A Single Homogeneous Form of ATP6 Protein Accumulates in Petunia Mitochondria Despite the Presence of Differentially Edited \textit{atp6} Transcripts

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Transcripts encoding ATP synthase subunit 6 (ATP6) in petunia mitochondria were shown to be edited at 15 sites, leading to 14 amino acid changes. Certain sites are partially edited, including a site that introduces a new translation termination codon that is 13 codons upstream of the genomically encoded stop codon. Transcripts lacking the new stop codon are present in an ~2.5:1 ratio to transcripts carrying the stop codon created by RNA editing. To investigate whether partially edited transcripts are represented as proteins, we generated an antibody against a 12-residue peptide that is specific for translation products of unedited transcripts. This antibody did not recognize any ATP6 protein in either total mitochondrial protein preparations or ATP6 samples purified by organic solvent extraction and reverse phase HPLC procedures. According to analysis by mass spectrometry, only one form of ATP6 protein accumulates in mitochondria despite the presence of abundant partially edited transcripts. Partially edited \textit{atp6} transcripts were associated with ribosomes, suggesting that a screening mechanism(s) acts cotranslationally or post-translationally to exclude the expression of incompletely edited transcripts.

INTRODUCTION

Primary transcripts of nuclear and organelle genes in a variety of organisms undergo editing, an alteration that changes the sequence of the RNA. Substitution, insertion, and/or modification of nucleotides may occur, depending on the organism and gene. Protein-coding genes exhibiting transcripts that undergo RNA editing are found in trypanosome mitochondria (Benne et al., 1986), \textit{Physarum} mitochondria (Mahendra et al., 1991), higher plant mitochondria (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989) and chloroplasts (Hoch et al., 1991), and the mammalian nucleus (Chen et al., 1987; Powell et al., 1987; Sommer et al., 1991; Higuchi et al., 1993; Sharma et al., 1994). The diversity of systems exhibiting editing indicates its importance in the regulation of gene expression. Additional genes and organisms in which RNA editing occurs probably await discovery.

Editing of higher plant mitochondrial RNAs results in the substitution of U residues for particular C residues present in the initial transcript (Rajasekhar and Mulligan, 1993). The plant mitochondrial protein sequences predicted from edited transcripts are usually more similar to homologous proteins from other organisms than are the proteins predicted from unedited transcripts (Gualberto et al., 1989; Conklin et al., 1991; Walbot, 1991; Wintz and Hanson, 1991). Editing can occur before both cis- and trans-splicing of plant mitochondrial transcripts (Conklin et al., 1991; Sutton et al., 1991; Yang and Mulligan, 1991).

Transcripts of some plant mitochondrial genes have C-to-U substitutions at all potential editing sites. Unedited transcripts of such genes, such as petunia and wheat ATP synthase subunit 9 (\textit{atp9}) and wheat cytochrome oxidase subunit 3 (\textit{coxIII}) and NADH dehydrogenase subunit 4 (\textit{nad4}), are either extremely low in abundance or completely undetectable (Begu et al., 1990; Gualberto et al., 1990; Lamattina and Grienberger, 1991; Wintz and Hanson, 1991). Transcripts of many other plant mitochondrial genes are less homogeneous; partially edited transcripts are readily detected and often are more abundant than transcripts that are fully edited (Covello and Gray, 1990; Schuster et al., 1990; Gualberto et al., 1991; Kempken et al., 1991; Salazar et al., 1991; Lu and Hanson, 1992). Such a transcript population has the potential of encoding multiple forms of the gene's protein product. In mammalian systems in which RNA editing occurs, both edited and unedited transcripts have been found to encode proteins. For example, mammalian apolipoprotein B (\textit{apoB}) mRNAs undergo a C-to-U editing event that creates a stop codon. The edited and unedited transcripts produce ApoB-100 and ApoB-48, two forms of the ApoB protein that differ in size. Tissue-specific regulation of editing results in different proportions of the two ApoB proteins in different tissues (reviewed in Hodges and Scott, 1992). In the human brain, differential RNA editing may be responsible for the different ion flow properties of two related classes of glutamate...
receptor channels (Sommer et al., 1991). RNA editing of transcripts of the Wilms' tumor susceptibility gene produces two different gene products (Sharma et al., 1994).

Whether certain individual plant mitochondrial proteins are present in multiple forms is not known. The hydrophobic nature of many such proteins has made characterization difficult. By typical gel electrophoretic techniques, proteins carrying editing-specified amino acid substitutions cannot readily be distinguished from proteins specified by unedited transcripts. Wheat ATP9 protein was successfully purified, and an N-terminal sequence and amino acid composition corresponding to edited transcripts have been obtained (Bégu et al., 1990). However, very few partially edited transcripts of wheat atp9 exist in the RNA population (Bégu et al., 1990); thus, proteins representing translation of unedited transcripts would not be detectable by the analytical methods used. Partially edited and intron-containing transcripts of other plant mitochondrial genes have been found in polysomal RNA preparations (Gualberto et al., 1991; Yang and Mulligan, 1993), raising the possibility that such transcripts may be translated to produce a spectrum of mitochondrial proteins encoded by the same gene.

To investigate whether partially edited transcripts could give rise to multiple plant mitochondrial proteins, we selected the petunia atp6 gene for analysis. Partial editing at a number of sites is observed in transcripts of this gene. Editing at one site introduces a stop codon that is 12 amino acids upstream of the genomically encoded stop codon; however, less than one-third of the transcripts are edited at this site. Translation of transcripts that are unedited versus edited at this site should give rise to proteins that can be distinguished both immunologically and by molecular mass. We have generated an antibody against a 12-amino acid "extension" peptide that would be predicted at the C terminus of proteins encoded by unedited transcripts. Although this antibody reacts with the Escherichia coli–expressed protein carrying the extension peptide, no ATP6 protein from unedited transcripts was detected in petunia mitochondria. We have also purified ATP6 protein biochemically and determined its molecular mass and degree of homogeneity using MALD (matrix-assisted laser desorption) mass spectrometry (Beavis and Chait, 1990). Our results show that petunia mitochondria accumulate only one form of ATP6 protein, which is lower in mass than the protein predicted from unedited transcripts. We further demonstrate that partially edited atp6 transcripts are associated with ribosomes. These findings suggest that expression of partially edited transcripts is regulated cotranslationally or post-translationally.

RESULTS

Isolation of the Petunia atp6 Gene and Transcript Analysis

To produce an atp6 probe, primers were designed from the maize atp6 sequence (Dewey et al., 1985) to amplify homologous DNA from the petunia mitochondrial genome. The polymerase chain reaction (PCR) product was used as a probe to screen a cosmid library (Folkerts and Hanson, 1991) of petunia mitochondrial DNA. Figure 1 shows the sequence of the petunia atp6 gene. The putative translation initiation codon is preceded by stop codons in all three reading frames. A potential ribosome binding site, identical to that found in the tobacco atp6 gene (Bland et al., 1987), is located near the initiation codon (Figure 1). The genomic petunia atp6 DNA sequence predicts a protein of 410 amino acids with a molecular mass of 45.5 kD.

Comparison of the deduced amino acid sequence with the cognate sequences from other plant and fungal species reveals that they share a highly conserved core region that is flanked by divergent N-terminal and C-terminal sequences (Figure 2). The core regions are 252 amino acids long in all plant species examined so far, whereas the N-terminal sequences range from 9 to 145 amino acids and the C-terminal sequences range from 0 to 35 amino acids (Figure 2). Isolation of yeast mitochondrial ATP6 protein and subsequent N-terminal sequencing revealed that ATP6 protein is post-translationally processed (Michon et al., 1988). A region homologous to the yeast processing site occurs at the beginning of the highly conserved region in petunia.

Filter hybridization of mitochondrial RNA isolated from suspension culture cells with an atp6 probe detected a major atp6 transcript of 1.6 kb (Figure 3A). The 5’ end of this transcript was identified by primer extension using a primer 50 bp downstream of the putative translation initiation site (Figure 3B). The 5’ terminus of the atp6 transcript follows a CGTA motif (Figure 1), which has been shown to be an essential promoter element of plant mitochondrial genes (Rapp and Stern, 1992).

Identification of RNA Editing Sites in Petunia atp6 Transcripts

A petunia mitochondrial cDNA library was screened with an atp6 gene–specific probe. Sequencing of 24 partial or complete cDNA clones revealed 15 RNA editing sites in atp6 transcripts that predict 14 amino acid changes. cDNA sequences that encompassed the 5’ untranslated region, the entire coding region, and almost all of the 3’ untranslated region were analyzed, but editing sites were found only in the coding region (Figure 1). Two editing sites occur within the same codon (codon 248). The last editing event changes a CAA glutamine codon (codon 396) to a UAA stop codon. Editing at codon 398 shortens the protein predicted from genomic DNA by 13 amino acids because the first genomically encoded stop codon is at codon 411. The molecular mass of the mature ATP6 protein predicted from edited RNA sequence is 28 kD. All RNA editing events detected occur within the conserved core region. Amino acid changes introduced by RNA editing increase the hydrophobicity of the predicted ATP6 protein; this might be important for its function as part of the ATPase proton channel.
Figure 1. Nucleotide and Deduced Amino Acid Sequences of Petunia atp6 Genomic DNA and cDNA.

Figure 2. Predicted Precursor and Mature Forms of Plant ATPG.

Table: Predicted Precursor and Mature Forms of Plant ATPG.

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<th>Plant</th>
<th>N-Extension</th>
<th>Core-region</th>
<th>C-Extension</th>
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<td>SPLDQ</td>
<td>PLEQ</td>
<td>LH</td>
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<tr>
<td>Oenothera</td>
<td>SPLDQ</td>
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<td>Tobacco</td>
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Figure 4 shows that the amino acids predicted after editing at the 14 sites are identical to the amino acids predicted from edited cDNAs of Oenothera and rapeseed and differ only by one residue from maize and sorgum. Some of the amino acids of maize, sorgum, and Oenothera shown in Figure 4 are encoded in the genomic DNA sequences of the respective atp6 genes, whereas others result from editing. Editing usually (Walbot, 1991), although not always (Conklin and Hanson, 1991; Schuster and Brennicke, 1991a), results in increased conservation of predicted amino acid sequence relative to sequences of homologous proteins in other organisms.

The genomic petunia atp6 DNA sequence is shown on the upper line, and the cDNA sequence is shown on the lower line. The complete amino acid sequence deduced from genomic DNA is shown above the DNA sequence. Amino acids deduced from the cDNA are shown below the cDNA sequence where they differ from the genomic DNA–predicted residues. Individual codons containing RNA editing sites and changed amino acids are boxed. The arrow indicates the transcript's 5' end. The boxed and underlined nucleotide sequences indicate the putative promoter element and putative ribosome binding site, respectively. The boxed amino acid sequence shows the N-terminal sequence of the petunia ATP6 protein as determined by microsequencing. The underlined amino acid sequence indicates the peptide sequence used to generate an anti-synthetic peptide antibody. Asterisks indicate translation stop codons.
was used to test the extent of RNA editing at this site. Total cDNA and unedited genomic DNA templates were mixed and clone or from unedited genomic DNA were also digested with Msel. PCR products amplified from a fully edited cDNA was amplified by PCR, and PCR products were then digested the cDNA surrounding the stop codon-producing editing site

Partial Editing at Certain Positions on the Transcript

When individual cDNA clones were sequenced, some were found to represent incompletely edited transcripts (Figure 5A). For example, clones 3 and 6 are unedited at single sites, whereas clone 5 is unedited at several sites, including the site that creates a stop codon. The editing event that produces a stop codon also generates a new Msel restriction site on the cDNA. Therefore, a reverse transcription (RT)-PCR technique that creates a stop codon. The editing event that produces a stop codon also generates a new Msel restriction site on

Partial editing of petunia atp6 near the stop codon was further analyzed by sequencing cDNA clones. RT-PCR products amplified from line 3688 total RNA (illustrated in Figure 5B, lane 5) were cloned. Fifteen such clones were randomly selected and sequenced (Figure 5C). Consistent with restriction analysis of PCR products, the stop codon site is partially edited, with only 5 of the 15 clones showing editing. Interestingly, two of the other three editing sites contained within the amplified region also show partial editing. The four edit sites are edited with different efficiency. One site (codon 377) shows the most efficient editing (15 of 15), and there is a tendency for the other three sites to be less edited the more distal they are to this site.

Production of an Antibody against the Unedited Transcript-Specific 12 Amino Acids and Analysis of Mitochondrial Proteins

The partially edited atp6 transcripts could theoretically be translated because all editing events are C-to-U modifications that do not disturb the reading frame and none of the edit sites affect the translation initiation codon or the 5' and 3' untranslated regions (UTRs). To test this possibility, we raised an antibody against a synthetic peptide containing the terminal 12 amino acids that could be present only in the ATP6 protein translated from transcripts unedited at the stop codon site. The specificity of this "anti-extension" antibody was demonstrated

Figure 4. Alignment of ATP6 Amino Acid Sequences at the 14 Codon Positions Affected by Editing in Petunia.

Amino acid sequences deduced from petunia atp6 genomic DNA and fully edited cDNA (this study) and from fully edited cDNAs of Oenothera (Schuster and Brennicke, 1991b), sorghum (Kempken et al., 1991), maize (Kumar and Levings, 1993), and rapeseed (Handa and Nakajima, 1992) are compared. Positions of the 14 codons are shown on the top. Asterisks indicate the stop codons introduced by RNA editing. Amino acids that are genetically encoded are underlined.

Figure 3. Transcript Analysis of Petunia atp6.

(A) RNA filter hybridization. Mitochondrial RNA isolated and processed as described in Methods was analyzed by hybridization with atp6 gene-specific probes. Lengths of RNA markers are shown at right in kilobases.

(B) Primer extension analysis. The atp6-7 primer end labeled with T4 kinase and total leaf RNAs were used in this analysis. The extension product was run on a 6% sequencing gel together with a dideoxy sequence ladder generated using the atp6-7 primer and plasmid pXH1.7. The nucleotide sequence surrounding the transcript end is depicted at right. The arrow indicates the 5' end of the transcript.
Figure 5. Assaying the Extent of RNA Editing by Sequencing and RT-PCR.

(A) A schematic of the sequence analysis of nine cDNA clones isolated from a petunia mitochondrial cDNA library. The codon positions of the 15 edit sites are shown at top. A filled oval represents a site that is edited; an open oval represents a site that is unedited.

(B) RT-PCR analysis showing partial editing at the site that generates a stop codon. On the left is a schematic diagram showing the positions of primers used in RT-PCR analysis, the four edit sites included in the amplified region, and the Msel restriction sites on cDNAs derived from edited and unedited atp6 transcripts. The asterisk indicates the Msel site that is created by RNA editing. At right is an ethidium bromide-stained gel of Msel-digested PCR products amplified from total RNAs of petunia lines 3677, 3699, 3704, 2423, 3688, and 11,127 (lanes 1 to 6) and from genomic DNA (lane 7) and a fully edited cDNA clone (lane 8). The lengths of the restriction fragments are shown at right in base pairs.

(C) Summary of sequencing analysis of 15 cDNA clones derived from the line 3688 RT-PCR product. The positions of the four sites are shown in base pairs.

Expression of Petunia atp6 Transcript

by its ability to distinguish two fusion proteins made by fusing 3' portions of atp6 genomic sequence or cDNA sequence to the glutathione S-transferase gene. The antibody reacts only with the fusion protein that contains the additional amino acids specified by unedited transcript (Figure 6).

As a control to test whether the anti-extension antibody could recognize the unedited transcript-specified 12 amino acids in the hydrophobic environment of ATP6, another Escherichia coli expression contract was designed to produce a hydrophobic protein consisting of approximately half of the mature ATP6 coding region and the unedited transcript-specified amino acid extension (Figure 7A). The anti-extension antibody effectively recognized this E. coli-expressed partial ATP6 protein (Figure 7B). As a further control to determine that petunia ATP6 was present on our immunoblots in an immunoreactive form, an immunoblot of total mitochondrial protein was reacted with an anti-Brassica ATP6 antibody (Y. L'Homme and G. Brown, unpublished data). Petunia ATP6 reacted with this anti-Brassica ATP6 antibody (Figure 7C).

When the purified anti-extension antibody was used to probe electrophoresed total mitochondrial proteins blotted to a filter, a reactive protein with a mobility corresponding to 40 kD was detected (Figure 8). Because the apparent size of this protein is close to the predicted size (45.5 kD) of a precursor ATP6 protein translated from unedited transcript, we decided to determine the identity of the 40-kD protein. Approximately 10 μg of the immunoreactive protein was purified by immunoprecipitation and run on a preparative SDS-polyacrylamide gel. Gel slices containing the 40-kD protein were cut out, partially digested with V8 protease in situ, and run on a second SDS-polyacrylamide gel. Digested protein fragments were then transferred to poly(vinylidene difluoride) (PVDF) membranes and microsequenced. Two of the peptide fragments gave an identical N-terminal amino acid sequence of AFLSDPSPLKLNLGVGAYRD. Data base search (Figure 9) shows that this peptide sequence is homologous to sequences in both plant and animal mitochondrial aspartate aminotransferase (MAAT). Therefore, the 40-kD immunoreactive protein is probably petunia MAAT.

Because the petunia maat gene has not been sequenced, we do not know which epitope of the protein is recognized by the antibody, although some similarity between the synthetic peptide and the lupine MAAT sequence can be detected (Figure 9). When fractionated mitochondrial proteins are probed with the anti-synthetic peptide antibody, the 40-kD protein is found solely in the soluble fraction (data not shown). MAATs...
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Figure 6. Immunoblot Analysis Showing Specificity of the Anti-Extension Antibody. At top are schematic structures of fusion proteins generated from pGEX/DNA and pGEX/cDNA constructs. The open bars represent glutathione S-transferase (GST) protein encoded by the vector, and the striped bars represent the C-terminal region of ATP6 protein (amino acids 369 to 398) fused to GST. The filled bar represents the 12-amino acid peptide used to generate anti-synthetic peptide antibody. Total £. coli proteins from cells induced (+) or uninduced (−) with isopropyl α-D-thiogalactopyranoside are made for each construct. Proteins transferred to a nitrocellulose filter were stained with Ponceau S (a) and immunodecorated with anti-synthetic peptide antibody after destaining (b). The identity of the cross-reactive £. coli protein that runs above the 29-kD fusion protein is unknown. The minor bands that exist only in induced cells transformed with pGEX/DNA construct are probably degradation products of the fusion protein. Protein molecular mass markers are shown at right in kilodaltons.

Figure 7. Specificity of the Anti-Extension Antibody and Anti-ATP6 Antibody. (A) A schematic diagram showing the pQE-ATP6 fusion protein that corresponds to the C-terminal half of the ATP6 core region, except for the 26 amino acids (a.a.) derived from the vector sequence. (B) Immunoblot showing reactivity of the pQE-ATP6 fusion protein with the anti-synthetic peptide antibody. SG13009[pREP4] cells containing the pQE-ATP6 construct grown in the presence (lane 2) or absence (lane 3) of isopropyl β-D-thiogalactopyranoside were used. The sizes of prestained molecular mass markers (lane 1) are shown at left in kilodaltons. (C) Detection of petunia mitochondrial ATP6 protein with anti-Brassica ATP6 antiserum. Total protein made from purified mitochondria of petunia line 3688 (lane 2) was run on a 15% SDS–polyacrylamide gel, transferred to a membrane, and probed with anti-Brassica ATP6 antiserum (kindly provided by G. Brown, McGill University, Montreal, Canada). The sizes of prestained molecular mass markers (lane 1) are shown at left in kilodaltons.

Only One Form of ATP6 Protein Can Be Extracted from Petunia Mitochondria Using Organic Solvents

The hydrophobic nature of mitochondrial ATP synthase F0 subunits (ATP6 and ATP9) enables them to be extracted from total mitochondrial proteins using organic solvents. Using a modified procedure for extracting yeast ATP6 protein (Michon et al., 1988), we have purified petunia mitochondrial ATP6 protein to homogeneity. A reverse phase HPLC elution profile of petunia mitochondrial extracts is shown in Figure 10A. Fraction II (33 min) contains a single protein, as determined by silver staining of an SDS–polyacrylamide gel (Figure 10B). This protein has the same gel mobility as yeast ATP6 protein and as petunia ATP6 identified with the anti-Brassica ATP6 antibody (Figure 7C). The discrepancy between the predicted (28 kD) and estimated (22 kD) molecular mass of ATP6 protein is probably due to its extreme hydrophobicity, because hydrophobic proteins often exhibit abnormal gel migration.

To confirm the identity of this protein, it was dot blotted onto PVDF membrane and microsequenced. The N-terminal 10 residues of this protein, SPLEQFEIIP, match perfectly with the predicted N-terminal sequence of mature petunia ATP6. To determine accurately the molecular mass of the extracted ATP6 protein, protein prepared from fraction II was subjected to MALD mass spectrometry. As seen in Figure 11A, only one form of ATP6 protein is detected by Lasermat analysis. In addition to
Expression of Petunia \textit{atp6} Transcript

A protein with a molecular mass of 7.7 kD was detected in fraction I by Lasermat (Figure 11B). The mass of ATP9 protein predicted from edited transcripts is 7577 D (Wintz and Hanson, 1991). Because unedited transcripts of the petunia \textit{atp9} gene are undetectable in the RNA population (Wintz and Hanson, 1991), uniformity of the ATP9 protein is expected. For petunia ATP9 as well as ATP6, the measured molecular mass is also somewhat larger than the mass calculated from the composition of fully edited transcripts, which is also possibly due to post-translational modification.

Protein prepared from fraction II was also subjected to immunoblot analysis by probing with the anti-extension antibody, and no reaction was detected (data not shown). This result together with Lasermat analysis confirm that ATP6 proteins corresponding to transcripts unedited at the stop codon site do not accumulate in petunia mitochondria.

Partially Edited \textit{atp6} Transcripts Are Associated with Polysomes

To determine whether transcripts unedited at the stop codon are prevented from access to the translation machinery, we examined the association of \textit{atp6} transcripts with mitochondrial ribosomes. Young leaf lysate was prepared under conditions that maintain intact ribosomes and was size-fractionated in analytical sucrose gradients. RNAs extracted from each fraction were assayed by RNA gel blot hybridization. As shown in Figure 12A, the distribution of \textit{atp6} transcripts in the sucrose gradient was similar to chloroplast ribulose bisphosphate carboxylase large subunit (rbcL) transcripts.

A protein specified from one of the transcripts edited at the stop codon but unedited at all observed partial editing sites would differ in molecular mass by 164 D from the protein derived from fully edited transcripts. Proteins with such mass differences (28.5 versus 28.7 kD) are resolvable by the Lasermat, but only one peak representing a singly protonated molecular ion was detected. Because of the added complication of possible post-translation modification, the measured molecular mass of 28.5 kD is larger than the 28-kD mass predicted for mature ATP6 protein derived from fully edited transcripts but is less than the 29.5-kD mass predicted from genomic DNA and unedited transcripts. As the precision of the Lasermat in this molecular mass range is ±30 D, the higher mass is not likely to be due to experimental error. Covalent attachment of lipid in vivo is a common modification of membrane proteins (Towler et al., 1988; Resh, 1994) and may explain the increase in molecular mass over that predicted from edited transcripts.

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The similarity of the 40-kD protein with plant aspartate aminotransferases is shown in Figure 9. The 40-kD protein shares significant similarity with the lupine mitochondrial aspartate aminotransferase (LupMAAT; Reynolds et al., 1992), alfalfa aspartate aminotransferase (AlfAAAT; Udvardi and Kahn, 1991), and carrot cytoplasmic aspartate aminotransferase (CarAAAT; Turano et al., 1992) sequences. Identical amino acids are in boldface letters, and colons indicate a conservative change.
analyses of hydrophobic proteins extracted from petunia mitochondria using organic solvents. (a) chromatographic analysis of organic solvent extracted hydrophobic proteins. Diethyl ether-precipitated hydrophobic proteins were fractionated on a C4 Ultrapore column (Beckman, Wakefield, MA). Proteins were detected by monitoring absorbance at 280 nm. The peak at 28 min is referred to as fraction I, and the peak at 33 min is referred to as fraction II. (b) SDS-PAGE of proteins contained in fractions I and II. Fractions I and II from HPLC were washed by adding water and chloroform, and proteins in the organic phase were dried, solubilized, and loaded onto a 15% SDS-polyacrylamide gel. The gel was silver stained after gel electrophoresis.

which have been shown to have tight association with poly- somes (Barkan, 1993). We then assayed the presence in the polysome fractions of *atp6* transcripts unedited at the stop codon site using the RT-PCR technique described earlier. As shown in Figure 12B, *atp6* transcripts unedited and edited at the stop codon were found in all the fractions. To exclude the possibility that these unedited transcripts are associated with other ribonucleoprotein complexes instead of ribosomes, we performed a control experiment in which mitochondrial poly- somes were subject to a high-salt puromycin reaction. High-salt puromycin treatment has been shown to dissociate organel- lar ribosomes into their subunits (Chua et al., 1973; Hanson and Bogorad, 1977). When a mitochondrial extract was treated with puromycin, *atp6* transcripts stayed on top of the sucrose gradient after ultracentrifugation. *atp6* transcripts were found in dense gradient fractions when an untreated portion of the same preparation was ultracentrifuged (Figure 12C). These results indicate that both fully edited and partially edited *atp6* transcripts are associated with ribosomes.

**DISCUSSION**

Our study of the petunia *atp6* gene indicates that, although differentially partially edited transcripts constitute a majority of the *atp6* transcript population, ATP6 protein is homogeneous. Furthermore, proteins carrying a 12-amino acid extension encoded by unedited transcripts are undetectable by immuno- logical analysis. There are two primary explanations for these observations: either multiple proteins are not produced from differentially edited transcripts, or they are rapidly degraded. Although most unedited transcripts of plant mitochondrial genes, including petunia *atp6*, have a correct reading frame, initiation codon, and a putative ribosome binding site, perhaps some filtration mechanism allows only fully edited transcripts to be translated. If so, editing efficiency of a particular mitochondrial gene may serve to regulate the amount of its functional mRNA. Alternatively, polypeptides synthesized from incom- pletely edited transcripts may be recognized in some way and then rapidly degraded.

Although comparable experiments have not yet been per- formed with plant systems, yeast mitochondrial gene expression has been observed to be regulated at the translational level (Costanzo and Fox, 1990). Translation of the yeast coxl gene, for example, involves recognition of the 5' UTR, and at least three nuclear-encoded proteins are implicated (Costanzo and Fox, 1990). An effect of the 5' UTR on ribosome binding may explain a discrepancy between our results with petunia *atp6* and studies of the wheat mitochondrial nad3-ribosomal protein S12 (rps12) transcription unit. Wheat polysome-enriched fractions (Gualberto et al., 1991) contained a much higher proportion of fully edited nad3-rps12 transcripts than is present in the total RNA population, whereas in petunia polysome fractions, the ratio of edited to unedited *atp6* transcripts does not vary throughout the gradient (Figure 12). The wheat nad3-rps12 locus specifies two transcripts that undergo editing, a larger one containing a 5' pseudo-tRNA sequence and a smaller transcript lacking the pseudo-tRNA sequence (Gualberto et al., 1991). Editing extent of the smaller transcript is much greater than that of the larger transcript. Perhaps the presence of the pseudo-tRNA prevents access of partially edited...
wheat nad3-rps12 transcripts to ribosomes. In contrast to the wheat transcripts, petunia atp6 transcripts are homogeneous at their 5' ends (Figure 3). If the 5' UTR is the major determinant of ribosome binding in the initial steps of translational initiation, fully edited and incompletely edited petunia atp6 transcripts should have equal access to the translation machinery, a result consistent with our observations (Figure 12).

The finding of polysome fractions containing partially edited petunia atp6 (this study) and unspliced maize cox2 transcripts (Yang and Mulligan, 1993) indicates nonselective recruitment of these RNAs by mitochondrial ribosomes. Because incompletely edited and unspliced transcripts are unlikely to encode functional proteins, there may be additional checkpoints that prevent the completion of translation of such transcripts. If such checkpoints do not exist and partially edited petunia atp6 transcripts are translated, the protein products must be degraded rapidly because they do not accumulate. If degradation is the mechanism preventing accumulation of polypeptides from unedited transcripts of plant mitochondrial genes, then a large number of multiple protein forms would need to be recognized and destroyed. For example, for petunia atp6, we have detected transcripts that predict at least seven distinct forms of the protein, but ATP6 protein is homogeneous by MALD spectrometry (Figure 9). For petunia nad3, fully edited transcripts represent <10% of the RNA population; if the abundant partially edited transcripts are translated, there should exist at least 14 different forms of the protein (Lu and Hanson, 1992; R. Wilson and M. Hanson, unpublished data). If plant mitochondria are able to degrade specifically proteins specified by unedited transcripts, then RNA editing could indirectly cause regulation of the abundance of a gene's product. For example, if more than two-thirds of the ATP6 protein translated in petunia mitochondria is from unedited transcripts and is rapidly degraded, then less than one-third of the atp6 transcripts present give rise to stable protein. Such a post-translational regulation of protein abundance seems likely to be less energetically efficient than regulation of transcript abundance or regulation of access of transcripts to ribosomes.

The association of incompletely edited plant mitochondrial transcripts with ribosomes (Yang and Mulligan, 1993; this study, Figure 12) raises the possibility that editing occurs during translation. We believe this is unlikely because editing is known to occur in plant mitochondrial pseudogene transcripts that contain stop codons near the initiation codon (Schuster and Brennicke, 1991a; Aubert et al., 1992).

Comparison of different species' mitochondrial gene sequences and cDNAs shows that sites where C residues are edited to U residues in one species are found to contain genomic T residues in another species (Hanson et al., 1993; Figure 4). Only one of the atp6 editing sites found in petunia, Oenothera, sorghum, and maize is retained in rapeseed (Figure 4); all others are represented by T residues in the rapeseed genomic DNA. Why only some editing sites have converted to T residues in plant mitochondrial genomic DNAs remains a topic of speculation; possibly RNA editing has an unidentified additional purpose other than sequence "correction."
Figure 12. Association of afp6 Transcripts Unedited at the Stop Codon Site with Ribosomes.

(A) RNA filter hybridization analysis showing association of afp6 mRNA with polysomes. Total extracts made from young petunia leaves were centrifuged on sucrose gradients as described in Methods. Fractions were collected from the bottom of the tube. Equal proportions of RNAs purified from each fraction were probed with an rbcL gene-specific probe (upper gel) or an afp6 gene-specific probe (lower gel). RNA length markers are shown at right in kilobases.

Whereas RNA editing in plant mitochondria could cause production of variant proteins from the same gene, partial editing of petunia atp6 transcripts evidently does not serve this purpose. Because the residues specified by unedited codons of many mitochondrial genes would predict nonfunctional proteins, tight controls on accumulation of such proteins may confer a selective advantage. Additional studies will be required to determine whether RNA editing has another role in gene regulation, such as controlling the abundance of translatable mRNA or of a gene’s protein product, or whether it is merely an evolutionary relic that is required to alter codons so that a functional gene product can be made.

METHODS

Isolation of the Petunia atp6 Gene

Petunia line 3688 mitochondrial DNA cosmid clone 88E3 hybridized to a polymerase chain reaction (PCR)-amplified probe generated by selecting ATP synthase subunit 6 (atp6)-specific primers based on the maize atp6 sequence (Dewey et al., 1985). The petunia gene was further localized to a Xhol fragment, which was then subcloned and sequenced. A single open reading frame with high similarity with other plant mitochondrial atp6 genes was found within the Xhol fragment.

Plant Genotypes

Lines 3688, 11,127, and 2423 contain different nuclear genomes but the same male sterility-encoding mitochondrial genome. Fertile lines 3677, 3699, and 3704 contain the same nuclear genomes as lines 2423, 3688, and 11,127, respectively. Line 3699 carries the Petunia parodii cytoplasmic genome, whereas lines 3677 and 3704 carry the P. hybrida cytoplasmic genomes. Lines 3688 and 3699 carry the P. parodii nuclear genome; 3704, 11,127, 3677, and 2423 are P. hybrida lines. The isonuclear lines were kindly provided by S. Izhar (Volcani Center, Israel).

DNA Templates

Plasmid pRBCL, which contains a 6-kb HindIII-SstI fragment of the rice chloroplast ribulose bisphosphate carboxylase large subunit (rbcL)

(B) Detection of atp6 transcripts unedited at the stop codon site in polysome fractions. RNAs purified from each fraction were subjected to RT-PCR analysis as described in Figure 5. RNA samples were treated with DNase I, and RT-PCR analysis of each sample was controlled to ensure no genomic DNA contamination. The lengths of the restriction fragments are shown at right in base pairs.

(C) Release of atp6 transcripts from polysome fractions after puromycin treatment. A mitochondrial extract made from petunia line 3704 was subjected to a high-salt puromycin reaction in the presence (+ puromycin) or absence (– puromycin) of puromycin and run on sucrose gradients. RNAs purified from each fraction were separated on an agarose gel, blotted, and probed with an atp6 gene-specific probe. The sizes of molecular length markers are shown at right in kilobases.
gene inserted into HindIII-digested pUC18, was a gift from E. Moon and R. Wu (Cornell University). To construct pGEXDNA and pGEXcDNA, PCR products amplified from petunia genomic DNA or an atp6 cDNA clone containing the edited stop codon using primers atp6-2 and atp6-3 were first cloned into the TA cloning vector (Invitrogen). The inserts were then isolated as BamHI-EcoRI fragments and cloned into-frame into the pGEX2T (Pharmacia) vector for production to the other tube, one-ninth volume of high-salt buffer was added. The inserts were then isolated as BamHI-EcoRI fragments and ninth volume of 10 mM puromycin (made in high-salt buffer) was added; and afp6-2 and afp6-3 were first cloned into the TA cloning vector (Invitro). The supernatant was divided equally into two tubes. To one tube, one-ninth volume of 10% Triton X-100. After spinning in a microcentrifuge for 10 min, the supernatant was divided equally into two tubes. To one tube, one-ninth volume of 10 mM puromycin (made in high-salt buffer) was added; and to the other tube, one-ninth volume of high-salt buffer was added. The mixtures were incubated at 37°C for 20 min and then loaded onto two 15 to 55% continuous sucrose gradients and analyzed as described above.

**Oligonucleotides**

The following oligonucleotides were synthesized at the Cornell Biotechnology Program Facility: atp6-2, 5'-TCTAGAGCAAATACCTGGAATGCT-CCACG-3' (complementary to nucleotides 1694 to 1718, containing a synthetic Xbal site at the 5' end); atp6-3, 5'-GGATCCGGTcTGcAA-3' and atp6-7 5'-GAGAGGATGTAGCACTGGAA-3' (complementary to nucleotides 1550 to 1568, containing a synthetic BamHI site at the 5' end); afp6-7 5'-GAGAGGATGTAGCACTGGAA-3'.

**Expression of Fusion Proteins in Escherichia coli**

pGEXDNA and pGEXcDNA constructs were transformed into the BL21 E. coli strain, and transformants were inoculated into 1 mL of Luria-Bertani medium (100 μg/mL ampicillin) and incubated at 37°C overnight. A 50-μL overnight culture was inoculated into 5 mL of Luria-Bertani medium containing 100 μg/mL ampicillin and grown at 37°C until OD600 reached 0.6. Isopropyl β-D-thiogalactopyranoside was then added to a concentration of 1 mM, and the culture was grown at 37°C for an additional 2 hr. The cells were pelleted, resuspended in SDS–gel loading buffer, and used for gel electrophoresis analysis. The pQE-ATP6 construct was transformed into SGI3009[pREP4] cells, and fusion protein induction and SDS-PAGE analysis were done as described above, except that the pelleted cells were resuspended in SDS–gel loading buffer containing 8 M urea.

**Anti-Synthetic Peptide Antibodies**

Antiserum to a synthetic peptide specified by a Brassica atp6 gene was provided by Greg Brown (Y. L’Homme and G. Brown, unpublished data). To produce the anti-extension antibody, a 12–amino acid peptide, specified by the DNA sequence beginning at the stop codon created by editing, was synthesized and coupled to the carrier protein keyhole limpet hemocyanin through glutaraldehyde linking. The conjugate was mixed with Freund’s complete adjuvant and injected into rabbits (Harlow and Lane, 1988). Antiserum were collected, and the anti-synthetic peptide antibody was affinity purified using the pGEX–DNA fusion protein as follows. Total proteins from induced E. coli cells were separated on preparative SDS–polyacrylamide gels and transferred to nitrocellulose filters; the filters were stained with Poncet S. The fusion protein bands were cut out, washed in Tris-saline (50 mM Tris-HCl, pH 7.4, 200 mM NaCl), blocked in blocking buffer (5% nonfat milk in Tris-saline) for 1 hr, and then incubated overnight in probe solution containing antiserum diluted in blocking buffer. After washing in Tris-saline for 5 min four times, the filters were washed with ice-cold elution buffer (0.2 M glycine, pH 2.8, 1 mM EGTA). The eluted antibody

**RNA Isolation**

Total leaf RNA was prepared as described by Ausubel et al. (1989). For mitochondrial RNA isolation, purified mitochondria were resuspended in 0.4 M mannitol, 10 mM Tricine, 10 μM EDTA, pH 7.5, and lysed by adding one-quarter volume of lysis buffer (10% SDS, 25 mM Tris-HCl, pH 7.5, 20 mM EDTA). The lysate was gently mixed, extracted with phenol, phenol–chloroform (1:1 [v/v]), and chloroform and ethanol precipitated.

**RNA Hybridization, Reverse Transcriptase–PCR, and Primer Extension**

RNA hybridization and reverse transcriptase (RT)–PCR were performed as previously reported (Lu and Hanson, 1992). The primer extension assay was performed essentially as described by Ausubel et al. (1989).

**Mitochondrial cDNA Library Screening**

A mitochondrial cDNA library constructed as described by Sutton et al. (1991) using young leaf tissue, polysomes were prepared from total leaf extract essentially as described by Barkan (1995). The supernatant was layered onto two tubes of 5-mL 15 to 55% continuous sucrose gradients, which were centrifuged at 39,000 rpm for 1 hr in a Beckman SW41Ti rotor. EDTA and SDS were added to each fraction to final concentrations of 20 mM and 0.5%, respectively. RNA was purified by phenol–chloroform extraction and ethanol precipitation and used in RNA filter hybridization and RT–PCR analysis. The high-salt puromycin reaction was done essentially as described by Hanson and Bogorad (1977). Purified petunia mitochondria were resuspended in high-salt buffer (50 mM Tris·HCl, pH 8.0, 500 mM KCl, 25 mM MgCl2, 5 mM DTT, 0.5 mg/mL heparin) and lysed by adding one-ninth volume of 10% Triton X-100. After spinning in a microcentrifuge for 10 min, the supernatant was divided equally into two tubes. To one tube, one-ninth volume of 10 mM puromycin (made in high-salt buffer) was added; and to the other tube, one-ninth volume of high-salt buffer was added. The mixtures were incubated at 37°C for 20 min and then loaded onto two 15 to 55% continuous sucrose gradients and analyzed as described above.

**Mitochondrial cDNA Library Screening**

A mitochondrial cDNA library constructed as described by Sutton et al. (1991) was used to isolate atp6 cDNA clones using standard procedures (Maniatis et al., 1982). Positive clones were converted into pBluescript KS+ plasmids by in vivo excision and sequenced using the U.S. Biochemical Sequenase double-stranded DNA sequencing system.

**Polysome Analysis**

Starting with 2 g of young leaf tissue, polysomes were prepared from total leaf extract essentially as described by Barkan (1995). The supernatant was layered onto two tubes of 5-mL 15 to 55% continuous sucrose gradients, which were centrifuged at 39,000 rpm for 1 hr in a Beckman SW41Ti rotor. EDTA and SDS were added to each fraction to final concentrations of 20 mM and 0.5%, respectively. RNA was purified by phenol–chloroform extraction and ethanol precipitation and used in RNA filter hybridization and RT–PCR analysis. The high-salt puromycin reaction was done essentially as described by Hanson and Bogorad (1977). Purified petunia mitochondria were resuspended in high-salt buffer (50 mM Tris·HCl, pH 8.0, 500 mM KCl, 25 mM MgCl2, 5 mM DTT, 0.5 mg/mL heparin) and lysed by adding one-ninth volume of 10% Triton X-100. After spinning in a microcentrifuge for 10 min, the supernatant was divided equally into two tubes. To one tube, one-ninth volume of 10 mM puromycin (made in high-salt buffer) was added; and to the other tube, one-ninth volume of high-salt buffer was added. The mixtures were incubated at 37°C for 20 min and then loaded onto two 15 to 55% continuous sucrose gradients and analyzed as described above.

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was neutralized by adding one-tenth volume of 1 M Tris and one-tenth volume of 10 x PBS and stored at 4°C.

Gel Electrophoresis, Blotting, and Immunological Detection of Proteins

Purified petunia mitochondria or E. coli cells were suspended in SDS–gel loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 5 min, loaded on 15% SDS–polyacrylamide gels, and electrophoresed. After gel electrophoresis, the proteins were electroblotted onto polyvinylidene difluoride (PVDF) membrane for 1 hr at 400 mA in transfer buffer (10 mM 3-(cyclohexylamino)-1-propane sulfonic acid, 10% methanol, pH 11). The membrane was then washed for 15 min in Tris-saline and blocked for 1 hr in blocking buffer. After incubation with primary antibody overnight at 4°C, the filter was washed for 5 min four times in Tris-saline, incubated with secondary antibody conjugated with alkaline phosphatase, washed, and developed in bromochloroindoly phosphate/nitroblue tetrazolium buffer.

Immunoprecipitation, V8 Protease Digestion, and Protein Microsequencing

Sucrose gradient–purified mitochondria were solubilized in 2% SDS, 60 mM Tris-HCl, pH 6.8, at a concentration of 10 μg/mL, boiled for 5 min, chilled on ice, and diluted with 20 vol of dilution buffer (1% Triton X-100, 0.3 M KCl, 10 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride). The lysate was spun at 10,000 rpm for 10 min in a Sorvall SS-34 rotor (Du Pont), and the supernatant was mixed with anti-synth-5 min, chilled on ice, and diluted with 20 VOI of dilution buffer (1% Triton X-100, 0.3 M KCl, 10 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride). The lysate was spun at 10,000 rpm for 10 min in a Sorvall SS-34 rotor (Du Pont), and the supernatant was mixed with anti-synthetic peptide antibody–protein A Sepharose slurry prepared by coupling antigen to protein A Sepharose beads (Sigma) using dimethyl-pimelimidate cross-linker (Harlow and Lane, 1988). After overnight incubation at 4°C, Sepharose beads were collected, washed four times in dilution buffer, and eluted with elution buffer. After elution, the Sepharose beads were neutralized, washed, and reused in immunoprecipitation experiments. Immunoprecipitates were concentrated by trichloroacetic acid precipitation. V8 protease digestion was performed according to procedures described by Cleveland et al. (1977). Digested proteins were transferred to PVDF membrane and stained with Coomassie Brilliant Blue R 250. Protein bands were cut from the membrane. Microsequencing was done with a gas phase sequencer (model 470, Applied Biosystems, Inc., Foster City, CA).

Organic Solvent Extraction, HPLC, and Lasermat

Hydrophobic protein extraction was done by the procedure of Michon et al. (1988) with some modifications. Hydrophobic proteins were precipitated twice with diethyl ether and used directly for HPLC analysis without dialysis. The organic phase and interphase were dried in a Speedvac (Savant Instruments, Inc., Farmingdale, NY), and the pellet was solubilized in hexafluoroisopropanol–99% formic acid (2:7 [v/v]). Approximately 1 μL of the solution given above and 3 μL of 50 mM sinapinic acid in 70% formic acid were mixed, placed on a stainless steel slide, and allowed to air dry. Matrix-associated laser desorption mass spectrometry was performed on a Finnigan Mat Lasermat analyzer (Hemmel-Hempstead, UK).

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A single homogeneous form of ATP6 protein accumulates in petunia mitochondria despite the presence of differentially edited atp6 transcripts.

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