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Mapping the mitochondrial DNA of *Zea mays*: Ribosomal gene localization

(mitochondrial rRNAs/restriction enzymes/hybridization/clone)

KEITH P. IAMS AND JOHN H. SINCLAIR

Biology Department, Indiana University, Bloomington, Indiana 47405

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ABSTRACT **We have located the 18S and 26S ribosomal genes on a 32.2-kilobase pair (kb) restriction map of *Zea mays* mitochondrial DNA. In a *Bam*HI restriction digest of mitochondrial DNA, band 4 carries all of the 26S gene whereas band 2 carries the 18S gene sequence. We have cloned and mapped bands 2 and 4 and show that they are contiguous in the genome. The 26S sequence is at one end of the 13.7-kb fragment 4, immediately adjacent to the junction with fragment 2. The 18S sequence is located at the far end of the 17.5-kb fragment 2, about 15 kb away from the 26S gene. A second region of 18S sequence homology is found on band 40. This region contains sequences that cross-hybridize with those in band 2. The nature of this apparent sequence repetition is unclear.**

The mitochondria of *Zea mays* and other higher plants have a genome size some 20–30 times as large as that of animals and some 5 or more times the size of yeast. In *Zea mays* mtDNA, this seems not to be a result of significant repetitive sequences (1). Determination of the size of this genome by summation of fragments produced by restriction enzymes is complicated by the large number of identical or similar-sized bands, and estimates of genome size from the literature vary from as small as 175 kilobase pairs (kb) (2) to as large as 600 kb (1). Our own estimates indicate a size of about 400 kb (unpublished data). Little is yet known about the physical organization of this large genome, although evidence from electron microscopy suggests that genes may be distributed among several circular molecules (2). If true, this will be the first instance of multiple molecules in organelle genomes.

In vivo protein synthesis experiments indicate that maize mtDNA codes for about 18 polypeptides (3). The gene for a part of cytochrome oxidase has been mapped and sequenced (4). Ribosomal RNAs have been identified for maize mtDNA and located on restriction patterns (5, 6).

To facilitate the mapping of the 18S and 26S genes, we cloned restriction enzyme-digested mtDNA in the plasmid pBR322. These clones were restriction mapped and used as probes to determine the orientation and copy number of these two genes. Only one gene codes for the 26S rRNA, and this is located approximately 15 kb from the 18S ribosomal sequence. However, the 18S sequence seems to be partially duplicated in other parts of the genome.

MATERIALS AND METHODS

Isolation of Mitochondria, mtDNA, mtRNA, and *Escherichia coli* rRNA. Mitochondria were isolated and mtDNA was extracted from sterile, etiolated seedlings of strain FRB 73 (Illinois Foundation Seeds, Inc.) by methods adapted from Levings and Pring (2). Mitochondria were prepared for RNA extraction in much the same way as for DNA up to the point of DNAse digestion. After this, RNA was extracted essentially as described by Pring (7).

E. coli rRNA was extracted from frozen cells. A 25-g pellet was suspended in 15 ml of buffer (50 mM Tris, pH 7.2/10 mM $MgCl_2$/30 mM NH_4Cl/1 mM dithiothreitol), digested with DNase 1 (10 μg/ml), and disrupted by three passes through a French pressure cell (103.4 MPa; 15,000 pounds per square inch); then the debris was removed on a Sorvall SS34 at 34,800 × g for 20 min. Ribosomes were pelleted in a Beckman vTi 50 rotor for 70 min at 170,000 × g. Ribosomal subunits were separated on a sucrose gradient, and RNAs were purified with phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol), followed by ethanol precipitation.

Radiolabeling RNAs and DNA Fragments. One microgram of 18S RNA was hydrolyzed 10 min in 10 mM Tris (pH 9.5) at 90°C in a sealed 20-μl micropipet. The 26S RNA was hydrolyzed for only 5 min. The labeling procedure was essentially as described by Maizels (8).

CsCl-purified cloned DNA and DNAs extracted from gels were nick-translated as described by Rigby *et al.* (9), followed by filtration on Sephadex G-50. DNAs were removed from soft agarose (Bethesda Research Laboratories) essentially as described by the manufacturer.

Restriction Digests of Cloned Fragments and mtDNA. Three-microgram digests of mtDNA were run overnight at 25 mA on 30-cm agarose gels (1%) and were stained with ethidium bromide. Cloned DNA was digested and run on 20-cm agarose gels (1%) for 3 hr at 75 mA. Transfer to nitrocellulose was by Southern blotting (10). Fragment sizes were determined from agarose gels by comparison to *Hin*dIII/*Bam*HI fragments of bacteriophage λ DNA. The sizes of 12 bands of phage λ were estimated from the data of P. Phillipson and R. W. Davis in the Bethesda Research Laboratories catalog.

RESULTS

Purification of RNAs and Restriction Fragment Localization. Fig. 1 compares *E. coli* RNA (lane A) with mtRNA (lane B). To limit degradation, mitochondria were vigorously lysed in a Virtis homogenizer in phenol. The 26S and 18S peaks were resolved on sucrose gradients before use as probes for each ribosomal gene. *E. coli* 16S and 23S rRNAs were used as unlabeled competitors to reduce subunit cross-contamination during hybridization. They were highly effective homologous competitors.

Fig. 2 shows the hybridization of mitochondrial 26S (lanes A, D, G, and J) and 18S (lanes C, F, I, and L) rRNAs to patterns of mtDNA produced by *Hin*dIII, *Bam*HI, *Pst* I, and *Sal* I. Each band of rRNA hybridization is designated by the initial of the endonuclease that produced the fragment and the band number

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Abbreviation: kb, kilobase pair(s).

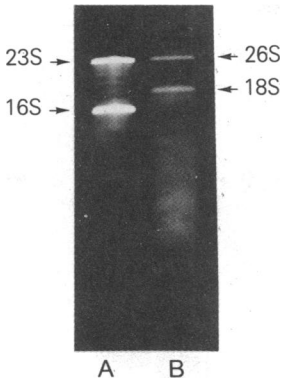

FIG. 1. Electrophoresis of *E. coli* RNA (lane A) and *Zea mays* mtRNA (lane B) run on a 1.5% agarose gel containing 6 M urea and 0.015 M iodoacetate. Gel was run for 3 hr at 100 V.

that it represents in the mtDNA restriction pattern (unpublished data). For example, "H21" defines band number 21 in the *Hin*dIII pattern of *Zea* mtDNA.

Hybridization patterns of 26S rRNA were highly reproducible. The *Bam*HI and *Pst* I patterns showed only one site of hybridization (B4 and P2), whereas the *Sal* I and *Hin*dIII patterns showed two sites each (S2 and S12, H19 and H47, respectively). These results suggest a single location for a 26S structural gene. This sequence contains one *Hin*dIII restriction site and one *Sal* I site but lacks both *Bam*HI and *Pst* I sites.

The 18S hybridization pattern was more complex. *Bam*HI and *Pst* I both produced only two sites of hybridization, suggesting a single restriction site for each enzyme within the gene. However, the *Hin*dIII and *Sal* I patterns contradict such a simple explanation because 18S rRNA hybridized to both of these patterns at three sites. The *Hin*dIII fragments were 12.5 kb (H2), 9 kb (H8), and 2.4 kb (H41) in length, whereas the *Sal* I fragments were 15.0 kb (S9), 12.5 kb (S12), and 9.2 kb (S19) in length. The sizes of these fragments make it difficult to postulate a single genomic location for the 18S gene unless unreasonably extensive regions of intervening sequence(s) are proposed.

Alternative possibilities are that there is more than one locus for the 18S gene, or else that there is an additional homologous sequence(s) in the genome not representing the functional 18S gene sequence.

Examination of several hybridization experiments reveals that bands H2 and H8, as well as S12 and S19, showed highly variable hybridization intensities, often being much less distinct than the third sites at H41 and S9. In Fig. 2 these variable bands show a higher-than-average relative intensity so that all three may be clearly seen. Variable hybridization was also characteristic of B40 and P23. Variability appeared to be related to post-hybridization wash conditions, and a short preincubation in hybridization buffer significantly reduced the intensity of these bands. These observations suggest that a single consistently hybridizing sequence accounts for the 18S gene and that additional variably hybridizing sequences have limited, but real, homology with the rRNA probe.

Isolation and Mapping of Cloned Fragments Carrying Ribosomal Genes. Three *Bam*HI fragments contain sequences homologous to rRNAs. B2 and B40 hybridize to 18S RNA (B40 being variable) and B4 to 26S rRNA. B2 and B4 were available from a clone bank, but we have been unable to identify a clone showing the B40 homology. Hybridization of *Hin*dIII digests of B4 and B2 (Fig. 3, lanes C and D, respectively) to RNA localized the 18S sequence to a 2.4-kb fragment within B2 (lane F) and the 26S sequence to two fragments of 5.5- and 1.9-kb length within B4 (lane A). There was no 26S RNA homology to B2 (lane B) or 18S RNA homology to B4 (lane E). *Sma* I digestion of B4 reduced the 26S sequence to a 2.6-kb segment of the 5.5-kb fragment (not shown). This localized the 26S ribosomal gene to 4.4 kb of the 14.7-kb B4 fragment and the 18S rRNA gene sequences to 2.4 kb of the 17.5-kb B2 fragment.

Alignment of the three rRNA homologous *Hin*dIII fragments with the *Hin*dIII pattern of mtDNA (not illustrated) identified them as H41, H19, and H47. These fragments represent the same bands to which 26S rRNA and 18S rRNA consistently hybridize (see Fig. 2). A restriction map produced by multiple digestion of B2 and B4 with *Bam*HI, *Hin*dIII, *Sal* I, *Pst* I, and *Sma* I locates the rRNA near the ends of each *Bam*HI fragment (Fig. 4). The order of the smaller *Hin*dIII fragments of B2 (sizes of 3.24, 2.0, 1.2, 0.9, 0.8, 0.6, 0.5, and 0.3 kb) is still not determined.

Reduction of the ribosomal genes to small segments of DNA (H41, H19, and H47) made it possible to test for the uniqueness of rRNA genes by DNA/DNA hybridization. H19 and H47 (extracted from soft agarose gels of *Hin*dIII-digested B4) and H41 (from a *Hin*dIII clone bank) were nick-translated and hybridized to Southern blots of mtDNA, which was cleaved with *Hin*dIII. Fig. 5 shows the hybridization patterns for H41 (lane B), H47 (lane C), and H19 (lane D). H19 and H47 are uniquely

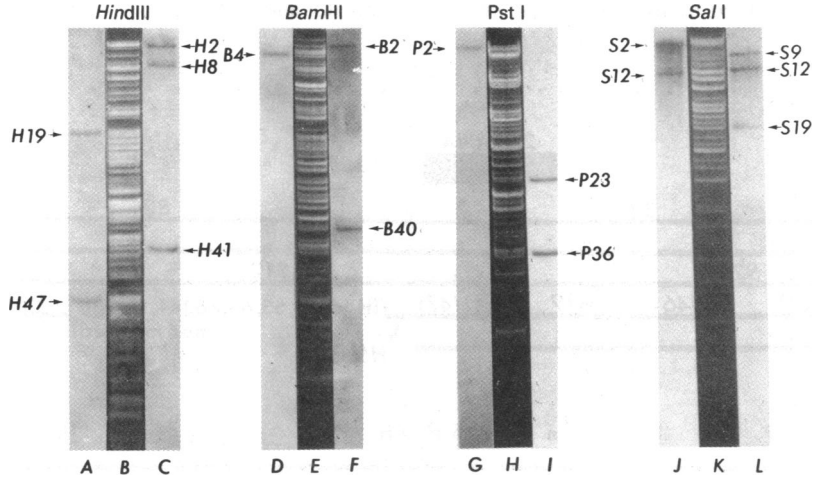

FIG. 2. Restriction patterns of *Zea mays* mtDNA produced by *Hin*dIII, *Bam*HI, *Pst* I, and *Sal* I (lanes B, E, H, and K, respectively). One percent agarose gels were run 16 hr at 60 V. Each restriction digest is aligned with the hybridization pattern produced by the radiolabeled 26S mitochondrial rRNA (lanes A, D, G, and J) and the 18S RNA (lanes C, F, I, and L).

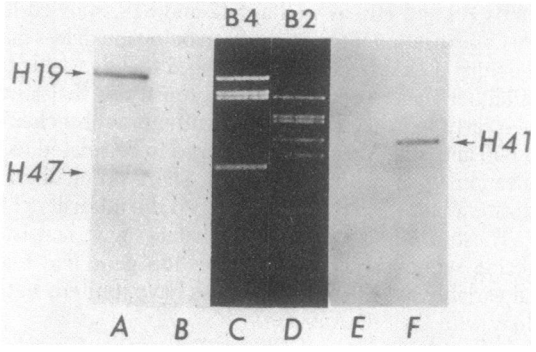

FIG. 3. Hybridization of *Zea mays* rRNAs to *Hin*dIII digests of cloned B2 and B4 fragments. The autoradiographic pattern of purified 26S rRNA (lanes A and B) is aligned with the patterns of B2 (lane C) and B4 (lane D). The 18S hybridization patterns to B4 (lane E) and B2 (lane F) are also shown. The 26S sequence is resolved to subfragments of B4, identifiable as *Hin*dIII fragments 19 and 47. The 18S sequence, located in B2, has been resolved to *Hin*dIII fragment 41.

homologous with their respective genomic fragments. However, H41, which carries the 18S ribosomal sequence, not only hybridized to the genomic band which it represents but also to two larger bands, H2 and H8. These are the same bands that hybridized with varying efficiency to 18S RNA (see Fig. 2), and this indicates a repetition of some sequences present on H41 at H2 and H8 fragment sites. This is consistent with the 18S rRNA hybridization results and with the conclusion that H2 and H8 contain nonunique sequences with limited homology to the 18S gene. Hybridization of the three probes to *Sal* I, *Pst* I, and *Bam*HI restriction patterns further confirmed the map of the 18S and 26S sequences illustrated in Fig. 4.

Cloned B2 and B4 DNAs were also nick-translated and hybridized to *Sal* I, *Hin*dIII, and *Pst* I mtDNA restriction patterns (not illustrated), thus identifying all bands contained within B2 and B4 and also identifying the fragments that span the sites at each end of B2 and B4. Common bands of hybridization to both B2 and B4 were seen for all enzymes, suggesting that the two DNAs are contiguous. To verify this, end fragments from the two DNAs were individually isolated for use as hybridization probes (Fig. 6).

When the 0.8-kb *Hin*dIII subfragment from the end of B2 (Fig. 4) was hybridized to a *Hin*dIII digest of mtDNA (lane B), it hybridized exclusively to H59 (lane A). The same subfragment also hybridized to S12 (lane D) of the *Sal* I pattern (lane E) and P2 (lane G) of the *Pst* I pattern (lane H). Confirmation that B2 and B4 are contiguous was provided by hybridizing the small

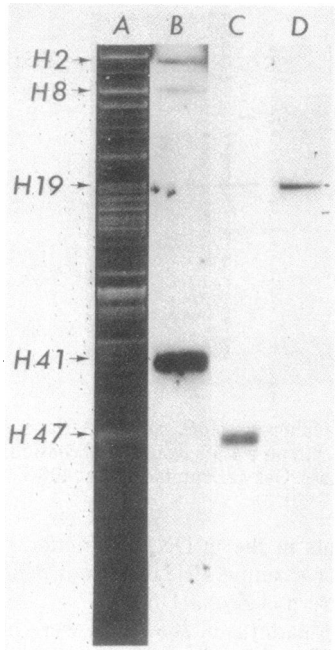

FIG. 5. Hybridization of *Hin*dIII fragments carrying ribosomal sequence to *Hin*dIII patterns of total mtDNA. Hybridization patterns produced by H41 (lane B), H47 (lane C), and H19 (lane D) are aligned with the *Hin*dIII pattern of total mtDNA (lane A). (Slight extraneous hybridization produced by H47 is due to contamination of the gel-extracted probe with the other *Hin*dIII fragments found within B4.)

Sal I subfragment from one end of B4 to the same restriction patterns. This fragment also hybridized to H59 (lane C), S12 (lane F), and P2 (lane I). Because H59 is a small fragment (1.0 kb) and because this is equal to the sizes of one end of B4 (0.2 kb) and one end of B2 (0.8 kb) (see Fig. 4), it is evident that the two fragments are located side-by-side. These results are summarized in the complete map of B4 and B2 (Fig. 4) which separates the rRNA genes by a distance of 15.3 kb. The entire mapped segment is 32.2 kb. Size estimates for all of the fragments shown, made from mtDNA restriction patterns run beside a standard of *Bam*HI/*Hin*dIII-digested phage λ DNA (unpublished data), are: B2, 17.5 kb; B4, 14.7; H19, 5.5; H21, 4.9; H34, 3.24; H36, 3.15; H41, 2.4; H45, 2.0; H46, 1.9; H47, 1.9; H55, 1.20; H59, 1.0; H63, 0.9; H>63, 0.8; H>>63, 0.6; H>>63, 0.5; H>>63, 0.3; P2, 23; P36, 3.52; P37, 3.48; S2, 25; S9, 15; and S12, 12.5.

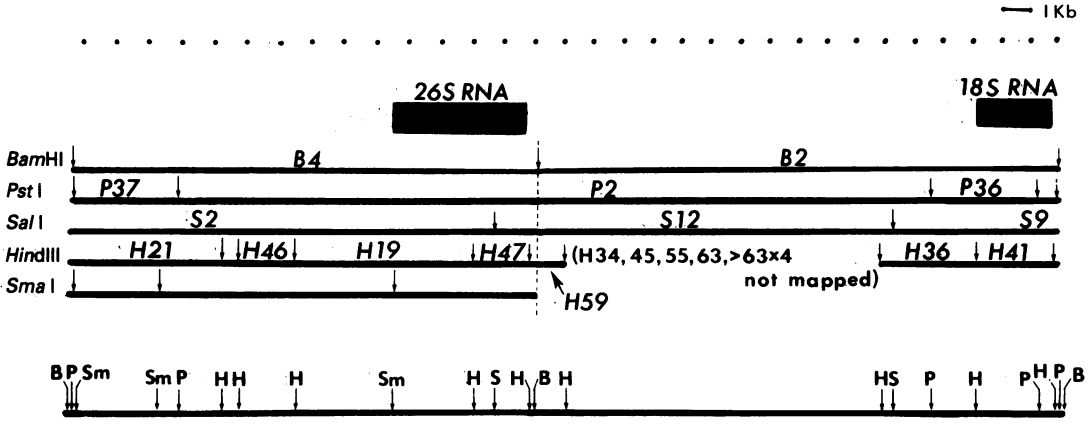

FIG. 4. Restriction maps of B2 and B4 fragments and localization of ribosomal genes within the maps. (The *Hin*dIII fragment order is still incomplete for B2.) The composite map of B2 and B4 illustrates fragments that span the *Bam*HI site. These include H58, H12, and P2. Other fragments within B2 and B4 are indicated.

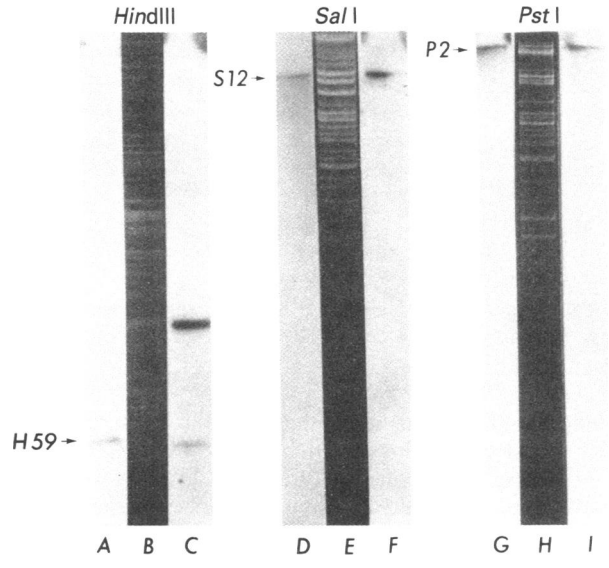

FIG. 6. Hybridization of the end fragments of cloned B2 and B4 to *Zea mays* mtDNA restriction patterns. *Hin*dIII, *Sal* I, and *Pst* I restriction patterns (lanes B, E, and H, respectively) are aligned to hybridization patterns produced by a *Hin*dIII end fragment of B2 (lanes A, D, and G) and by a *Sal* I end fragment of B4 (lanes C, F, and I).

DISCUSSION

Genes for the large (26S) and small (18S) RNAs of *Zea mays* mitochondria have been localized on a 32.2-kb segment of DNA. A restriction map locating the *Bam*HI, *Pst* I, *Sal* I, and most *Hin*dIII sites has been completed for this segment. The 26S RNA is found on *Bam* fragment 4 (14.7 kb), and the 18S gene is on *Bam* fragment 2 (17.5 kb). B2 and B4 are contiguous with 15.3 kb of sequence separating the two genes. This separation distance is considerably less than the 32 kb separating rRNAs of *Saccharomyces cerevisiae* (11) but is much greater than the 5–6 kb of *Neurospora crassa* (12) or the 0.5-kb spacer that separates HeLa rRNA genes (13). The large distance between the rRNAs of *Zea mays* makes it highly unlikely that transcription of both genes is initiated at a single promoter.

The extent of the 26S rRNA sequence has been reduced to a 4.4-kb region bounded by a *Sma* I site and a *Hin*dIII site, and the gene appears to occur only once in the genome. Assuming a continuous 26S sequence occupies 3.8 kb (7), we cannot rule out the possibility of intronic regions within the gene. Such introns are absent from the rRNA genes of mammalian and most other animal mtDNA, but have been found in chloroplast rRNAs of *Chlamydomonas* (14) and *Zea mays* (15) and in the mitochondrial genomes of *N. Crassa* (12) and *S. cerevisiae* (16).

The complete 18S gene should occupy a sequence of 2.3 kb (8), which is nearly the full extent of our reduced 2.4-kb locus. However, we do find that a few regions other than the 2.4-kb *Hin*dIII fragment hybridize to the 18S rRNA. The fact that a purified *Hin*dIII DNA fragment (H41) also hybridizes to the same additional regions shows that some degree of homology is real. Because these regions show considerable sensitivity to variation in hybridization conditions, it may be that only limited sequence homology is present. Our data do not yet permit speculation regarding whether these homologous segments represent parts of genes, pseudogenes, or other less interpretable sequences.

Our results confirm that *Zea mays* mitochondrial rRNAs share homology with *E. coli* rRNA (5). Such sequence homology with prokaryote rRNAs is more commonly a characteristic of chloroplast genomes, including that of *Zea mays* itself, which exhibits a surprising 74% homology. However, aside from the organelle DNAs of higher plants, extensive similarity between eukaryotic and prokaryotic rRNA sequences has only been reported for the mitochondrial genome of *S. cerevisiae* (17).

The exceptionally large size of the corn mitochondrial genome makes the finding of unique sequences for the rRNA genes of particular interest. Both the 15-kb mammalian and the 75-kb yeast mtDNAs have unique ribosomal genes, whereas the larger (135 kb) organelle genomes of chloroplasts often contain multiple copies of these genes (14, 15). At 400 kb, *Zea mtDNA* has a coding capacity 10% as large as the *E. coli* genome which contains seven copies of the ribosomal genes. However, despite its large size, current estimates of the *Zea* mitochondrial gene products show them to be no more than twice as numerous as those of yeast and mammalian mitochondrial products (3). If these estimates are correct, single copies of rRNA genes in higher plants should be adequate to support all necessary protein synthesis.

A unique sequence for the ribosomal genome adds to several other kinds of evidence that the exceptionally large mitochondrial genome of *Zea mays* is comprised primarily of unique sequences. Bonen and Gray (18) report single ribosomal genes in wheat mtDNA, and a single copy of the *OXI 1* gene is present in maize mtDNA (4). Renaturation kinetics give no evidence for redundancy (1), a result confirmed by hybridization of extensive banks of cloned fragments of mtDNA to mtDNA restriction patterns (19). Our own work has involved hybridizations of well over half of all *Bam*HI, *Hin*dIII, and *Pst* I fragments to restriction patterns of four different enzymes, and only a very few fragments show evidence of significant redundancy.

Note Added in Proof. Since our work was completed, similar conclusions regarding the organization of the ribosomal genes of maize mtDNA have been published (20).

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