Novel Recombinations in the Maize Mitochondrial Genome Produce a Unique Transcriptional Unit in the Texas Male-Sterile Cytoplasm

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Summary

We have characterized a 3547 bp DNA fragment from male-sterile (cms-T) maize mitochondria, designated TURF 2H3, selected because of its unique and abundant transcripts. Sequence analysis indicated that TURF 2H3 originated by recombinations among portions of the flanking and/or coding regions of the maize mitochondrial 26S ribosomal gene, the ATPase subunit 6 gene, and the chloroplast tRNA-Arg gene. TURF 2H3 contains two long open reading frames that could encode polypeptides of 12,961 Mr and 24,675 Mr. The larger open reading frame hybridizes to transcripts in all maize cytoplasms, the smaller to transcripts only in T cytoplasm. TURF 2H3 transcripts appear to be uniquely altered in cms-T plants restored to fertility by the nuclear restorer genes Rf1 and Rf2. A possible relationship between TURF 2H3, nuclear restorer genes, and the male sterility trait in T cytoplasm is suggested.

Introduction

Mitochondrial genomes of higher plants are larger and more complex than those of other eukaryotic organisms (Levings, 1983a). The complexity is due in part to intramolecular recombinational events in which populations of subgenomic circles are generated from a master genomic circle (Palmer and Shields, 1984; Lonsdale et al., 1984). Large repeated sequences often appear to be the sites of intramolecular recombination (Lonsdale et al., 1984). Intergenomic rearrangements involving the exchange of DNA between the chloroplast, nuclear, and mitochondrial genomes of higher plants have also been reported (Stern and Lonsdale, 1982; Kemble et al., 1983; Stern and Palmer, 1984).

The mitochondrial genome of Zea mays from normal (male-fertile) cytoplasm is estimated to be 570 kilobases (kb) in length by cosmid mapping (Lonsdale et al., 1984). Restriction endonuclease digestion analysis of the mitochondrial DNA (mtDNA) from normal and mutant cytoplasms of maize, however, has revealed considerable heterogeneity among the different cytoplasms (Pring and Levings, 1978). Restriction analysis of various members of the genus Zea shows that recombination is the major force accounting for mtDNA heterogeneity among the species (Sederoff et al., 1981).

Abundant evidence indicates that the mitochondrial genome contains the genetic information responsible for the cytoplasmic male-sterile (cms) trait (see review, Levings, 1983b). Soon after tassel emergence, male-fertile (normal) maize plants exert anthers and shed viable pollen. In contrast, plants containing male-sterile cytoplasms do not exert anthers and viable pollen is not produced. The cms trait has been used extensively in hybrid seed production to avoid the costly procedure of hand emasculation. The three major types of maize male-sterile cytoplasms, cms-T, cms-S, and cms-C, are distinguished on the basis of the specific nuclear genes that restore pollen fertility. The Texas male-sterile cytoplasm (cms-T) is unique in that an inseparable association appears to exist between disease susceptibility to the fungal pathogen Bipolaris maydis (Helminthosporium maydis, race T, and male sterility in this cytoplasm (Hooker et al., 1970; Gengenbach et al., 1977; Brettell et al., 1979). Cms-T was widely used in commercial hybrid maize production before severe disease outbreaks in 1970 forced the industry to curtail its use.

We have isolated and characterized a fragment of cms-T mtDNA from maize, designated TURF 2H3, that presumably arose through a series of novel recombinations within and between the mitochondrial and chloroplast genome. The DNA organization of TURF 2H3 is unique to T cytoplasm and part of the fragment is uniquely transcribed in T. The sequence contains two open reading frames that could encode proteins of 12,961 Mr and 24,675 Mr. Our evidence indicates that the TURF 2H3 sequence is a part of a 6.6 kb Xho I fragment whose alteration is associated with reversion to male fertility (Umbeck and Gengenbach, 1983). Furthermore, the processing of the TURF 2H3 message appears to differ in the presence of nuclear genes that restore cms-T to male fertility. We suggest that the TURF 2H3 sequence may be associated with the T type of male sterility because of the chimeric and unstable nature of the sequence, the novel and abundant transcripts, the open reading frames, and the apparent differential processing of its transcript by nuclear restorer genes.

Results

Identification of a Highly Transcribed mtDNA Clone in cms-T

End labeled mtRNA (oms T) was hybridized to a Bam HI maize mtDNA library from T cytoplasm to identify mtDNA clones containing sequences actively participating in mitochondrial transcription. Also, duplicate filters of the same library were hybridized to end-labeled mtRNA from normal cytoplasm. A 9.0 kb Bam HI fragment, designated TURF 2B, was identified that hybridized more intensely to mtRNA from T cytoplasm than to mtRNA from normal. Hybridization of end-labeled mtRNA (cms-T) to restriction digests of TURF 2B revealed that significant hybridization was confined to three Hind III fragments within the 9.0 kb TURF 2B clone. The Hind III fragments were found to be in consecutive alignment (Figure 1) and the complete
Figure 1. Restriction Map of TURF 2H3

Open reading frames are indicated by boxed regions. Arrows below the map show the direction and extent of sequence analysis from each restriction site. Clones cited in the text are designated with numbers. Restriction sites are indicated by vertical lines: a, Alu I; H, Hind III; s, Sau 3A; Sm, Sma I; T, Taq I.
of reading frames ORF 13 and ORF 25 are translated according to the mitochondrial genetic code of higher plants (Fox and Leaver, 1961), beginning at the first ATG codon.

Analysis of TURF 2H3 Nucleotide Sequence

Significant sequence homology was observed between the 5’ end of TURF 2H3 and the 5’ flanking region of the maize ATPase subunit 6 gene. ORF 2H3 and ORF 25 are translated according to the mitochondrial genetic code of higher plants (Fox and Leaver, 1961), beginning at the first ATG codon.

Recombination in Maize mtDNA of ems-T

ORF 25 is expressed at a lower level than ORF 2H3 and ORF 25 is expressed at a lower level than ORF 2H3. The predicted amino acid sequence of ORF 2H3 is as published (Dale et al., 1964; Kato et al., 1965). Points of recombination are designated with arrows. The predicted amino acid sequences of the 26S ribosomal RNA-Arg genes are as published (Dale et al., 1964; Kato et al., 1965). Points of recombination are designated with arrows. The predicted amino acid sequences of the 26S ribosomal RNA-Arg genes are as published (Dale et al., 1964; Kato et al., 1965).

Tobacco chloroplast tRNA-Arg

mitochondrial genome in cms-T. In Bam HI digests of T mtDNA, both the 9.0 kb TURF 2H fragment and the 6.5 kb fragment that contains atp 6 (Jewey et al., 1985b) hybridize strongly to probes interior to the repeat, whereas in digests of normal only the atp 6 fragment is detected (Figure 3). Similarly, cms-S and cms-C genomes contain only one copy of this DNA segment (data not shown). The extent of the repeat is unknown beyond the sequenced region in cms-T.

The nucleotide sequence of TURF 2H3 was compared with the corresponding E. coli sequence in this region (Dale et al., 1964). TURF 2H3 matches 44 of 53 bp (83%) with the corresponding E. coli sequence in this region (data not shown).

Computer searches of the N.I.H. GenBank and E.M.B.L. gene libraries also revealed significant homologies between a fragment of TURF 2H3 and the chloroplast tRNA-Arg genes of tobacco, Spirodela oligorhiza, and Euglena gracilis (Kato et al., 1965; Keus et al., 1984; Orozco and Hallick, 1982). The homology extends from positions 713 to 812 of tobacco, corresponding with positions 2260 to 2363 of the TURF 2H3 sequence (Figure 2). This region of TURF 2H3 is homologous with 37 bp of the tobacco tRNA-Arg 5' flank, along with all of the coding region except the last 10 bp. A homology of 90% is detected between these segments of DNA. Five mismatches in this region result from a short duplication of the sequence AGCTC in TURF 2H3 at position 2309. The homologies between the S. oligorhiza and E. gracilis tRNA-Arg genes and TURF 2H3 are 89% and 83%, respectively. However, no homology is found in the 5' flanking sequence of the E. gracilis gene as in the tobacco and S. oligorhiza genes. The homology at the 3' end with the tobacco, S. oligorhiza and E. gracilis genes ends in the stem of the T loop. The maize sequence is therefore unable to complete pairing in the T stem and the aminoacyl stem (Figure 5). The significant homology with chloroplast tRNAs from various species suggests that this portion of TURF 2H3 originated from the maize chloroplast genome. TURF 2H3 subclones containing the RNA-like region hybridize to the same 12.7 kb Eco RI maize chloroplast fragment on which Selden et al. (1985) have mapped a tRNA-Arg (data not shown).

Since the nucleotide sequence of this maize chloroplast tRNA-Arg has not been determined, it is not possible for us to compare the maize chloroplast tRNA-Arg sequence directly with the homologous sequences of TURF 2H3. Nevertheless, due to its probable chloroplast origin and inability to complete pairing, the tRNA-Arg found in TURF 2H3 must be regarded as a pseudogene.

The extensive sequence homologies between TURF 2H3 and the 5' flank of atp 6, various portions of the flanking and coding regions of the 26S ribosomal gene, and a chloroplast tRNA gene indicate that several recombination events have occurred to form this fragment. Assuming the TURF 2H3 sequence arose via recombination, a minimum of seven recombination points (positions 1146, 1160, 1478, 1506, 1565, 2259, and 2364 of Figure 2) are identified.

Transcript Analysis of TURF 2H3

When the 9.0 kb TURF 2B clone was hybridized to Northern blots of maize mtRNA from cms-C, normal, cms-S, and cms-T, a complex pattern was observed (Figure 6A). Hybridization to cms-T mtRNA, however, was much greater than to the other three cytoplasts. Hybridization of subclones of TURF 2A in Northern blots revealed that significant transcription was limited to the area of TURF 2B contained in TURF 2H3 (data not shown). Single-stranded
M13 subclones of TURF 2H3 complementary to the sense strand from the Taq I site at position 1123 to the Sma I site at position 3137 (Figure 2) demonstrated positive hybridization to the same set of transcripts in cms-T as the 9.0 kb TURF 2B fragment. Probes outside of this region did not significantly hybridize to mtRNA blots. M13 subclones of the noncomplementary strand throughout the entire TURF 2H3 fragment showed no detectable hybridization to Northern blots (data not shown).

To study the transcripts of TURF 2H3, individual subclones of the transcribed region were hybridized to Northern blots. When clone 913T-41 (Figure 1) was hybridized to maize mtRNA blots of all four cytoplasms, transcripts were seen only in cms-T mtRNA preparations (Figure 6B). Clone 913T-41 contains the DNA sequences homologous to maize mtRNA blots of all four cytoplasms transcripts (Figure 6C). Different transcripts appeared in mtRNA preparations of omo C, normal, and omo S when a more 3′ distal clone (clone 45Sma-6, Figure 1) was used as a hybridization probe, the 26S ribosomal RNA transcript was visualized in all cytoplasms along with other minor transcripts (Figure 6C). Different transcripts appeared in mtRNA preparations of omo C, normal, and omo S when a more 3′ distal clone (clone 45Sma-6, Figure 1) was used as a probe (Figure 6D). In cms-T, the largest detectable transcript is approximately 3900 nucleotides long. Transcripts of approximately 2000 nucleotides, 1600 nucleotides, 1500 nucleotides, and 1100 nucleotides are also seen (Figure 6). The 3900 nucleotide transcript is considerably larger than the length of DNA in TURF 2H3 that hybridizes positively to Northern blots (approximately 2 kb). No other fragment within TURF 2B, however, hybridizes to the 3900 nucleotide transcript. The explanation of this discrepancy is unknown.

**Open Reading Frames of TURF 2H3**

Two large open reading frames are situated in the transcribed region of TURF 2H3 (Figure 2). The first frame, designated ORF 13, is located from positions 1161 to 1559, where a TAG stop codon occurs. According to recent studies, TAG is a termination codon in higher plant mitochondria (Schuster and Brennicke, 1965; Braun and Levings, 1985). Assuming translation begins with an AUG codon, ORF 13 could start at position 1215 (Figure 2), and encode a polypeptide 115 amino acids long with a predicted molecular weight of 12,961. A smaller polypeptide is predicted if initiation occurs at the next ATG codon at position 1326. Because the complex rearrangements of DNA from the 26S ribosomal gene and its flank occur in this region, the complete open reading frame is unique to cms-T. ORF 13 is also in the segment of TURF 2H3 that is uniquely transcribed in T cytoplasm (Figure 6B).

The second open reading frame, designated ORF 25, extends from positions 1547 to 2363, where a TAG stop codon is encountered. ORF 25 occurs in a different reading frame than ORF 13 and overlaps with ORF 13 from positions 1547 to 1559. The first ATG codon, however, is located at position 1640. Assuming translation begins with the ATG codon at position 1640 (Figure 2), ORF 25 could code for a protein 221 amino acids long with a predicted molecular weight of 24,675. The DNA sequences encoding ORF 25 hybridize to transcripts in all four cytoplasms (Figure 6D). Hybridization of ORF 25-specific subclones to Southern blots of mtDNA from bean, wheat, pea, and rice shows that homologous sequences are present in the mitochondrial genomes of these higher plants (data not shown). Interestingly, the TAG termination codon of ORF 25 is situated at the beginning of the chloroplast tRNA-Arg pseudogene (Figure 5). Computer searches of the predicted translation products of ORF 13 and ORF 25 to amino acid sequences in the NBRF protein library detected no significant homologies.

**Effect of Restorer Genes on TURF 2H3 Transcript**

Nuclear genes that countermand the cms trait in maize by restoring it to full pollen fertility are called restorers (Rf). Two dominant restorer genes, designated Rf1 and Rf2, are required to re-establish fertility in the cms-T phenotype (Duvick, 1965). That is, cms-T is restored to pollen fertility by the nuclear genotype Rf1 _ Rf2.

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**Figure 4. Sequence Homologies between TURF 2H3 and 5′ Flanking Sequences of atp 6 and the 26S Ribosomal Gene**

Sequences are numbered as described in Figure 2. A 5 bp repeat in atp 6 is boxed.

**Figure 5. PseudotRNA-Arg Gene in TURF 2H3**

Positions 2296 to 2363. Sequences nonhomologous with tobacco chloroplast tRNA-Arg gene are boxed, including a 5 bp repeat in TURF 2H3. Stop codon of reading frame ORF 25 is indicated by bracket.
Figure 6. Northern Blot Analysis of TURF 2B
Approximate sizes are indicated in kilonucleotides. Each lane contains equal amounts of RNA from cms-C, normal (N), cms-Vg (S), or cms-T mt preparations. Cms-Vg cytoplasm is a member of the S group of male-sterile cytoplasms. The following clones were used as hybridization probes: A, 9.0 kb TURF 2B clone; B, 913T-41 (Figure 1); C, 913S-50 (Figure 1); D, 45Sma-6 (Figure 1).

... but is not restored by Rf1, Rf2, Rf7, Rf2, or Rf1 Rf2 Rf2. To determine the effects of restorer genes on TURF 2H3 transcripts, we have examined TURF 2H3 transcripts in restored (fertile) and nonrestored (sterile) lines of cms-T. Portions of TURF 2H3 were hybridized to Northern blots of total mtRNA from the male-sterile line, B37 cms-T (sterile) and the fertility-restored line, B37 cms-T (Rf1 Rf1 Rf2 Rf2) (Figure 7). The lines were grown simultaneously and mtRNAs were isolated under identical conditions. Two major differences were detected in the RNA banding patterns between the two lines. Clone 913T-41, containing most of ORF 13 (Figure 1), hybridized to a 1600 nucleotide mtRNA species in the cms-T (restored) but not in the cms-T (sterile) mitochondria. In contrast, 913T-41 hybridized to a 1500 nucleotide RNA species of cms-T (sterile) but not in cms-T (restored). In addition, a unique RNA species approximately 600 nucleotides long was detected in cms-T (restored) mtRNA preparations that was absent in cms-T (sterile). Hybridization of ORF 25 specific probe also distinguished the 1500 nucleotide RNA in cms-T (sterile) and the 1600 nucleotide species in cms-T (restored), yet did not appear to hybridize to the unique 600 nucleotide RNA species found in the cms-T (restored) (Figure 7B). Similarly, clones containing maize mitochondrial genes atp 9 (Dewey et al., 1965a) and atp 6 (Dewey et al., 1965b) were hybridized to mtRNA blots of restored and sterile cms-T. Unlike TURF 2H3, no alterations were detected in the transcripts of these mitochondrial genes when in a nuclear genotype containing Rf1 and Rf2 (data not shown).

To further characterize the differences in the transcripts of TURF 2H3 between restored and sterile cms-T mitochondria, two distinct oligonucleotides complementary to the message were prepared and hybridized to Northern blots of total mtRNA. A 17 base long oligonucleotide, complementary to positions 1400 to 1416 of TURF 2H3 and located in the middle of ORF 13, gave the same hybridization pattern as clone 913T-41 (data not shown). A 21 base long oligonucleotide, complementary to positions 1235 to 1255, located near the 5' end of ORF 13 and 161 bp 5' of the 17 base long oligonucleotide, was also hybridized to mtRNA blots. This 21 base long oligonucleotide hybridized to the same set of transcripts in cms-T (restored) as the 17 base long oligonucleotide and clone 913T-41, yet failed to hybridize to the 1500 nucleotide RNA species in cms-T (sterile) preparations (Figure 7C). Similar results were observed using the maize cross B73 × Ky21 (cms-T) Rf1 Rf2 Rf2 as a source of restored cytoplasm (data not shown). These data indicate that TURF 2H3 transcripts are altered by the same restorer genes (Rf1 and Rf2), which are able to suppress the male-sterility trait of cms-T.
Recombination in Maize mtDNA of cms-T

A

\[
\begin{array}{ccc}
3.9 & 2.0 & 1.8 \\
1.6 & 1.5 & 0.6
\end{array}
\]

B

\[
\begin{array}{ccc}
3.9 & 2.0 & 1.8 \\
1.6 & 1.5 & 0.6
\end{array}
\]

C

\[
\begin{array}{ccc}
3.9 & 2.0 & 1.8 \\
1.6 & 1.5 & 0.6
\end{array}
\]

Discussion

The complexity of plant mitochondrial genomes has been demonstrated in numerous studies. These studies have described rearrangements involving intramolecular recombination within the mitochondrial genome and intermolecular recombination between the mitochondrial genome and plasmid-like DNAs, and between the chloroplast and mitochondrial genomes (Lonsdale et al., 1984; Schardl et al., 1984; Stern and Palmer, 1984). For the most part, hybridization techniques have been used to delineate these rearrangements. In this study we characterized, by nucleotide sequence analysis, a fragment of maize mtDNA from T cytoplasm that originated from intramolecular recombinations as well as intermolecular recombinations between the chloroplast and mitochondrial genomes.

Substantial sequence homologies between various regions of TURF 2H3 and portions of flanking and/or coding sequences of the 26S ribosomal gene, atp 6, and chloroplast tRNA gene indicate that multiple recombinational events have occurred to form this fragment. In fact, at least seven points of recombination are indicated in the fragment. The first site of recombination (position 1146, Figure 2) may be due to the duplication of a large segment of DNA 3' of the atp 6 gene, creating the repeat unique to cms-T (Figure 3). Since this region is repeated only in cms-T and not in normal, cms-C, or cms-S, we favor a duplication rather than a deletion to explain this rearrangement.

Rearrangements involving the 26S ribosomal gene and the chloroplast tRNA gene have most likely arisen either by nonhomologous recombination or by homologous recombination involving small areas of nucleotide homology. We cannot discriminate between these alternatives with our data. Because of the large amount of chloroplast DNA found in the mitochondria of maize, it is possible that the chloroplast tRNA sequence in TURF 2H3 came about indirectly from the chloroplast genome via intramolecular recombination between chloroplast-like sequences already present in the mitochondrial genome, rather than through a direct intermolecular recombinational event. Although several instances of intermolecular recombination between chloroplast and mitochondrial genomes have been described in maize, the molecular mechanism is not yet determined.

The maize mitochondrial genome of normal cytoplasm contains six major sequence reiterations, approximately 1 kb, 2 kb, 3 kb, 10 kb, 12 kb, and 14 kb in length (Lonsdale et al., 1984). Each repeat is found twice within the master genomic circle and all except the 10 kb repeat are implicated in the formation of smaller subgenomic circles. We have identified and partially sequenced a large repeat unique to the T cytoplasm (Figure 3). The presence of a unique repeat suggests that a novel set of subgenomic cir-
Figure 6. Hybridization of Clones

(A) Hybridization of clone TURF 2H3 to a Bam HI mtDNA blot of normal maize cytoplasm. Approximate sizes are indicated in kb. (B-D) Hybridization of clones 913T-41, 45Sm-a-6, and 913T-17 (Figure 1), respectively, to Bam HI mtDNA digests of normal maize.

clones could arise from recombination with this repeat in T cytoplasm. Indeed, electron microscopy studies have shown differences in the sizes and distributions of subgenomic circles between T and normal cytoplasms (Lavings et al., 1979).

Sequence comparisons of TURF 2H3 with homologous sequences from the 26S ribosomal gene and the tobacco chloroplast tRNA-Arg gene reveal three short 5 bp repeats in these regions of homology. Similar short repeats are found in the nucleotide sequence of the 18S and 5S ribosomal gene in Oenothera mitochondria and in flanking sequences of ribosomal genes and the rbcl gene of maize and barley chloroplasts (Brennicke et al., 1965; Takaiwa and Sugiura, 1962; Zurawski et al., 1964). Misalignment of sequences during replication through mispairing, formation of stem-loops, or slippage can result in short direct tandem repeats (Drake et al., 1963).

Three segments of TURF 2H3 have sequence homology with the maize 26S ribosomal gene. The first region of homology is interesting because the homologous sequences occur in the 5' flanking region of three different genes. Twenty-nine of 30 nucleotides are conserved between sequences located 5' of the 26S ribosomal gene, atp 6, and the open reading frames of TURF 2H3 (Figure 4). The location and conservation of these sequences suggests that they could play a role in the initiation and/or regulation of transcription.

Two long open reading frames, designated ORF 13 and ORF 25, are located in the transcribed region of TURF 2H3. ORF 13 could encode a polypeptide of 115 amino acids with an estimated molecular weight of 12,961. Because this open reading frame consists primarily of sequences derived from the 3' flank and an interior region of the 26S ribosomal gene, it seems unusual that it would produce a functional protein product. Moreover, since the 5' end of the transcript is not precisely mapped, it is uncertain whether transcription initiates before or after the first in-frame ATG codon shown in Figure 2. If ORF 13 is a functional reading frame, it would produce a protein product exclusive to the T cytoplasm because this region of TURF 2H3 is uniquely transcribed in cms-T.

In vitro protein synthesis studies with isolated mitochondria have revealed differences in the proteins produced by normal and T cytoplasms (Forde and Leaver, 1980). One-dimensional SDS gel electrophoresis has shown a 13,000 Mr protein product unique to T cytoplasm. In addition, a 21,000 Mr polypeptide distinguished in normal mitochondria is absent in T. The functions of these translational products have not been determined. The 13,000 Mr protein is apparently subject to nuclear regulation, since fertility restoration of the T cytoplasm with nuclear restorer genes Rf7 and 172 significantly suppresses synthesis of the 13,000 Mr product (Forde and Leaver, 1980). Leaver and coworkers have proposed that the 13,000 Mr polypeptide may play a part in the Texas type of cytoplasmic male sterility. It is interesting that the protein predicted by ORF 13 is similar in size to the 13,000 Mr polypeptide unique to cms-T discovered by Forde and Leaver (1980).

DNA and RNA hybridization studies suggest ORF 25 is an active plant mitochondrial gene. This view is supported by the fact that ORF 25 probes hybridize to mitochondrial transcripts in all four maize cytoplasms (normal, C, S, and T, Figure 6D) and that sequences homologous to ORF 25 are detectable in mtDNA digests of such diverse species as bean, pea, wheat, and rice. The latter result indicates that the reading frame is common to plant mitochondrial genomes. ORF 25 could code for a protein of 221 amino acids with a molecular weight of 24,675. Computer searches have failed to identify a similar protein among the data banks, so the function of this putative gene product is unknown. Hydropathy plots indicate that the predicted ORF 25 protein is hydrophilic; therefore, it is unlikely to be a membrane polypeptide. Interestingly, the 5' end of the reading frame is still open at the point of recom-
bination with the interior segment of the 26S gene (position 1565, Figure 2). Consequently, the reading frame of the unrecombined form of this gene in normal maize cytoplasm may extend farther 5' producing a polypeptide larger than ORF 25. Moreover, it is possible that ORF 25 may not be properly translated in TURF 2H3 if important regulatory sequences are interrupted by recombination in the 5' end of the gene. Forde and Leaver (1980) have reported a polypeptide synthesized in normal cytoplasm (approximately 21,000 Mr) that is not present in cms-T.

ORF 25 is novel in that the last 14 amino acids are coded by DNA derived from the chloroplast genome. Although chloroplast-like sequences are prevalent in the mitochondrial genome, UV-2H3 is distinct in that these incorporated chloroplast sequences are transcribed as part of a mitochondrial message. ORF 25 terminates with a TAG codon located in the 5' aminoacyl stem of the chloroplast tRNA-Arg pseudogene (Figure 5). The pseudogene location is analogous to the organization of animal mitochondrial genomes in which genes are frequently terminated by functional tRNA genes (Anderson et al., 1981). In animal mitochondria, 3'-terminal tRNA genes are thought to aid in transcriptional processing (Attardi et al., 1982).

A complex transcriptional pattern is observed when TURF 2B is hybridized to Northern blots of maize mRNA (Figure 5A). Part of the complex pattern results from cross hybridization of TURF 2B with transcripts produced by the 26S ribosomal gene. Similarly, complex mRNA hybridization patterns are reported for maize genes coding for cytochrome oxidase subunit II (Fox and Leaver, 1981), apocytochrome b (Dawson et al., 1984), ATPase subunits (Dewey et al., 1985a), and ATPase subunit 6 (Dewey et al., 1985b). Recombinations between transcribed regions of different mitochondrial genes may partially explain the complex transcriptional patterns of these and other maize mitochondrial genes.

Regeneration studies with tissue cultures of cms-T maize have discovered interesting changes in disease resistance and the cma phenotype. Reversion from male sterility to male fertility often occurs in plants regenerated from callus culture (Gengenbach et al., 1977; Brettell et al., 1979). Reversion is also invariably associated with a newly acquired resistance to Bipolaris maydis (Helminthosporium maydis), race T, and its fungal toxin (T toxin). Molecular changes are also associated with the reversion phenomenon. Significant suppression or total loss of the 13,000 Mr polypeptide unique to cms-T is reported in revertant plants (Dixon et al., 1982). At the DNA level, the disappearance of a 6.8 kb Xho I fragment in mtDNA digests is associated with reversion. In contrast, plants that maintain the male-sterile, susceptible phenotype during culture do not lose the 6.8 kb Xho I fragment. This specific alteration, presumably via rearrangements, deletions, or base substitutions leading to new restriction sites, is reported in 15 of 16 revertants examined, prompting speculation that this change may be associated with fertility restoration (Umbeck and Gengenbach, 1983). Wise et al. (1985) report that this 6.8 kb Xho I fragment is internal to a 9.0 kb Bam HI fragment and a 4.5 kb Xho I fragment in mtDNA digests of both T and normal cytoplasms. We have observed the same hybridization pattern for TURF 2B (Figure 3, Figure 9), strongly suggesting that the 6.6 kb Xho I fragment located within the 9.0 kb TURF 2B molecule is the same fragment that disappears in revertant maize plants. The entire 3.5 kb TURF 2H3 fragment is internal to a 6.6 kb Xho I digestion fragment of cms-T mtDNA. Since the chimeric sequence, TURF 2H3, has been the site of numerous previous rearrangements, it is not surprising to find it associated with yet another change in the mtDNA. In any event, it is clear that the sequence has some unusual properties making it prone to change. At the moment, the evidence linking the loss of a 6.6 kb Xho I fragment with reversion to fertility and to the possibility that this region may be responsible for the T type of male sterility is circumstantial. Nevertheless, the chimeric and unstable nature of the sequence, the novel and abundant transcripts, and the open reading frames suggest a relationship between TURF 2H3 and the male sterility trait.

Evidence linking TURF 2H3 to the cms-T phenotype is also indicated by the effect of the nuclear restorer genes Rf1 and Rf2 on the TURF 2H3 transcripts. When cms-T plants are restored to fertility by the nuclear genes Rf1 and Rf2, changes occur in the mitochondrial transcripts of TURF 2H3 (Figure 7). Nuclear genes affecting proper transcriptional processing of mitochondrial messages have been described in yeast (Pillar et al., 1983; Faye and Simon, 1984). Rf1 and Rf2, however, have no apparent effect on transcripts of the mitochondrial genes coding for ATPase subunits 6 and 9. TURF 2H3 transcripts from cms-T that have been restored to fertility with Rf1 and Rf2 are distinguished from cms-T (sterile) by the presence of transcripts approximately 1600 and 600 nucleotides long, and the absence of a 1500 nucleotide RNA species. A 21 base long oligonucleotide, complementary to the message located near the 5' end of ORF 13, hybridizes to the 1600 nucleotide RNA species in restored cms-T, but not to the 1500 nucleotide RNA species in sterile cms-T. Probes located farther 3' to the 21 base long oligonucleotide hybridize to both RNAs. These results suggest that the size difference between the 1600 and 1500 nucleotide RNAs is caused by additional 5' flanking nucleotides in the 1600 nucleotide species. In our view, the differences are most likely due to differential processing of the TURF 2H3 transcript. This proposal suggests that the gene product of either one or both of the nuclear restorer genes, Rf1 and Rf2, are involved in RNA processing, perhaps as an RNA processing enzyme. Further studies will be required to determine the specific role of Rf1 and Rf2, and the relationship between TURF 2H3 and the male sterility trait in cms-T.

Experimental Procedures

Isolation of Nucleic Acids

Mitochondrial RNA (mRNA) and mtDNA were prepared from 6 to 7 day old, dark grown seedlings of Zea mays L., as previously described (Ping and Levings, 1978; Schuster et al., 1983). The following sterile (nonrestored) maize lines or crosses were used as sources of male sterile cytoplasms: B73 x Mo17 (cma-C), B73 (cma-Wy), B73 (cma-T), and
quences were determined by the chain-termination method of Sanger.

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. (1979). MRNA was heat denatured and fractionated by electrophoresis in 1.2% agarose gel containing 0% formaldehyde and blotted to nitrocellulose as described by Thomas (1980). Double-stranded DNA was labeled with [γ-32P]dATP (NEN, 3200 Ci/mmol) by nick translation (Rigby et al., 1977). Single-stranded DNA clones in the bacteriophage M13 were labeled by the backpriming technique of Hu and Messing (1982).

Nucleic acid hybridizations were performed under conditions already described (Dewey et al., 1985a). The 18S (1986 nucleotides) and 26S (5346 nucleotides) ribosomal RNAs of maize mitochondria were used as markers for estimating RNA sizes. Hind III digest of bacteriophage lambda DNA were used as markers for estimating DNA sizes.

Oligonucleotide Synthesis and Hybridization

Oligonucleotide probes were prepared with the Applied Biosystems 380A DNA Synthesizer according to the manufacturer's instructions. Oligonucleotides were 5'-end labeled with [γ-32P]ATP (ICN, 7000 Ci/mmol) using T4 polynucleotide kinase (Maxam and Gilbert, 1960). Northern blots were prehybridized in a solution containing 6x NET (0.75 M NaCl, 75 mM Tris-HCl, pH 7.8, 1 mM EDTA), 5x Denhardt's (0.1% bovine serum albumin, 0.1% Ficol, 0.1% polyvinylpyrrolidone), 0.1% SDS, and 100 mg/ml salmon sperm DNA for 5 hr at 66°C. Labeled oligonucleotides were hybridized at 53°C in a solution containing 6x NET, 5x Denhardt's, and 0.1% SDS. Filters were washed in 6x SSC, 0.1% SDS at room temperature.

DNA Sequence Analysis

Cloning for sequence analysis was carried out using M13 bacteriophage vectors mp10 and mp11 (Messing, 1982). Ligation and transformation procedures were as outlined by New England Biolabs. DNA sequences were determined by the chain-termination method of Sanger et al. (1977) with a universal primer (PBL Biochemicals). Sequencing gels were either 6% or 8% polyacrylamide and 0.4 mm thick. Sequencing strategies are shown in Figure 1.

Nucleotide and amino acid sequence analyses were performed by computer programs furnished by Bionet. Bionet accesses the National Institutes of Health (GenBank) and European Molecular Biology Laboratories DNA sequence libraries and the National Biomedical Research Foundation protein sequence database.

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