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Nucleotide sequence of F_0-ATPase proteolipid (subunit 9) gene of maize mitochondria

(dicyclohexylcarbodiimide-binding protein/amino acid sequence)

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ABSTRACT **The F_0-ATPase proteolipid, also referred to as subunit 9 or the dicyclohexylcarbodiimide-binding protein, is encoded by a mitochondrial gene in maize that we have designated *atp* 9. The clone containing *atp* 9 was selected for investigation from a mitochondrial DNA library because of its abundant transcript in total maize mitochondrial RNA preparations. Sequence analysis of the clone revealed an open reading frame that was readily identified by its nucleotide homology with the ATPase subunit 9 gene of yeast. As deduced from the nucleotide sequence, the maize ATPase subunit 9 protein contains 74 amino acids with a molecular weight of 7368. Substantial amino acid sequence homology is conserved among maize, yeast, bovine, and *Neurospora* mitochondrial ATPase subunit 9 proteins, regardless of whether the gene is nuclearly encoded (bovine and *Neurospora*) or mitochondrially encoded (yeast and maize). RNA transfer blot analysis indicated that the gene sequence is actively transcribed, producing an initial transcript that is large and extensively processed.**

The mitochondrial genomes of higher plants are much larger and more complex than those of animals and fungi (1). Although plant mitochondria appear to synthesize a larger number of polypeptides than do the mitochondria of other organisms, this alone does not explain the size differences (2). In higher plants, DNA sequences have been reported for a single protein-encoding gene, cytochrome oxidase subunit II (3, 4), for the 18S and 5S rRNA genes (5, 6), and for a single tRNA gene (7). In addition, mitochondrial protein analysis has indicated that the F_1-ATPase subunit α is translated in the mitochondria of *Vicia faba* and maize (8, 9). Tentative identifications suggest that cytochrome oxidase subunit I, apocytochrome *b*, and ATPase subunit 9 are mitochondrial gene products in maize (9).

The oligomycin-sensitive ATPase is a complex of many subunits located in the inner mitochondrial membrane. It essentially consists of three components designated F_0, F_1, and the oligomycin-sensitivity-conferring protein (10). The polypeptides that make up the ATPase complex are encoded by mitochondrial and nuclear genes. Interestingly, the genes for at least two functional ATPase subunits may be either nuclear or mitochondrial depending upon the organism. In yeast, mammals, and *Neurospora* the α-subunit of the F_1 component is nuclearly encoded (11), whereas in maize it is mitochondrially encoded (8, 9). ATPase subunit 9, which is also called the proteolipid and the *N,N'*-dicyclohexylcarbodiimide (DCCD)-binding protein, is a nuclear gene in mammals and *Neurospora* (12) but is a mitochondrial gene in yeast (13). *Neurospora* also contains a nonfunctional ATPase subunit 9 gene within its mitochondrial genome (14).

Based on *in vitro* mitochondrial protein synthesis and DCCD-binding studies, it has been tentatively proposed that subunit 9 is encoded by a mitochondrial gene in maize (9). In this report we present additional results that indicate that this is indeed the case. We have isolated and sequenced the maize mitochondrial ATPase subunit 9 gene from the maize mitochondrial genome. Moreover, we have provided evidence that it is actively transcribed.

MATERIALS AND METHODS

Isolation of Nucleic Acids. Mitochondrial DNA (mtDNA) and RNA (mtRNA) were isolated from 6- to 7-day-old, dark-grown seedlings of *Zea mays* L., B73XMo17, as described (15, 16).

Cloning of mtDNA and Hybridization with Labeled RNA. A DNA library was constructed from *Eco*RI digests of total maize mtDNA cloned into the plasmid vector pUC 9 (17). Ligated DNA was used to transform *Escherichia coli* strain JM83. Ampicillin-resistant, lac$^-$ colonies were selected, replicated, and fixed onto nitrocellulose filters (18). Total mtRNA was 5′ end-labeled with [γ-^{32}P]ATP (ICN, 7000 Ci/mmol; 1 Ci = 37 GBq) using T4 polynucleotide kinase (19) and hybridized to the fixed colonies at 50°C in a hybridization solution containing 0.75 M NaCl, 75 mM sodium citrate, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1 mg of salmon sperm DNA per ml, 20 mM sodium phosphate (pH 6.5), and 50% formamide. Electrophoresis of DNA was performed on 0.8% agarose gels in TPE buffer (0.08 M Tris phosphate/0.008 M EDTA). DNA was transferred to nitrocellulose and hybridized according to Wahl *et al.* (20).

RNA Electrophoresis, Transfer, and Hybridization. mtRNA was heat denatured and fractionated by electrophoresis in 1.2% agarose gels containing 6% formaldehyde (21). Gels were blotted to nitrocellulose as described by Thomas (22).

DNA was labeled with [α-^{32}P]dATP (New England Nuclear, 3200 Ci/mmol) by nick-translation (23) or M13 backpriming (24) and hybridized to RNA blots as described for DNA hybridizations.

DNA Sequence Analysis. Cloning for sequence analysis was carried out by using the M13 bacteriophage vectors mp10 and mp11 (25). Preparations of the plasmid 15X, defined later, digested with *Bam*HI, *Hae* III, *Hin*dIII, *Sau*3A, *Xba* I, and *Xho* I, were ligated into the appropriate M13 restriction sites. Ligation and transformation procedures were as outlined by New England Biolabs.

DNA sequences were determined by the dideoxynucleotide chain-termination method of Sanger *et al.* (26) with a universal primer (P-L Biochemicals). Sequencing gels were either 6% or 8% polyacrylamide and 0.4 mm thick. Se-

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Abbreviations: mtDNA and mtRNA, mitochondrial DNA and RNA; DCCD, *N,N'*-dicyclohexylcarbodiimide; kb, kilobase(s).

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quences were analyzed and compared to mtDNA sequences of yeast and *Neurospora* by using a dot matrix computer program provided by M. Edgell (University of North Carolina, Chapel Hill).

RESULTS

Identification of an Actively Transcribed Mitochondrial Gene. To locate functional maize mitochondrial genes, we screened an *Eco*RI library of mtDNA with ^{32}P-labeled mtRNA. Clones demonstrating the greatest amount of hybridization to the RNA were selected. Since we were primarily interested in genes coding for mRNAs, positive clones were subsequently screened to eliminate those encoding rRNA genes. DNA clones containing genes for the 18S and 5S rRNAs and the 26S rRNA were used as probes to identify the rDNA clones in our library (27).

A 4.5-kilobase (kb) *Eco*RI clone, designated 15E, was of particular interest. It hybridized strongly with the labeled total mtRNA yet was not a rRNA gene. Clone 15E showed significantly greater hybridization intensity with labeled mtRNA than did clone pZmE1 (3), which contained the maize mitochondrial gene for cytochrome oxidase subunit II (data not shown). Hybridizations of 15E to *Eco*RI digests of total mtDNA showed a single band of 4.5 kb, indicating that the fragment exists within the genome as a single copy. Hybridization of 15E to *Bam*HI digests of total mtDNA also supported this conclusion.

To define the coding region of the 4.5-kb *Eco*RI fragment of clone 15E, a double digest of 15E was performed with restriction endonucleases *Eco*RI and *Xba* I. This digestion yielded three DNA fragments: 0.9-kb and 1.4-kb *Eco*RI–*Xba* I end fragments and a 2.2-kb *Xba* I internal fragment. When Southern blots of these digests were hybridized with a total mtRNA probe, detectable hybridization was confined to the 2.2-kb *Xba* I internal DNA fragment. This fragment was subcloned into the plasmid vector pUC 13 and designated 15X. A detailed restriction map of the 2.2-kb *Xba* I fragment of clone 15X is given in Fig. 1.

M13 Cloning Strategy and the DNA Sequence. The DNA sequence was determined from the 2.2-kb *Xba* I insert of clone 15X. Two cloning strategies were employed. First, both *Hae* III and *Sau*3A restriction fragments were "shotgun" cloned into the M13 vectors and subjected to sequence analysis. The second strategy involved the forced cloning of specific restriction fragments. The entire 2.2-kb *Xba* I, the 1.7-kb *Bam*HI–*Hin*dIII, the 0.8-kb *Xho* I–*Hin*dIII, and the 1.0-kb *Xho* I–*Bam*HI fragments (see Fig. 1) were cloned into

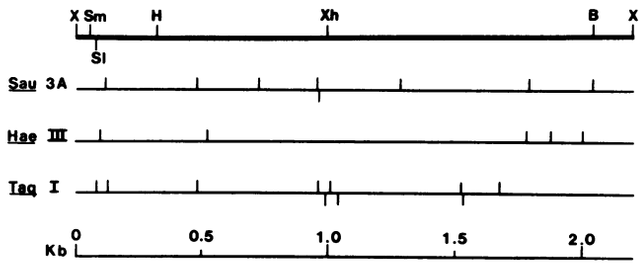

FIG. 1. Restriction map of the 2.2-kb *Xba* I clone, 15X. Restriction sites are indicated by vertical lines: B, *Bam*HI; H, *Hin*dIII; Sl, *Sal* I; Sm, *Sma* I; X, *Xba* I; Xh, *Xho* I. The restriction map was derived from the nucleotide sequence.

M13 mp10 and 11 and sequenced. Together, these two methods provided the alignment of the DNA sequences in relation to each other.

Computer analysis was performed with a dot matrix program in which the DNA sequence of the 2.2-kb *Xba* I fragment was compared to DNA sequences of mitochondrial genes of yeast (cytochrome oxidase subunits I–III, apocytochrome *b*, *var*1, and ATPase subunits 6, 8, and 9). The analysis revealed that a portion of the 2.2-kb fragment had extensive homology to the ATPase subunit 9 gene of yeast mitochondria (13). This gene or its gene product is also commonly referred to as *oli*1, the ATPase proteolipid, or the DCCD-binding protein. The sequence of the maize gene and portions of the 5′ and 3′ flanking sequences are shown in Fig. 2. DNA sequence homology between the maize and yeast mitochondrial ATPase subunit 9 genes is 56%. The colinearity between the maize and yeast ATPase subunit 9 sequences also suggests that the maize gene, like the yeast gene, contains no introns. The symbol, *atp* 9, was selected to designate the maize gene.

Amino Acid Sequence. The amino acid sequence of *atp* 9 contains 74 amino acids, as deduced from the nucleotide sequence. The predicted protein sequence is the same regardless of whether the universal code or the higher plant mitochondrial code is used (3). The positions of both the amino and carboxyl termini of the protein are well defined. The amino terminus is indicated by an ATG codon (Fig. 2); no other ATG codons are present before an in-frame stop codon at position −51. The carboxyl terminus is predicted by a TAA stop codon at position +223. These termini are either identical to or in close proximity to the termini of ATPase subunit 9 proteins found in bovine and yeast (Fig. 3). The

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                -51     5'-TAGTTGCGAAGGAAAAGCGTGAAACCCGACAATGTCAACTCTCAACTCTAC

          MET leu glu gly ala lys ser ile gly ala gly ala ala thr ile ala leu ala gly ala
    1     ATG TTA GAA GGA GCT AAA TCA ATA GGT GCT GGA GCT GCT ACA ATT GCT TTA GCG GGA GCT

          ala val gly ile gly asn val leu ser ser ser ile his ser val ala arg asn pro ser
   61     GCT GTC GGT ATT GGA AAC GTT CTC AGT TCT TCG ATT CAT TCC GTG GCG CGA AAT CCT TCA

          leu ala lys gln ser phe gly tyr ala ile leu gly phe ala leu thr glu ala ile ala
  121     TTG GCT AAA CAA TCA TTC GGT TAT GCC ATT TTG GGC TTT GCT CTC ACC GAA GCT ATT GCA

          ser phe ala pro met met ala phe leu ile ser phe val phe
  181     TCG TTT GCC CCA ATG ATG GCC TTT CTG ATC TCA TTC GTT TTC TAA TTCGTAAAAATAAGGTGTA

  245     GTTTCTCCGTCTTCCTGGGAAGGAAAATA-3'
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FIG. 2. Nucleotide sequence of the maize ATPase subunit 9 gene. The standard genetic code was used to deduce the amino acid sequence.

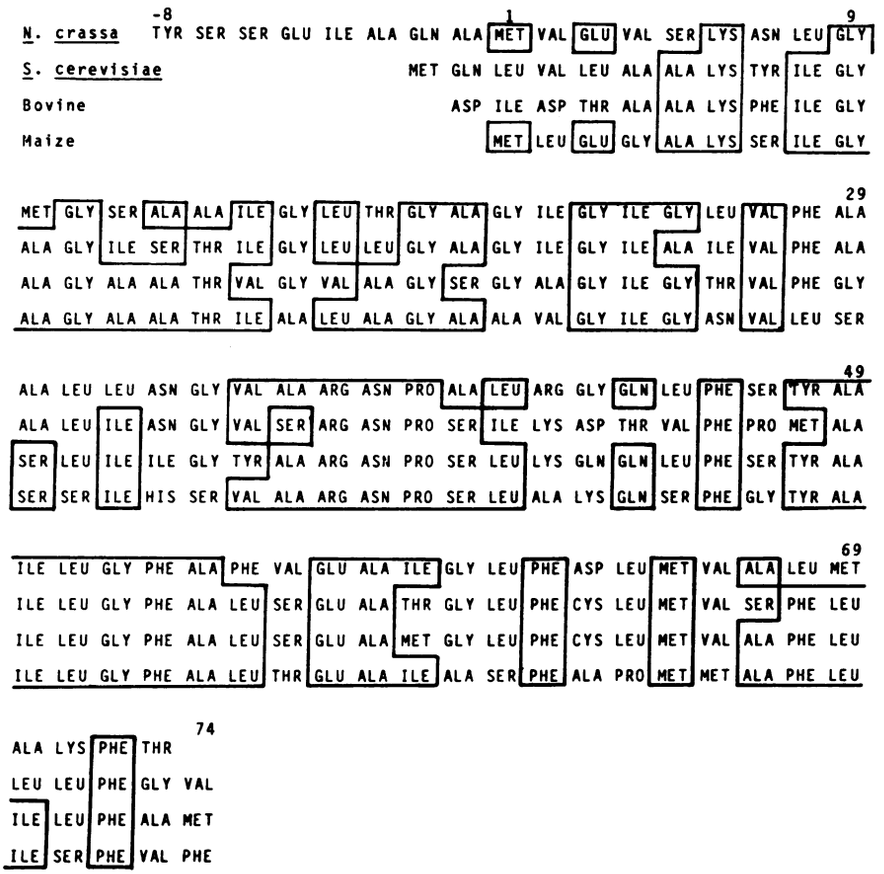

FIG. 3. Comparison of the predicted amino acid sequence of maize ATPase subunit 9 with the corresponding *Neurospora*, yeast, and bovine sequences. The *Neurospora* and bovine proteins are nuclearly encoded. The yeast and maize proteins are mitochondrially encoded. Boxes indicate amino acids in agreement with the maize sequence.

Neurospora protein contains eight more residues at the amino terminus than the predicted maize protein. The amino acid sequence homology between maize and yeast is 46%. This is very similar to the 47% homology found between the maize and yeast cytochrome oxidase subunit II mitochondrial proteins (3). Comparisons of the maize protein to the nuclearly encoded ATPase subunit 9 protein sequences from *Neurospora crassa* and bovines (12) show homologies of 44% and 56%, respectively. The spinach chloroplast proteolipid and the analogous proteins from *E. coli* and the thermophilic bacteria PS-3 (12) have homologies of 20%, 22%, and 24%, respectively, with the maize protein (data not shown). Amino acids at positions 19, 23, 37, 39, and 58 are perfectly conserved among the seven species. Comparisons among the four mitochondrial proteins, maize, bovine, yeast, and *Neurospora*, show 22 amino acids (30%) perfectly conserved (Fig. 3). Also of note is the conservation of the glutamic acid residue at position 57. This is the actual DCCD-binding amino acid and it is conserved in all species thus far examined, except *E. coli*, in which an aspartic acid residue exists (12). Finally, the close correspondence between the amino acid sequence of the subunit 9 proteins of bovines, *Neurospora*, and yeast with the predicted sequence of maize eliminates the possibility that the maize gene contains introns.

The maize ATPase subunit 9 has a molecular mass of 7368 daltons, as predicted from the DNA sequence. Leaver and co-workers have tentatively identified a mitochondrially synthesized protein in maize as ATPase subunit 9 because of its solubility in butanol and its specificity in binding DCCD (28). Based on sodium dodecyl sulfate gel electrophoresis, they estimated a molecular mass of 8000 daltons for the subunit 9 protein; their estimate is in reasonable agreement with our predicted value of 7368 daltons.

A summary of codon usage for *atp* 9 is given in Fig. 4.

Transcriptional Processing of the *atp* 9 Message. To determine if *atp* 9 is transcribed, we searched for RNA transcripts of this DNA sequence in total mtRNA preparations of maize. When the 2.2-kb *Xba* I fragment of clone 15X was hybridized to an RNA transfer blot of total mtRNA, a complex hybridization pattern was revealed (Fig. 5, lane A). This result suggested that the 2.2-kb fragment was transcribed and that the transcript was processed. Even more complicated RNA processing was observed for the transcript of another maize mitochondrial gene, cytochrome oxidase subunit II, which contains a single intron (3). However, since the maize subunit 9 does not contain introns, all processing must occur through cleavage of 5′ and 3′ flanking sequences. Although the possibility of a polycistronic message has not been ruled out, open reading frames larger than 270 base pairs are not found within the 2.2-kb *Xba* I fragment. Moreover, computer analysis has not detected significant homologies between these sequences and the other yeast mitochondrial genes compared.

Transcriptional processing has been analyzed by using successive M13 clones complementary to the sense strand (Fig. 2) as hybridization probes against RNA transfer blots (Fig. 5, lanes B–G). The study identified transcripts that corresponded with the subunit 9 coding sequence as well as 5′ and 3′ flanking sequences of the 2.2-kb *Xba* I fragment. Transcripts were not detected by hybridizations with single-stranded probes of the noncomplementary strand of the 2.2-kb *Xba* I fragment (data not shown). The largest detectable

	U		C		A		G	
U	Phe	UUU: 3	Ser	UCU: 1	Tyr	UAU: 1	Cys	UGU: -
		UUC: 3		UCC: 1		UAC: -		UGC: -
	Leu	UUA: 2		UCA: 4		UAA: 1		UGA: -
		UUG: 2		UCG: 2		UAG: -	Trp	UGG: -
C	Leu	CUU: -	Pro	CCU: 1	His	CAU: 1	Arg	CGU: -
		CUC: 2		CCC: -		CAC: -		CGC: -
		CUA: -		CCA: 1	Gln	CAA: 1		CGA: 1
		CUG: 1		CCG: -		CAG: -		CGG: -
A	Ile	AUU: 5	Thr	ACU: -	Asn	AAU: 1	Ser	AGU: 1
		AUC: 1		ACC: 1		AAC: 1		AGC: -
		AUA: 1		ACA: 1	Lys	AAA: 2	Arg	AGA: -
	Met	AUG: 3		ACG: -		AAG: -		AGG: -
G	Val	GUU: 2	Ala	GCU: 10	Asp	GAU: -	Gly	GGU: 3
		GUC: 1		GCC: 3		GAC: -		GGC: 1
		GUA: -		GCA: 1	Glu	GAA: 2		GGA: 4
		GUG: 1		GCG: 2		GAG: -		GGG: -

FIG. 4. Codon usage in the *atp* 9 gene.

transcript is ≈1950 nucleotides in length and may be the primary transcript. The predominant form of the transcript is ≈1000 nucleotides in length. From this study, we cannot unambiguously identify the mature messenger or deduce the set of processing and/or degradation events that lead to the complex patterns. The yeast mitochondrial ATPase subunit 9 mitochondrial gene also yields a large initial transcript that contains no introns but is processed to give a smaller mature transcript (13, 29). Although much remains to be learned about transcriptional units in the maize mitochondria, it appears from the two genes studied that the primary transcripts are large and require extensive processing.

DISCUSSION

We have identified the DNA sequence of the ATPase subunit 9 gene from maize by its nucleotide homology with the corresponding yeast mitochondrial gene. Substantial evidence suggests that the maize mtDNA sequence is an active gene. First, the predicted protein sequence is homologous and similar in size to ATPase subunit 9 proteins of bovines, *Neurospora*, and yeast. Second, it has been demonstrated that the proteolipid protein is mitochondrially translated in maize (9) and that the molecular mass of the translated protein (8000 daltons) is similar to the molecular weight calculated from our predicted amino acid sequence (7368). There also appears to be a correlation between the unusually high abundance of message present in the mitochondria and the large amounts of protein product produced by the mitochondria. ATPase subunit 9 is the most abundant subunit of the F_0 membrane factor because it is present in an oligomeric form, most likely as a hexamer (30). Third, the DNA sequence coding for this protein is present as a single copy within the maize mitochondrial genome. Finally, the sequence is actively transcribed and the transcript appears to be processed within the mitochondria.

Fox and Leaver suggest from their analysis of the maize cytochrome oxidase subunit II gene that TGA may not code for tryptophan as in other mitochondrial systems and that CGG may code for tryptophan in maize rather than arginine as predicted by the universal code (3). These deviations in the code are also supported by the sequence analysis of the cytochrome oxidase subunit II gene in *Oenothera* (4). Since maize ATPase subunit 9 contains neither of these codons, it is impossible to confirm these potential changes in codon usage (Fig. 4).

The mitochondrial ATPase subunit 9 amino acid sequences are well conserved among distantly related organisms. There appears to be no significant difference in the degree of conservation as to whether the mitochondrial protein is nuclearly or mitochondrially encoded. The distribution of hydrophobic and hydrophilic residues is also well conserved. Two long hydrophobic segments (Fig. 3, positions 8–32 and 45–72) characterize all known ATPase subunit 9 proteins. In maize most substitutions in these regions are isofunctional, maintaining the hydrophobicity of the segments. The hydrophilic amino acids are localized primarily at the amino terminus and throughout positions 33–44 (Fig. 3). One exception to these generalizations is the location of the DCCD-binding glutamic acid residue at position 57. This highly conserved residue is located in the middle of the second hydrophobic region. An uncharacteristic lack of conservation is found at positions 7, 26, and 43, along with both

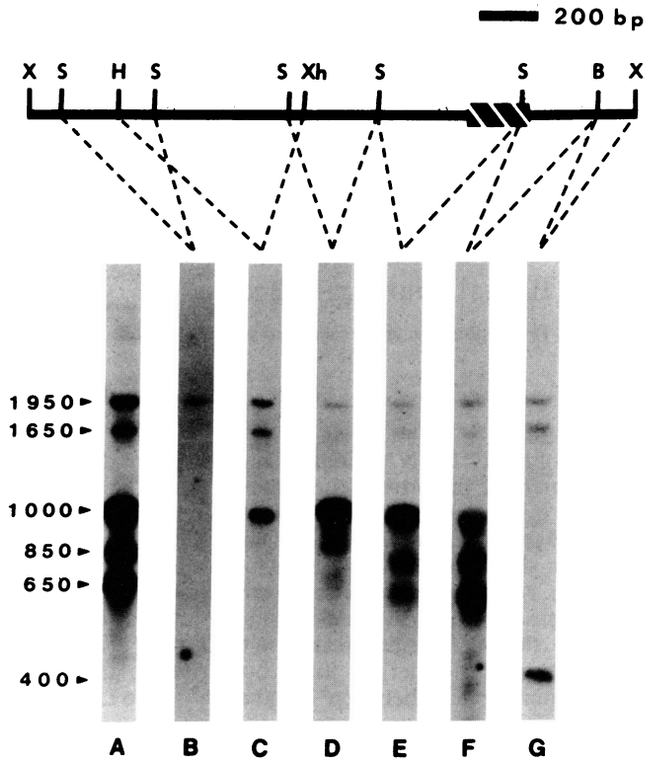

FIG. 5. Hybridization of M13 clones of 15X to RNA transfer blots of maize mtRNA. Restriction sites are indicated by vertical lines: B, *Bam*HI; H, *Hin*dIII; S, *Sau*3A; X, *Xba* I; Xh, *Xho* I. Translation is from left to right. Individual clones are defined by dashed lines. Striped box designates the predicted amino acid coding region. Approximate transcript sizes are indicated in nucleotides. Lane A, entire *Xba* I fragment hybridized to a RNA transfer blot of maize mtRNA; lanes B–G, individual M13 clones hybridized to RNA transfer blots. Each lane contains equal amounts of RNA hybridized under similar conditions. Exposure times were similar, except for lane G, which required longer exposure to visualize the bands. bp, Base pairs.

termini, where the amino acids are different among all four mitochondrial proteins compared.

The maize sequence is peculiar in that at seven locations a unique serine residue exists. Maize also shows unusually poor homology with the other three mitochondrial proteins at positions 28–31, where three of the serine substitutions occur. Finally, it is notable that the maize protein shows greatest homology with the bovine sequence, even though they are the most distantly related.

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