Ci/mmol]). At each pulse period, samples were withdrawn, and the reaction was stopped as described. mtDNA was then fractionated by PFGE. For pulse-and-chase experiment, after a 1 h [α-^{32}P]dCTP pulse-labeling period, a 1000-fold molar excess of unlabeled dCTP was added, and the incubation continued for various durations. Aurintricarboxylic acid (ATA) treatment during *in organelle* mtDNA synthesis was performed as follows: after 1 h of synthesis, ATA at a final concentration of 100 μM was added to the reaction mixture, and DNA synthesis was continued. At 1, 2, 4, 6 and 8 h incubation, aliquots were withdrawn and subjected to PFGE.

Mitochondrial DNA synthesis *in vivo*

Two hundred mung bean seeds were surface sterilized with 0.75% sodium hypochlorite (NaOCl) for 5 min with shaking followed by rinsing with distill water three times. The surface-sterilized seeds were then soaked in sterilized water in the dark at 27°C overnight. For each time point, 20 budding seeds were selected and transferred to a beaker on 10 layers of paper towel soaked in 100 ml of sterilized tap water. Seven individual beakers each containing 20 budding seeds were then kept in the dark at 30°C for 2 days. Twenty two-day-old seedlings from each beaker were transferred into a separate sterilized glass beaker with 50 ml of sterilized tap water containing 50 μCi of [α-^{32}P]dCTP (3000 Ci/mmol). The seedlings weighted between 0.33 and 0.45 g, and were between 4.2 and 5.3 cm long. The seedling DNA synthesis was carried out for 5 pulse and 2 pulse-and-chase periods. The pulse duration was 0, 0.5, 2, 4 and 8 h. The two pulse-and-chase samples were pulsed for 4 h each and then chased for 2 and 24 h, respectively. After 24 h chase, the seedlings weighted between 0.66 and 0.78 g, and were between 4.53 and 6.8 cm long. In total, seven reactions were performed in seven independent beakers to avoid over crowding. At the end of each pulse and pulse-chase time, 1000× excess of cold dCTP and 10 mM EDTA (final concentration) were added. Mitochondria were isolated from 20 whole seedlings after removing the root immediately after each time point. Total mitochondria isolated from 20 seedlings were embedded in a single plug for PFGE analysis. Each lane in Figure 9 loaded with a plug containing mitochondria isolated from whole 20 seedlings as described. The entire experiment was conducted in the dark until the seedlings were harvested for mitochondrial isolation.

RNA synthesis *in vitro*

The method for RNA synthesis was as described previously (32) with modification. Mitochondrial nucleoids isolated from 1 mg protein-equivalent of mitochondria were incubated in a 200 μl reaction mixture containing 10 mM Tris–HCl, pH 8.5, 5 mM MgCl$_2$, 1 mM DTT, 0.1 mg/ml BSA, 120 μM each of CTP, GTP, ATP, 0.1 μM [α-^{32}P]UTP (3000 Ci/mmol) and

200 U RNAsin. After 25 min incubation at 25°C, unlabeled UTP was added to 20 µM, and the incubation was stopped 5 min later by adding 1% SDS, 30 mM diaminocyclo-hexanetetra-acetic acid (CDTA) and 20 µg carrier tRNA. RNA was then isolated by treatment with proteinase K (200 µg/ml, in the presence of 1% sarkosyl, 20 mM CDTA, 50 mM Tris–HCl, pH 8.0) at 55°C for 30 min followed by extraction with phenol/chloroform:isoamylalcohol.

Reverse PCR

Total RNA samples purified from mitochondrial nucleoids or mitochondria were first treated with RNase-free DNase to remove contaminating DNA and used as templates. Four sets of primers (cx3-34U, gtagatccaagtccatggcct; cx3-458L, gcatgatgggcccaagttacggc; a15-495U, gcggtgtgaaaggaatag; a15-911L, cacaatacaa tgtctcactctc; cx2-527U, tcccdcaaa-grattgttcatgg; cx2-764L, ccyaattctaratcatcttctg; *rbc*L-301U, ttggactgatggacttaccagtcttgatcg; *rbc*L-840L, tcttcgcatgtacct-gcagtcgcattcaag) were used for RNA-template-directed PCR. These primer sets were designed to detect the presence of mitochondrion-encoded *cox3* and ATPase α, nucleus-encoded *cox 2* and chloroplast-encoded *rbc*L. These primer sets generated a 448, 439, 260 and 570 bp PCR product, respectively, from mRNA and/or RNA transcripts. The first-strand cDNA synthesis was initiated by incubating the RNA template and the anti-sense PCR primer in a final volume of 5 µl at 72°C for 2 min, followed by 2 min cooling on ice. A total of 2 µl of a 5× first-strand buffer, 1 µl of 20 mM DTT, 1 µl of 10 mM each of dATP, dGTP, dCTP and dTTP, and 1 µl MMLV reverse transcriptase (200 U/µl) were then added and the mixture incubated at 42°C for 1 h. The resulting first-strand cDNAs were amplified by PCR with an initial 95°C, 1.5 min heating period, followed by 30 cycles of 94°C for 30 s; 55°C (51°C for *ATPase* α) for 30 s; 72°C for 1 min, and terminated by 72°C for 5 min.

RESULTS

Membrane association and chromatin-like structure *in vitro* and *in situ*

Isolated mung bean mitochondrial nucleoids were visualized by thin-section electron microscopy to have an electron-dense chromatin-like structure associated with a vesicle component (Figure 1); most of the chromatin-like elements appeared to be linked to or located immediately next to the vesicles. Similar chromatin-like elements of comparable dimension were identified in plant tissues (Figure 2C and D, arrows). The chromatin-like element underwent a distinctive change to a fibril-like structure (Figure 2E and F, arrows) during the early stages of cotyledon development. Table 1 summarizes the changes in mitochondrial-nucleoid organization observed at the early cotyledon developmental stages. Since EM observations of random thin sections do not necessarily represent the whole picture of a given mitochondrion, we adapted a standardized experimental procedure by attempting to take micrographs always near the middle section of each mitochondrion. Among all the cotyledon tissues obtained from seeds immersed for 12 h at 4°C we examined (totaling 476 mitochondria), no chromatin- or fibril-like structure was found
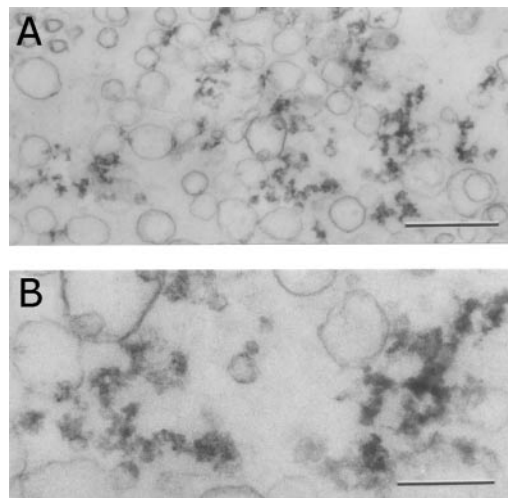

Figure 1. Ultrastructure of isolated mitochondrial nucleoids. Freshly isolated mitochondrial-nucleoid samples from highly purified mitochondria were imbedded in agarose, fixed, dehydrated and infiltrated with Spur resin. This resin block was sectioned, stained and examined under an electron microscope. (**A**) A representative view. (**B**) Enlarged part of (A). Bars equal to 100 nm in (A) and 200 nm in (B).

(Table 1 and Figure 2A and B). However, out of 294 mitochondria examined, 106 chromatin-like nucleoids were found in cotyledons of 12 h–27°C immersed seeds (Table 1 and Figure 2C and D, arrows). After 1 day growth in vermiculite of the 12 h–27°C immersed seeds, chromatin-like mitochondrial nucleoids were no longer seen, but fibril-like nucleoids structure was observed in 114 out of 330 mitochondria examined (Table 1 and compare Figure 2E and F with Figure 2C and D, arrows). Only a few (12 out of 444) fibril-like nucleoids were found in mitochondria of cotyledons from 3-day-old seedlings. Similarly, only 3 fibril-like nucleoids were found among 128 mitochondria examined in hook cortical cells of 3-day-old seedlings (data not included in Table 1). This observation suggests that mung bean mitochondrial nucleoids are dynamic entities, whose organization undergoes function-related changes at different stages during seedling development (compare Figure 2C and D with 2A, B and 2E, F; Table 1).

The contents of phospholipids in mitochondrial nucleoids varied quantitatively according to their isolation conditions. For instance, the phospholipid content decreased quantitatively with an increasing concentration of NP-40, a nonionic detergent used routinely for mitochondrial nucleoids isolation, whereas no significant qualitative change was observed (Figure 3, lanes 2–4). Since constituents of mitochondrial nucleoids are sensitive to detergent concentrations used for their isolation, we standardized our preparation at an NP-40 concentration of 0.5% in order to be consistent with the work of other investigators (8,9,12,13,27). Nucleoids isolated with 0.5% NP-40 had a phospholipid composition similar to that of whole mitochondrial membrane (Figure 3, compare lanes 3 and 5). Under over-loading conditions for thin-layer chromatography, which distorted the separation pattern and retarded the mobility of cardiolipin, no other phospholipid was detected (data not shown). These results suggest that the composition of phospholipids of mitochondrial nucleoids is qualitatively the same as that of whole mitochondria.
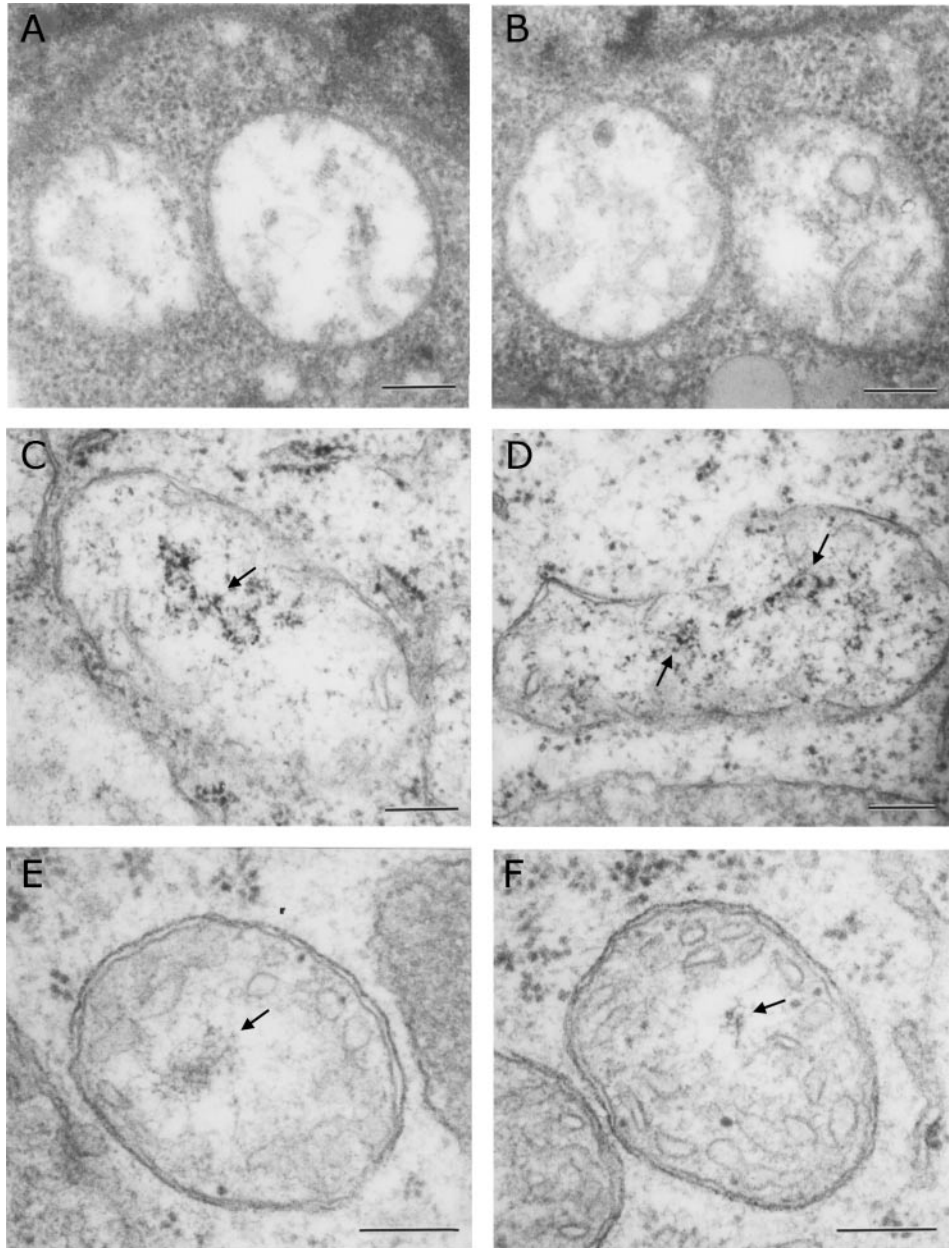
Figure 2. Ultrastructure of chromatin- and fibril-like structures in mitochondria of developing cotyledon tissue. Cotyledons from mung bean seeds immersed in water at 4°C for 12 h (**A** and **B**), at 27°C for 12 h (**C** and **D**) and at 27°C for 12 h followed by 1 day growth on vermiculite at 27°C (**E** and **F**) were fixed, dehydrated and infiltrated with Spur resin, sectioned and stained for electron microscope examination. Mitochondrial nucleoids exhibiting a chromatin-like organization (C and D) or a fibril-like organization (E and F) at different cotyledon developing stages are indicated by arrows. Bars equal to 200 nm.

Association of protein with mitochondrial nucleoids

The protein contents of mitochondrial nucleoids varied qualitatitively and quantitatively with the detergent concentration used for their isolation. The association of some proteins with mitochondrial nucleoids appeared considerably more resistant to NP-40 concentration than others. For instance, COXII, α subunit of ATPase, porin and actin were all detected in nucleoids isolated with 0.25% NP-40 (Figure 4). At 0.5% NP-40, COXII was eliminated from the nucleoids. The α subunit of ATPase was gradually removed by increasing the detergent concentration to 1.0%, whereas the proportion of porin and actin remained essentially unchanged.

This differential association of proteins with mitochondrial nucleoids as a function of detergent concentration may reflect whether the association may have certain structural/functional significance or is merely fortuitous. In this regard, the persistent presence of actin irrespective of the detergent concentration tested was unexpected. To ascertain whether mitochondrial-nucleoid-associated actin was derived from cytoplasmic actin by its adhesion to the organelle, highly purified mitochondria were pre-treated with actin depolymerizing agent, cytochalasin B (CB) followed by additional proteinase K treatment. The treated mitochondria were subjected to an *in organelle* cross-linking procedure with formaldehyde (12) prior to the isolation of nucleoids. The resulting nucleoids

Table 1. Mitochondrial nucleoids organizational dynamics in developing cotyledon tissue

Age of cotyledon	Cell type	Epidermis	Hypodermis	Storage parachyma	Sieve tube member	Companion cell	Phoem parachyma	Xylem parchyma	Total
Immersed seed (12 h 4°C)	Total[a]	32	11	129	6	89	123	86	476
	Chromatin-like[b]	0	0	0	0	0	0	0	0
	Fibril-like[c]	0	0	0	0	0	0	0	0
Immersed seed (12 h 27°C)	Total	71	41	52	12	45	24	49	294
	Chromatin-like	30	21	31	2	11	4	7	106
	Fibril-like	0	0	0	0	0	0	0	0
Day 1[d] seedling	Total	134	64	36	14	41	32	9	330
	Chromatin-like	0	0	0	0	0	0	0	0
	Fibril-like	57	24	7	0	15	9	2	114
Day 3[d] seedling	Total	49	54	60	10	153	67	51	444
	Chromatin-like	0	0	0	0	0	0	0	0
	Fibril-like	0	0	2	0	3	4	3	12

[a]Total: total mitochondria examined.
[b]Mitochondrial nucleoids organized into a chromatin-like structure, as shown in Figure 2C and 2D.
[c]Mitochondrial nucleoids organized into a fibril-like structure, as shown in Figure 2E and 2F.
[d]Day 1 and Day 3 seedlings represent the immersed seeds (at 27°C for 12 h) followed by sowing the seeds on vermiculite and growing at 27°C for 1 day and 3 days, respectively.
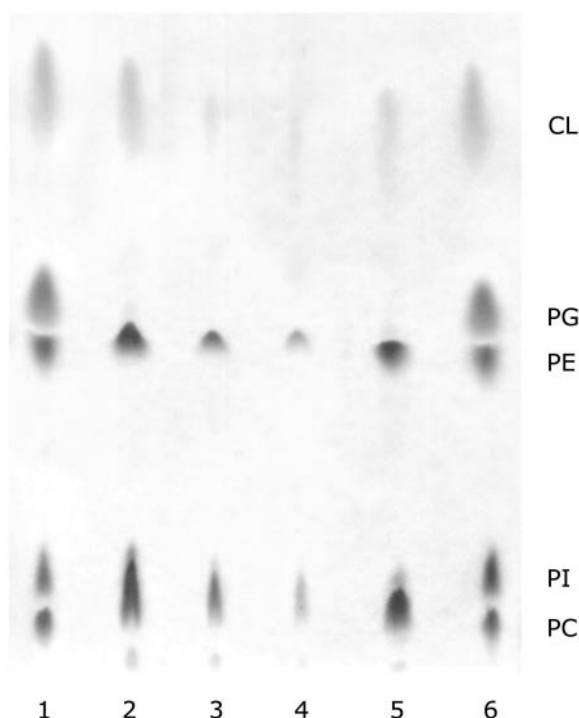

Figure 3. Phospholipid composition of mitochondrial nucleoids as a function of their isolation condition. Phospholipids extracted from three mitochondrial-nucleoid samples, each obtained from 25 mg protein equivalent of mitochondria at a NP-40 concentration of 0.25, 0.5 and 1.0% were loaded in lanes 2, 3 and 4, respectively, of a thin-layer chromatography chromatogram. A phospholipid sample extracted from 0.5 mg protein equivalent mitochondria was loaded in lane 5 as a control. A mixture of phospholipid markers, cardiolipin (CL, 20 µg), phosphoglycerol (PG, 10 µg), phosphatidylethanolamine (PE, 10 µg), phosphatidylinositol (PI, 20 µg) and phosphatidylcholine (PC, 10 µg) were loaded in lanes 1 and 6. The chromatogram was developed and stained as described in Materials and Methods.

Figure 4. Immunoblotting analysis of mitochondrial-nucleoid proteins as a function of their isolation condition. Five mitochondrial-nucleoid samples prepared at a NP-40 concentration of 0, 0.25, 0.5, 0.75 and 1%, were loaded in lanes 2, 3, 4, 5 and 6, respectively, for SDS–PAGE. Each nucleoids sample loaded was obtained from 50 µg protein equivalent of mitochondria. A total of 0.5 µg protein equivalent of lysed mitochondria was loaded in lane 1 as a control. The following antibodies were used for the ensuing immunoblots: anti-α subunit of ATPase, anti-COXII, anti-porin and anti-actin.

were then lysed and fractionated by CsCl-density centrifugation to separate free and DNA cross-linked proteins (Figure 5). After fraction collection, the distribution of mtDNA was determined by dot hybridization, with a *cox 3* DNA fragment used as the probe (Figure 5A). The distribution of free protein
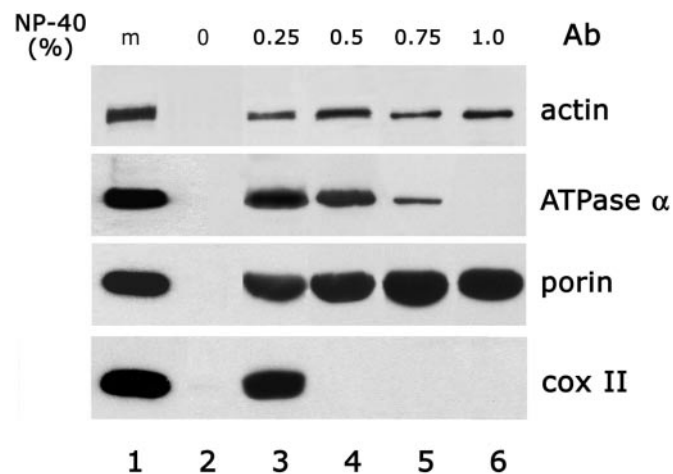
and cross-linked protein–mtDNA complex was detected by immunoblotting (Figure 5B–D). In the nonformaldehyde-treated control, actin, α subunit of ATPase and porin migrated as free proteins to fractions 4–8 (Figure 5B). After cross-linking, actin and α subunit of ATPase not only appeared as free proteins but also banded in fraction 13 as protein–mtDNA complexes (Figure 5C and D). Either proteinase K treatment alone or cytochalasin B/proteinase K double treatment did not eliminate actin from cross-linking with mtDNA in nucleoids preparations (Figure 5C and D, respectively). This result suggests that mtDNA-associated actin molecules in mitochondrial nucleoids are protected by mitochondrial membranes from depolymerization and/or proteolytic degradation by cytochalasin B and proteinase K, respectively. In contrast to α subunit of ATPase and actin, no porin was detected as mtDNA-cross-linked protein
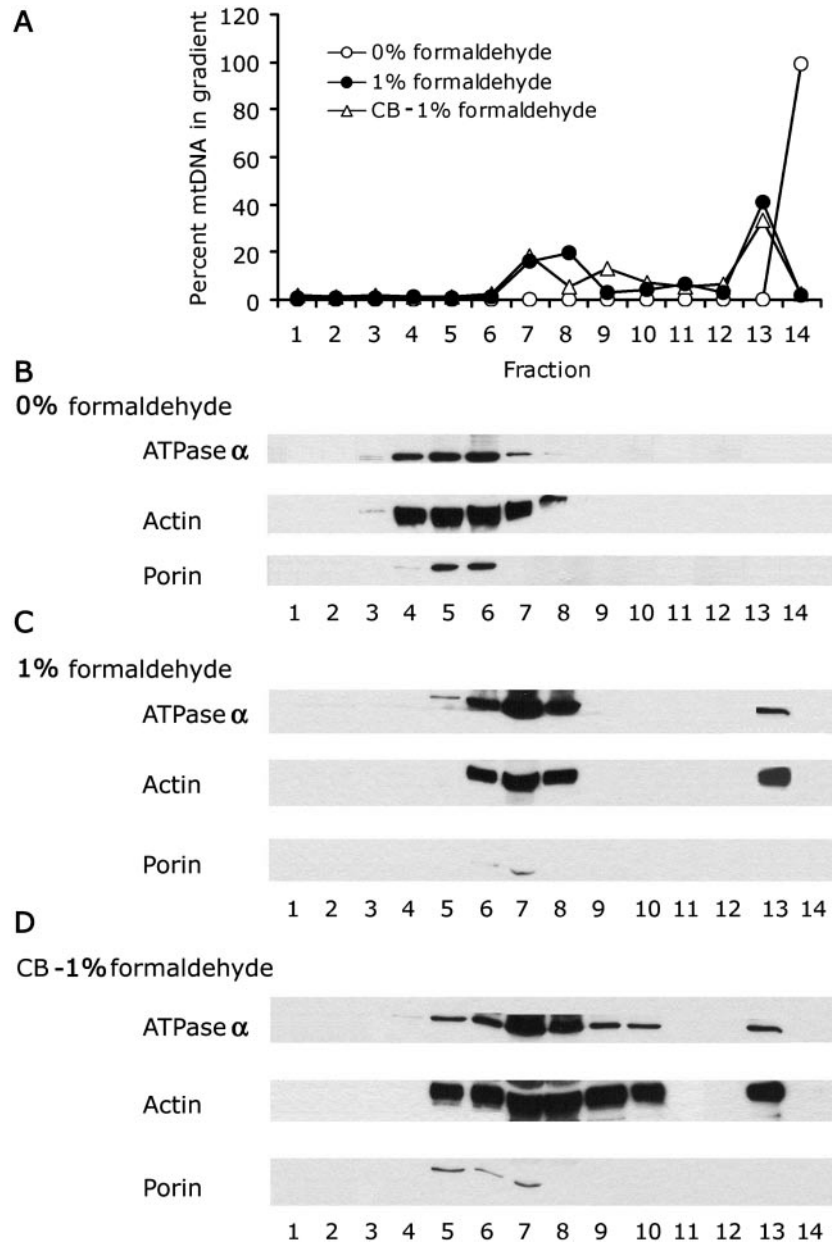
Figure 5. Cross-linking of actin with mtDNA in mitochondrial nucleoids. Highly purified mitochondria with or without cytochalasin B (CB) pre-treatment were subjected to sequential DNase I and proteinase K treatment followed by 1% paraformaldehyde cross-linking for 8 h. After quenching with glycine, mitochondrial nucleoids were isolated, homogenized and treated with 1% sarkosyl and RNase A (50 µg/ml). The final mixture was then subjected to CsCl gradient fractionation. (**A**) The distribution of mtDNA across the gradient. Each fraction was dot blotted with a ^{32}P-labeled *cox* 3 probe. The density increases from left to right (fraction 14 being the most dense fraction). (**B–D**) The free and mtDNA cross-linked protein by immunoblot analysis. Each fraction was immuno blotted with anti-ATPase α, anti-actin and anti-porin antibody, respectively. Results shown in (B) were obtained from control mitochondria without cross-linking. Results shown in (C and D) were obtained from formaldehyde cross-linked mitochondria. Results shown in (D and C) were obtained from mitochondria that were pre-treated with and without cytochalasin B, respectively, in addition to proteinase K and DNase I before cross-linking. The co-existence of actin with mitochondrial nucleoids DNA is shown in fraction 13 of (C and D).

complex after the *in organello* formaldehyde cross-linking step (Figure 5C and D).

Molecular complexity of mitochondrial-nucleoid DNA

PFGE revealed a considerable amount of heterogeneity among DNA molecules present in isolated mung bean mitochondrial nucleoids. Under a PFGE condition capable of fractionating 50–2200 kb linear DNA, most of the DNA did not migrate out

of the sample-loading well. This result indicates that the well-bound (wb) component had an apparent molecular mass >2200 kb and/or a complex conformation that retarded its migration (Figure 6). The other major DNA component appeared as a prominent zone without distinct bands, spanning a range equivalent to ~50–200 kb of linear DNA and was termed the fast-moving (fm) component (Figure 6, lanes 2–5). The relative content of the wb and fm components remained essentially unchanged, despite a substantial difference in
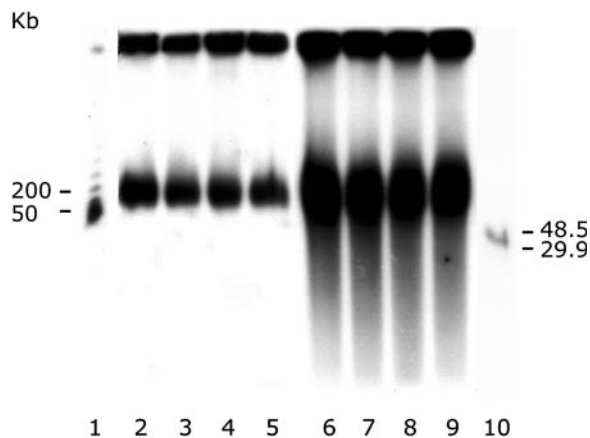
Figure 6. Characterization of DNA heterogeneity in mitochondrial nucleoids by PFGE. Three agarose-imbedded nucleoids samples, each isolated from 2 mg protein equivalent of mitochondria, were incubated with self-digested proteinase K at a concentration of 0.2, 1 and 5 mg/ml and loaded in lanes 2, 3 and 4, respectively. An identical nucleoids sample was incubated with 1 mg/ml of proteinase K (without self-digestion) and loaded in lane 5. Three agarose-imbedded mitochondrial samples (2 mg protein equivalent each) were incubated with self-digested proteinase K at a concentration of 0.2, 1 and 5 mg/ml and loaded in lanes 6, 7 and 8, respectively. Another identical mitochondrial sample was incubated with 1 mg/ml proteinase K (without self-digestion) and loaded in lane 9. After PFGE, the gel was blotted and hybridized with a ^{32}P-labeled probe of pure mtDNA. Exposure time was 10 h for lanes 2–5 and 5 h for lanes 6–9. Size markers are in lanes 1 and 10. The same result was obtained with a ^{32}P-labeled probe obtained from a PCR-amplified *cox 3*-gene fragment (data not shown).

proteolytic digestion to remove bound proteins before fractionating the resulting DNA (Figure 6, lanes 2–5). This result suggests that the immobility of the wb form was unlikely caused by inadequate removal of protein moiety from mtDNA. Under the same conditions, DNA from whole mitochondria exhibited the same electrophoretic pattern as that of isolated mitochondrial nucleoids, except that the higher relative content of some smear components migrated just ahead of the fm-component (Figure 6, lanes 6–9). This pattern of heterogeneity of mung bean mtDNA molecules is consistent with the pattern of other higher plant and yeast mtDNAs (17,19,33).

Mitochondrial nucleoids DNA synthesis *in vitro*

To ascertain whether mitochondrial nucleoids are capable of self-directed DNA synthesis, pulse-labeling experiments were carried out. In our preliminary experiments, [α-^{32}P]labeled nucleoside triphosphates were shown to incorporate well into isolated mitochondria and mitochondrial nucleoids. DNA synthesis activity of isolated mitochondrial nucleoids *in vitro* measured by the incorporation of [α-^{32}P]dCTP was essentially linear for ~2 h before settling into a plateau lasting for several hours (Figure 7A). Under this regime, mitochondrial-nucleoid DNA synthesis can be studied efficiently by pulse labeling, followed by PFGE, autoradiography and counting the sliced gel fragments for label incorporation. After 30 min pulse, newly synthesized mtDNA manifested as both wb and fm forms (Figure 7B, lane 1). As the pulse period lengthened, the amount of the label increased in both the wb and fm zones (Figure 7B, lanes 2–5).

At the onset of the pulse-labeling experiments, the ratio of the newly synthesized wb-form to the total newly synthesized

DNA exhibited a significantly decrease, whereas the reverse was true for the fm-form. Approximately 2 h later these ratios remained about the same (Figure 7C). This short-lived divergent labeling pattern for the two mtDNA forms was consistent in four independent pulse-labeling experiments. This pattern most probably have caused by different labeling kinetics or relative stabilities of the newly synthesized wb and fm forms, although the possibility that some newly synthesized wb may be converted to fm-form during the initial labeling phase cannot be excluded. Figure 7D represents the electrophoretic mobility pattern of wb and fm DNA during the chase period ranging from 10 min to 4 h after 1 h of pulse labeling. The ratio of pulse-labeled wb and fm form to the total newly synthesized DNA, respectively, showed a slight divergent trend at the very beginning of the chase period (Figure 7E), which is reminiscent of the result of the pulse-labeling experiment described above.

The effect of DNA polymerase inhibitors on mitochondrial-nucleoid DNA synthesis activity was determined. As summarized in Table 2, the DNA synthesis activity of mitochondrial nucleoids was highly resistant to aphidicolin; at 40 μM, no inhibition was detected; even at 100 μM, the inhibition was <10%. However, the nucleoids DNA synthesis activity was sensitive to NEM. The 60% inhibition of mitochondrial-nucleoid DNA synthesis by NEM at 2 mM (Table 2) is comparable with that shown in other higher plants in which 2 mM of NEM inhibited ~80% of mtDNA synthesis (34,35). Complete inhibition was achieved at an NEM concentration of 10 mM. Mitochondrial DNA polymerases of higher plants are known for their reduced sensitivity to ddCTP (7,34,35). We found that this is also the case for mung bean. At a ddCTP/dCTP ratio of 10, the inhibition of mung bean's mitochondrial DNA polymerase activity was ~10%; even at a ddCTP/dCTP ratio of 1000, only ~90% of the activity was inhibited (Table 2). The insensitivity to aphidicolin and sensitivity to NEM are characteristics of γ-type DNA polymerase. Thus, similar to that of other higher plants, mung bean mitochondrial DNA polymerase appears to be a γ-type DNA polymerase. This γ-type DNA polymerase of mung bean mitochondrial nucleoids, however, appeared to be more resistant to ddCTP inhibition than other plant mitochondrial DNA polymerase.

DNA synthesis in isolated mitochondria and *in vivo*

DNA synthesis in intact mitochondria was examined under similar experimental conditions used for mitochondrial-nucleoid DNA synthesis *in vitro*. Unlike DNA synthesis in mitochondrial nucleoids (Figure 7B and D), the newly synthesized wb form in isolated mitochondria exhibited a massive loss of previously incorporated label after 8 h pulse or 1 h pulse followed by 4 h chase (Figure 8A and B, lane 5). This apparent degradation of newly synthesized wb form was reversed in the presence of the nuclease inhibitor, ATA (Figure 8C). An ATA-sensitive nuclease activity (or activities) is thus implicated in processing of newly synthesized DNA in isolated mitochondria. Interestingly, such an activity is apparently very low or absent in mitochondrial nucleoids.

To ascertain whether mtDNA synthesis *in vivo* follows the same pattern, ^{32}P-labeling experiment for mtDNA synthesis was also carried out in mung bean seedlings. Mitochondria were purified from ^{32}P-labeled seedlings after different pulse
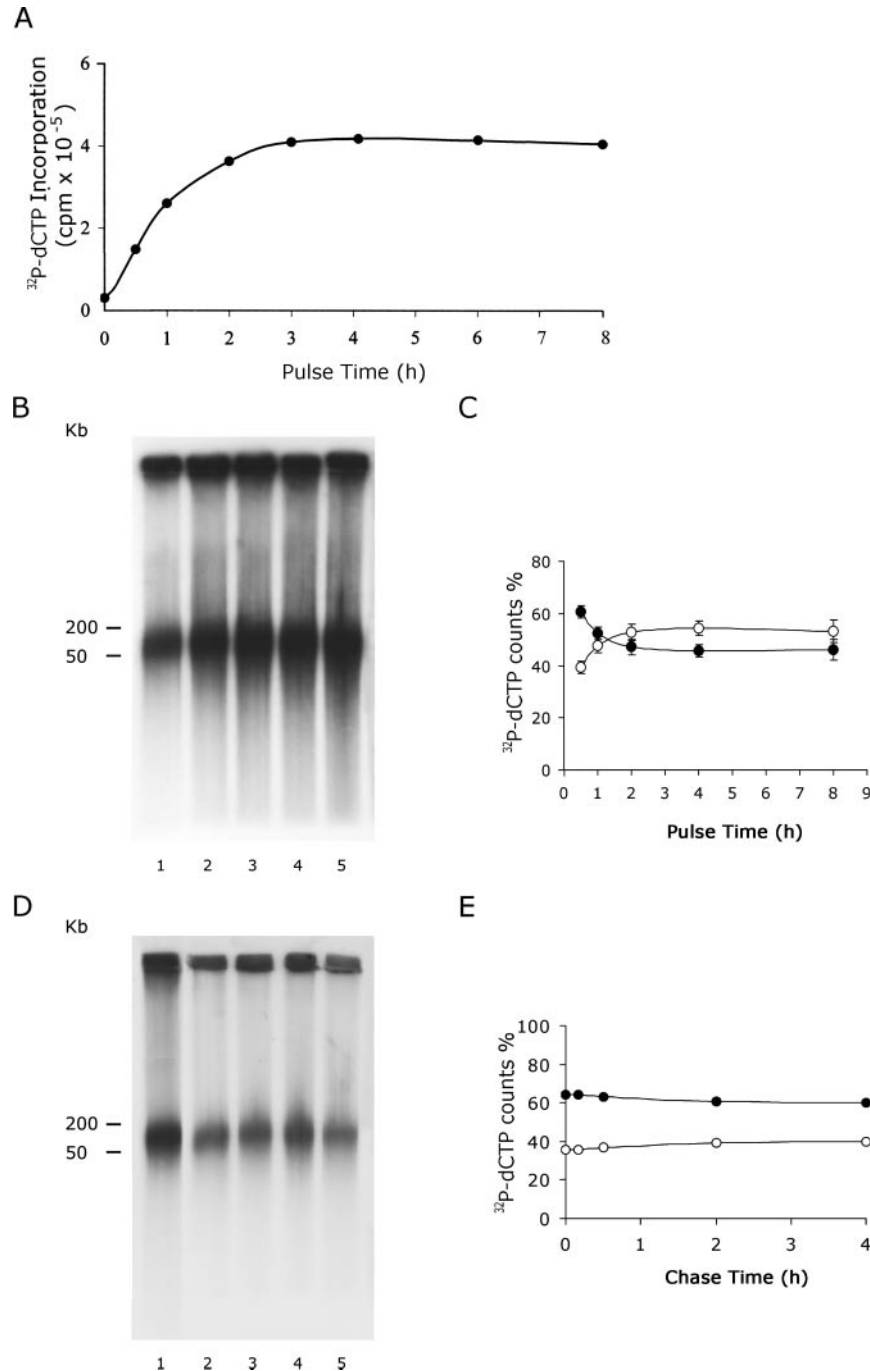
Figure 7. *In vitro* mitochondrial-nucleoid DNA synthesis with different pulse and chase periods. Nucleoids samples isolated from mitochondria equivalent to 2 mg protein were incubated at 30°C in a DNA-synthesis reaction mixture in the presence of ^{32}P-dCTP. At 0, 0.5, 1, 2, 3, 4, 6 and 8 h, duplicate aliquots were taken, one was used for ^{32}P-dCTP incorporation determination and the other was processed for fractionation analysis. (**A**) Kinetics of mitochondrial-nucleoid DNA synthesis *in vitro*. (**B**) PFGE autoradiogram of mitochondrial-nucleoid DNA synthesized at various pulse time. Lanes 1–5 present samples taken at 0.5, 1, 2, 4 and 8 h pulse-labeling time, respectively. (**C**) Graphic representation of the newly synthesized well-bound form (closed circle) and fast-moving form (open circle) were plotted over the total of the newly synthesized mitochondrial-nucleoid DNA. Fractions of well-bound (wb) and 50–200 kb fast-moving (fm) DNA were cut out from the gel and counted. The percentage of newly synthesized wb DNA was calculated as (c.p.m. of wb DNA)/(c.p.m. of wb DNA + c.p.m. of fm DNA). The percentage of newly synthesized fm form was calculated similarly. The average results of four repeated experiments are shown with the standard deviation (vertical bar). (**D**) PFGE autoradiogram of newly synthesized mtDNA after various chase periods. Lanes 1–5 represent 0, 10, 30 min, 2 and 4 h chase, respectively, after an 1 h pulse. (**E**) Proportion of newly synthesized wb and fm DNA corresponding to (D).

and pulse-and-chase period and then subjected to PFGE analysis. Consistent with the pattern observed in mitochondria and mitochondrial nucleoids, the newly synthesized mtDNA *in vivo* separated into in wb and fm forms after PFGE

fractionation (Figure 9, lanes 2–5). A complete disappearance of both newly synthesized wb and fm of mtDNA within 24 h of chase after 4 h pulse labeling indicates that an active turn over of newly synthesized mtDNA *in vivo* is operative during rapid

Table 2. Effects of inhibitors on mitochondrial-nucleoid DNA synthesis

Inhibitors	Mitochondrial-nucleoid DNA synthesis Percentage (%)	
	30 min	60 min
Control	100	100
Aphidicolin (20 μM)	118	99
Aphidicolin (40 μM)	114	105
Aphidicolin (100 μM)	98.4	91.1
NEM (2 mM)	44	41.1
NEM (10 mM)	0.106	0.03
ddCTP/dCTP (1:1)	99	94.8
ddCTP/dCTP (10:1)	90	94.5
ddCTP/dCTP (100:1)	44.3	42.2
ddCTP/dCTP (1000:1)	11.3	13.5

Control represents 20 000–30 000 c.p.m. for each set of reactions.

seedling growth (Figure 9, compare lanes 5 and 7). It is not known whether the nuclease responsible for this mtDNA turns over *in vivo* is the same or different from the ATA-sensitive nuclease that degraded the newly synthesized wb DNA in isolated organelle as seen in Figure 8A and B, lane 5.

RNA transcription

To ascertain whether mung bean mitochondrial nucleoids possess a mechanism for gene expression, we performed RNA synthesis experiment. The specificity of the nucleoid's newly synthesized, [32P]UTP-labeled RNA was evaluated first by its homology to total mtDNA as well as mitochondrion-, chloroplast- and nucleus-encoded gene fragments (Figure 10A, II). 32P-labeled RNA synthesized by isolated intact mitochondria was also evaluated as a control (Figure 10A, I). It is evident that mitochondrial nucleoids are capable of directing RNA synthesis, and the products of such synthesis are homologous to mtDNA and mitochondrion-encoded but not nucleus- or chloroplast-encoded genes (Figure 10A, II), since it is difficult to completely eliminate plastids (at varying developmental stages) from mitochondria preparations. Thus, the hybridization of RNA synthesized by mitochondria preparations to chloroplast-encoded *rbc*L sequence (Figure 10A, I, rbcL row) can be explained by the presence of minor contamination of transcription-active plastids in mung bean mitochondria preparations. The fact that 32P-labeled RNA synthesized by mitochondria nucleoids did not hybridize to chloroplast-encoded *rbc*L or nucleus-encoded *cox2* (Figure 10A, II, rbc L or *cox2* row) indicates that our mitochondrial-nucleoid preparations were essentially free of nuclear fragment and plastid-nucleoid contamination.

To assess the stability and specificity of RNA transcripts regularly present in isolated mitochondrial nucleoids, their total RNA was used as the template for reverse PCR to test amplification of mitochondrion-encoded *cox 3*, α subunit of ATPase, nucleus-encoded *cox 2* and chloroplast-encoded *rbc* L genes. Figure 10B demonstrates that RNA transcripts of mitochondrion-specific *cox* 3 and α subunit of ATPase were present in mitochondrial nucleoids but not nucleus- and chloroplast-encoded mRNA/transcripts.

To better characterize the *in vitro* nucleic acid synthetic processes exhibited by isolated mitochondrial nucleoids, their newly synthesized, 32P-labeled DNA and RNA products were used separately to probe two identical DNA blots each
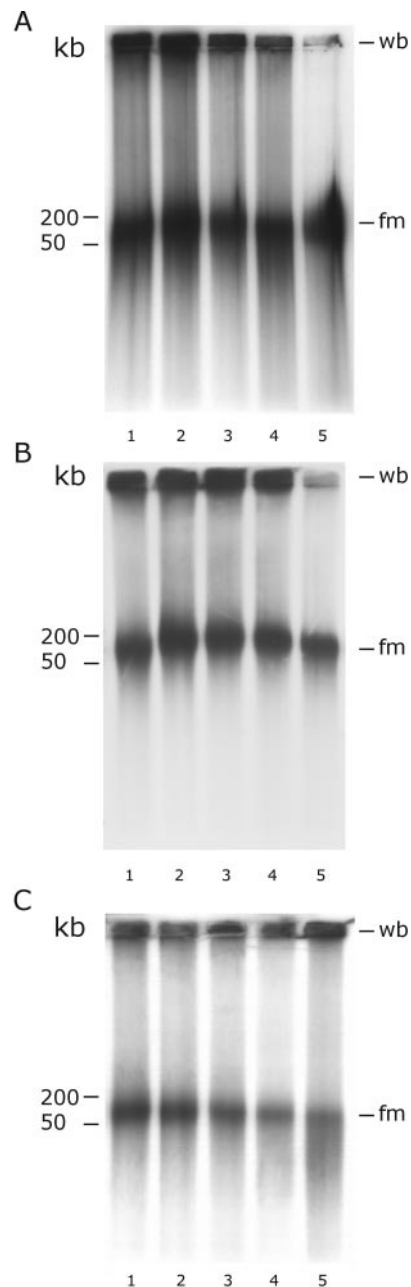

Figure 8. *In organello* mitochondrial DNA synthesis with different pulse and chase periods. Mitochondrial DNA synthesis was carried out as described in Materials and Methods. At 0.5, 1, 2, 4 and 8 h pulse period, respectively, an aliquot was taken from the reaction mixture and mitochondria (equivalent to 0.5 mg protein) were recovered by centrifugation, and subjected to PFGE and autoradiographic analysis. (**A**) PFGE autoradiogram of newly synthesized mtDNA at different pulse periods. Lanes 1–5 represent 0.5, 1, 2, 4 and 8 h pulse period, respectively. (**B**) PFGE-autoradiogram of newly synthesized mtDNA after different chase periods. Lanes 1–5 represent 0, 10, 30 min, 2 and 4 h chase periods, respectively, after an 1 h pulse. (**C**) The effect of ATA on mitochondrial DNA synthesis. This experiment is the same as that shown in (A), except 100 μM ATA was added to the reaction mixture 1 h after the start of the DNA synthesis reaction. Lanes 1–5 present samples taken at 1, 2, 4, 6 and 8 h, respectively, following the addition of ATA.

containing EcoRI fragments of highly purified mtDNA. With the newly synthesized mitochondrial-nucleoid DNA probe, the intensity of the hybridization signal was stoichiometrically distributed among the EcoRI-digested mtDNA fragments
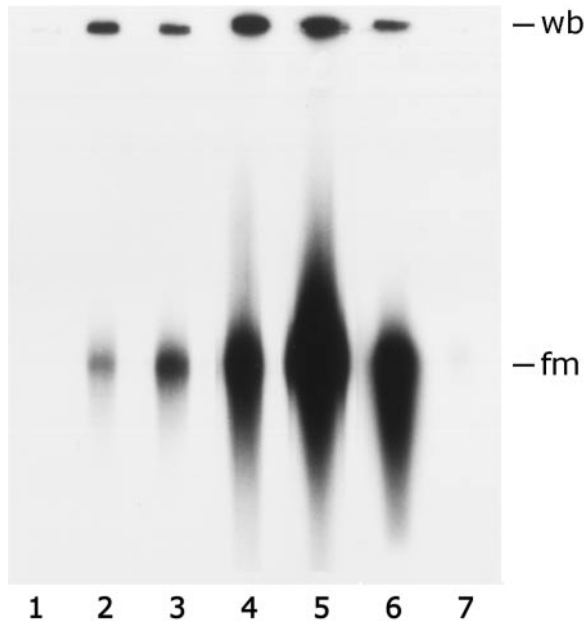
Figure 9. Mitochondrial DNA synthesis in mung bean seedlings. Mitochondrial DNA synthesis in mung bean seedlings was carried out as described in Materials and Methods. The seedlings were incubated with $[\alpha\text{-}^{32}p]dCTP$ for 0, 0.5, 2, 4 and 8 h (lanes 1–5, respectively). Two additional 4 h pulse labelings were performed and one was subjected to 2 h chase (lane 6) and the other a 24 h chase (lane 7). At the end of each pulse or pulse-chase period, EDTA was added to stop the reaction and mitochondria were isolated immediately from 20 labeled seedlings and subjected to PFGE fractionation. Each plug containing mitochondria isolated from whole 20 seedlings was loaded for PFGE.

according to their respective molecular sizes (Figure 10C, lanes 3 and 4). This hybridization pattern mimiced the *in organello* mtDNA labeling pattern (Figure 10C, lane 5) and the ethidium-bromide staining pattern (Figure 10C, lane 1). However, with the newly synthesized mitochondrial-nucleoid RNA probe, the hybridization pattern was nonstoichiometrical, because excessive or little hybridization was accorded selectively to a few EcoRI-cleaved mtDNA fragments (Figure 10C, lane 2, asterisks). Thus, mitochondrial-nucleoid RNA is transcribed *in vitro* in a nonrandom fashion apparently under certain regulations, whereas mitochondrial-nucleoid DNA is synthesized evenly.

DISCUSSION

Results presented above indicate that plant mitochondrial nucleoids are dynamic entities in structure and function and may participate actively in the biogenesis of the organelle. In addition to being self-sufficient for DNA replication and RNA transcription, mitochondrial nucleoids and their DNA apparently are associated with actin (Figures 4 and 5). These results suggest strongly that mitochondrial nucleoids may be the basic component of cytoskeleton-driven mtDNA segregation machinery during mitochondrial propagation. Our findings in mung bean are consistent with those shown in yeast and animal cells (4–6,14,15,36).

The distinct change of mitochondrial-nucleoid ultrastructure *in situ* during cotyledon development provided the initial evidence supporting the dynamic nature of plant mitochondrial nucleoids (Figures 1 and 2). The change of

mtDNA organization from an inconspicuous state to a chromatin-like structure and then to a loosely packed, thin-fibril-like organization was observed during early development of the cotyledon (Table 1 and Figure 2). To our knowledge, structural changes of mitochondrial nucleoids during tissue development have not been reported in other organisms. Under etiolated conditions, respiration is the only energy conversion pathway available to dormant seeds for germination and further development. The activation of mitochondrial genetic function in preparation for rapid organelle proliferation must be mandatory. Thus, the observed structural changes of mitochondrial nucleoids appear to be coordinated with mitochondrial biogenesis.

It is of interest to note that α subunit of ATPase, an inner membrane protein, was cross-linked to mtDNA *in organello* by treating intact mitochondria with formaldehyde prior to nucleoids isolation (Figure 5). The same phenomenon was reported in yeast (12). Thus, mtDNA molecules in mitochondrial nucleoids appear to locate in close proximity to ATP synthase in the organelle. The reason for this association is not known at present. Although α subunit of ATPase was routinely associated with isolated mitochondrial nucleoids prepared with 0.25–0.75% NP-40, this association was abolished at an NP-40 concentration of 1% (Figure 4). This result suggests that there is a range of detergent concentration suitable for mitochondrial nucleoids isolation beyond which the *in organello* organization of nucleoids may no longer be preserved. Most intriguing is that a sizable fraction of porin was still associated with nucleoids prepared with 1% NP-40, which completely eliminated inner-membrane proteins, α subunit of ATPase and COX II (Figure 4). In yeast, porin was largely eliminated from isolated mitochondrial nucleoids by subjecting them to an extra round of NP-40 treatment (9), but this was not the case in our study. Hence, the possibility that the membrane associated with isolated nucleoids, or a significant portion of it, is derived from the inter-membrane contact sites rich in porin should be entertained. The association of mitochondrial nucleoids with porin, with its newly found properties of translocating double-stranded DNA and of auto-directed self insertion (37,38), may have not-yet-appreciated functional significance with respect to mitochondrial biogenesis.

In addition, our finding that nucleoids isolated with NP-40 possess a phospholipid composition similar to those of whole mitochondrial membrane (Figure 3, lanes 3 and 5) may also be explained by the co-existence of inner and outer membranes in isolated nucleoids. This interpretation is consistent with and supports the finding that actively replicating nucleoids in yeast were associated *in situ* with a discrete proteinaceous structure that spanned the outer and inner mitochondrial membrane (15). Taken together, these findings imply that there may be a defining relationship between structural organization and functional integrity for isolated mitochondrial nucleoids. Although the structural domain of the nucleoids membrane is yet to be characterized, the association of mtDNA with an outer membrane protein complex, mdm10p-mdm12p and mmm1p, has been shown in yeast (14,15), which suggests that mitochondrial nucleoids in yeast may also be associated with some components of the outer membrane.

The finding that actin's association with mitochondrial nucleoids was more resistant to nonionic detergent than that of certain mitochondrial proteins routinely found with
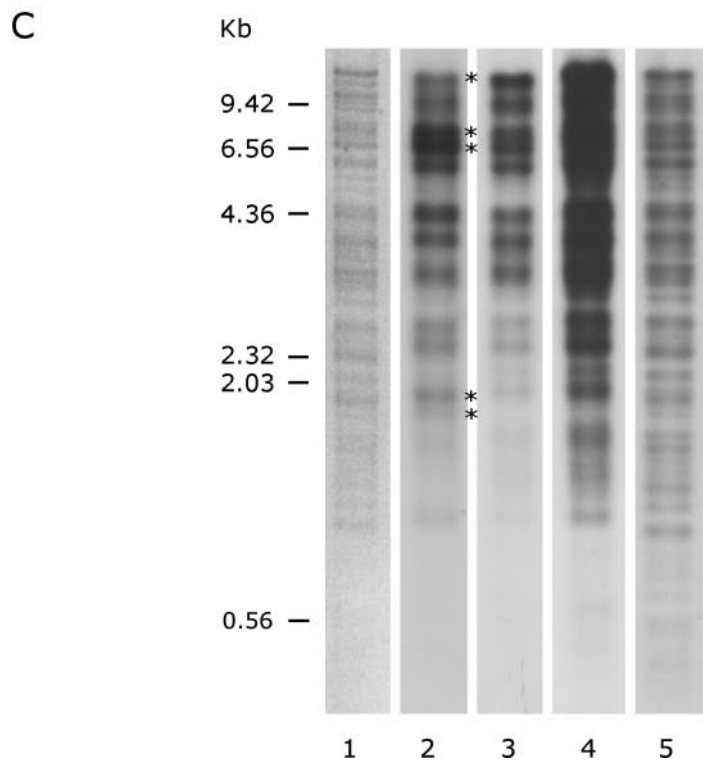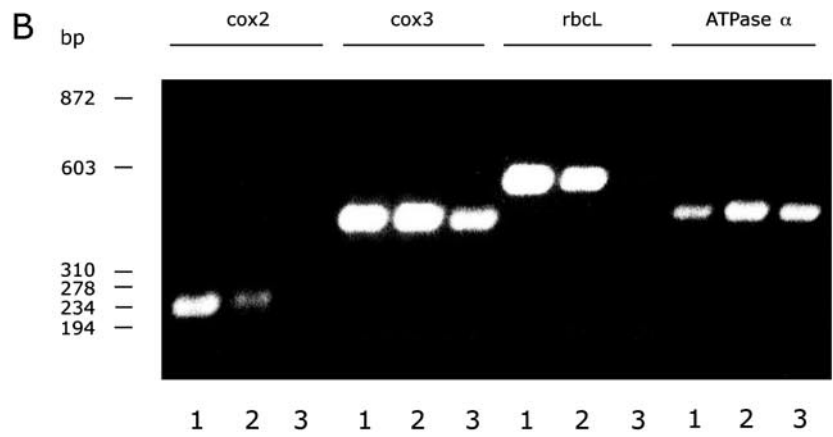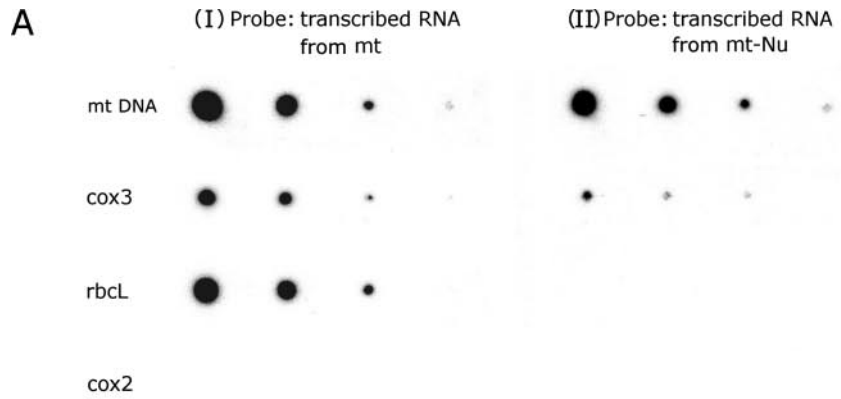
mitochondrial nucleoids (Figure 4) was unexpected. *In organello* cross-linking of actin to mtDNA in cytochalasin B- and proteinase K- pre-treated mitochondria (Figure 5) has provided strong support for the interaction of actin with mitochondrial nucleoids *in organello*. These mutually reinforcing results suggest that actin is involved in the segregation of mtDNA and of mitochondrial nucleoids in higher plants. In yeast, mtDNA was shown to be associated with the protein complex, mdm10p/mdm12p/mmm1p, and its linkage with actin was postulated to be involved in the transfer of mtDNA during propagation (14). Tubulin was shown to be an inherent component of human mitochondrial membrane by its resistance to high-salt wash and by its immuno-coprecipitation with porin (VADC) (39). Human mitochondrial nucleoids were shown to be connected to cytoskeletal tubulin structure exhibiting a mobile and dynamic characteristic, which presumably ensures the transmission of mtDNA to daughter mitochondria during mitochondrial growth and division (6). Our preliminary work also showed that high-salt treatment could not strip away tubulin from purified mung bean mitochondria, as was the case for actin (unpublished data). Further investigations are necessary to dissect the significance of porin's association with the cytoskeleton and the functional role of actin and tubulin in mitochondrial development and biogenesis.

The efficient incorporation of ^{32}P-labeled deoxyribonucleotides into mitochondrial nucleoids allowed us to characterize mtDNA synthesis by [α-^{32}P]dCTP pulse-labeling followed by PFGE and autoradiography. From this approach, it is clear that isolated mung bean mitochondrial nucleoids are self-sufficient in terms of template, primer, enzymes and other factors necessary to direct DNA synthesis *in vitro* (Figure 7). Although we could not rule out the possibility that the DNA synthesis we detected in mitochondrial nucleoids was due in part to DNA repair, a number of collateral lines of evidence suggest that such DNA synthesis was mainly the result of DNA replication. First, ^{32}P-labeled nucleotides were incorporated stoichiometrically into newly synthesized mtDNA fragments (Figure 10C, lane 3). Second, the mtDNA synthesis pattern in isolated nucleoids was the same as that in isolated mitochondria (Figures 7B and 8A and Figure 10C, lanes 3–5). Third, under virtually the same experimental conditions used for the present work, both DNA polymerase and topoisomerase I activities were found to co-localize with a fast-sedimenting wheat mtDNA fraction that exhibited endogenous DNA synthesis activity (23). Recent studies in human cells showed that BrdU was incorporated into mitochondrial

nucleoids *in situ* and mtDNA polymerase could also be co-purified with BrdU-containing mitochondrial nucleoids (4–6). These results are analogous to and support our interpretation that DNA synthesis in isolated mitochondrial nucleoids detected by ^{32}P-dCTP pulse labeling *in vitro* (Figure 7) is originated from the mtDNA replication process. Thus, mung bean mitochondrial nucleoids appear to possess a full complement of physical basis to maintain the genetic continuity of the mitochondrial genome.

The resistance to aphidicolin and sensitivity to NEM we demonstrated for mung bean mitochondrial-nucleoid DNA synthesis *in vitro* (Table 2) are consistent with other plant mtDNA synthesis studies (7,22,34,35). Our result suggests that in so far as sensitivity to inhibitors is concerned, mung bean mitochondrial DNA polymerase like its counterpart in other plants is similar to DNA polymerase γ of nonplant eukaryotes. Plant mitochondrial DNA polymerases are known to differ from that of nonplant eukaryotes with respect to their lower sensitivity to ddCTP. We found that this is also the case for mung bean. In fact, DNA-synthesis sensitivity to ddCTP in mung bean mitochondrial nucleoids was exceptionally low (Table 2), which implies that this enzyme may have an unusual structural characteristic.

Yeast and higher plant mitochondria contain a sizable amount of DNA that would not migrate out of the sample-loading well in PFGE (16,18,21,33,40). Our results demonstrate that the same is true for DNA of mung bean mitochondrial nucleoids and intact mitochondria (Figure 6). The reason for the immobility of the well bound mtDNA remains unsettled. Molecular details of wb and fm forms in terms of their physical structure, template activity for DNA synthesis and possibly also their differential accessibility/sensitivity to endogeneous nucleases remain largely unknown. Two postulations have been advanced for the wb form: it is resulted from replicating rolling circular mtDNA molecules that may be too large and/or too complex to be mobile in the pulsed electrical field, or alternatively, the immobility may have resulted from an unusual mtDNA structure produced by multiple recombination events involving branch points that connect multigenomic concatemers (19,21).

For ^{32}P-dCTP pulse labeling up to 4 h in duration, the pattern of DNA synthesis in isolated mung bean mitochondrial nucleoids is essentially the same as that of intact mitochondria. Significantly, *in vivo* mung bean mtDNA replicates in seedling tissues by the same pattern (Figure 9). In all three cases, the newly synthesized DNA manifested in the wb and in fm form

Figure 10. Characterization of mitochondrial-nucleoid RNA synthesis *in vitro*. (**A**) The specificity of newly synthesized RNA. (I): As a control, a newly synthesized, ^{32}P-labeled RNA isolated from 1 mg protein equivalent of purified mitochondria was used to probe a duplicate membrane described in (II). (II): A nucleoids sample isolated from 1 mg protein equivalent of mitochondria was subjected to RNA synthesis *in vitro* in the presence of [α-^{32}P]UTP. The newly synthesized, ^{32}P-labeled RNA was then isolated to probe serially diluted DNA templates immobilized on a membrane. The DNA templates are: mtDNA and PCR-amplified gene fragment of *cox 3*, *rbc* L, and *cox 2*. (**B**) Specificity of mitochondrial-nucleoid RNA. The RNA templates used for reserve PCR were derived, from whole mung bean seedlings (lane 1), mitochondria (lane 2) and isolated mitochondrial nucleoids (lane 3). Four sets of primers (see Materials and Methods) each designed to amplify a coding region of nucleus-encoded *cox 2*, mitochondrion-encoded *cox 3*, chloroplast-encoded *rbc* L and mitochondrion-encoded α-subunit of *ATPase* were used for standard reverse PCRs. The product of these 12 individual PCRs was analyzed on an agarose gel. The size of double-stranded DNA markers is indicated on the left. Approximately a billion-fold amplification revealed the presence of contaminating chloroplast RNA transcripts in the mitochondrial RNA samples (lane 2 in rbcL sector) isolated from mitochondria. (**C**) A comparison of newly synthesized RNA and DNA products of mitochondrial nucleoids. Two nucleoids samples, each isolated from 5 mg protein equivalent of mitochondria, were subjected to RNA synthesis in the presence of [α-^{32}P]UTP and to DNA synthesis in the presence of [α-^{32}P]dCTP. The newly synthesized RNA was then purified and used to probe a DNA blot containing EcoRI-fragments of CsCl-gradient-purified mtDNA (lane 2). An identical blot was probed by the newly synthesized, ^{32}P-labeled DNA (lane 3). Asterisks shown in lane 2 indicate the nonstoichiometrical bands resulting from hybridization of newly synthesized RNA with mtDNA-EcoRI fragments. Lane 4 (48 h exposure) came from the same blot as lane 3 (5 h exposure). EcoRI fragments of ^{32}P-labeled, newly synthesized DNA isolated from mitochondria are shown in lane 5 as a control. The EtBr-stained, mtDNA-EcoRI-fragment banding pattern is shown in lane 1.

(Figures 7B, 8A and 9). This synthesis pattern is, in general, congruent with the mtDNA synthesis pattern obtained by labeling plant cell cultures with ^{3}H-thymidine (17,21). For longer pulse or pulse-and-chase period, a significant difference in stability between the newly synthesized wb and fm form was observed in intact mitochondria but not in mitochondrial nucleoids (lane 5 in Figure 8A and B and Figure 7B and D). The fact that this stability difference could be abolished by an exogenous nuclease inhibitor, ATA, suggests that the accessibility and/or substrate specificity to the nuclease for newly synthesized wb and fm DNA may not be the same in intact mitochondria and this ATA-sensitive nuclease was either absent or inactive in nucleoids. It is of interest to note that both wb and fm forms of newly synthesized mtDNA in mung bean seedlings turned over completely in 24 h (Figure 9). To our knowledge, this is the first demonstration of a complete turn over for plants mtDNA *in vivo*.

By BrU incorporation and immunofluorescent staining, mitochondrial nucleoids in human cells were shown to synthesize RNA *in situ* and the mitochondrial transcription factor was shown to co-purify with mitochondrial nucleoids (4–6). RNA synthesis *in vitro* was also reported for mitochondrial nucleoids obtained from cultured tobacco cells (7). Consistent with these findings, we have demonstrated the presence of a self-sufficient mitochondrial transcriptional system and of mitochondria-encoded gene transcripts in isolated mung bean mitochondrial nucleoids (Figure 10).

On the basis of the structural and functional characteristics of higher plant mitochondrial nucleoids elucidated in the present study, together with results from mitochondrial nucleoids studies in yeast and mammalian cells, our results clearly establish the important paradigm that mitochondrial nucleoids in eukaryotes are more than mere mtDNA packaging and segregation entities but are centers of mtDNA maintenance and expression. Even though we found that mitochondrial nucleoids possessed no protein translation capability (data not shown), mitochondrial nucleoids were implicated in organizing the translation machineries on both sides of the mitochondrial membrane and could enhance assembling efficiency of nucleus-encoded and mitochondrion-encoded proteins into a mitochondrial complex (6). Mung bean mitochondrial nucleoid**s** appear to be structurally and functionally competent to constitute the rudimentary origin for mitochondrial biogenesis and they may play an essential role in the non-binary propagation of plant mitochondria in rapidly grown mung bean tissues as we have postulated in our earlier work (29). In this regard, our present finding on the association of mitochondrial nucleoids with actin is of particular interest.

ACKNOWLEDGEMENTS

Conflict of interest statement. None declared.

REFERENCES

1. Nass,M.M. (1969) Mitochondrial DNA. I. Intramitochondrial distribution and structural relations of single- and double-length circular DNA. *J. Mol. Biol.*, **42**, 521–528.
2. Stevens,B. (1981) Mitochondrial structure. In Strathern,J.M., Jones,E.W. and Broach,J.R. (eds), *The Molecular Biology of the Yeast Saccharomyces*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 471–504.
3. Spelbrink,J.N., Li,F.Y., Tiranti,V., Nikali,K., Yuan,Q.P., Tariq,M., Wanrooij,S., Garrido,N., Comi,G., Morandi,L. *et al.* (2001) Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nature Genet.*, **28**, 223–231.
4. Garrido,N., Griparic,L., Jokitalo,E., Wartiovaara,J., van der Bliek,A.M. and Spelbrink,J.N. (2003) Composition and Dynamics of human mitochondrial nucleoids. *Mol. Biol. Cell*, **14**, 1583–1596.
5. Legros,F., Malka,F., Frachon,P., Lombes,A. and Rojo,M. (2004) Organization and dynamics of human mitochondrial DNA. *J. Cell Sci.*, **117**, 2653–2662.
6. Iborra,F.J., Kimura,H. and Cook,P.R. (2004) The functional organization of mitochondrial genomes in human cells. *BMC Biol.*, **2**, 9.
7. Sakai,A., Takano,H. and Kuroiwa,T. (2004) Organelle nuclei in higher plants: structures, composition, function, and evolution. *Int. Rev. Cytol.*, **238**, 59–118.
8. Miyakawa,I., Sando,N., Kawano,S., Nakamura,S. and Kuroiwa,T. (1987) Isolation of morphologically intact mitochondrial nucleoids from the yeast, *Saccharomyces cerevisiae*. *J. Cell Sci.*, **88**, 431–439.
9. Newman,S.M., Zelenaya-Troitskaya,O., Perlman,P.S. and Butow,R.A. (1996) Analysis of mitochondrial DNA nucleoids in wild-type and a mutant strain of *Saccharomyces cerevisiae* that lacks the mitochondrial HMG box protein Abf2p. *Nucleic Acids Res.*, **24**, 386–393.
10. Miyakawa,I., Fumoto,S., Kuroiwa,T. and Sando,N. (1995) Characterization of DNA-binding proteins involved in the assembly of mitochondrial nucleoids in the yeast *Saccharomyces cerevisiae*. *Plant Cell Physiol.*, **36**, 1179–1188.
11. Meeusen,S., Tieu,Q., Wong,E., Weiss,E., Schieltz,D., Yates,J.R. and Nunnari,J. (1999) Mgm101p is a novel component of the mitochondrial nucleoid that binds DNA and is required for the repair of oxidatively damaged mitochondrial DNA. *J. Cell Biol.*, **145**, 291–304.
12. Kaufman,B.A., Newman,S.M., Hallberg,R.L., Slaughter,C.A., Perlman,P.S. and Butow,R.A. (2000) In organello formaldehyde crosslinking of proteins to mtDNA: identification of bifunctional proteins. *Proc. Natl Acad. Sci. USA*, **97**, 7772–7777.
13. MacAlpine,D.M., Perlman,P.S. and Butow,R.A. (2000) The numbers of individual mitochondrial DNA molecules and mitochondrial DNA nucleoids in yeast are co-regulated by the general amino acid control pathway. *EMBO J.*, **19**, 767–775.
14. Boldogh,I.R., Nowakowski,D.W., Yang,H.C., Chung,H., Karmon,S., Royes,P. and Pon,L.A. (2003) A protein complex containing Mdm10p, Mdm12p, and Mmm1p links mitochondrial membranes and DNA to the cytoskeleton-based segregation machinery. *Mol. Biol. Cell*, **14**, 4618–4627.
15. Meeusen,S. and Nunnari,J. (2003) Evidence for a two membrane-spanning autonomous mitochondrial DNA replisome. *J. Cell Biol.*, **163**, 503–510.
16. Andre,C.P. and Walbot,V. (1995) Pulsed-field gel mapping of maize mitochondrial chromosomes. *Mol. Gen. Genet.*, **247**, 255–263.
17. Oldenburg,D.J. and Bendich,A.J. (1996) Size and structure of replicating mitochondrial DNA in cultured tobacco cells. *Plant Cell*, **8**, 447–461.
18. Maleszka,R., Skelly,P.J. and Clark-Walker,G.D. (1991) Rolling circle replication of DNA in yeast mitochondria. *EMBO J.*, **10**, 3923–3929.
19. Backert,S., Dorfel,P., Lurz,R. and Borner,T. (1996) Rolling-circle replication of mitochondrial DNA in the higher plant Chenopodium album (L.). *Mol. Cell. Biol.*, **16**, 6285–6294.
20. Kreuzer,K.N. and Morrical,S.W. (1994) Initiation of DNA replication. In Daram,J.D. (ed.), *Molecular Biology of Bacteriophage T₄*. American Society for Microbiology Press, Washington D.C., pp. 28–42.
21. Oldenburg,D.J. and Bendich,A.J. (1998) The structure of mitochondrial DNA from the liverwort, Marchantia polymorpha. *J. Mol. Biol.*, **276**, 745–758.
22. Sasak,N., Sasaki,A., Kawano,S., Kuroiwa,H. and Kuroiwa,T. (1999) DNA synthesis in isolated mitochondrial nucleoids from plasmodia of Physarum polycephalum. *Protoplasma*, **203**, 221–231.

23. Echeverria,M., Robert,D., Carde,J.P. and Litvak,S. (1991) Isolation from wheat mitochondria of a membrane-associated high molecular weight complex involved in DNA synthesis. *Plant Mol. Biol.*, **16**, 301–315.

24. Fey,J., Vermel,M., Grienenberger,J., Marechal-Drouard,L. and Gualberto,J.M. (1999) Characterization of a plant mitochondrial active chromosome. *FEBS Lett.*, **458**, 124–128.

25. Kuroiwa,T. (1982) Mitochondrial nuclei. *Int. Rev. Cytol.*, **75**, 1–59.

26. Nunnari,J., Marshall,W.F., Straight,A., Murray,A., Sedat,J.W. and Walter,P. (1997) Mitochondrial transmission during mating in *Saccharomyces cerevisiae* is determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. *Mol. Biol. Cell*, **8**, 1233–1242.

27. Okamoto,K., Perlman,P.S. and Butow,R.A. (1998) The sorting of mitochondrial DNA and mitochondrial proteins in zygotes:preferential transmission of mitochondrial DNA to the medial bud. *J. Cell Biol.*, **142**, 613–623.

28. Dai,H., Lo,Y.S., Wu,C.Y., Tsou,C.L., Hsu,G.S., Chern,C.G., Ruddat,M. and Chiang,K.S. (1991) Protein synthesis in isolated mitochondria of rice (Oryza sativa L.) seedlings. *Plant Physiol.*, **96**, 319–323.

29. Dai,H., Lo,Y.S., Jane,W.N., Lee,L.W. and Chiang,K.S. (1998) Population heterogeneity of higher-plant mitochondria in structure and function. *Eur. J. Cell Biol.*, **75**, 198–209.

30. Higgins,J.A. (1990) Separation and analysis of membrane lipid components. *Biological Membranes: A Practical Approach.* Oxford University Press, NY, pp. 103–137.

31. Sambrook,J., Fritsch,E. and Maniatis,T. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 382–389.

32. Martin,M.T., Echeverria,M., Litvak,S. and Araya,A. (1987) Studies of transcription in isolated wheat mitochondria and organelle extracts. *Plant Sci.*, **49**, 199–207.

33. Bendich,A.J. (1996) Structural analysis of mitochondrial DNA molecules from fungi and plants using moving pictures and pulsed-field gel electrophoresis. *J. Mol. Biol.*, **255**, 564–588.

34. Heinhorst,S., Cannon,G.C. and Weissbach,A. (1990) Chloroplast and mitochondrial DNA polymerase from cultured soybean cells. *Plant Physiol.*, **92**, 939–945.

35. Sato,K. and Fukuda,H. (1996) Purification and characterization of a mitochondrial DNA polymerase from cultured tobacco cells. *Plant Cell Physiol.*, **37**, 989–995.

36. Hobbs,A.E., Srinivasan,M., McCaffery,J.M. and Jensen,R.E. (2001) Mmm1p, a mitochondrial outer membrane protein, is connected to mitochondrial DNA (mtDNA) nucleoids and required for mtDNA stability. *J. Cell Biol.*, **152**, 401–410.

37. Xu,X. and Colombini,M. (1997) Autodirected insertion: preinserted VDAC channels greatly shorten the delay to the insertion of new channels. *Biophys. J.*, **72**, 2129–2136.

38. Szabo,I., Bathori,G., Tombola,F., Coppola,A., Schmehl,I., Brini,M., Ghazi,A., De Pinto,V. and Zoratti,M. (1998) Double-stranded DNA can be translocated across a planar membrane containing purified mitochondrial porin. *FASEB J.*, **12**, 495–502.

39. Carre,M., Andre,N., Carles,G., Borghi,H., Brichese,L., Briand,C. and Braguer,D. (2002) Tubulin is an inherent component of mitochondrial membranes that interacts with the voltage-dependent anion channel. *J. Biol. Chem.*, **277**, 33664–33669.

40. Bendich,A.J. (1993) Reaching for the ring: the study of mitochondrial genome structure. *Curr. Genet.*, **24**, 279–290.