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urf13-T of T cytoplasm maize mitochondria encodes a 13 kD polypeptide

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Abstract

Polyspecific antibody to a 17 amino acid synthetic peptide from the maize T-cytoplasm *urf13-T* mitochondrial open reading frame immunoprecipitated a 13 kD polypeptide from ^{35}S-methionine incorporations of T cytoplasm maize. Male-fertile, toxin-insensitive mutants in which the *urf13-T* sequence is deleted do not synthesize the 13 kD polypeptide. A mutant designated T-4, which carries a 5 bp insertion and a premature stop codon, synthesizes a truncated polypeptide, corresponding to an open reading frame of 8.3 kD. Thus the 13 kD polypeptide is trunctated or absent in mutants expressing male fertility and toxin insensitivity in T-cytoplasm maize.

Introduction

Studies of tissue culture-derived mutants of T cytoplasm maize have shown that the mitochondrial gene *urf13-T* is associated with cytoplasmic male sterility (cms) and sensitivity to host-selective fungal toxins produced by race T of *Cochliobolus heterostrophus* Drechsler and *Phyllosticta maydis* Arny and Nelson. Those studies demonstrated that the *urf13-T* reading frame [3] was either truncated or deleted in male-fertile, toxin-insensitive mutants [16, 17]. A polypeptide of apparent molecular mass 12961 (13 kD) was predicted from the *urf13-T* sequence [3], which correlates with a T-specific 13 kD mitochondrial translation product [5] absent in most mutants [4]. A mutant designated T-4 contains a 5 bp insertion, placing a TGA stop codon in frame 4 bp from the insertion and truncating the predicted polypeptide at 8.3 kD [16, 17]; we have designated this gene *urf8.3-T4*.

Antibodies directed against synthetic peptides derived from the predicted amino acid sequence of open reading frames have been utilized in the identification of gene products [15]. A number of mitochondrial proteins were identified by this method [1, 12, 13, 14]. We have taken this approach as a first step in the biochemical characterization of *urf13-T*.

Materials and methods

Maize lines

A188(N), A188(T), a deletion mutant A188(T-7), and the insertion mutant A188(T-4), were previously described [16, 17].

^{35}S-methionine incorporation by isolated mitochondria

^{35}S-methionine incorporation into isolated maize mitochondria was performed by the method of Leaver *et al.* [10]. Mitochondria were isolated from five-day old dark-grown coleoptiles by sucrose gradient purification and were allowed to incorporate ^{35}S-methionine using either 10 mM sodium succinate and 2 mM ADP-ATP generated by respiratory chain-linked phosphorylations or 8 mM phosphocreatine, 25 μg creatine phosphokinase, and 6 mM ATP-ATP generated externally. After incubation for 90 min, the reaction was stopped and mitochondria were pelleted in an Eppendorf Microfuge, frozen in liquid nitrogen, and stored at −80 °C. For electrophoretic analysis, the mitochondrial pellets were thawed and 50 μl of sample buffer (2% SDS, 62.5 mM Tris pH 6.8, 5% V/V 2-mercaptoethanol, 10% sucrose, 0.1 mM bromphenol blue) was added and incubated for 3 h at 37 °C [5]. The samples were subsequently electrophoresed on 12 to 18% linear polyacrylamide gradient gels for 18 h at 16 mA according to Laemmli [9]. ^{14}C-Labeled low molecular weight protein standards (Bethesda Research Laboratories) were used as internal molecular weight markers.

Preparation of antibody

A 17 amino acid sequence corresponding to *urf13-T/urf8.3-T4* nucleotides 94–144 [17] was chosen for its hydrophilic nature [7]. The synthetic peptide was synthesized by the Applied Biosystems synthesizer. The peptide (PEP17) was coupled to keyhole limpet hemocyanin (KLH) (Cal Biochem) through an added cysteine residue at the amino terminus, with *m*-maleimidobenzoyl-N-hydroxy-succinimide ester (MBS) as the coupling agent [11]. Typically 4 mg of KLH was coupled to 5 mg of peptide for 3 h at 25 °C. The extent of coupling (50 molecules peptide/molecule KLH) was determined by amino acid analysis. The PEP17-KLH conjugate (1 mg of peptide equivalent) was injected into a New Zealand White rabbit with equal volume Freund's complete adjuvant and booster injections were given subsequently at 7–10 day intervals with Freund's incomplete adjuvant. Rabbits were ear bled (7–10 days after booster injections) and serum was stored at −20 °C.

Immunoprecipitation of mitochondrial polypeptides

The anionic tenside SDS and non-ionic tensides Triton X-100, Thesit, and Tween 20 were evaluated for their ability to solubilize mitochondrial polypeptides for subsequent immunoprecipitation with anti-PEP17. Immunoprecipitation protocols using SDS showed no precipitation of any maize mitochondrial translation products, whereas Thesit or Tween 20 solubilization displayed high levels of non-specific precipitation. For optimized immunoprecipitation with anti-PEP17 we used Triton X-100 (Boehringer Mannheim) [1] as a solubilizing agent.

All manipulations were carried out at 4 °C unless otherwise noted. Mitochondria (600–800 μg of protein, 4×10^6 cpm) were lysed in the presence of 1% Triton X-100 in buffer A (100 mM NaH_2PO_4, pH 7.5, 150 mM NaCl, 5 mM Na_2EDTA, 1 mg per ml bovine serum albumin, 0.1 mM phenylmethylsulfonylfluoride, 2 mM methionine) for 30 min at room temperature. The suspension was subsequently diluted with 19 volumes of A resulting in a final concentration of 0.05% Triton X-100. After a 60 min incubation with 100 μl of formaldehyde-fixed *Staphylococcus aureus* Cowan strain I suspension (Pansorbin, Cal Biochem), prewashed according to Kessler [8], the mixtures were centrifuged in an Eppendorf Microfuge for 2 min to remove insoluble materials. Immune or preimmune serum (200 μl) was added to the supernatant and incubated overnight at 4 °C with end over end shaking. Pansorbin (100 μl) was added and allowed to incubate for an additional 60–90 min. The cells were pelleted in the microfuge for 2 min and were subsequently washed twice with buffer A containing 0.05% Triton X-100 followed by a final wash with buffer A without Triton. The pellet was

resuspended in 50 μl of sample buffer, incubated 60 min at 37 °C followed by 4 min at 95 °C, and electrophoresed as described above.

Results

To evaluate the synthesis of the 13 kD polypeptide, *in vitro* ^{35}S-methionine incorporations by mitochondria isolated from A188(T), A188(T-4), A188(T-7) (a deletion mutant), and A188(N) (normal, male fertile) were examined by polyacrylamide gradient gel electrophoresis and fluorography (Fig. 1). As previously established [5], A188(T) was characterized by a prominent polypeptide which in our electrophoretic system migrated at 15 kD, which we refer to as the 13 kD polypeptide. A188(N) and A188(T-7) did not synthesize this polypeptide, and A188(T-4) was characterized by absence of the 13 kD polypeptide and the appearance of a unique polypeptide at approximately 8 kD. This smaller protein is of the size predicted from the truncated open reading frame (222 bp) in T-4 [17].

The amount of the 8 kD polypeptide produced by mitochondria isolated from T-4 is reduced compared to the wild type 13 kD polypeptide. This may be due to instability of the truncated polypeptide or reduced translation efficiency, since transcription of the *urf13-T* region appears to be unaltered in T-4 [17].

A specific 17 amino acid sequence from *urf13-T/urf8.3-T4* was selected for chemical synthesis on the basis of hydrophilic character [7]. A restriction map of the *urf13-T/urf8.3-T4* region with location of the synthetic peptide is shown in Fig. 2. Rabbit antiserum was raised against the synthetic

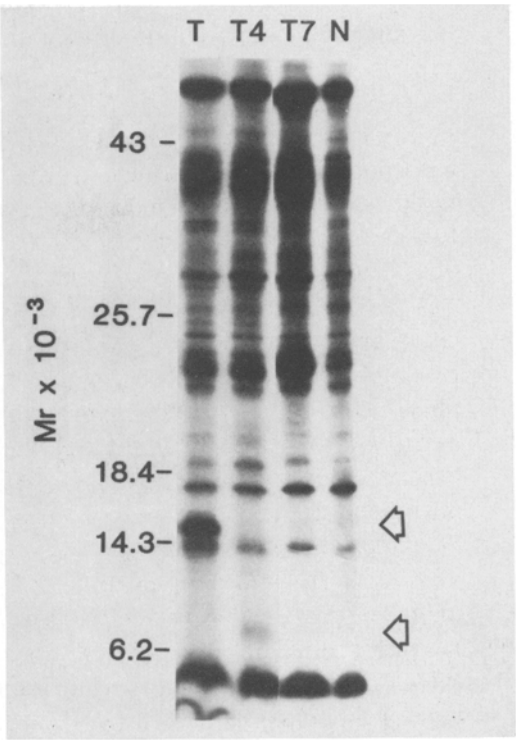

Fig. 1. Electrophoretic pattern of ^{35}S-methionine labeled polypeptides synthesized by isolated maize mitochondria. A188(T) displays the characteristic 13 kD polypeptide (arrow), while a unique polypeptide at approximately 8 kD was observed in the T-4 mutant (arrow). A deletion mutant (T-7) and a normal male-fertile cytoplasm, A188(N), do not contain either the 13 kD or the 8 kD polypeptides.

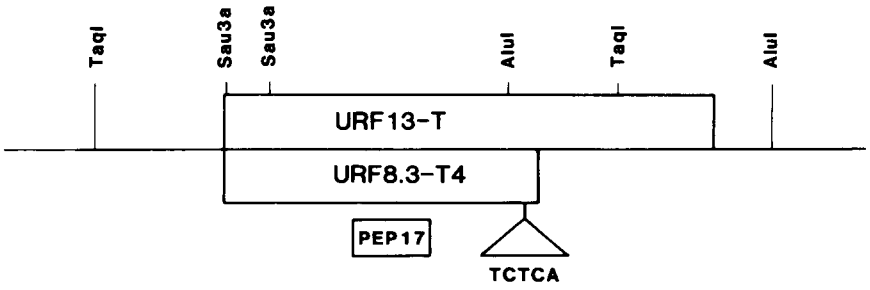

Fig. 2. urf13-T/urf8.3-T4 region of T cytoplasm maize mitochondrial DNA. The location of PEP17 corresponding to the amino acid sequence KGYLRKMDDSYLAQLSE is indicated. The site of the TCTCA insertion in T-4, generating the truncated reading frame in *urf8.3-T4*, is shown.

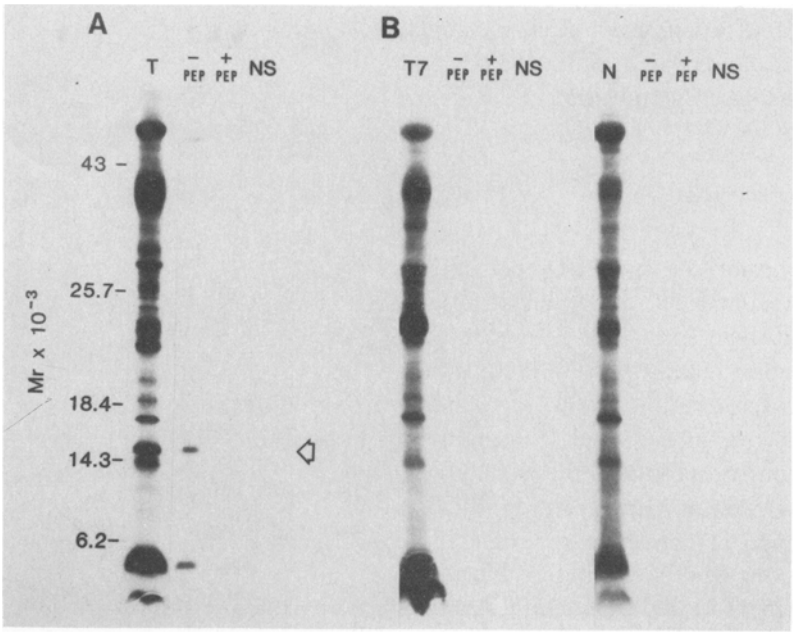

Fig. 3. Immunoprecipitation of the 13 kD (arrow) mitochondrial translation product from A188(T) (A) by anti-PEP17, and absence of precipitation from A188(T-7) or A188(N) (B). PEP17 (PEP+) was added to 100 μg in competition assays. Normal serum (NS) demonstrated no precipitation with A188(T), A188(T-7), or A188(N).

peptide (PEP17) and was evaluated by immunoprecipitation of mitochondrial translation products from T-cytoplasm maize.

Immunoprecipitations of *in vitro*-synthesized polypeptides from A188(T), T-4, T-7, and N were examined. Anti-PEP17 specifically precipitated the 13 kD mitochondrial translation product from A188(T) (Fig. 3A) and a major 6 kD polypeptide, previously identified as the DCCD-binding protein [10] encoded by the gene *atp9* [2]. Addition of PEP17 (100 μg) effectively competed with these polypeptides in the precipitation reaction. The normal serum control did not precipitate the 13 kD or the DCCD polypeptides. The DCCD-binding protein was not precipitated by anti-PEP17 from mitochondrial amino acid incorporations of T-7 and N (Fig. 3B), indicating lack of epitope homology between the DCCD-binding protein and PEP17. These results suggest that the polypeptides may be associated with each other as a complex in the mitochondrion. Attempts at precipitation of the unique 8 kD polypeptide from T-4 *in vitro* amino acid incorporations were inconclusive, possibly due to the decreased amount or an altered configuration of the protein. Thus we can only suggest that the 8 kD polypeptide is a gene product of *urf8.3-T4*.

Discussion

The co-precipitation of the T-cytoplasm specific 13 kD protein and the DCCD-binding protein could be due to epitope homology between PEP17 and the DCCD-binding protein or to the precipitation of part of a mitochondrial complex. Chomyn *et al.* [1] demonstrated that antibodies directed against the carboxy-terminal heptapeptide of the human mitochondrial URF4 and URF4L were effective in the co-precipitation of several components of human mitochondrial respiratory chain NADH dehydrogenase from Triton X-100 lysates. Using similar protocols, antibodies against the carboxy-terminal undecapeptide of subunit II of cytochrome c oxidase precipitated the entire complex [12]. Since the DCCD-binding protein was not precipitated by anti-PEP17 from mitochondrial amino acid incorporations of T-7 or N, we suggest that the polypeptides may be associated with each

other as part of the F_1F_0-ATPase complex in the mitochondrion. Alternatively, the 6 kD polypeptide may be a premature translation product, detected by antibody internal to the open reading frame.

Several lines of evidence suggest a role of the 13 kD polypeptide in cms and disease toxin sensitivity. Synthesis of the polypeptide by isolated T cytoplasm mitochondria is reduced in lines restored to fertility [6]. In our evaluation, fertility restoration of A188(T) reduced synthesis of the 13 kD polypeptide by ca.70% (data not shown). Fertility restoration also significantly reduces the effect of purified toxin on malate oxidation in T cytoplasm maize mitochondria (S. J. Danko, J. M. Daly and B. G. Gengenbach, personal communication). The extent of the restoration effect varied depending on inbred line, toxin concentration, and malate oxidation assay used, but reductions of 50% were not uncommon. These cumulative data suggest a correlation of toxin response with levels of the 13 kD polypeptide. *urf13-T* is deleted in four male-fertile, toxin-insensitive mutants, and truncated in the T-4 insertion mutant [16, 17], correlated with absence of the 13 kD polypeptide in the former and the presence of the 8 kD polypeptide in the latter. The immunoprecipitation data demonstrate that the 13 kD polypeptide, implicated in cms and disease toxin sensitivity, is the gene product of *urf13-T*. Similar results have been obtained in other laboratories (R. E. Dewey and C. S. Levings, III, personal communication; W. H. Rottmann, D. M. Lonsdale and C. J. Leaver, personal communication). Hydrophilicity plots of the predicted amino acid sequence of *urf13-T* show strong hydrophilic and hydrophobic regions, consistent with a possible membrane site of the polypeptide. If the 13 kD polypeptide indeed plays a role in sensitivity to the toxins it may function through recognition, binding, complexing, or other processes on mitochondrial membranes, possibly involving the F_1F_0-ATPase complex.

Acknowledgements

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