Short Communication

Nucleotide Sequence of ATPase Subunit 6 Gene of Maize Mitochondria

R. E. Dewey, Charles S. Levingis III*, and D. H. Timothy

Departments of Crop Science (R.E.D., D.H.T.) and Genetics (G.S.L.), North Carolina State University, Raleigh, North Carolina 27695

ABSTRACT

The ATPase subunit 6, located in the inner mitochondrial membrane, is encoded by mitochondrial genomes in animals and fungi. We have isolated and characterized a mitochondrial gene, designated atp 6, that encodes the subunit 6 polypeptide of Zea mays. Nucleotide and predicted amino acid sequence comparisons have revealed a homology of 44.6 and 33.2% with the yeast ATPase subunit 6 gene and polypeptide, respectively. The predicted protein in maize contains 291 amino acids with a molecular weight of 31,721. Hydrophathy profiles generated for the maize and yeast polypeptides are very similar and contain large hydrophobic domains, characteristic of membrane bound proteins. RNA transfer blot analysis indicates that atp 6 is actively transcribed. Interestingly, 122 base pairs of nucleotide sequence interior to atp 6 have extensive homology with the 5' end of the cytochrome oxidase subunit II gene of maize mitochondria, suggesting recombination between the two genes.

The mt2 ATPase complex, located in the inner mt membrane, consists of three components designated Fo, Fi, and the oligomycin-sensitivity-conferring protein (OSCP) (27). The various subunits making up the complex are encoded either by the nuclear or mt genomes. In yeast, subunits 6, 8, and 9 of the Fo component are mt gene products while the other subunits are of nuclear origin (16, 27, 28). Animal systems and certain fungi differ in that subunit 9 is encoded within the nucleus (25). Higher plant mt genomes contain a gene coding for ATPase subunit 9 (8), yet differ from both animals and fungi in that they also code for the alpha subunit of the Fi component (4, 11).

Two different methods have been used to identify protein encoding genes of the maize mt genome. The Cyt oxidase subunit II and apocytochrome b genes were located with heterologous probes of the corresponding genes from Saccharomyces cerevisiae and Kluyveromyces lactis, respectively (7, 9). The other approach involved the isolation and sequencing of an actively transcribed clone selected from a mtDNA library, followed by computer searches of gene banks to identify the gene encoded by the clone. The ATPase subunit 9 gene of maize mitochondria was identified in this manner (8). Using the latter method, we have isolated and identified the maize mt Fo-ATPase subunit 6 gene. We present the nucleotide sequence of the subunit 6 gene and evidence that it is actively transcribed.

MATERIALS AND METHODS

Isolation of Nucleic Acids. Mitochondrial DNA and RNA were isolated from 6 to 7 d old dark-grown seedlings of Zea mays L, W182BN cms-SC or B73 cms-T as previously described (21, 24). The cms-SC cytoplasm is a member of the T (Texas) group of male-sterile cytoplasm (10).

Construction of Mitochondrial DNA Library. BamHI digests of total maize mtDNA were ligated into the plasmid vector pUC 8 (29), and transformed into Escherichia coli strain JM 83. Ampicillin-resistant, lac- colonies were selected, replicated and fixed onto nitrocellulose filters (17).

Radioactive Labeling of DNA and RNA. Double-stranded DNA was labeled with [α-32P]dATP (NEN, 3200 Ci/mmol) by nick translation (22). Single-stranded DNA clones in bacteriophage M13 were labeled using the back priming technique of Hu and Messing (13). Total mtRNA was 5' end-labeled with [γ-32P] ATP (ICN, 7000 Ci/mmol) using T4 polynucleotide kinase (18).

Gel Electrophoresis and Nucleic Acid Hybridizations. DNA fragments were separated by electrophoresis on 0.8% agarose gels in TPE buffer (80 mm Tris-phosphate, 8 mm EDTA (pH 7.8)) and transferred to nitrocellulose according to Wahl et al. (30). mtRNA was heat denatured and fractionated by electrophoresis in 1.2% agarose gels containing 6% formaldehyde and blotted to nitrocellulose as described by Thomas (26). The 18S (1986 nt) and 26S (3546 nt) ribosomal RNAs of maize mitochondria were used as markers for estimating RNA sizes.

All nucleic acid hybridizations were performed under conditions previously described (8).

DNA Sequence Analysis. Cloning for sequence analysis was carried out using M13 bacteriophage vectors mp10 and mp11 (18). Ligation and transformation procedures were as outlined by New England Biolabs. DNA sequences were determined by the chain-termination method of Sanger et al. (23) with a universal primer (PL Biochemicals). Sequencing gels were either 6 or 8% polyacrylamide and 0.4 mm thick. The sequencing strategy is shown in Figure 1.

Sequence analyses were performed with computer programs furnished by Bionet or with a dot matrix computer program provided by M. Edgell (University of North Carolina, Chapel Hill).

RESULTS

Identification and Analysis of the Maize ATPase Subunit 6 Gene. To locate mtDNA clones actively involved in transcription, end-labeled mtRNA was hybridized to a BamHI mtDNA

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2 Abbreviations: mt, mitochondrial; kb, kilobase(s); nt, nucleotides; bp, base pairs
library from SC cytoplasm, a maize T-type male-sterile cytoplasm (10). Among the clones exhibiting positive hybridization was a 6.5 kb BamHI clone designated T25B. Hybridization of end-labeled mtRNA to Southern blots of restriction digests of T25B revealed that significant hybridization was confined to a 2.7 kb HindIII fragment interior to the 6.5 kb BamHI clone. This fragment was inserted into plasmid vector pUC 13 and designated T25H. T25H was also cloned into the viral vector M13 and the complete nucleotide sequence of 2583 bp was determined. A restriction map and sequencing strategy of T25H are given in Figure 1.

Using a dot matrix computer program (M. Edgell, University of North Carolina, Chapel Hill) the nucleotide sequence of T25H was compared with the mtDNA sequences of yeast. Sequence homology was found between a segment of T25H and the yeast mitochondrial gene coding for ATPase subunit 6; no other yeast gene contained significant sequence homology with T25H. The nucleotide sequence of the maize gene is shown in Figure 2. DNA sequence homology between the maize and yeast ATPase subunit 6 genes is 44.6%. Based on this homology we have concluded that this sequence codes for the ATPase subunit 6 gene and have selected the symbol atp 6 to designate the gene in maize. Unlike the cytochrome oxidase subunit II gene in maize mitochondria (9), atp 6 does not appear to contain intervening sequences. Due to low homologies at the terminal regions of the gene, however, we cannot exlude the possibility that introns exist near the 5' or 3' ends of the gene.

**Amino Acid Sequence.** As a translational initiation site for the atp 6 gene, we have selected the ATG codon closest to the initiator methionine of the homologous gene in yeast and *Aspergillus*. This ATG site (beginning at position 1 in Fig. 2) is distanty located from the next adjacent in frame ATG codons in both the 3' and 5' directions. In the 5' direction, the next ATG codon begins at position -294 (Fig. 2) and would increase the size of the polypeptide by 98 amino acids. These additional amino acids are not homologous with ATPase subunit 6 protein sequences from other organisms and would generate a polypeptide much larger than observed in other organisms. In the 3' direction, the next ATG codon starts at position 162 (Fig. 2) and would decrease the polypeptide by 53 amino acids, portions of which contain significant homology with the yeast protein. It has not been unequivocally demonstrated, however, that translation always begins with AUG in maize mitochondria. In mammalian mitochondria the entire AUN family is capable of translational initiation (1, 2).

Assuming translation initites as proposed in Figure 2, the protein sequence of atp 6 contains 291 amino acids. The predicted protein sequence is the same regardless of whether the universal code or the higher plant mitochondrial code is used (9). The predicted maize protein is 32 amino acids longer than the predicted yeast protein.

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**FIG. 1.** Restriction map of the maize mitochondrial ATPase subunit 6 gene and flanking sequences. Arrows below the map show the direction and extent of sequence analysis from each restriction site. Restriction sites are indicated by vertical lines: E, EcoRI; H, HindIII; S, Sau 3A; T, Taq I.

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**TABLE 2.** Nucleotide sequence of the maize ATPase subunit 6 gene. The predicted amino acid sequence is translated according to the higher plant mitochondrial code (9) and is indicated in Roman type. The amino acid sequence of the open reading frame extending beyond the putative ATG initiation codon is in italics.
the corresponding yeast protein with most of the additional amino acids located at both the amino and carboxyl termini (Fig. 3). The 5' end of the *atp 6* open reading frame extends 408 nucleotides upstream of the putative ATG start site shown in Figure 2. However, analysis of the DNA sequence and predicted protein sequence of this region reveals no significant homology with other DNA or protein sequences in the sequence libraries of NIH GenBank or National Biomedical Research Foundation. The carboxyl terminus is predicted by a TAG stop codon at position 873, 45 nucleotides beyond the stop site of the yeast gene. A mol wt of 31,721 is calculated from the predicted protein sequence.

The maize and yeast proteins share an amino acid sequence homology of 33.2% (Fig. 3). When conservative replacements are included (Asn-Gln), (Lys-Arg), (Ser-Thr), (Phe-Tyr-Trp), (Ile-Leu-Val-Met), the homology increases to 48.6%. Comparisons of the maize protein to the predicted mitochondrial proteins from *Aspergillus nidulans*, Drosophila yakuba, and mouse (2, 6, 19) show amino acid homologies of 35.6, 20.5, and 20.2%, respectively (data not shown). A homology of 16.7% exists between the maize ATPase subunit 6 protein and the analogous bacterial protein from *Escherichia coli* (20).

As expected for membrane associated proteins, the predicted amino acid sequence of maize ATPase subunit 6 contains a majority of hydrophobic residues and relatively few charged amino acids. To analyze the distribution of these residues, a hydrophathy profile was constructed according to the values of Kyte and Doolittle (Fig. 4) (15). Hydrophobic domains located throughout the protein indicate the portions of the molecule most likely to lie within the membrane. The maize *atp 6* profile is similar to the plot of the yeast ATPase subunit 6 protein with

![Hydropathy profiles of the predicted maize and yeast ATPase subunit 6 proteins.](https://www.plantphysiol.org/)

**Fig. 4.** Hydropathy profiles of the predicted maize and yeast ATPase subunit 6 proteins. The x axis represents arbitrary hydrophobic values (15). The y axis indicates the positions of the individual amino acids. Area above the line shows domains with increased probability of being located in the lipid bilayer.

![Comparison of predicted amino acid sequence of maize ATPase subunit 6 with corresponding protein from yeast.](https://www.plantphysiol.org/)

**Fig. 3.** A comparison of the predicted amino acid sequence of maize ATPase subunit 6 with the corresponding protein from yeast. Boxed regions indicate amino acids that are conserved. A dash indicates an amino acid that is absent.
Aspergillus ATPase

The secondary structure of the maize ATPase subunit 6 protein was deduced from the amino acid sequence by the method of Chou and Fasman (5). A large amount of β-sheet conformation (61%) is predicted by this procedure. Analysis of yeast and Aspergillus ATPase subunit 6 proteins also predicts the β-sheet conformation to be the most prevalent secondary structure, with estimates of 73 and 66%, respectively. In contrast, the predicted secondary structure of the amino acid sequence extending beyond the putative start methionine contains relatively low β-sheet conformation (30%).

A summary of codon usage for atp 6 is given in Figure 5.

Homology Between atp 6 and the Cytochrome Oxidase Subunit II Gene of Maize Mitochondria. Nucleotide sequence comparison of the atp 6 gene to the NIH GenBank sequence library revealed extensive homology to a region of the Cyt oxidase subunit II (COII) gene in male-fertile maize mitochondria (9). The homology extends from the EcoRI site, where the published COII sequence begins, to a position 8 bp 5' of an ATG codon designated by Fox and Leaver (9) as the "second possible initiation codon" (positions -104 to -8, Fig. 6). This corresponds to positions 97 to 194 of atp 6 (Figs. 2, 6). To examine the extent of the homology 5' of the EcoRI site, we cloned and sequenced this portion of the COII gene from cms-T maize cytoplasm. Homology was found to continue 25 bp 5' of the EcoRI site, indicating a continuous homologous region of 122 bp. The atp 6 and COII sequences in this segment differ only by 3 bp substitutions and a single insertion/deletion of the sequence TATCAA at position 88 of atp 6 (Fig. 6). Comparison of the predicted amino acid sequences in this region shows 40 of 43 amino acids (93%) are conserved. The portion of atp 6 that is homologous with the maize COII gene is located in the coding region (positions 73-194, Fig. 2). The predicted amino acid sequence and hydropathy profiles of this region are homologous to the yeast and Aspergillus ATPase subunit 6 proteins, whereas no homology is seen with the yeast Cyt oxidase subunit II protein (data not shown). Thus, it is probable that the maize COII gene derived the homologous sequences from recombination with atp 6 and thereby may be considered a chimeric gene.

Southern Blot Analysis. Hybridization of clones containing atp 6 to Southern blots of BamHI and HindIII mtDNA digests revealed intense hybridization to a 6.5 kb BamHI fragment and a 2.7 kb HindIII fragment, respectively (data not shown). In addition, several fragments showed weak hybridization after long exposure in both digests. The weakly hybridizing bands are probably due to poorly matched or short homologous sequences. In fact, we have previously described a short sequence (122 bp)

**Figure 5.** Codon usage in the atp 6 gene.

**Figure 6.** Nucleotide and amino acid sequence homology between atp 6 and COII genes of maize mitochondria. Nucleotide positions of atp 6 are as described in Figure 2. Positions of COII nucleotides are in relation to the possible ATG initiator codon indicated with a bracket. Homologous nucleotides are indicated with an asterisk. Amino acids of COII nonhomologous with atp 6 are boxed. Proposed points of recombination are designated with arrows.
in the COII gene with substantial homology to the *atp 6* gene. These results, together with the transcriptional studies given below, suggest that the complete *atp 6* gene is present as a single copy in this genome.

**Transcriptional Processing of the atp 6 Message.** When the *atp 6* sequence was hybridized to a Northern blot of total maize mtRNA, a strong and complex hybridization pattern was revealed (Fig. 7). The largest detectable transcript is approximately 6800 nt in length and may be the primary transcript. The most predominant forms of the transcript are approximately 4500, 1900, and 1600 nt long. Further studies are needed to determine unequivocally the primary and mature forms of the message. As expected, single-stranded M13 probes of the noncomplementary strand showed no detectable hybridization to RNA blots (data not shown). These results indicate that the *atp 6* sequence is an actively transcribed gene.

**DISCUSSION**

Subunit 6 of the mitochondrial ATPase is an inner membrane polypeptide of the F$_o$ component, encoded within the mitochondrial genomes of all eukaryotic organisms examined to date. The nucleotides and amino acid sequence homologies of *atp 6* with the ATPase subunit 6 genes from yeast and other organisms, along with evidence of active transcription, indicates that the ATPase subunit 6 is also encoded by a mitochondrial gene in maize. Our characterization of the maize *atp 6* nucleotide sequence is the first evidence that ATPase subunit 6 is mitochondrially encoded in higher plants.

It has been proposed that the code in higher plant mitochondria differs from that found in yeast; the triplet CGG is translated as tryptophan rather than arginine, and TGA codons are non-translatable rather than specifying tryptophan residues (9). The *atp 6* sequence contains no TGA codons, thus supporting the view that it is a nonsense codon in higher plant mitochondria. The triplet CGG is also absent, making it impossible to confirm its usage as either a tryptophan or arginine residue in the mitochondrial genome of maize.

The amino acid homology between the maize and yeast ATPase subunit 6 proteins (32.2%) is less than that found between the other maize genes and their yeast counterparts. This is not surprising considering the general lack of conservation among ATPase subunit 6 proteins of distantly related species (6). For example, the amino acid homology between *Drosophila* and yeast ATPase subunit 6 proteins is 23.0%. The homology between the *Drosophila* and mouse polypeptides is 35.7% (6). Of particular interest is the overall size differences observed among the species. The maize protein is 32 amino acids longer than the yeast protein and 55 amino acids longer than the corresponding protein from mouse. Almost all of these additional amino acids are located at the terminal regions and not within the interior of the protein. Interestingly, the open reading frame containing the maize ATPase subunit 6 protein extends 408 base pairs upstream beyond the putative ATG initiation codon. Thus the maize protein could be even larger than the 291 amino acids proposed here. It is unlikely, however, that these additional amino acids could be part of the mature ATPase subunit 6 polypeptide since these amino acids are very hydrophilic. It is possible that ATPase subunit 6 in maize mitochondria is translated as a precursor, with the hydrophilic amino acids at the amino terminus undergoing cleavage to produce the mature form of the protein.

Extensive nucleotide and amino acid homology is observed between a portion of *atp 6* and the 5' end of the COII gene in maize mitochondria (Fig. 6). This homology is presumably due to recombination between the two genes. Homology among mitochondrial COII proteins of *Oenothera*, rice, and wheat, (3, 12, 14) with the predicted maize COII sequence begins at the 'possible ATG initiator codon' indicated in Figure 6 and does not include any of the amino acids homologous with *atp 6*. Although *Oenothera*, rice, and wheat share nucleotide homology in the 5' flanking region of the Cyto oxidase subunit II gene, no homology is observed with the corresponding maize sequence where the recombination with *atp 6* has occurred. This recombination, therefore, does not appear to be a common characteristic of higher plants. Nucleotide sequence analysis of the COII gene of *Zea diploperennis*, a wild relative of maize, indicates that the recombination with *atp 6* is also found in this species (R. E. Dewey, C. S. Levings III, D. H. Timothy, unpublished results). It is therefore likely that this phenomenon is common to the genus *Zea*.

A complex hybridization pattern is observed when the *atp 6* gene is hybridized to Northern blots of total mtRNA. Complex RNA hybridization patterns are also observed for the COII, apocychrome b, and ATPase subunit 9 genes of maize mitochondria (7–9). Part of the *atp 6* hybridization complexity may
be due to cross-hybridization of the \textit{atp} 6 sequence with the transcript produced by the COII gene, since \textit{atp} 6 and the COII gene contain nucleotide homology (Fig. 6). Likewise, some of the complexity observed when the COII gene is hybridized to RNA blots may be caused by cross-hybridization to \textit{atp} 6 transcripts. Because intramolecular recombination is relatively common in the maize mitochondrial genome, rearrangements may be partially responsible for the complex hybridization patterns detected by Northern blot analysis.

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