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Nuclear and cytoplasmic genes controlling synthesis of variant mitochondrial polypeptides in male-sterile maize

(cytoplasmic male sterility/restoration of fertility/nuclear–mitochondrial interaction)

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ABSTRACT **The polypeptides synthesized *in vitro* by mitochondria isolated from etiolated maize shoots of a number of different nuclear and cytoplasmic genotypes have been compared by using polyacrylamide gel electrophoresis. We have previously shown that mitochondria from maize plants carrying the T or C forms of cytoplasmically inherited male sterility (*cms*-T and *cms*-C mitochondria) can be distinguished from each other and from the mitochondria of normal (N) plants by the synthesis of a single additional or variant polypeptide species. Using lines that carry the T cytoplasm, and that differ principally in the presence or absence of nuclear "restorer" alleles that suppress the male-sterile phenotype, we find that these nuclear genes specifically suppress synthesis of the 13,000 M_r variant polypeptide. A 21,000 M_r polypeptide that is synthesized by N mitochondria is not detectable among the translation products of *cms*-T mitochondria from either restored or nonrestored lines. Results obtained with a number of lines possessing dominant restorer alleles from different sources indicate that it is the restorer gene at the Rf_1 locus that is primarily responsible for regulating synthesis of the 13,000 M_r polypeptide. Mitochondria from lines with the S form of cytoplasmic male sterility (*cms*-S) were found to synthesize a group of minor polypeptides, ranging in molecular weight from 42,000 to 88,000, which were not detected in N, *cms*-T, or *cms*-C mitochondria. In the case of the S and C forms of male sterility no differences were found between the translation products of mitochondria from restored and nonrestored lines.**

Mitochondrial biogenesis depends upon the interaction between two distinct genetic systems, one located in the nucleus and the other in the mitochondrion itself. The need for coordination between these two systems implies the existence of mechanisms by which the nucleus can control the expression of mitochondrial genes and vice versa. In yeast, evidence has been obtained that proteins coded in the nucleus control the synthesis or integration of mitochondrially made subunits of cytochrome oxidase (1, 2). Nuclear mutants that suppress cytoplasmically inherited mitochondrial defects have been isolated in both *Neurospora crassa* (3, 4) and *Saccharomyces cerevisiae* (5); in *S. cerevisiae* (6) and in *Paramecium* (7) the converse effect has also been reported.

In maize (*Zea mays* Linnaeus), cytoplasmically inherited male sterility has been used extensively in the production of hybrid seed because its presence in the seed-plant parent prevents self-fertilization (8). Although this trait was described in 1931 (9), its commercial exploitation began only when it was discovered that certain maize lines carry nuclear genes, known as restorer genes (*Rf*), which are able to suppress the male-sterile phenotype (10). Subsequent genetic analysis has shown that there are at least three types of male sterility, designated T, C, and S, each of which is suppressed by different nuclear genes (8, 11). Several lines of evidence indicate that the genetic determinants that control cytoplasmic male sterility in maize are carried by the mitochondrion. Restriction endonuclease analysis has revealed differences between the mitochondrial DNAs of normal (N), *cms*-T, *cms*-S, and *cms*-C plants (12). However, with the exception of *cms*-S plants, the respective chloroplast DNAs were indistinguishable (13). In the sterile anthers of *cms*-T plants, ultrastructural studies have shown that mitochondrial degeneration is the first sign of abnormality during pollen development (14). Mitochondria from *cms*-T plants can be distinguished from N mitochondria by their sensitivity to the toxin of *Helminthosporium maydis*, race T (15–18). Finally, we have recently shown that there are discrete qualitative differences among the polypeptides synthesized by N, *cms*-T, and *cms*-C mitochondria (19). In the present paper we report that *cms*-S mitochondria also differ from N mitochondria (and from *cms*-T and *cms*-C mitochondria) in synthesizing a group of minor polypeptide species of high molecular weight. As part of an investigation into the possible involvement of the variant polypeptides in male sterility, we have examined the effect of *Rf* genes on the translation products of mitochondria from the T, C, and S cytoplasms. If the variant polypeptides are responsible for triggering pollen abortion, then the *Rf* genes may restore fertility by suppressing their synthesis.

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MATERIALS AND METHODS

Maize Lines. Normal and male-sterile versions of 10 inbred lines or crosses were used as indicated in the text. In some lines restorer alleles from various sources had been transferred into the nuclear backgrounds of the male-sterile cytoplasms by recurrent backcrossing and selecting for restoration of fertility in each generation. The sources of restorer alleles for the *cms*-T lines are given in Table 1, along with the number of backcrosses used in their transferral.

Isolation of Mitochondria. Mitochondria were isolated from 4-day-old etiolated shoots by the procedures described previously (19), with the following modifications. The discontinuous sucrose gradient used for purifying the mitochondrial fraction was replaced by a continuous 20–60% (wt/vol) sucrose gradient. After centrifugation at 40,000 × g_{av} for 60 min, the mitochondria were recovered from a band at approximately 42% sucrose.

Protein Synthesis by Isolated Mitochondria and Analysis of *In Vitro* Translation Products. The conditions used for labeling mitochondrial translation products with [^{35}S]methionine and the procedures used for their analysis by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide slab gel electrophoresis and autoradiography have been described (19).

RESULTS

T Cytoplasm. Mitochondria were isolated from etiolated maize shoots of three closely related genotypes and their translation products were labeled *in vitro* with [^{35}S]methionine. Two of the lines (Pioneer Inbred 4 N and Pioneer Inbred 4 T)

Table 1. Source of restorer alleles in the TRf lines and their effect on pollen fertility

Inbred line	Source of *Rf* alleles*	Strength of Rf_1 allele†	Dominant modifier alleles†	Degree of restoration†
Pioneer Inbred 4	Ky21 (9)	Strong	+	Good
A239	A293 (5)	?	?	Good
WF9	Ky21 (12)	Strong	+	Good
Pioneer Inbred 6	F5DD1 (6)	Strong	−	Poor
Pioneer Inbred 7	Ky21 (7)	Strong	−	Poor
A632	940‡	Strong	?	Poor

* Figures in parentheses indicate the number of backcrosses used in transferring the *Rf* alleles into each inbred line.

† As reported in personal communications from F. Salamini (inbred line A239 TRf) and R. Kleese (other TRf lines).

‡ Inbred line number 940 from the University of Illinois Experiment Station.

are isonuclear and differ only in having N or T cytoplasm. The third line (Pioneer Inbred 4 TRf) carries the T cytoplasm and has the same nuclear genotype as the other lines except that it is homozygous for nuclear restorer alleles.

Electrophoresis of the translation products of N and *cms*-T mitochondria revealed almost identical sets of approximately 20 polypeptide species, ranging in molecular weight from 10,000 to 54,000 (Fig. 1*A*). However, as reported (19), *cms*-T mitochondria synthesize a 13,000 M_r polypeptide that is not detected in N mitochondria. In addition, Fig. 1*A* shows that a 21,000 M_r polypeptide is synthesized by N mitochondria but not by *cms*-T mitochondria. This polypeptide, which was not resolved in earlier experiments (19), is also synthesized by *cms*-C and *cms*-S mitochondria.

The effect of the *Rf* alleles on the polypeptides translated *in vitro* by *cms*-T mitochondria was found to be specific to the variant 13,000 M_r polypeptide. When compared with those from the nonrestored (T) line the mitochondria from the TRf line synthesized the 13,000 M_r polypeptide at a greatly reduced rate (Fig. 1*A*).

An examination of the stained gel from which the autoradiographs in Fig. 1*A* were taken confirms the effect of the *Rf* alleles on the 13,000 M_r polypeptide (Fig. 1*B*). Because only a fraction of the 100 or so mitochondrial proteins are translated on mitochondrial ribosomes, many more polypeptides are detected by staining with Coomassie blue than by autoradiography of the labeled products *in vitro*. Nevertheless, among the proteins of *cms*-T mitochondria we have resolved a stained band of 13,000 M_r that was not detectable in mitochondria from the N or TRf lines (Fig. 1*B*). This stained band co-electrophoresed with the radioactively labeled variant polypeptide.

We have also studied the mitochondrial translation products of five additional inbred lines of maize that contain *Rf* alleles derived from several different sources (Table 1). In two of these lines, as in Pioneer Inbred 4 TRf above, pollen fertility has been almost fully restored. In the other three lines, however, fertility restoration is poor (personal communications from R. Kleese and F. Salamini).

In A239 TRf (Fig. 2*A*) and WF9 TRf (Fig. 2*B*), the two lines in which restoration is good, the degree to which synthesis of the 13,000 M_r polypeptide was reduced is similar to that in Pioneer Inbred 4 TRf, as revealed by comparison with densitometer scans of Fig. 1*A* (not shown). The same result was obtained with two of the poorly restored lines, Pioneer Inbred 6 TRf (Fig. 2*C*) and Pioneer Inbred 7 TRf (not shown). In a contrast, a much less effective suppression of the 13,000 M_r polypeptide was observed with the third poorly restored line, A632 TRf (Fig. 2*D*). There was therefore no simple correlation between the extent of fertility restoration and the extent of suppression of the variant polypeptide.

Synthesis of the 21,000 M_r polypeptide that is characteristically absent from *cms*-T mitochondria was not detected in mitochondria isolated from Pioneer Inbred 4 TRf (Fig. 1*A*) or from any of the five other restored lines examined.

C Cytoplasm. In the translation products of *cms*-C mitochondria, synthesis of a 15,500 M_r polypeptide that is detected in N mitochondria has been replaced by synthesis of a polypeptide with slightly lower electrophoretic mobility in 15% polyacrylamide gels (Fig. 3 and ref. 19). The difference in mobility does not necessarily imply a difference in molecular

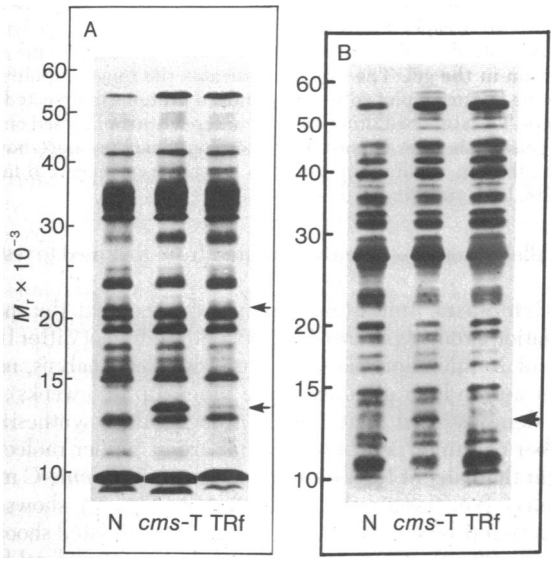

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of mitochondrial polypeptides from normal (N), male-sterile (*cms*-T), and fertility-restored (TRf) lines of maize (Pioneer Inbred 4). Mitochondria were isolated and incubated for 90 min in a medium containing [^{35}S]methionine and an energy-generating system. The mitochondrial polypeptides were fractionated by NaDodSO$_4$ slab gel electrophoresis and the gel was stained with Coomassie brilliant blue and dried onto Whatman 3MM chromatography paper. (*A*) Autoradiograph of the radioactively labeled mitochondrial translation products. (*B*) Photograph of the stained gel. Arrows indicate polypeptides not common to all three lines.

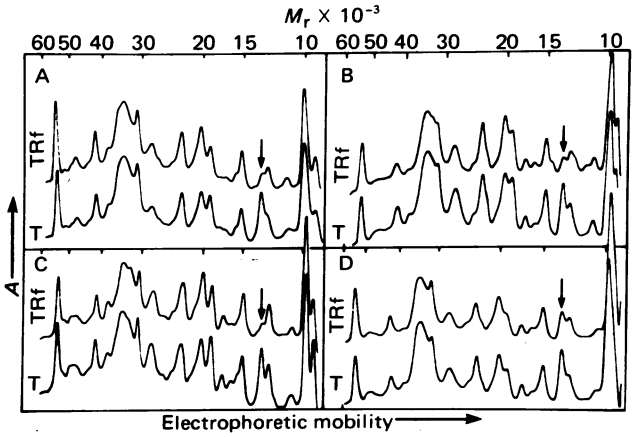

FIG. 2. Polyacrylamide gel profiles of polypeptides synthesized by mitochondria from male-sterile (*cms*-T) and fertility-restored (TRf) versions of four inbred lines. Autoradiographs of labeled mitochondrial translation products were obtained as for Fig. 1 and scanned with a densitometer. The maize lines used were A239 (*A*), WF9 (*B*), Pioneer Inbred 6 (*C*), and A632 (*D*). The arrows indicate the 13,000 M_r variant polypeptide that is associated with the T cytoplasm.

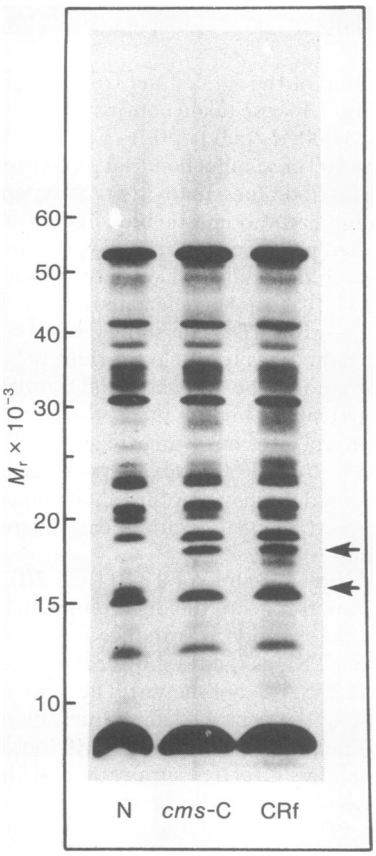

FIG. 3. Electrophoresis of polypeptides synthesized by mitochondria from normal (N), male-sterile (*cms*-C), and fertility-restored (CRf) lines of maize (A632). Autoradiographs of labeled mitochondrial translation products were obtained as for Fig. 1. The arrows indicate polypeptides not common to all three lines.

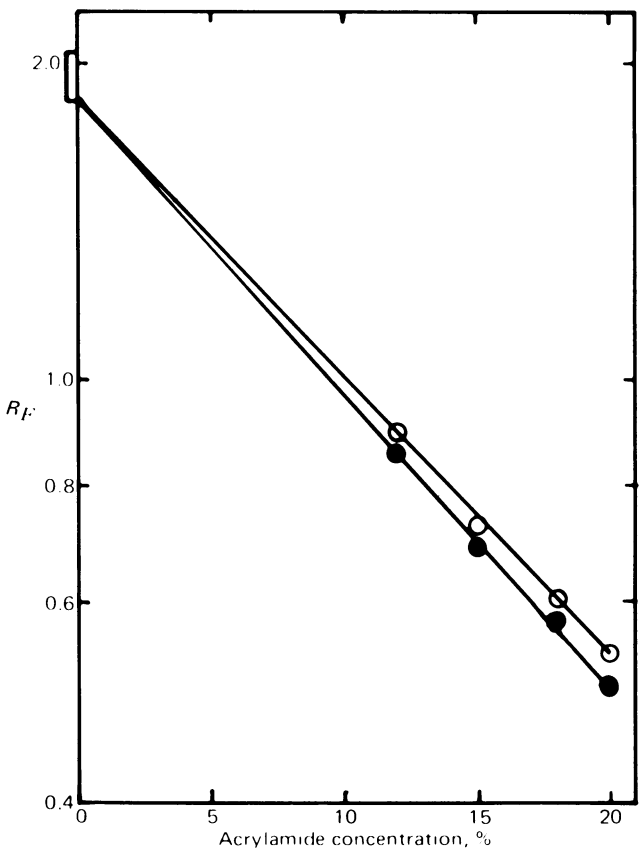

FIG. 4. Ferguson plot of the variant polypeptide synthesized by *cms*-C mitochondria (●) and of the polypeptide synthesized by N mitochondria that is missing from *cms*-C mitochondria (○). Labeled translation products of mitochondria from the lines A632 N and A632 C (see legend to Fig. 3) were electrophoresed on a series of polyacrylamide gels with different percentages of monomer (and a constant ratio of acrylamide to bisacrylamide of 150:1). After staining, the gels were autoradiographed. The relative mobility (R_F) of each polypeptide was plotted on a logarithmic scale against the acrylamide concentration in the gel. The bracket indicates the range of points at which the Ferguson plots of seven standard proteins intersected the ordinate. The standard proteins, which were electrophoresed on the same gels as the mitochondrial translation products, were bovine serum albumin, ovalbumin, creatine kinase, trypsinogen, β-lactoglobulin, lysozyme, and cytochrome *c*.

weight. Although for most proteins there is a linear relationship between the logarithm of the molecular weight and the relative mobility in $NaDodSO_4$ gels, this is not true of glycoproteins, proteins with an abnormal binding capacity for $NaDodSO_4$, or proteins having an unusual conformation (20). In such cases it has been shown that the free electrophoretic mobility deviates from that of standard water-soluble proteins (21–23). The free electrophoretic mobility (i.e., the mobility at an acrylamide concentration of zero) is estimated by using a series of gels of different pore sizes and plotting the logarithm of the relative mobility against acrylamide concentration (24). For the two polypeptides in question it was found that a line through each set of points intersected the ordinate (at 0% acrylamide) at the same point and within the range of free electrophoretic mobilities of seven standard proteins (Fig. 4). This demonstrates the absence of any anomalous electrophoretic behavior by either polypeptide and indicates that the variant polypeptide synthesized by *cms*-C mitochondria is indeed larger than the 15,500 M_r polypeptide. By comparison with the standard proteins used in Fig. 4, we now estimate the molecular weight of this variant polypeptide to be 17,500.

To investigate the effect of restoration of fertility on synthesis of the 17,500 M_r polypeptide, we compared the translation products of mitochondria from the inbred line A632 C with those of mitochondria from the same inbred line, also carrying the C cytoplasm but restored for pollen fertility by homozygous nuclear genes (Fig. 3). In contrast to the result obtained with the T cytoplasm, we found no apparent effect of restorer alleles on synthesis of the variant polypeptide. This result has been confirmed in a second inbred line (B37), which possessed restorer alleles from a source that differed from that used to restore A632.

S Cytoplasm. Initial experiments showed that the major translation products of *cms*-S mitochondria did not differ from those of N mitochondria (19). More detailed analysis, using longer autoradiographic exposure times (up to 3 weeks), has now demonstrated that *cms*-S mitochondria synthesize a number of minor polypeptides that are of higher molecular weight than any of the products of N, *cms*-T, or *cms*-C mitochondria (i.e., larger than 54,000 M_r). Fig. 5*A* shows the translation products of mitochondria from etiolated shoots of the lines B37 N, B37 S, and B37 SRf. In the stained gel from which this autoradiograph was taken (not shown), a polypeptide of approximately 85,000 M_r was detected that co-electrophoresed with the slowest migrating of the translation products of *cms*-S mitochondria. This stained polypeptide, and the other additional high molecular weight translation products (Fig. 5*A*), were observed in mitochondria from both nonrestored (*cms*-S) and restored (SRf) lines but not in N mitochondria. A similar group of high molecular weight translation products has now been detected in 3 other lines or crosses carrying the S cytoplasm

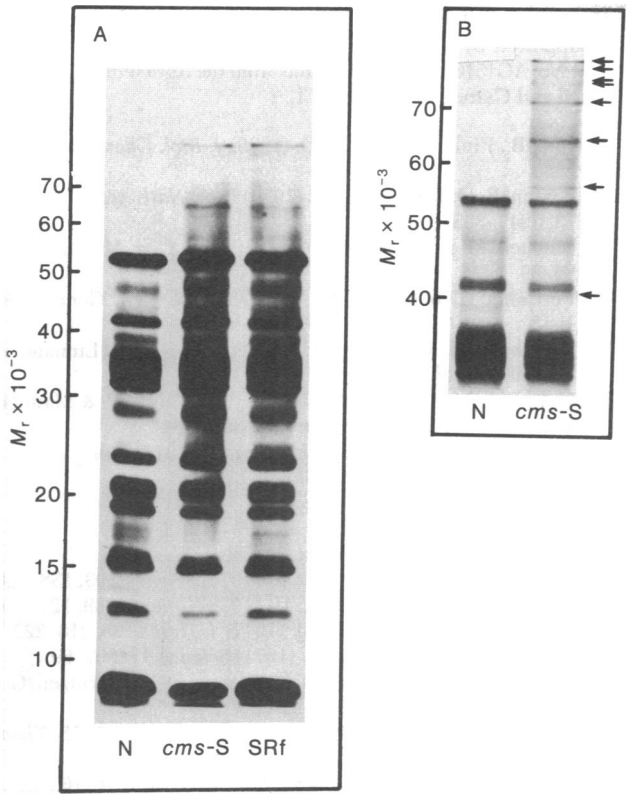

FIG. 5. Electrophoresis of polypeptides synthesized by mitochondria from normal (N), male-sterile (*cms*-S), and fertility-restored (SRf) lines. (*A*) Autoradiographs of the labeled translation products of mitochondria from the three closely related genotypes B37 N, B37 S, and B37 SRf were obtained as for Fig. 1. (*B*) An enlarged portion of an autoradiograph of the labeled translation products of mitochondria from the crosses CO192 (N) × WJ and CO192 (S) × WJ, showing the additional polypeptides synthesized by *cms*-S mitochondria (arrows).

(W64A, WM13, and CO192 × WJ) and in 18 other sources of the S form of male sterility (unpublished observations). In mitochondria from the hybrid CO192 (S) × WJ we have resolved a total of seven additional translation products of >54,000 M_r and a further additional polypeptide of 42,000 M_r (Fig. 5*B*).

DISCUSSION

Mitochondrial synthesis of a 13,000 M_r variant polypeptide is a cytoplasmically inherited trait that is associated with the T form of cytoplasmic male sterility in maize (19). Translation of the variant polypeptide on mitochondrial ribosomes, together with the cytoplasmic mode of its inheritance, is a strong indication that its amino acid sequence is encoded on mitochondrial DNA. In yeast it has now been shown that all proteins made on mitochondrial ribosomes are encoded by mitochondrial genes, and there is no evidence that messenger RNA is imported from the nucleus (25). The nature of the mutation responsible for synthesis of the variant polypeptide in *cms*-T mitochondria is not known. However, the observation that *cms*-T mitochondria fail to synthesize a 21,000 M_r polypeptide that is detected in N mitochondria (Fig. 1*A*) suggests that a deletion or other mutation in the structural gene for this larger polypeptide may have led to the synthesis of an abbreviated form of the protein.

The evidence presented in Figs. 1 and 2 indicates that, in addition to being controlled by cytoplasmic genes, the synthesis of the 13,000 M_r polypeptide is under the specific control of certain nuclear genes. When alleles that restore fertility are introduced into the nuclear background of lines carrying the T cytoplasm there is a specific reduction in the rate of synthesis of the 13,000 M_r polypeptide by isolated mitochondria (Figs. 1*A* and 2), and the polypeptide is no longer detectable in gels stained for protein (Fig. 1*B*). However, in none of the restored lines examined was synthesis of the 13,000 M_r polypeptide completely suppressed, nor was synthesis of the 21,000 M_r polypeptide even partially recovered. Therefore, even in lines that had been fully restored to fertility, the mitochondrial translation products can be clearly distinguished from those of N mitochondria.

Among the six restored lines tested it is known that there are differences in the degree to which the fertility is restored (Table 1). There is, however, no simple correlation between the strength of restoration and the degree to which synthesis of the 13,000 M_r polypeptide is suppressed (Figs. 1 and 2). The Pioneer Inbred lines 4 TRf and 6 TRf, for example, show strong and weak restoration, respectively, yet in both lines synthesis of the variant polypeptide is equally strongly suppressed (Fig. 2). In contrast, a second poorly restored line (A632 TRf) shows only moderate suppression of the variant polypeptide. These results could indicate that the variant polypeptide has no direct involvement in the process of pollen abortion, but an explanation for the apparent anomaly may lie in the complexity of the genetics of fertility restoration. Suppression of the T form of cytoplasmic male sterility is controlled by dominant genes at two loci, Rf_1 and Rf_2 (8). Dominant alleles, in either the homozygous or heterozygous state, must be present at both loci in order to obtain any restoration of fertility. However, because most Corn Belt inbreds are homozygous dominant at the Rf_2 locus, only the Rf_1 locus need be considered here. A number of different dominant alleles are known to exist at the Rf_1 locus, ranging in their restoring effect from weak to strong (8). For complete restoration of fertility in all environments, however, a strong allele at the Rf_1 locus must be accompanied by an unknown number of "modifier" genes. Therefore, although the restored lines WF9, Pioneer Inbred 4, Pioneer Inbred 6, and Pioneer Inbred 7 each have a strong allele at the Rf_1 locus, only the first two carry the necessary modifier alleles and are strongly restored (Table 1). Because in each of these four restored lines suppression of the 13,000 M_r polypeptide is equally effective (Figs. 1 and 2), regardless of the presence or absence of dominant modifier alleles, we suggest that it is the Rf_1 gene (or genes closely linked to it) that is primarily responsible for this suppression. Other factors may also be involved in controlling synthesis of the variant polypeptide, as indicated by the result obtained with the A632 TRf line. Suppression of the 13,000 M_r polypeptide in this line was less effective than in any of the other TRf lines (Figs. 1 and 2) even though, like the four lines listed above, it carries a strong allele at the Rf_1 locus. However, restoration of fertility in A632 TRf is also poor, despite the fact that the line from which the restorer alleles were transferred gives good restoration when used as a restorer in other inbred lines (R. Kleese, personal communication). The weak suppression of synthesis of the 13,000 M_r polypeptide, like the poor restoration of fertility, may therefore result from an effect of the background genotype of A632.

Mitochondria from *cms*-T lines have been shown to be susceptible to the host-specific toxin of *H. maydis*, race T, although N mitochondria are insensitive (15–18). An indication that pollen abortion and susceptibility to the toxin are expressions of a single defect is given by the finding that mitochondria from TRf lines are significantly less sensitive to the toxin (26). If it can be shown conclusively that suppression of synthesis of the 13,000 M_r polypeptide and reduction in the sensitivity to the toxin are under the control of the same nuclear restorer gene(s),

this will provide strong circumstantial evidence that the variant polypeptide is involved in both male sterility and susceptibility to *H. maydis*, race T.

There are a number of possible mechanisms by which a nuclear gene could specifically regulate the synthesis of the 13,000 M_r polypeptide. These include a direct interaction between the nuclear gene product and the mitochondrial protein-synthesizing system (1, 2) or an indirect effect operating through changes in the concentration of an effector molecule (4).

Although variant polypeptides are also synthesized by mitochondria from *cms*-C and *cms*-S lines of maize, no effect of restorer genes on these products was observed in either case (Figs. 3 and 5). However, because these experiments were carried out with mitochondria isolated from etiolated shoots, we cannot rule out the possibility that the *Rf* genes that restore fertility to *cms*-C and *cms*-S plants exert an effect on mitochondrial translation at a later stage of plant growth, perhaps only in the developing anthers.

The additional high molecular weight polypeptides that are synthesized by *cms*-S mitochondria (Fig. 5) may result from a fault in the processing of messenger RNA or protein precursors. For example, the recent demonstration that there are intervening sequences in a number of yeast mitochondrial genes (25) suggests that the initial gene transcript may contain one or more sequences that are not normally expressed in the final gene product. If a mutation occurred that reduced the efficiency of processing of a precursor RNA, a series of polypeptides of molecular weight higher than that of the normal gene product might then be produced from the same structural gene (27). An alternative explanation, however, may be provided by the finding that *cms*-S mitochondria possess two unique plasmid-like DNA species (28). The role, if any, of these small DNAs in causing male sterility has not been identified, nor is it known if they are expressed *in vivo*. Evidence for at least partial transcription of a small (2-μm) circular DNA in yeast cells has been obtained by RNA·DNA hybridization studies (29), and on insertion into *Escherichia coli* minicells the same DNA coded for a number of low molecular weight polypeptides (30). We must therefore consider the possibility that the high molecular weight translation products that are characteristic of *cms*-S mitochondria are encoded by the small DNA species uniquely associated with these mitochondria.

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