The NCS3 mutation: genetic evidence for the expression of ribosomal protein genes in *Zea mays* mitochondria

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A deletion eliminating part of a transcribed region of mitochondrial DNA (mtDNA) has been found in the maize nonchromosomal stripe 3 (NCS3) mutant. This results in the specific loss of a set of three mitochondrial RNAs consisting, in normal plants, of a 4.9 kb transcript, its 1.8 kb intron and the resulting processed mRNA of ~2.9 kb. In the NCS3 mitochondrial genome the DNA encoding the putative promoter and 5' end of the affected RNAs is missing. This transcribed region of normal maize mtDNA has been sequenced and the intron splice junction has been determined. The 2.9 kb processed mRNA carries two overlapping open reading frames (ORFs) with predicted amino acid sequences that show similarity to two Escherichia coli ribosomal proteins, S3 (rps3) and L16 (rpl16). The presence of severe stunting and striping in NCS3 plants correlates absolutely with the molecular changes described here. This fact and the impaired ability for mitochondrial protein synthesis by NCS3 plants indicate that one or both of these reading frames are translated to functional ribosomal proteins in normal maize mitochondria.

Key words: L16/maize/mitochondria/ribosome/S3

Introduction

Although most mitochondrial proteins are encoded by nuclear genes and imported into the organelles posttranslationally, mitochondria of all species have a distinct genetic identity. Their genomes encode a number of important proteins and they contain the transcription and translation apparatus necessary for the expression of those proteins.

Because higher plant mitochondrial genomes are generally larger and more complex than those of other species, they may possess more functional genes. In *Brassica campestris*, 24 abundant transcripts covering 30% of the 218 kb genome can be detected, and a much larger percentage of the genome gives rise to less abundant transcripts (Makaroff and Palmer, 1987). Extensive transcription has also been described in *Brassica napus* (Carlson *et al.*, 1986) and cucurbit mitochondria (Stern and Newton, 1985a). Functions for many of these transcripts have not been determined. To date, most of the genes in plant mitochondria have been identified by their similarity to genes in other species. It is not yet known if the uncharacterized transcripts in plant mitochondria are in fact translated into protein products.

One approach to the identification of functional regions of a genome is to analyze mutations that cause detectable phenotypes. We are using a group of maize mitochondrial mutants to search for actively expressed genes in the plant mitochondrial genome. These mutants have been called 'nonchromosomal stripe' (NCS) mutants, reflecting both their non-Mendelian mode of inheritance and the pattern of sectoring that arises on the leaves. Each independently isolated mutant shows a unique change in the arrangement of its mitochondrial DNA (Newton and Coe, 1986; Lauer *et al.*, 1990; Newton *et al.*, 1990).

One of the NCS mutations, NCS3, produces severely stunted plants with sectors of undeveloped tissue on both the leaves and the ears. Both phenotypic and molecular evidence suggest that NCS3 plants are heteroplasmic, carrying a mixture of normal and mutant mitochondria that varies from plant to plant. Sectoring results from somatic segregation of mutant from normal mitochondria during development.

The molecular change associated with the NCS3 mutation involves a decrease in the abundance of a 16 kb *XhoI* fragment of mitochondrial DNA and the concomitant appearance of a novel 20 kb *XhoI* fragment (Newton and Coe, 1986). Although the disappearance of the normal fragment from heteroplasmic mutant tissue is not complete, there is an absolute correlation between this DNA alteration and the expression of the NCS3 mutant phenotype: NCS3 plants always exhibit the novel 20 kb fragment on agarose gels, and highly affected plants show a relatively more abundant 20 kb fragment and less of the 16 kb fragment than very slightly affected plants (Newton and Coe, 1986).

We report here that this restriction fragment change, associated with the NCS3 phenotype, is the result of a deletion in the mitochondrial genome that has eliminated part of a transcribed region of DNA. This region contains two overlapping open reading frames that show similarity to the *Escherichia coli* and chloroplast S3 and L16 ribosomal proteins, two genes not found in the mitochondrial genomes of animals and fungi. A loss of ribosome function would be expected with the disruption of such genes, and our observation that NCS3 mutant mitochondria apparently fail to synthesize protein is consistent with this expectation.

Results

The NCS3 DNA rearrangement has caused a deletion in the mitochondrial genome

The NCS3 mutation arose in a maize line carrying CMS-T male-sterile mitochondria (Coe, 1983). In CMS-T mitochondria, the 16 kb *XhoI* fragment that is affected by the NCS3 mutation lies between map positions 171 and 187 on the map of the 540 kb chromosome (Fauron *et al.*, 1989).



Fig. 1. Origin of the 20 kb XhoI fragment found in NCS3. (A) Partial restriction maps of the normal 14 and 16 kb XhoI fragments aligned with the 20 kb XhoI fragment that appears in NCS3 mutants. XhoI-digested NCS3 and normal mtDNA samples were probed with subfragments of pMX5-7 and pMX5-30 on a series of identical Southern blots. Stippled boxes on the restriction maps represent probes which showed homology to the 20 kb XhoI fragment in NCS3. Probes represented by diagonally hatched boxes did not (see panel B). The open box (no fill) shows the location of pSB1 on the 20 kb XhoI fragment. Only relevant restriction enzyme sites are shown. Abbreviations for these are: B, BamHI; C, HincII; H, HindIII; N, NdeI; P, PvuII; R, EcoRI; S, SmaI; T, StyI; X, XhoI. The region bounded by a broken line represents the end of an 11 kb repeated region on the CMS-T genome. The dotted arrow indicates the NCS3 junction point on the three XhoI fragments shown here. (B) Southern blots of NCS3 (3) and normal (n) mtDNA resulting from hybridization with probes 1-7 (see panel A). Fragment sizes are designated to the left of the blots.

This fragment has been cloned as plasmid pMX5-7. The 20 kb XhoI fragment, unique to the NCS3 mutant genome, shares homology with, and is partly composed of, sequences from the 16 kb fragment. In order to identify other sequences that are included in the novel restriction fragment, a portion of the 20 kb XhoI fragment (clone pSB1) was used as a hybridization probe on Southern blots of XhoI-digested mtDNA. In addition to the expected 16 kb fragment, a 14 kb XhoI fragment (subsequently cloned as pMX5-30) was identified. This 14 kb fragment maps at the zero point (from co-ordinate 536 to 10) of the CMS-T map (Fauron et al., 1989). Those portions of the two progenitor fragments that give rise to the new 20 kb XhoI fragment are shown in Figure 1. As illustrated in the figure, only sequences from the left portion of pMX5-30 and from the right side of pMX5-7 contribute to the formation of the 20 kb fragment.

Because part of the 16 kb fragment lies within the large 11 kb repeated region of CMS-T (Fauron *et al.*, 1989), it shares homology with a 13 kb *Xho*I fragment that includes part of the other 11 kb repeat. The 13 kb fragment (located between map positions 352 and 365) is unchanged by the NCS3 rearrangement. A probe from the left end of pMX5-7 in Figure 1 (probe 4) shows homology to this 13 kb fragment in both mutant and non-mutant samples, as expected (Figure 1B). However, hybridization with this probe shows that the abundance of the 16 kb band is reduced in the NCS3 sample only. In addition, a 0.9 kb *SmaI-StyI* subfragment of pMX5-7, which lies outside the 11 kb repeated region, but to the left of the mutation junction (probe 5 in Figure 1), shows no homology to any region of the mutant genome

(Figure 1B). The fact that these probes detect no newlyformed fragments in NCS3 mtDNA indicates that the formation of the 20 kb mutant fragment, linking sequences present on 14 and 16 kb *XhoI* fragments in normal DNA, has been accompanied by a deletion. This deletion is bounded on one side by the NCS3 mutation junction within the 16 kb *XhoI* fragment, and it extends at least to the *XhoI* site at the left end of that fragment in Figure 1.

Although no homology between the 14 and 16 kb fragments has been detected by Southern blot analysis, such experiments do not reveal short regions of homology that could serve as recombination sites. The composition of the 20 kb fragment suggests that recombination between the two progenitor *XhoI* fragments has played a role in the generation of the mutation. To investigate this, the junction regions from pMX5-7, pMX5-30 and pSB1 were sequenced, revealing a 12 bp reiterated sequence that lies precisely at the mutation junction in all three fragments (Figure 2).

The entire sequence of events giving rise to the NCS3 mutant genome is not yet known. Although the 16 kb fragment essentially disappears from NCS3 DNA, the 14 kb fragment is not markedly reduced in abundance (see Figure 1). It appears, therefore, that a simple reciprocal recombination event cannot entirely account for the generation of the NCS3 genome.

In a search for other rearrangements or deleted regions in NCS3, mtDNAs from mutant and normal plants were probed sequentially with nick-translated cosmids spanning the entire CMS-T genome (described by Fauron *et al.*, 1990). No other changes in the NCS3 genome were found

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TTACACGGCTCACAAACTCTGC CTGGGGTGGGGC TACCTATTATTCGTAGGCGGTAC ..pMX5-30
TTACACGGCTCACAAACTCTGC CTGGGGTGGGGC GTTTACTTGAAAATGACAACCGC ..pSB1
CCAATAGAGCGAGGGCGTCCTT CTGGGGTGGGGC GTTTACTTGAAAATGACAACCGC ..pMX5-7
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Fig. 2. DNA sequences at the junction regions of pSB1 and the two progenitor fragments, pMX5-30 and pMX5-7. The 12 base sequence at the NCS3 junction is underlined.

(C.M.R.Fauron and M.D.Hunt, unpublished data). The only unique sequence eliminated from the NCS3 genome appears to be a region of ~ 1 kb (represented by probe 5 in Figure 1). Although the deletion extends into the adjacent 11 kb repeat, all repeated sequences and the unique sequences on the other side of the 11 kb repeat are still present in some context in the mutant genome (Hunt, 1990). A more precise analysis of the rearrangement will require the construction of a map of the complete NCS3 mitochondrial genome but, taken together, the Southern analyses strongly suggest that it is the loss of sequences from the 16 kb region that is associated with the mutant phenotype.

Transcript analysis

The discovery of a deletion in the NCS3 mitochondrial genome led to a search for transcribed sequences in this region of normal mtDNA. In general, transcription in NCS3 mutants appears unaltered. Northern blot hybridizations, using previously characterized and cloned mitochondrial genes as probes, indicated that transcripts from these genes were not affected by the NCS3 mutation (Hunt, 1990).

The same is true of the portion of the 14 kb XhoI fragment that is involved in the NCS3 recombination. This region of the genome is transcribed in both mutant and normal plants and there is little difference between the transcript patterns of the two types (Figure 3). This result is consistent with the fact that an intact copy of the 14 kb DNA fragment is still present in NCS3 plants (Figure 1).

In contrast, a portion of the 16 kb progenitor fragment that spans the mutation junction identifies a specific set of three transcripts, with sizes of ~4.9, 2.9 and 1.8 kb, that are severely reduced in abundance in NCS3 plants (Figure 3). Because the mutant plants retain some normal mitochondria, an over-exposure of panel 16 in Figure 3 reveals small amounts of these three RNA species (not shown). Two other bands (~2.3 kb and 3.5 kb) can often be seen on Northern blots hybridized with probes from this region, particularly if the stringency of hybridization is lowered. The source of these signals is not known, but on a given blot, mutant and normal samples are similar in appearance, indicating that these transcripts are expressed equally in mutant and normal tissue and that they are not affected by the NCS3 mutation.

Neither these transcripts nor the NCS3-affected transcripts arise from within the 13 kb *XhoI* fragment that overlaps the other 11 kb repeat. No stable transcripts of any size hybridize with probes from the unique (i.e. non-repeated) portion of the 13 kb fragment (Figure 3).

A more detailed examination of the transcribed region within the 16 kb *XhoI* fragment of normal mtDNA (Figure 4) shows the location and orientation of the three NCS3-affected RNAs. The use of several small DNA probes (Figure 4B) indicates that the 4.9 kb transcript hybridizes only to probes from within a 5.3 kb region bounded by the *PstI* site and an *Eco*RI site shown in Figure 4A. The same region also encodes the two smaller NCS3-affected RNAs. These are the only transcripts detected from within the



Fig. 3. Identical Northern blots of NCS3 (3) and normal (n) RNAs hybridized with ³²P-labeled DNA from regions associated with the NCS3 DNA rearrangement. The probes used in each panel are listed below. 14: A 0.9 kb *HindIII*-*HincII* fragment spanning the mutation breakpoint on the 14 kb XhoI fragment referred to in the text. See Figure 1 for the location of the *HindIII* and *HincII* sites. 16: A 4 kb KpnI-*Eco*RI fragment from normal DNA that crosses the mutation junction on the 16 kb XhoI fragment. See Figure 4A for the location of the *KpnI* and *Eco*RI sites. 13: A 2.3 kb XhoI-*HindIII* probe from pMX5-32 (the 13 kb XhoI fragment referred to in the text) that lies just outside one of the 11 kb repeats. rrn18: The 18S ribosomal RNA from maize mitochondria (Maloney and Walbot, 1990). The blot in panel '16' was stripped and reprobed with *rrn18* to show equal loading in the two lanes.

boundaries of the 16 kb fragment (data not shown) and they are transcribed from the same strand of DNA (Figure 4C). The relative positions of the transcripts suggest that the 1.8 kb RNA is excised as an intron from the 4.9 kb transcript.

Genomic and cDNA sequence analysis

Figure 5 shows the DNA sequence between the *PstI* and *EcoRI* sites that serve as approximate boundaries of this transcribed region in normal maize mitochondria. That the 1.8 kb RNA does, in fact, represent an intervening sequence was verified by PCR amplification and cloning of cDNA fragments from the region of mRNA between primers a and h in Figure 4. Two clones derived from the processed RNA (287 bp) were sequenced to determine the precise location of the splice junction. This cDNA sequence is aligned with the genome sequence in Figure 5.

Each of the two independently isolated cDNA clones that were selected for sequencing showed specific deviations from the corresponding genome sequence. At two points (nucleotides 64 and 1935 in Figure 5) both cDNAs have a thymine substituted for a cytosine found in the DNA sequence. At a third point (nucleotide 69), one of the cDNAs has a thymine while the other retains the cytosine found at this position on the DNA. These changes conform to the recently reported pattern of RNA editing in plant mitochondria (Covello and Gray, 1989; Gualberto *et al.*, 1989; Heisel *et al.*, 1989; Schuster *et al.*, 1990).



Fig. 4. The location and orientation, in normal mitochondria, of the NCS3-depleted transcripts. (A) A restriction map of pMX5-7 is shown with the transcribed region enlarged below it. Stippled regions indicate the part of this region that is within the CMS-T 11 kb repeat. The segment of DNA that has been deleted in NCS3 and an interpretation of the location of each of the transcripts are indicated above the map. Abbreviations for restriction enzyme sites are: B, *Bam*HI; H, *Hind*III; C, *Hinc*II; K, *Kpn*I; V, *Pvu*II; P, *Pst*I; R, *Eco*RI; S, *Sal*I; M, *Sma*I; T, *Sty*I. (B) DNA restriction fragments (numbered 1-10 and drawn below the map in panel A) were used as probes in a 'Northern walk' along the transcribed region of pMX5-7. Only normal mtRNA was used in these Northern blots. (C) Eight custom-synthesized oligonucleotides from the transcribed region of pMX5-7 were end-labeled and used as probes on blots containing normal RNA. The locations and orientations of each of these primers are shown as arrowheads on the map in panel A.

The 1843 bp intervening sequence contains no extensive open reading frames. Its 3' region (beginning with base 1788 in Figure 5) has a primary and potential secondary structure that is consistent with features of domains 5 and 6 of group II introns (Michel and Dujon, 1983).

The 12 bp repeated sequence that lies at the NCS3 recombination junction (Figure 2) is found within the intron (see Figure 5). The sequence that precedes the recombination junction in Figure 5 is part of the region deleted from NCS3 DNA. This means that DNA encoding the 5' flanking sequence, the first exon and part of the intron of this transcript has been lost from the mutant genome.

Coding potential of the 2.9 kb RNA

With the excision of the intron from the primary transcript, a long open reading frame (ORF1) is formed. It begins 74 bp upstream from the intron junction and continues through the splice site for another 1603 bp. If this ORF were to be translated from the first methionine codon, it would produce a 64.5 kd protein. Further sequencing revealed a second 561 bp open reading frame (ORF2) that overlaps (by 149 bp) the end of ORF1 and could encode a protein of ~ 21 kd.

The NBRF-PIR protein database was searched for polypeptides showing similarity to the deduced amino acid sequences of ORF1 and ORF2. Although a translation of the first portion of ORF1 bears no resemblance to anything in the database, the amino acid sequence derived from the last 702 nucleotides of this reading frame (beginning with

1048

base 2819 in Figure 5, or amino acid 326 in Figure 6) is similar to the S3 ribosomal proteins of *E. coli* and higher plant chloroplasts (Figure 6A). The deduced amino acid sequence of this portion of ORF1 shows 27% identity with the *E. coli* S3 sequence and includes one absolutely conserved block (amino acids 494-506). When conservative amino acid changes are taken into account, the overall similarity rises to 57%.

A more striking resemblance is seen between the ORF2 amino acid sequence and the L16 ribosomal protein of *E. coli* or chloroplasts (Figure 6B) with 50% identity between the *E. coli* and maize mitochondrial genes. Another 20% of the ORF2 sequence is composed of amino acids that have undergone conservative changes. ORF2 begins 149 bp before the stop codon of ORF1, but similarity to *E. coli* L16 begins at a valine (GTG) codon at position 3495, 26 bases upstream from the ORF1 stop codons (Figure 5).

Protein synthesis by NCS3 mitochondria

The synthesis of proteins by mitochondria of NCS3 and non-mutant plants were compared by [³⁵S]methionine labeling of nascent polypeptides in isolated mitochondria (Leaver *et al.*, 1983). Figure 7 shows an electrophoretic profile of total mitochondrial protein and labeled proteins from normal and highly affected NCS3 plants. The vast majority of mitochondrial proteins are products of nuclear genes and are synthesized outside the organelle. Total mitochondrial protein includes these, predominantly, as well

CTCTTTTGA <u>CTGCAG</u>	ACCCGTAGGAGGAAG	GTGGAGTTAGTTAGA	TGAATTGCACCCGAC	ATGGCTCGATGCGTC	- 5 9 3		
GCAGCACGCCATCCG	TGGAAGAACACACAT	GAATAGAATAGGCCT	CCATACTTGCCCAGC	CCACAGCAAGCATCA	-518	TTCCTGATAGAAAAT GACGACTCCTTCATA AAAACAAAGTTATTC AAGTTCTTTTTTA CCAAAGAAGIUCCCCC 2	2982
GTGGTGGTCACAAAA	CCTGCACATTGTAAT	TCGATCATAATGTGA	GTGCTTCCCCAGAGG	CAGAAGCTGAGTCTA	-443	FLIEN DDSFI KTKLF KFFFL PKKSK	
CAAAAAGGTGGGAGC	ATAAAATGTAGGTGA	GTCAATTGCGTGTGG	CCTTCAAGTATTTCG	TATTGTAACAATATT	-368	TCTGACGGCCCGACG AGTCATCTACTAAAA AGGACCCTCCCCGCGCT GTGCGCCCCTTCG AATTATTCGGTCATG 2	2557
CAATCGGCATCCAAA	CAAAGGTGCATGTAC	GGTTCCTAAGGGATA	CAATTTTTGTCCTAAT	CAACCAAACAAGCAA	-293	S D G P T S H L L K R T L P A V R P S L N Y S V M	
CGGATTGAGCAACTA	CGCTAGGCGCATCCG	TTTTCTTGCTTCATC	GAGAAAGATTAAATA	AGTTGATAGCGCGAT	-218	CAATACTTTTTTAAT ACAAAGAACAAAATG CATTTCGACCCCGTC GTAGTTCTCAATCAT TTCGTGGCACCGGGT 2	2632
CTCGTACTAACACAT	ACTCTCTAAATATTG	AAGAACTTGCATGCG	GCCACAACCGCGGTA	TGAGTTCTGATCTCA	-143	QYFFN TKNKM HFDPV VVLNH FVAPG	
GACTTTGTTTGGGGC	TGCTTGCCCCTTCGC	GTCGACAAGGAAACT	GTGGACGACAATGGG	TTTAGAATTAGAATA	- 6.8	GTGGCTGAACCATCT ACGATGGGGGGGGGGGGGGGGGGG	2707
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GRARAGGRARTCORR	TTTCCCTAACACTTC		CAGATCCAACTCCCT	TCAGTGAGGGGACC	8.2	ANDLR FAGTT KTTIS LFPFF GATFF	
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						PTYCPNTMCKDKVMELTEKFIDLGR	
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AAAAGAGACCTTTTT	CGCTCGGNNCCGCAC	TATGCTCCGCTTCAA	CAAATGAGAGTTTTTC	GGTGGAACCGGTGAA	607	SVKIKSVYQSASLIAQDISFQUKARA	2207
CCACGCGAGCTGGTT	AGATGCGTGGGGGCAG	AGGGCTCGTAGTACC	TGATAGCGCAGCTAT	GCAGTGGAAGGGAAG	682	CCAATCTCATTTCGT TCCATTTTTTAGTAAA ATAGTTAAGGATATT CCATTAATAATGCCA AAAGGGGTGGAGGGGG	3307
GAGCCTAAATCGACC		TATTCAAAGACATAA	AAGCACAGTACAAAG	AGATTGGACTCTCGG	757	PISFRSIFSKIVKDIPLIMPKGVEG	2200
ATGAGACCTTGCAGC	TACCGTTCCGACGCG	CCCCTGACCCTATCT	TGATTAAAAAAAACC	GAAAACCCTCTCTAA	832	ATACGTATTTGTTGT TCTGGTCGATTAGGG GGTGCGGAAATAGCT AGAACTGAATGCGGA AAGTATGGAAAAACA	3382
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CTCGCTCAGTAAAGA	GAGTTGTAGATTCGT	AGAAGAGGATCAGAG	TACGCGCGCTACAAA	AGGCAGCTAGCCAAT	982	ORF2 M E K H	
GAAAGTAAGTGGAAA	GAATATAGTACTTTT	GTACTGACCCCTCCG	GAGCCCCCGGTTGTT	TTGGCGCGCCCTACC		TCTTGTAATGTATTT AACCAGAAAATCGAT TATGCTCCTGCGGAA GTATCTACTCGTGAC GGAATTTCAGGTGTC	3457
CCAACGTTAGACTAT	TGCCTTTTTAGCCTA	CGCTTGCTGCTTGCA	GCATGGATTCGATAG	ATCTATCGAATCCAT	1132	S C N V F N Q K I D Y A P A E V S T R D G I S G V	
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CTTALCOTALCOTA	TCACCACCACCOTTC	CTRCTTRATCRCCCCCC	COLORADOTOCOL	A GTA TOCOCCA A A CO	1730	RAGRL SYRAI EAARR ATIGQ FRRAM	
ATCAAGCCCTTTTAC	TERCORCOROCOTTO	COCHTCCACCACACA	TTCCCTTCCA	CTCTC & TCCCCTCA CT	1007	GAGCGGACAATTCCG AAGAAATTGTAAGAT ATGGGTAAGAGTTCT CGCAGATCTTCCTAT TACGGGGAAACCCCCC	3757
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GTEEE	RNEVR	GRGAG	KRVES	IDREK		GIGUTATTIGIAUTA TIATITTAGIAGUCA LUGUCLAUGIGUADA GATTAAUGUGUGAAC AGACUTCACUTUGIA	4507
CAAAACGAAATCAGG	ATTTGGCCGAAAAAG	ATGCAACGTTATGGA	TACCATGACCGTACA	CCATCGAGAAAGAAG	2332	AACGACGAGGGGAAG ATGAAATTCHTGACT TACCCCAAAGGGGAA TTTGGAAACGGGGGA GTCATCATCCTCCGG	45.00
QNEIR	I W P K K	MQRYG	YHDRT	PSRKK		TCAACCEGGAAGCCC CACAAATCGAGGGG TCECGGAAGCCCCTC AACTAGAGGCGGGGG CTCCAGTGGCTTCCC	4582
AATTTTTTTCTAAATCA	CTTCGGGTGAGCGGG	GCCTTCAAGCATCCG	AAATACGCCGGGGTT	GTAAATGACATAGCC	2407	CCGTCCAAGTAGAAG AAGTGAAGCAAGAAG GACACCAGAAATGCA GGGACATAATTG <u>GAA TTC</u>	4645
NESKS	LRVSR	AFKHP	KYAGV	VNDTA		ECORI	

Fig. 5. The DNA sequence of the transcribed region affected by NCS3. The *PstI* and *Eco*RI sites near either end of the transcribed region are indicated. Two potential protein sequences from the open reading frames, ORF1 and ORF2 (beginning at nucleotide positions 1 and 3372, respectively), are aligned with the DNA sequence. The sequence of a cDNA fragment, beginning at an underlined *Sal1* site, is shown below the corresponding genomic DNA sequence. Asterisks indicate agreement between cDNA and genomic sequences. At points where the cDNA differs from the genomic DNA, the appropriate letters are substituted (Y = either cytosine or thymine). Ambiguous bases in non-coding portions of the sequence are designated by N (= any base) or S (= C or G). The twelve-base NCS3 recombination site in the intron is shaded. The G residue at position 263 (bold face and underlined) is the last base within the 11 kb repeated region.

as proteins made within the mitochondria. On Coomassiestained gels, there is no visible difference between the protein complements of NCS3 and normal mitochondria (Figure 7A). When they are compared with their normal counterparts, however, the mutants consistently show a marked reduction in the amount of label incorporated into mitochondrially synthesized proteins (Figure 7B).

A prolonged exposure of the fluorograms of these gels (Figure 7C), to allow viewing of labeled proteins in the mutant lanes, reveals no specific alteration in any mitochondrially synthesized protein in NCS3 tissue. The difference lies in the overall amount of protein produced within the organelles. Since even severely affected NCS3 plants are heteroplasmic and, therefore, carry a mixture of mitochondrial types, we attribute the low level of protein synthesis in the mutants to the residual normal mitochondria. A genetic lesion affecting one or more ribosomal protein genes might be expected to prevent the formation of functioning ribosomes. The reduction in protein synthesis that we observe in NCS3 is a result consistent with that expectation.

Discussion

Short, directly repeated sequences have been implicated in the generation of deletions in circular genomes of other species (deZamaroczy *et al.*, 1983; Manna and Brennicke, 1986; Holt *et al.*, 1988; Johns *et al.*, 1989; Shoffner *et al.*, 1989; Zeviani *et al.*, 1989). They also appear to be important in the generation of NCS mutations in maize mitochondria.

In the case of NCS3, the progenitor 14 and 16 kb XhoI fragments are separated by a great distance (>100 kb)on the published map of the CMS-T genome, making a slipped-mispairing deletion mechanism (Efstratiadis et al., 1980) improbable. Since homologous recombination is very active in plant mitochondria, it seems more likely that intramolecular recombination between short reiterated sequences has led to the deletion of a region of the CMS-T mitochondrial genome. The mutation event cannot have been a single reciprocal recombination event, because one of the parental fragments and only one of the reciprocal recombination products are recovered in the mutant genome. This situation is consistent with a model proposed by Small et al. (1989) in which two separate subgenomic circular molecules can reintegrate to form a new 'master genome' that contains a large duplication and a small deletion relative to the progenitor genome. This mechanism has recently been demonstrated in the reversion of a CMS-T tissue culture line to fertility (Fauron et al., 1990).

The NCS3 deletion eliminates DNA encoding the promoter region, first exon and a portion of the intron of a normally abundant transcript. The 5' end of this transcript lies within one of the 11 kb repeats in CMS-T mitochondria. (Although these sequences, including the putative promoter, are present on the other copy of the 11 kb repeat, they do not give rise to stable transcripts in that context.) The

Α.	<u>S3 SEQUENCE RONOLOGIES</u>	
OPEI		50
ORFI	XTFGFRIGRCITLHFPKRTFIHFFLPRRPLRLKRRDKSRPGKOKGRWWAF	100
ORF1	GKVGP IGCLHSSEGTEEERNEVRGRGAGKRVESIDREKONEIRIWPKKMO	150
ORF1	RYGYHDRTPSRKKNFSKSLRVSXAFKHPKYAGVVNDIAFLIENDDSFIKT	200
ORF1	KLFKFFFLPKKSRSDGPTSHLLKRTLPAVRPSLNYSVMQYFFNTKNKMHF	250
ORF1	DPVVVLNHFVAPGVAEPSTMGGAKGGSLDKRIRSRIAFFVESSTSDKKCL	300
E. coli	MGQKVHPNGIRLGIVKPWNSTWFAN G * * * *	25
ORF1	ARAKKRLIHFIRQANDLRFAGTTKTTISLFPFFGATFFFSRDGVGVYNNP	350
	I**F * R **	
М. р.	MGQKINPLGFRLGITQNHRSYWFANKKY	28
E. coli	TKEFA-DNLDSDFKVRQYLTKELAKASVSRIV-IERPAKSIR-VTI	68
	E*A **L ***** R* * K* * *** * R *K I* * I	
ORF1	FYEYAREQLLGQLRIKCRNLMGKDKVMELIEKFIDLGRIGKLIKGIEMMI * ** * * * * K* *** R LI* *E*	400
М. р.	SKVFEEDKKIRDCIELYVQKHIKNSSNYGGIARVEIKRKTDLIQ-VEIYT	77
E. coli	HTA-R-PGIVIGKKGEDVEKLRKVVADIAGVPAQINIAEVRKPELDAK * R I G ** **V*K*R *** * I* ** A	114
ORF1	EIILRKRRIPYGYNSYLNEVQKMRSFLSNRTNTNTLIESVKIKSVYQSAS * *L R G * *VQ** S *R TLIE K Y *	450
М. р.	GFPALLVESRGQ-GIEQLKLNVQNILSS-EDRRLRMTLIEIAKPYGEPK	124
E. coli	LVADSITSQL-ERRVMFRRAMKRAVQN-AMRLGAKGIKVEVSGRLGGA L*A* I* QL * * FR * V** ** * G GI** SGRLGGA	160
ORF1	LIAQDISFQLRNNPISFRSIFSKIVKDIPLIMPKGVEGIRICCSGRLGGA **A* I***L ** **FR K I L * GI*I *GRL GA	500
М. р.	ILAKKIALKL-ESRVAFRRTMKKAIELAKKGNIKGIKIQIAGRLNGA	170
E. coli	EIARTEWYREGRVPLHTLRADIDYNTSEAHTTYGVIGVKVWIFKGEILGG Fiarte - G IDY - F. T. G. Gykvei G	210
ORF1	EIARTECGKYGKTSCNVFNQKIDYAPAEVSTRDGISGVKVRISYSQNKKG	550
М. р.	EIARVEWAREGRVPLQTIRARINYCYYAAQTIYGVLGIKVWIFQDEE.	217
E. coli	MAAVEQPEKPAAQPKKQQRKGRK.	233
	AEE	
ORF1	RAISETYEI	559

E. coli ORF2 Z. m. E. coli	MLQPKRTKF -L PKRTT* MEKHLVMYLTRKSIMLLRKYLLVTEFQVSKCGSHIVKRRDVPKRTKY -L PKRT** MLSPKRTRF	9 50 6
ORF2 Z. m. E. coli	 L PKRT* MEKHLVMYLTRKSIMLIRKYLLVTEFQVSKCGSHIVKIRGVPKTKY L PKRT** MLSPKRTRF 	50 6
ORF2 Z. m. E. coli	MEKHLVMYLTRKSIMLLRKYLLVTEFQVSKCGSHIVKIRRDVLYPKRTKY *L PKRT** MLSPKRTF	50 6
Z. m. E. coli	*L PKRT** Mlspkrtrf	6
Z. m. E. coli	MLSPKRTRF	6
E. coli		
	RKMHKGRN RGL-AQGTDVSFGSFGLKAVGRGRLTARQIEAARRA-MT	55
	K K R *RG **GT***FG *G K* *RGRL* R IEAARRA *	
ORF2	SKYSKCRCSRGREPDGTQLGFGRIGTRSSRAGRLSIRAILAARRAIIGUF	100
-	K W R "G" G W FGRI W THE R ILA-RRA	
Z. m.	RKQHRGR-HKGKSCKGNHICFGKIALQVLEPAWITARQILAGKKA-HIRI	24
E. coli	-RAVKRQGKIWIRVFPDKPITEKPLAVRMGKGKGNVEYWVALIOPG	99
	RA* *R* KIW*RV**D PIT KP VRNG*GKGN W*A * G	
ORF	RRAMSGQFRRNCKIWVRVLADLPITGKPAEVRNGRGKGNPTGWIARVSTG	150
	RR KIWVR***D P*T *P*E RMG GKG*P W*A V G	
Z. m.	ARRGGKIWVRIFPDKPVTIRPTETRNGSGKGSPEYWVAVVKPG	97
E. coli	KVLYEMDGVPEELAREAFKLAAAKLPIKTTFVTKTVM	136
	** *ENDGV AR A *LAA K T FV *	
ORF2	QIPFEMDGVSLSNARQAARLAAHKPCSSTKFVQNS	185
	*I *EM GVS * AR A *AA K **F**	
Z. m.	RILYEMSGVSETVARAAISIAASKMPIRSQFLRLEI	133

L16 SEQUENCE HOMOLOGIES

Fig. 6. (A) The protein sequence derived from ORF1 is aligned with homologous sequences from *E.coli* and liverwort (*Marchantia polymorpha*) chloroplast, designated M.p. in the figure. Between the sequence lines, asterisks indicate conservative changes and letters show points where the amino acids are identical. Amino acid numbering (starting at the first methionine codon in ORF1) is shown in the right margin. (B) The ORF2-derived amino acid sequence aligned with homologous sequences from *E.coli* and from *Zea mays* chloroplast (*Z.m.*). Designations are the same as described for panel A.

В.

rps 3- and *rpl* 16-like sequences lie downstream from the deleted sequences within the unique part of the 16 kb fragment. That region is not directly affected by the mutation, but the loss of 5' sequences prevents expression of both genes.

Defining the coding regions of ORFs 1 and 2

Comparisons of deduced protein sequences indicate that the NCS3-affected transcripts could encode ribosomal proteins analogous to S3 and L16 in prokaryotes and chloroplasts. The point at which translation actually begins in either reading frame is uncertain since the exact sizes of these proteins in plant mitochondria are not known. The deduced protein sequence from ORF2 is highly conserved with respect to the L16 proteins of *E. coli* and chloroplasts. The region of apparent homology does not begin at a methionine codon, but with a valine (GUG), although there are three in-frame methionine codons which precede, and are not far upstream from, that GUG codon in ORF2.

Similarity between the S3 proteins of *E. coli* and chloroplasts, and the S3-like region found on ORF1 is somewhat weaker than that found for the L16 protein, and is confined to the last portion ($\sim 40\%$) of the large open reading frame. Within this region, however, the resemblance to other S3 proteins is significant. (The similarity scores for S3 proteins when compared with ORF1 ranged from 13 to 30 standard deviations above the mean score.)

Within ORF1, there are no in-frame methionine codons near the point where similarity to other S3 proteins begins. The nearest AUG codons are 168 bp upstream and 135 bp downstream from the start of homology, and RNA editing

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of the sort described in plant mitochondria could not create a nearby AUG codon. It is possible that a functional S3-like mitochondrial protein is larger than its homolog in other organisms, or is derived by post-translational processing from a larger precursor. Alternatively, a substitute for the canonical AUG start codon should not, perhaps, be ruled out in plant mitochondria until direct sequencing of more mitochondrial proteins has been performed. In bacteria, for example, GUG can serve as an initiator of translation, and mammalian mitochondria can accept other AUN codons as initiators (Anderson *et al.*, 1981; Kozak, 1983).

Transcripts from the NCS3-altered region are edited

Recently, several laboratories have reported the posttranscriptional alteration of mRNA in plant mitochondria (Covello and Gray, 1989; Gualberto et al., 1989; Heisel et al., 1989; Schuster et al., 1990). This editing generally involves the deamination of specific cytosines to leave uracil residues. The comparison of cDNA and genomic sequences in this paper provides further evidence for the generality of this sort of post-transcriptional editing in plant mitochondria. The two individually isolated cDNA clones described here show two $C \rightarrow U$ conversions in identical places. At a third point, one of the clones has a uracil residue while the other retains the cytosine found in the DNA sequence. This is consistent with the observation by Schuster et al. (1990) that several cDNA clones of the nad3 gene in Oenothera exhibited only partial editing, indicating that editing may be a rather slow or inefficient post-transcriptional process.

The existence of RNA editing in plant mitochondria means that the cDNA sequence, rather than the genome sequence,



Fig. 7. A 12-18% SDS-polyacrylamide gradient gel showing proteins from isolated mitochondria that had been incubated in the presence of [³⁵S]methionine. In all panels, lanes 1 and 3 contain samples from NCS3-affected plants, and lanes 2 and 4 contain proteins made by normal mitochondria. Paired samples (1 with 2, and 3 with 4) were prepared simultaneously. (A) Total mitochondrial protein stained with Coomassie blue. (B) Fluorogram of the gel shown in panel (A). (C) An overexposure of lanes 3 and 4 from the same gel.

must be used to infer an amino acid sequence from a given coding region. Complete cDNA sequencing of the ORF1 and ORF2 regions will probably alter the deduced amino acid sequences, and it may uncover translation control regions, including initiation codons and ribosome binding sites, that are not apparent from surveying the genome sequence.

The maize S3- and L16-like ORFs overlap

In E. coli, the rps3 and rpl16 genes are found together on the S10 operon with rpl16 immediately downstream from rps3 (Nomura et al., 1984). This arrangement is highly conserved among prokaryotic and chloroplast genomes (see Christopher and Hallick, 1990), and raises the possibility that physical coupling of the two genes may be important in the coordination of their expression at the translational level. In E.coli, a 15 bp spacer, containing a ribosome binding site for L16 translation, separates the two genes (Zurawski and Zurawski, 1985), but in maize mitochondria, the reading frames overlap by 149 bp. The rps 3/rpl 16 region of maize mtDNA may function as a ribosomal operon, but the mechanism of translational coupling of these overlapping reading frames is not known. Genes for ribosomal proteins similar to others encoded on the E. coli S10 operon have not been reported in plant mitochondria, and we find no apparent homology to any of those genes on the 2.9 kb RNA described here.

In addition to the S3-like sequence that we report, several sequences similar to proteins of the small ribosomal subunit in prokaryotes and chloroplasts—S4, S12, S13 and S14— have been found in some plant mitochondrial genomes (Bland *et al.*, 1986; Schuster and Brennicke, 1987; Gualberto *et al.*, 1988; Wahleithner and Wolstenholme, 1988). The

mitochondrial S4-like sequence is an incomplete copy of a plastid gene, and is non-functional (Schuster and Brennicke, 1987). Whether the other three genes are expressed is unknown. Translation products have not been found and the presence of these genes within the mitochondrial genome appears to vary from species to species (Levings and Brown, 1989). No mitochondrial genes for proteins of the large ribosomal subunit had been reported previously.

There are several reasons to believe that one or both of the ribosomal protein genes described in this paper are, in fact, expressed in plant mitochondria: (i) NCS3-affected plants exhibit a severely stunted phenotype indicating that a functional gene has been disrupted; (ii) the deleted region of the genome that is associated with this phenotype is, in normal plants, transcribed into a mRNA with the potential coding capacity for L16- and S3-like ribosomal proteins; (iii) NCS3 mitochondria appear to be incapable of synthesizing proteins—an expected result if the mutation prevents formation of functional ribosomes.

These observations constitute strong genetic evidence that normal maize mitochondria carry at least one functional ribosomal protein gene.

Materials and methods

Plant material

The nuclear genotype of the WF9 inbred line in maize is associated with a relatively high frequency of mitochondrial mutations (Duvick, 1965; Coe, 1983). NCS3 arose in a family carrying WF9 nuclear genes and CMS-T mitochondria (Coe, 1983). Current stocks have been removed from the destabilizing effects of WF9 nuclear genes by repeated outcrosses with pollen of other inbred lines (B37, A619 or Ky21). Normal plants have arisen from NCS3 families by the segregation of normal from mutant mitochondria. These have been propagated in the same inbred lines and are used as the normal control plants in our studies.

Mitochondria were isolated from unpollinated ear shoots 1-4 days after silk emergence.

Oligonucleotide primers

Synthetic oligonucleotides were synthesized at the University of Missouri-Columbia DNA Core Facility. The sequences of primers a-h used in Figure 4 are shown below. The locations of these primers on the sequence in Figure 5 are given in parentheses $(5' \rightarrow 3')$.

Primer a; 5'-TCGCGTCGACAAGGAAACTGT-3' $(-114 \rightarrow -96)$ b: 5'-ACAGTTTCCTTGTCGACGCGA-3' $(-96 \rightarrow -114)$

c: 5'-GAG AGG ACG TTG CAG ATG TCC-3' (147-167) d: 5'-GGA CAT CTG CAA CGT CCT CTC-3' (167-147) e: 5'-ATA GAG CGA GGG CGT CCT TCT-3' (1269-1289) f: 5'-AGA AGG ACG CCC TCG CTC TAT-3' (1289-1269) g: 5'-CGA TAC GTC CAC CTA CGA GAC-3' (1974-1994) h: 5'-GTC TCG TAG GTG GAC GTA TCG-3' (1994-1974)

DNA isolation and analysis

Methods for mitochondrial isolation and the preparation and electrophoretic analysis of mtDNA have been presented by Newton and Coe (1986). The DNA blotting and hybridization methods have been described by Feiler and Newton (1987).

Isolation and analysis of mtRNA

Intact mitochondria were collected from the 47/35% interface of a 60/47/35/20% sucrose step gradient, and mtRNA was isolated as described (Stern and Newton, 1985b) except that RNA to be used for cDNA cloning was not exposed to the RNase inhibitor, aurintricarboxylic acid.

RNA was fractionated on 2.2 M formaldehyde -1.2% agarose gels and blotted using standard procedures (Maniatis *et al.*, 1982). Blots were hybridized with oligo-labeled DNA probes (Feiler and Newton, 1987).

Single-stranded probes were generated by end-labeling oligonucleotides with T4 polynucleotide kinase obtained from Promega (Maniatis *et al.*, 1982). Hybridizations were carried out at 50°C in 5 × SSC, 10 × Denhardt's solution, 7% SDS, 20 mM Na phosphate (pH 7) and 100 μ g/ml salmon sperm DNA. Blots were washed at room temperature in 6 × SSC, ending with a single 2 min incubation at 50°C.

Cloning

Restriction fragments of mitochondrial DNA were cloned into one of the following appropriately cut plasmid vectors: pUC19 (Vieira and Messing, 1982), pUC8c1 (see Stern and Newton, 1985a), pIBI24 (International Biotechnologies, Inc.) or Bluescript (Stratagene Cloning Systems). Nested deletions of plasmids were generated using *E.coli* exonuclease III from Bethesda Research Laboratories (Henikoff, 1984).

First strand cDNA was synthesized in a 25 μ l reaction using 1.5 μ g of mtRNA. The cDNA synthesis kit and protocol supplied by Amersham was employed except that 75 ng of specific primer (primer h in Figure 4) was substituted for that supplied with the kit. Residual RNA was hydrolyzed with NaOH after polymerization of the first strand cDNA.

Second strand synthesis and polymerase chain reaction (PCR) amplification (Saiki *et al.*, 1988) of the cDNA segment were performed together in a 50 μ l volume as described by Kocher *et al.* (1989) using the entire first strand DNA sample and 1 μ M each of primers h and a. The PCR samples were cycled at 94°C for 1 min, 50°C for 1.5 min and 72°C for 1.5 min. These conditions favored production of the shorter (processed) cDNA fragment. After 30 cycles, any remaining overhanging ends were removed with 2.5 U of Klenow fragment (Pharmacia) added directly to the PCR reaction and incubated for 5 min at 37°C. Since primer a contains a *Sal1* site, the PCR-amplified product was digested with *Sal1*, ligated into *Smal-Sal1* cut pUC19 or pIBI24 and analyzed for the appropriate insert.

DNA sequence analysis

Sequence analysis was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using double-stranded template, $[\alpha^{.35}S]dATP$ (New England Nuclear, >1000 Ci/mmol), and template-specific primers (UMC DNA Core Facility) or M13 sequencing primers (United States Biochemical Corp.). The kit and protocol, using T7 DNA polymerase (Tabor and Richardson, 1977), were supplied by Pharmacia, and sequences were analyzed on 6% polyacrylamide-8 M urea gels. Sequence analysis was performed using programs provided by DNAstar, Inc. (Madison, WI).

Protein synthesis by isolated mitochondria

Intact mitochondria were removed from the 35/47% interface of the sucrose step gradient, diluted slowly to avoid osmotic shock and used immediately for the labeling of nascent mitochondrial proteins (Leaver *et al.*, 1983; Newton and Walbot, 1985) with [³⁵S]methionine (New England Nuclear, >1000 Ci/mmol). Erythromycin and streptomycin (40 μ g/ml) were used to inhibit protein synthesis by any contaminating plastids and bacteria. Gel electrophoresis of labeled mitochondrial proteins and fluorography have been described (Cooper and Newton, 1989).

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Note added in proof

Overlapping *rps* 3 and *rpl* 16 genes, similar to those described here, have been found independently in *Petunia* mitochondria (K.D.Pruitt, Ph.D. dissertation, Cornell University, 1990).