Plant Physiol. (1991) 95, 861–870
0032-0889/91/95/0861/10/$01.00/0

Received for publication August 6, 1990
Accepted November 16, 1990

T-URF13 Protein from Mitochondria of Texas Male-Sterile Maize (*Zea mays* L.)[1]

Its Purification and Submitochondrial Localization, and Immunogold Labeling in Anther Tapetum during Microsporogenesis

Ethan Hack[2], Chentao Lin[3], Hongyun Yang[3], and Harry T. Horner*

Department of Botany, Iowa State University, Ames, Iowa 50011–1020

ABSTRACT

The protein T-URF13 (URF13) is specific to mitochondria of maize (*Zea mays* L.) with Texas (T) male-sterile cytoplasm and has been implicated in causing male sterility and susceptibility to T-cytoplasm-specific fungal diseases. T-URF13 was purified from isolated mitochondria from maize (line B73) with T cytoplasm by gel filtration and a quasi two-dimensional polyacrylamide gel electrophoresis system. Antibodies to the purified and denatured protein were produced in rabbits. Anti-T-URF13 antiserum was used to show that T-URF13 is in the inner membrane of mitochondria and behaves as an integral membrane protein when mitochondria are fractionated with sodium carbonate or Triton X-114. The antiserum and protein A tagged with 20-nanometer-gold particles were used to localize T-URF13 in T mitochondria by electron microscopy of sections of isolated mitochondria from etiolated shoots and sections of roots and of tapetal cells at pre- and post-degeneration stages of microsporogenesis. The microscopic study confirms that T-URF13 is specifically localized in the mitochondrial membranes of all of the T mitochondria tested, notably those in the tapetum from the meiocyte stage to the late-microspore stage. No change in the amount of labeled T-URF13 protein in the mitochondria of aging tapetal cells was detected.

Maize (*Zea mays* L.) lines with T[4] cytoplasm are completely male sterile and were widely used for producing hybrid seeds until 1970. Subsequently, use of T cytoplasm was largely abandoned because T plants were found to be highly susceptible to two new fungal diseases, southern corn leaf blight, caused by race T of *Bipolaris* (*Helminthosporium*) *maydis* (teleomorph *Cochliobolus heterostrophus*), and yellow leaf blight, caused by *Phyllosticta maydis* (teleomorph *Mycosphaerella zeae-maydis*). Both these fungi produce host-specific toxins called, respectively, BmT (or HmT) toxin and Pm toxin, that specifically affect T mitochondria, apparently by permeabilizing the inner mitochondrial membrane (see ref. 26 for a recent review). Methomyl, an insecticide, affects T mitochondria similarly to the toxins. The mechanism by which the toxins and methomyl produce their effects is unknown.

Mitochondria of T maize plants contain a unique gene called T-*urf13* (9), coding for a 13-kD polypeptide (T-URF13 or URF13) that is found only in T mitochondria (11, 15, 28). Several lines of indirect evidence implicate T-URF13 in causing both male sterility and susceptibility to BmT toxin, Pm toxin, and methomyl in T maize (26). Fertile, toxin-resistant mutant plants can be regenerated from T-cytoplasm tissue cultures; the T-*urf13* gene is deleted or mutated in these plants, so that they do not synthesize T-URF13. The nuclear genes *Rf1* and *Rf2* in combination restore fertility to plants with T cytoplasm, and the amount of T-URF13 in mitochondria of these plants is significantly lower than in near-isogenic male-sterile lines. Only the *Rf1* gene is necessary for this suppression of T-URF13 synthesis (11), however, so that a reduction in T-URF13 levels is not sufficient to restore fertility. Also, plants restored to fertility by the action of *Rf1* and *Rf2* are still affected by BmT toxin (3).

Recently, direct evidence that T-URF13 causes sensitivity to toxins and methomyl has been obtained: expression of the T-*urf13* gene in *Escherichia coli* (5, 10) and in yeast (20) confers sensitivity on these organisms. Moreover, studies with *E. coli* cells synthesizing T-URF13 indicate that the fungal toxins bind to this polypeptide (6). Because T-URF13 is evidently responsible for susceptibility to two important diseases and may also have a role in causing male sterility, it is important to learn more about the properties of this protein. In the present report, we describe a method for the purification of T-URF13 from maize mitochondria. Using antibodies to the purified protein, we examine its location in mitochondria

[1] Supported by National Science Foundation grant DCB-8409797, by a grant from Enichem Americas, Inc., Biotechnology Laboratory, and by the Department of Botany and the Graduate College, Iowa State University, Ames.

[2] Present address: Department of Agricultural and Environmental Science, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, England.

[3] Present address: Genetics Program, Michigan State University, East Lansing, MI 48824.

[4] Abbreviations: T, Texas male-sterile (maize cytoplasm); BmT (or HmT) toxin, toxin produced by fungus *Bipolaris* (*Helminthosporium*) *maydis* race T; N, normal (maize cytoplasm); PBST, 10 mM sodium phosphate (pH 7.2), 0.15 M sodium chloride, 0.05% (v/v) Tween 20; Pm toxin, toxin produced by fungus *Phyllosticta maydis*; SP, 0.3 M sucrose, 10 mM potassium phosphate buffer (pH 7.2); T_{Rf}, Texas male-sterile cytoplasm with fertility restored by *Rf1* and *Rf2* nuclear genes; T-*urf13*, T mitochondrial gene coding for T-URF13 polypeptide; T-URF13 (or URF13), 13-kD polypeptide found in T mitochondrial membranes.

and show by immunocytochemistry that it is present in mitochondrial membranes of tapetal cells of T anthers.

MATERIALS AND METHODS

Plant Material

Seeds of the maize lines B73(N) (inbred line B73 with normal cytoplasm) and B73(T) (line B73 with T cytoplasm) were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA); W64A and B37 (N, T, and T_{Rf}) were obtained from Dr. C. A. Martinson (Department of Plant Pathology, Iowa State University). Seeds of B73(N) and B73(T) were also harvested from plants propagated in the field. Mitochondria were isolated either from etiolated seedling shoots or, for T-URF13 purification, unfertilized cobs.

To obtain etiolated seedlings, seeds were surface-sterilized with 1% (w/v) sodium hypochlorite for 10 min, rinsed several times with tap water, and imbibed overnight in running tap water. Seedlings were grown for 3 to 4 d in vermiculite (watered with tap water), in loosely covered trays in the dark at 30°C. Immature, unfertilized cobs weighing less than 30 g were obtained from field-grown B73(T) plants. To obtain anthers at different stages of development, B73(N) and B73(T) plants were grown either in the field or in the greenhouse under long days.

Mitochondria

For purification of T-URF13, mitochondria were isolated (21) from etiolated shoots or unfertilized cobs of field-grown plants by differential and sucrose density gradient centrifugation. For fractionation, mitochondria were isolated from etiolated shoots and self-generating 30% (v/v) Percoll (Pharmacia) gradients were used instead of sucrose gradients. Gradients were run as described (21), except that the centrifugation was for 90 min at $35{,}000g_{max}$ in a Sorvall SS-34 rotor. This long centrifugation improved the resolution of mitochondria with the batch of Percoll used.

Purification of T-URF13

T-URF13 was isolated from purified T mitochondria by gel filtration and a quasi two-dimensional PAGE system. A similar mock preparation from normal mitochondria was performed to help in identifying T-URF13 at each step in the purification.

Gel Filtration

Low molecular-mass proteins of mitochondria were first isolated by gel filtration chromatography on Sephacryl S-200 in the presence of SDS in order to reduce the amount of protein to be fractionated subsequently. Mitochondria (10–30 mg protein) were lysed by incubation in 10% (w/v) SDS and 5 mM DTT at 100°C for 2 min. The lysate was applied at a flow rate of 45 mL h^{-1} to a 2.5 × 120 cm column of Sephacryl S-200 (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1% (w/v) SDS. Proteins were eluted with the same buffer at a flow rate of 10 mL h^{-1}. Aliquots of fractions eluting between 160 and 280 mL were analyzed by electrophoresis on 15% SDS-polyacrylamide gels. Fractions containing low-relative-molecular-mass (low M_r) proteins (M_r about 5,000 to 20,000) were pooled and concentrated by ultrafiltration (Diaflo YM10 membrane, Amicon).

Preparative Gel Electrophoresis

The concentrate containing low-M_r proteins was fractionated by electrophoresis on 27-cm-long, 12 to 18% linear gradient SDS-polyacrylamide gels. The best resolution of T-URF13 was obtained when the gels were run in a refrigerator at 4°C and the bromophenol blue marker dye was allowed to run off the end of the gel (about 16 h at 18 mA for 14 cm wide × 1.5 mm thick gels). At this stage, T-URF13 was observable by staining with brilliant blue R (C.I. 42660) (25) as a prominent band that was not present in the low-M_r proteins of N mitochondria fractionated by the same procedure. The region of the gel containing T-URF13 was excised. Gel slices were squashed and fragmented in elution buffer (35 mM Tris-HCl [pH 8], 0.5% [w/v] SDS, 5 mM DTT). The homogenate was stirred at room temperature for approximately 24 h, during which time the elution buffer was changed twice, then the acrylamide pieces were removed by brief centrifugation. Diffusion from squashed slices allowed easy handling of large amounts of gel slices and gave much better recovery of T-URF13 (>60%) than electrophoretic elution. The solution containing eluted protein was concentrated by ultrafiltration, and the protein was precipitated by addition of 5 volumes of cold acetone (−20°C).

T-URF13 was further purified by urea-SDS gel electrophoresis in phosphate buffer (2). The separation gel (11.5 cm long × 1 cm wide × 1.5 mm thick) contained 6 M urea, 0.1% (w/v) SDS, 0.1 M sodium phosphate (pH 7.3), 0.4% (w/v) bis, and 15% (w/v) acrylamide. A 3% (w/v) acrylamide stacking gel (2 cm high) was formed on top of the separation gel using the same buffer. The electrophoresis buffer was 0.1 M sodium phosphate (pH 7.3), 0.1% (w/v) SDS. Protein samples were dissolved in sample buffer (6 M urea, 0.1% [w/v] SDS, 0.1% [v/v] 2-mercaptoethanol, 0.1 M sodium phosphate [pH 7.3], and 0.05% [w/v] bromophenol blue). Electrophoresis was at 250 V for 6 h in a water-cooled electrophoresis unit (Hoefer). After electrophoresis, the gel was stained briefly with brilliant blue R and then washed with deionized water. The T-URF13 polypeptide was recovered from the gel by excision, elution, ultrafiltration, and acetone precipitation in the same way as after gradient gel electrophoresis.

Preparation of Antibodies

Purified and denatured T-URF13 was prepared as described above, and the protein pellet (acetone precipitate) was redissolved in 0.2% (w/v) SDS, 10 mM sodium phosphate (pH 7.3), 0.14 M NaCl. Two rabbits received an initial subcutaneous injection of approximately 80 μg protein in 1 mL emulsified with 1 mL Freund's complete adjuvant. Each rabbit was boosted with about 80 μg T-URF13 emulsified with Freund's incomplete adjuvant 6 weeks after the initial injection, and again with about 100 μg T-URF13 4 weeks after the first boost. For the experiments described here, antisera were obtained 1 to 3 weeks after the second boost

and used directly without further purification. The specificity and activity of antisera obtained from the two rabbits immunized with T-URF13 were similar.

Mitochondrial Fractionation

A modification of the method of Mannella (22) was used to isolate mitochondrial fractions enriched in matrix, outer membrane, and inner membrane. Isolated mitochondria were resuspended to a protein concentration of approximately 40 mg mL^{-1} in SP buffer, and swollen by dilution with 49 volumes 4.2 mM sucrose to give a sucrose concentration of 10 mM. The mitochondrial suspension was stirred on ice for about 15 min, then 0.17 volume of 2 M sucrose was added, giving a concentration of 0.3 M, and the suspension was stirred further for about 15 min. Mitoplasts (mitochondria whose outer membranes have been stripped off) were collected by centrifugation at 27,000g_{max} for 12 min in a Beckman JA-17 rotor and crude outer membranes collected from the supernatant by centrifugation at 230,000g_{max} for 90 min in a Beckman 70.1 Ti rotor. The outer membrane pellet was suspended in SP buffer and layered on a 0.6 to 0.9 M sucrose step gradient in 10 mM potassium phosphate (pH 7.2) (1.5 mL per layer). The gradient was centrifuged at 54,000g_{max} for 70 min in a Beckman SW 55 Ti rotor. The material in the 0.6 M layer and at the 0.6 to 0.9 M interface was collected and diluted with 2 volumes 10 mM potassium phosphate (pH 7.2). PMSF was added to 1 mM, and the suspension centrifuged at 230,000g_{max} for 70 min. The pellet was suspended in SP buffer to give the outer membrane fraction (protein concentration 1.7 mg mL^{-1}).

The mitoplast fraction was suspended in SP buffer to the same volume as the initial mitochondrial suspension and stored at −75°C. A 0.1-mL portion of this suspension was thawed, diluted fivefold with SP buffer, adjusted to 1 mM PMSF, and then sonicated with 6 × 5-s bursts at 25-s intervals, with a Bronwill Biosonik sonicator at 70% full power; the tube was cooled in an ice-ethanol bath. Unbroken mitochondria and aggregated material were removed by centrifugation for 10 min in a micro centrifuge at 15,600g_{max}, then the supernatant was centrifuged at 230,000g_{max} for 70 min to give matrix (soluble) and inner membrane (pellet) fractions. The pellet was suspended in 0.25 mL SP buffer.

To resolve peripheral (with contaminating soluble) and integral membrane proteins, the inner membrane fraction was treated with 0.1 M sodium carbonate (17) or subjected to phase fractionation with Triton X-114 (7). For sodium carbonate treatment, an inner membrane pellet was suspended to a protein concentration of 0.5 mg mL^{-1} in 0.1 M sodium carbonate. The suspension was incubated in ice for about 40 min and centrifuged at 230,000g_{max} for 60 min to give solubilized and insoluble fractions. Triton X-114 phase fractionation was based on the method of Bricker and Sherman (7) for chloroplast membranes. An inner membrane pellet was suspended to 2 mg mL^{-1} in 10 mM Tris-HCl (pH 7.2), 150 mM sodium chloride, 1 mM PMSF, 5 mM *p*-aminobenzamidine·$(HCl)_2$ neutralized with 5 mM KOH. To the suspension was added 0.1 volume of precondensed 10.9% (v/v) Triton X-114. The solution was incubated for 30 min on ice and centrifuged for 4 min in an Eppendorf microcentrifuge at 15,600g_{max} to remove insoluble material. The supernatant was incubated for 5 min at 37°C, then centrifuged for 5 min in a micro centrifuge swing-out rotor at room temperature to separate the phases. The upper phase was removed and 0.1 volume 10.9% (v/v) Triton X-114 was added to it. The lower phase, which included precipitated protein, was brought back to the starting volume with the original buffer. The phase separation was repeated for both phases, and the wash phase from the second separation was discarded.

Protein Synthesis by Isolated Mitochondria

Isolated mitochondria were allowed to incorporate [^{35}S] methionine into protein (21). Immunoprecipitation was carried out essentially as described by Anderson and Blobel (1) except that fixed cells of *Staphylococcus aureus* strain Cowan 1 were used instead of protein A-Sepharose to collect immune complexes.

Immunoblotting

Proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose. Antibody binding was detected either with [^{125}I]protein A (27) or with alkaline-phosphatase-coupled anti-rabbit or anti-mouse IgG (4). With alkaline-phosphatase-coupled second antibody, nitrocellulose was blocked with PBST containing 1% (w/v) BSA, incubated with antibody in PBST, washed twice with PBST, incubated with second antibody in PBST, and washed twice with PBST. To detect alkaline phosphatase activity, filters were washed with 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$, then incubated in the same buffer containing 0.08 mg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate and 0.16 mg mL^{-1} nitro blue tetrazolium. To stop the reaction, filters were washed twice with PBST.

Enzyme Assays

Antimycin-A sensitive and insensitive NADH-Cyt *c* oxidoreductases were assayed as described by Douce *et al.* (12).

Microscopy

Roots from 3- to 4-d-old seedlings were rinsed with tap water, and the terminal 5 mm of each tip was excised with a razor blade. Anthers were excised from developing florets. One of the three anthers in each floret was squashed in a drop of water; a coverslip was applied, and the anthers were viewed with phase contrast microscopy to determine the stage of microsporogenesis (meiocyte through microspore stages). The remaining two anthers were cut through the connective tissue between the pairs of adjacent locules with a clean razor blade to aid in fixative penetration.

The roots and anthers were immediately immersed in 5% glutaraldehyde/4% paraformaldehyde in a 0.05 M sodium cacodylate buffer at pH 7.2, with added 0.05% calcium chloride. Fixation was carried out for 2 h at 20°C. Mitochondria isolated from shoots by density gradient centrifugation were suspended in 2% glutaraldehyde/1% paraformaldehyde in a 0.1 M sodium/potassium phosphate buffer at pH 7.2 for 60 min at 4°C. After fixation, the mitochondria were centrifuged

at 14,000g_{max} for 6 min at 4°C. Pellet fragments were quickly embedded in 1.5% agar. The agar containing the fragments was cut into small blocks. All three materials (roots, anthers, and pellets) were rinsed in their respective buffers three times, 5 min each, and dehydrated in a graded ethanol series (25%, v/v, at 0°C, 50, 75, 85, and 95%, v/v, at −20°C), 1 h for each step. The dehydrated materials were infiltrated with Lowicryl K 4M resin containing 86.1% monomer, 13.4% cross-linker, and 0.5% initiator. Infiltration was performed in the dark at −20°C in a 1:3 (v/v) mixture of ethanol and resin for 8 h, then for 16 h in the pure resin (two changes). Samples were loaded into Beem capsules, filled with fresh resin, and capped to preclude contact with air. The capsulated material was polymerized by illumination with long-wavelength ultraviolet light for 24 h at −20°C in a cryostat unit, followed by ultraviolet light for 48 h at room temperature. The resin blocks were stored in capped vials in a desiccator to avoid hygroscopic softening before sectioning.

Thin sections of roots, anthers, and mitochondria were all cut onto double-distilled water with a diamond knife. Sections were picked up on 300-mesh nickel grids that had been previously coated with a film made from 0.075% Formvar (w/v) dissolved in 1,2-dichloroethane. This film was coated with a thin layer of carbon.

Immunolabeling

All incubation and wash steps were carried out on Parafilm on top of a wet filter paper in a Petri dish at room temperature. The grids, with section side down, were floated on drops of the solutions placed on the Parafilm. The sections were preincubated for 10 min with 10% SDS to improve antigen-antibody interactions, since the antibodies were prepared against SDS-denatured T-URF13. The sections were rinsed in washing solution (0.01 M sodium/potassium phosphate buffer [pH 7.4], 0.15 M sodium chloride, 0.02% polyethylene glycol 20, and 0.02% sodium azide) three times for 5 min each. Sections were then transferred to normal (preimmune) rabbit serum (Sigma) diluted 1:20 with washing solution for 30 min. Following five rinses, 2 min each, the grids were incubated in protein A (Sigma) diluted 1:1 with washing solution for 30 min, and rinsed with washing solution. Sections were then incubated for 45 min in rabbit anti-T-URF13 antiserum diluted 1:3,600 with washing solution and rinsed as described. The treatments with normal rabbit serum and protein A were expected to decrease nonspecific binding. Also, the antiserum against T-URF13 was preincubated with small pieces of nitrocellulose filters that had been adsorbed with total normal mitochondrial protein, for one week at 4°C, for the same purpose.

Sections were placed on a drop of protein-A-gold (20 nm) solution (Bioclin; diluted 1:20 with washing solution) for 1.5 h and rinsed thoroughly (six times for 2 min each) with double-deionized water. After drying in air, the sections were double stained with uranyl acetate and lead citrate. In order to obtain statistically valid data, more than 27 field views were observed for each labeling experiment and stage using a transmission electron microscope. More than 10 micrographs were made for analysis for each stage of microsporogenesis.

RESULTS

Purification of T-URF13

The T-URF13 protein has no known enzymatic activity. It is associated with membranes (11), but whether it interacts with other membrane proteins is unknown. Therefore, we purified T-URF13 from mitochondrial proteins solubilized and denatured with SDS. This was possible because the protein is specific to T mitochondria, so that it can be identified by comparison of patterns of proteins obtained from T and N mitochondria. When products of mitochondrial protein synthesis are analyzed by SDS-PAGE and autoradiography, T-URF13 is clearly detectable by autoradiography in T mitochondria but not in N mitochondria (Fig. 1B; 15). Provided that the time of electrophoresis is long enough (about 16 h at 18 mA for a 27-cm long 12–18% polyacrylamide gradient gel), T-URF13 can be distinguished among proteins stained with brilliant blue R as a band specific to T mitochondria (Fig. 1A; 14).

To eliminate contaminating polypeptides that coincidentally migrated with T-URF13 in the SDS-polyacrylamide gradient gel electrophoresis, a further purification step was necessary. Neither isoelectric focusing nor urea gels with the

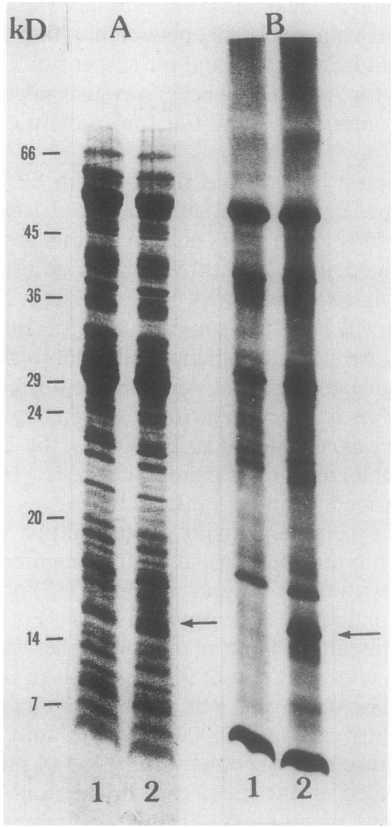

Figure 1. Total mitochondrial proteins and products of protein synthesis by isolated mitochondria from B73(N) and B73(T) maize. Total mitochondrial proteins (100 μg containing 4 × 10^5 cpm in each lane) were fractionated in a 12 to 18% SDS-polyacrylamide gradient gel. Arrow indicates T-URF13. A, Stained proteins; B, autoradiograph of same lanes shown in A. Lanes 1, N proteins; lanes 2, T proteins.

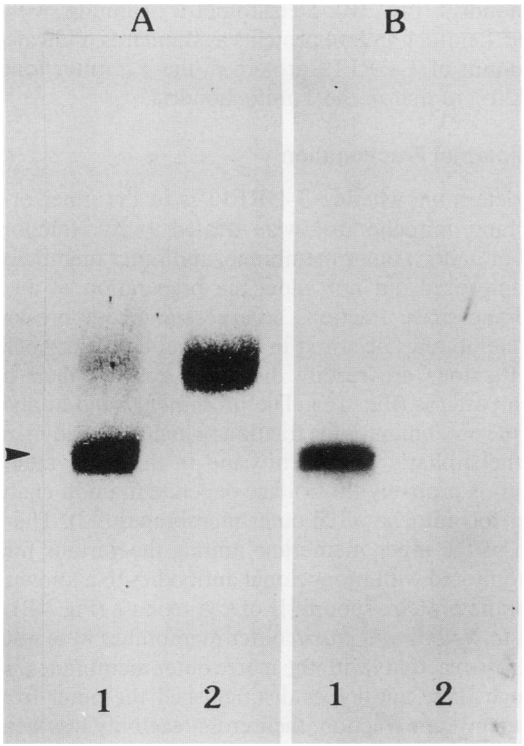

Figure 2. Purification of T-URF13 by SDS-urea polyacrylamide gel electrophoresis. Proteins, purified in 12 to 18% SDS-polyacrylamide gels from mitochondria that had been allowed to carry out protein synthesis with [^{35}S]methionine, were fractionated in an SDS-urea gel. A, Stained proteins; B, autoradiograph of same lanes shown in A. Lanes 1, proteins from approximately 1 mg T mitochondrial protein; lanes 2, proteins from approximately 8 mg N mitochondrial protein. Specific activities of the total T and N mitochondrial proteins were similar.

Laemmli buffer system (19) gave a distinct spot or band of T-URF13 (not shown). A urea-SDS-polyacrylamide gel with a phosphate buffer system (2), however, cleanly separated T-URF13 from contaminants. The preparation of T-URF13 recovered from the gradient gel was separated into two predominant bands in the urea-SDS-polyacrylamide gel system (Fig. 2A). The lower of these two bands was T-URF13, since a mock preparation of normal mitochondrial proteins contained the upper band but not the lower one (Fig. 2A), and only the lower band was radioactively labeled when T-URF13 was isolated from mitochondria that had been allowed to carry out protein synthesis with [^{35}S]methionine (Fig. 2B).

The amount of T-URF13 was estimated in gels stained with brilliant blue R by comparing the density of the blue color of the T-URF13 band with that of a known amount of a molecular mass standard of similar size, α-lactalbumin (14 kD). As estimated by this procedure, about 0.1 mg of T-URF13 could be isolated from 100 mg of mitochondrial protein (corresponding to about 1 kg fresh weight of tissue).

Antibodies to T-URF13

When proteins from N, T, and T_{Rf} mitochondria were resolved by SDS-PAGE, blotted to nitrocellulose, and incubated with antiserum to T-URF13, a single band was detected in T and T_{Rf} mitochondrial protein; no bands were detected in N mitochondrial protein (Fig. 3A). Only one major polypeptide was immunoprecipitated by anti-T-URF13 from total products of protein synthesis by isolated T or T_{Rf} mitochondria, and no similar protein was immunoprecipitated from the products of protein synthesis by normal mitochondria (Fig. 3B). Complete immunoprecipitation of T-URF13 required approximately 1 μL anti-T-URF13 antiserum for 20 μg total mitochondrial protein (not shown).

Consistent with previous observations (11, 14), less T-URF13 protein was present in mitochondria of T_{Rf} maize than T maize (Fig. 3A), and less was synthesized by isolated

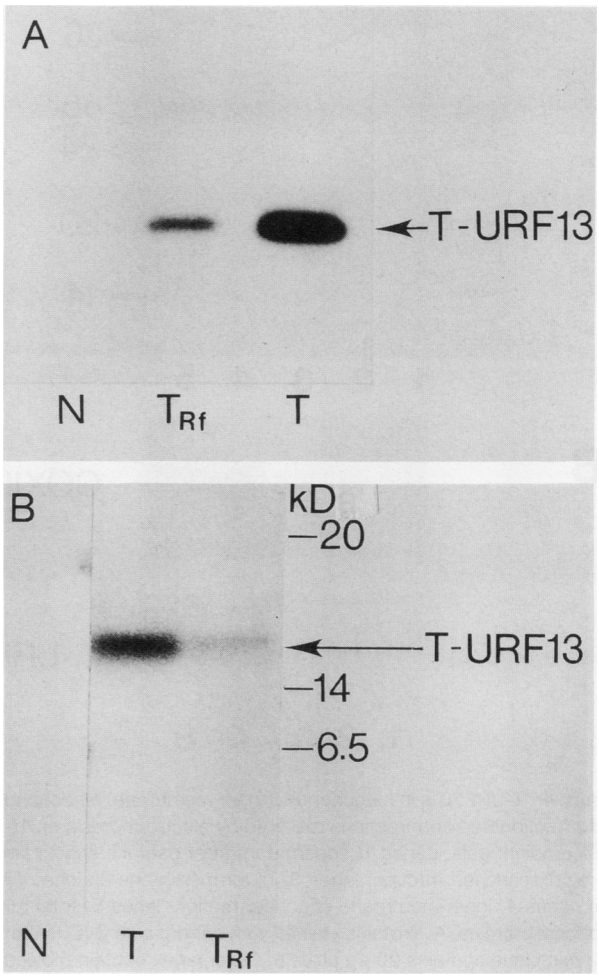

Figure 3. Detection of T-URF13 in mitochondrial protein and in products of mitochondrial protein synthesis [B37(N), B37(T), and B37(T_{Rf})]. A, Immunoblot of total mitochondrial protein. Proteins (30 μg) from N, T_{Rf}, and T mitochondria were isolated, resolved by electrophoresis in a 15% acrylamide minigel, transferred to nitrocellulose, and incubated with antibodies to T-URF13, diluted 1:1,000, and with ^{125}I-protein A; location of binding was detected by autoradiography. B, Labeled proteins immunoprecipitated by antibody to T-URF13 from products of protein synthesis by isolated mitochondria. N immunoprecipitate was from 1×10^5 cpm, T, T_{Rf} immunoprecipitates from 1.7×10^5 cpm; all lanes are from a single exposure of a single autoradiograph.

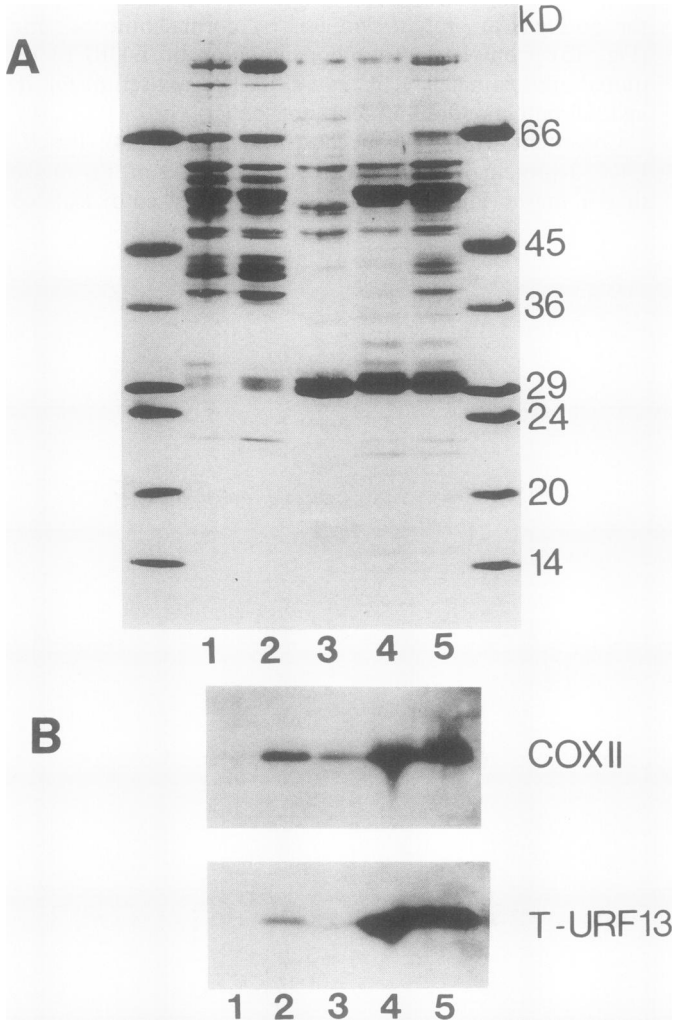

Figure 4. T-URF13 is in mitochondrial inner membrane. Mitochondria were fractionated and fractions resolved by electrophoresis on 10 to 15% gradient gels. Lanes 1, supernatant after osmotic shock; lanes 2, matrix-enriched fraction; lanes 3, outer-membrane-enriched fraction; lanes 4, inner-membrane-enriched fraction; lanes 5, total mitochondrial proteins. A, Proteins stained with brilliant blue G (C.I. 42655; 24); each lane contains 20 μg protein. Outer lanes contain molecular mass markers (Sigma). B, Proteins were blotted to nitrocellulose, and strips containing proteins of appropriate size were cut out and probed with antibodies to yeast Cyt oxidase subunit II, diluted 1:125, (COXII) or to T-URF13, diluted 1:10,000; each lane contains 10 μg protein. Antibody binding was detected by incubation with alkaline-phosphatase-conjugated second antibody and then with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

mitochondria (Fig. 3B). Densitometric scanning, with dilutions of T mitochondrial proteins as standards, indicated that the amount of T-URF13 protein in the T_{Rf} mitochondria is about 20% of that in the T mitochondria.

Mitochondrial Fractionation

To determine whether T-URF13 is in the inner or outer membrane, mitochondria were treated to give fractions enriched in matrix, outer membrane, and inner membrane; the procedure used did not allow the preparation of an intermembrane space fraction, because significant breakage of inner membranes occurred in the initial osmotic shock. The three fractions are readily distinguishable by their overall protein patterns (Fig. 4A). The prominent band at about 29 kD in the outer membrane fraction (which differs in migration from the similarly prominent band in the inner membrane fraction) is probably the voltage-dependent anion channel, a marker for mitochondrial outer membranes (22). The distribution of the inner membrane among the various fractions was monitored with monoclonal antibodies to a known inner membrane protein, subunit II of Cyt oxidase (Fig. 4B). Antibodies to *Neurospora crassa* outer membranes were tested for their cross-reactivity with the maize outer membrane fraction. Although these antibodies distinguished the outer from the inner membrane fraction, their cross-reactivity was weak and was comparable with their reactivity with a molecular mass standard (not shown). Therefore, the activities of antimycin-A-sensitive and antimycin-A-insensitive NADH-Cyt *c* oxidoreductase were used to confirm the identity of the outer membrane fraction and estimate the cross-contamination between outer and inner membrane fractions; the antimycin-sensitive activity marks the inner membrane and the antimycin-insensitive the outer membrane (12, 22). The antimycin-insensitive specific activity was 5.8-fold higher in the outer membrane fraction than in the inner membrane fraction (Table I); this value probably underestimates the degree of purification of the outer membrane, since the inner membrane fraction also has antimycin-insensitive activity once broken (22). The distribution of T-URF13 parallels that of Cyt oxidase subunit II, and there is no enrichment of it in the outer membrane fraction (Fig. 4B). Thus, T-URF13 is located in the inner mitochondrial membrane.

Resolution of Integral and Peripheral Membrane Proteins

In order to determine whether T-URF13 is an integral or a peripheral membrane protein, the inner membrane fraction

Table I. *Activities of Antimycin-A-Sensitive and Antimycin-A-Insensitve NADH:Cyt c Oxidoreductase in Inner and Outer Membrane Fractions from Maize Mitochondria*

Values are the means of three or four measurements.

	Sensitive	Insensitive	Insensitive/ Sensitive
	nmol s^{-1} mg^{-1}		
Inner membrane fraction	18.3	1.5	0.080
Outer membrane fraction	1.9	8.6	4.6

was partially solubilized with 0.1 M Na_2CO_3; the mitochondrial membrane lipid bilayer and integral membrane proteins within it are insoluble in this reagent (17). The protein patterns of the soluble and insoluble fractions were quite distinct (Fig. 5A, lanes 4 and 5). Cyt oxidase subunit II, which is believed to have a highly conserved structure containing two membrane-spanning helices (8, 16), was found only in the sodium-carbonate-insoluble fraction. In contrast, the β-subunit of ATP synthase, which is a component of the peripheral catalytic (F1) portion of this enzyme (23), was almost entirely in the soluble fraction (Fig. 5B). Like Cyt oxidase subunit II, T-URF13 was completely insoluble in sodium carbonate (Fig. 5B).

An alternative to sodium carbonate for separating integral and peripheral membrane proteins is phase fractionation with Triton X-114 (7). Solutions of this detergent separate into lower detergent-rich and upper detergent-poor phases at 30°C. Peripheral membrane proteins are predominantly found in the upper phase and integral membrane proteins in the lower phase. When the maize mitochondrial inner membrane fraction was subjected to phase partitioning, the overall patterns of proteins in the two phases (Fig. 5A, lanes 2 and 3) were similar to those in the two corresponding fractions produced by sodium carbonate treatment. Cyt oxidase subunit II partitioned to the lower phase, and ATP synthase β-subunit to the upper (Fig. 5B). T-URF13 partitioned to the lower phase. Thus, T-URF13 behaves as an integral membrane protein when membranes are fractionated with Triton X-114 as well as with sodium carbonate.

Detection of T-URF13 by Immunoelectron Microscopy

To investigate the subcellular location of T-URF13 directly and to look for this protein in specific cells during plant development, T-URF13 was localized in tissue sections by immunoelectron microscopy with the anti-T-URF13 antibodies and protein A-gold. Root and shoot materials were about the same ages, whereas anther material spanned four stages of development (meiocyte, dyad, tetrad, and microspore) encompassing the stages during which changes leading to male cell abortion take place in the B73(T) line. The quality of fixation of root and anther materials by both conventional and antigenicity-preserving procedures was monitored by both light and transmission electron microscopy; isolated mitochondria were observed solely by electron microscopy.

The specificity of the anti-T-URF13 antiserum in the immunolabeling protocol was checked in roots of seedlings and isolated mitochondria from etiolated shoots of N and T maize. Under the optimal conditions outlined in "Materials and Methods," both root mitochondria (Fig. 6A) and isolated shoot mitochondria (Fig. 6B) from B73(T) seedlings were specifically labeled. Generally, one to several gold particles could be seen per mitochondrial section. There was little to no background label. The N mitochondria from the same sources did not show any label (not shown).

Mitochondria were labeled in T anther tissues from all stages observed. The tapetal tissue was of primary interest in this study and its mitochondria (meiocyte, Fig. 6C; dyad, Fig. 6D; tetrad, Fig. 6E; early-microspore, Fig. 6F; midmicrospore, Fig. 6G; and late-microspore, Fig. 6H) were labeled similarly

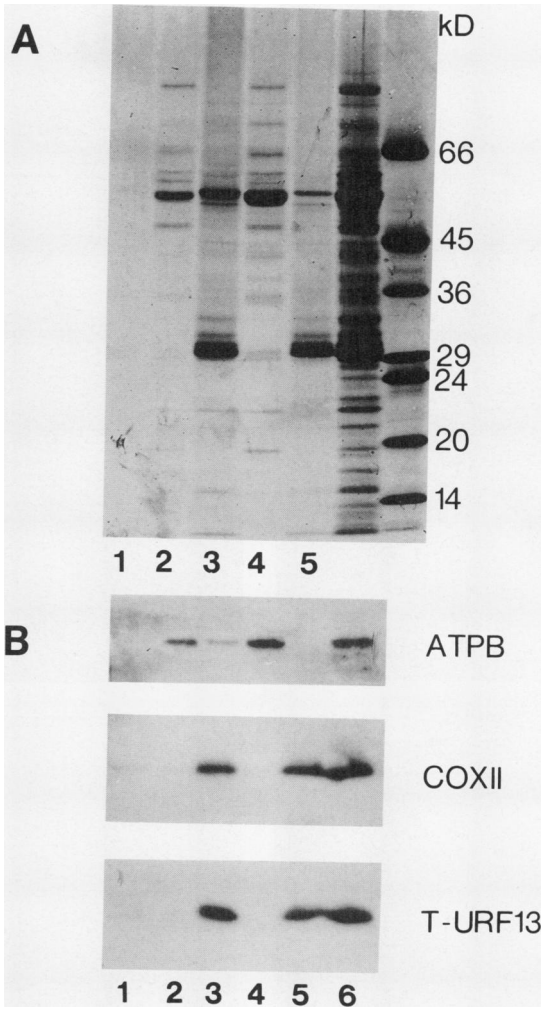

Figure 5. T-URF13 is an integral membrane protein. Inner membrane fraction was fractionated by phase partitioning with Triton X-114 or by treatment with 0.1 M sodium carbonate, and fractions were resolved by electrophoresis in 10 to 15% gradient gels. Lanes 1, Triton X-114 fractionation, insoluble fraction; lanes 2, Triton X-114 fractionation, upper phase; lanes 3, Triton X-114 fractionation, lower phase; lanes 4, sodium carbonate fractionation, soluble proteins; lanes 5, sodium carbonate fractionation, insoluble proteins; lanes 6, total inner membrane fraction. A, Proteins stained as in Figure 4; each lane contains protein derived from 20 μg of inner membrane fraction. Right-side lane contains molecular mass markers. B, Proteins were blotted to nitrocellulose, and strips containing proteins of appropriate size were cut out and probed with antibodies to yeast mitochondrial F1-ATPase, diluted 1:5,000 (ATPB), yeast Cyt oxidase subunit II, diluted 1:125, (COXII), or T-URF13, diluted 1:10,000; each lane contains protein derived from 10 μg of inner membrane fraction. Antibody to F1-ATPase reacts predominantly with β-subunit (not shown). Proteins were detected as in Figure 4.

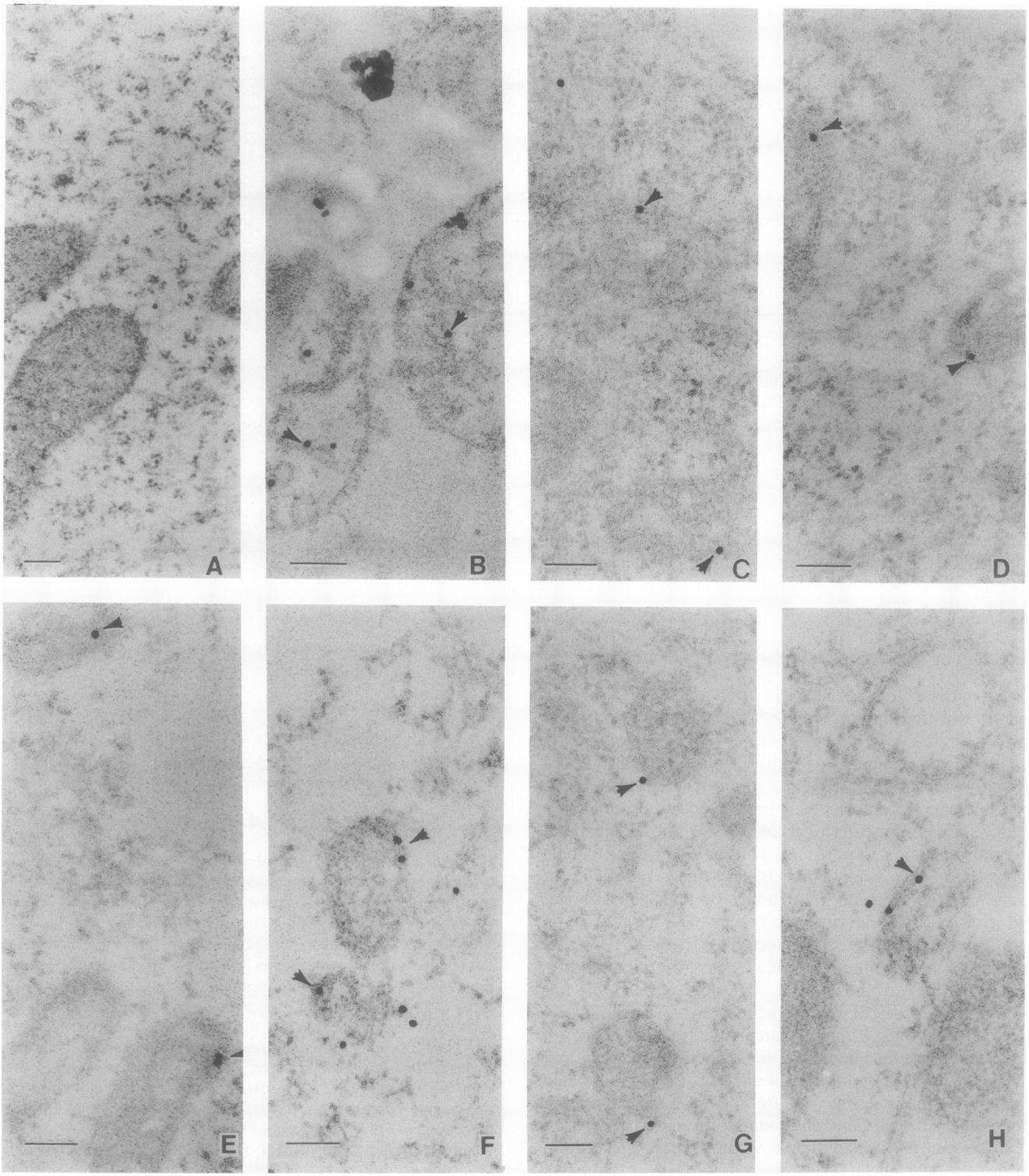

Figure 6. Electron microscopic immunocytochemical localization using 1:3,600 diluted anti-T-URF13 tagged with 20-nm-gold particles on T mitochondria in maize roots and anther tapetal cells, and isolated mitochondria from coleoptiles; bars = 200 nm. A, Root cortical mitochondria with gold label at periphery of mitochondria; B, isolated coleoptile mitochondria with gold label associated with cristae and boundary membranes; C, labeled tapetal mitochondria at meiocyte stage; D, labeled tapetal mitochondria at dyad stage; E, labeled tapetal mitochondria at tetrad stage; F, labeled tapetal mitochondria at early microspore stage; G, labeled tapetal mitochondria at midmicrospore stage; H, labeled tapetal mitochondria at late-microspore stage.

to the other tissues. One to three gold particles per mitochondrial section were observed throughout all of the stages examined, slightly fewer than for the T mitochondria from roots and coleoptiles. There was no labeling of mitochondria at any stage in N anthers, or in the other N tissues.

Tissues used for immunolabeling were not postfixed with osmium tetroxide because of potential interference with the antibody-antigen binding reaction (18). This omission caused a reduction in the general contrast in the sectioned materials, making it difficult to define organelle membranes clearly. Therefore, it was difficult to determine whether gold particles at the periphery of the mitochondria were associated with the inner or outer mitochondrial membrane or both. Some particles were definitely associated with the cristae membrane. There did not appear to be any localization in the mitochondrial matrix.

DISCUSSION

The combination of gel filtration with electrophoresis of denatured proteins provides an effective means of purifying the T-URF13 protein and avoids the association with other membrane components that could occur with a nondenaturing procedure. The initial gel filtration step removes the majority of mitochondrial proteins, whose molecular masses are greater than 20 kD, and thereby substantially increases the amount of small proteins that can be loaded onto a single preparative polyacrylamide gel. The capacity of the gradient gels is the principal limitation to the capacity of the purification procedure; running gels thicker than 1.5 mm greatly reduced resolution. Despite its excellent resolution, the gradient gel does not separate T-URF13 from all other proteins. The SDS-urea gel has poorer resolution, but complements the gradient gel. Although both electrophoretic systems separate proteins primarily by molecular mass, the presence or absence of urea and the different buffer systems evidently change the relative migrations of different proteins enough to separate T-URF13 from almost all contaminants, so that the final preparation is pure enough to yield monospecific antibodies when injected into rabbits.

The apparent molecular mass of T-URF13 was reported to be 13 kD by Forde *et al.* (15), and the open reading frame of the gene coding for T-URF13 (T-*urf13*) codes for a polypeptide of molecular mass 12,961 kD (9). In all the gel systems used in this study, the apparent molecular mass of T-URF13 was approximately 14.5 kD. Newton and Walbot (24) also found that T-URF13 migrated in a 12 to 18% SDS-polyacrylamide gradient gel at 14 to 15 kD rather than 13 kD. The consistent differences in the apparent molecular mass of T-URF13 reported by different research groups could be due to the use of different marker proteins, while the difference between the theoretical and observed molecular masses probably reflects the unreliability of SDS-PAGE for measuring the molecular mass of membrane proteins.

The differences between the proteins of plant mitochondrial inner and outer membranes have received considerable attention (22), but the distribution of inner membrane proteins between integral and peripheral constituents has been studied less. Our results are of general significance in showing that two very different methods of separating integral and peripheral proteins, Triton X-114 phase fractionation and sodium carbonate fractionation, give similar distributions of proteins between the corresponding fractions. In agreement with the results of Fujiki *et al.* (17) for animal mitochondria, the peripheral protein fraction contains a higher proportion of large proteins than the integral protein fraction. There is some resemblance between the polypeptide profiles of the peripheral membrane protein fraction and the matrix fraction, which is also enriched in large polypeptides. This resemblance is probably both because some peripheral membrane proteins are released from the membrane into the matrix fraction when mitochondria are sonicated and because some matrix proteins are trapped in the inner membrane vesicles. Since these trapped proteins are released when the inner membrane is fractionated, the integral protein fraction should be purer than the peripheral protein fraction.

That T-URF13 is located in the inner mitochondrial membrane and is an integral membrane protein is typical of mitochondrial gene products, even though the T-URF13 open reading frame probably evolved by recombination of segments of the mitochondrial genome that normally do not code for proteins (9). These properties help to explain its known biological activity. T-URF13 confers sensitivity to fungal toxins (BmT and Pm) and to methomyl when the gene coding for it is expressed in *E. coli* (5, 10), and also confers sensitivity on yeast cells, provided that it is targeted to mitochondria (20). Both toxins and methomyl dissipate the membrane potential of mitochondria and cause leakage of ions. The simplest explanation of their effects is that they bind to the T-URF13 protein and the combination forms a channel through the inner membrane. This hypothesis is consistent with the properties of T-URF13 that we report here. It is also supported by the results of Braun *et al.* (6), who provided direct evidence for binding of toxins to T-URF13 protein.

Our results indicate that the amount of T-URF13 protein in T_{Rf} mitochondria is approximately fivefold less than in T mitochondria, but Braun *et al.* (6) reported that the binding of toxins by T_{Rf} mitochondria was only about 25% less than the binding by T mitochondria. The same inbred line (B37) was used in our experiment as in those of Braun *et al.* (6). Perhaps more of the protein in T_{Rf} mitochondria than in T mitochondria is able to bind toxin; Braun *et al.* (6) proposed an analogous explanation for the discrepancy they observed between binding and protein levels in *E. coli* compared to T maize mitochondria.

That T-URF13 is an integral membrane protein is consistent with its predicted amino acid sequence (9, 11): the protein has a highly hydrophobic amino terminus long enough to form a single membrane-spanning helix. The hydrophobic moment prediction algorithm of Eisenberg *et al.* (13) can be used to predict associations between membrane-spanning helices. By the criteria of Eisenberg *et al.* (13), the amino terminal membrane-spanning helix of T-URF13 has the combination of amphiphilicity and hydrophobicity characteristic of helices in oligomeric membrane proteins (using the terminology of Eisenberg *et al.*, the maximum value of the mean hydrophobic moment per residue for an 11-residue segment of the predicted membrane-spanning helix is 0.356 and the corresponding mean hydrophobicity is 0.556). Thus, T-URF13 may form oligomers. Preliminary evidence for the existence of oligomers

comes from the observation of a small amount of an anomalously migrating aggregate, probably a dimer, in heavily exposed immuno blots of T mitochondrial protein (20). Moreover, purified T-URF13 protein associates with itself even when stored in SDS (our unpublished data). The existence of such oligomers in mitochondrial membranes would make the formation of a membrane channel easier to explain.

Although it is now clear that T-URF13 is the receptor for BmT and Pm toxins, its role in causing sterility remains uncertain. Our results provide direct evidence that the protein is present in mitochondria of tapetal cells, even after the mitochondria have begun to degenerate. In *Petunia,* as in T maize, a chimeric gene has been implicated in causing sterility. Expression of this gene, S-*pcf,* is greatest in anthers (29), and it is possible that accumulation of an excessive amount of the abnormal protein is responsible for sterility. No increase in the amount of T-URF13 during T microsporogenesis was detectable in our experiments, but more precise measurements will be necessary to determine conclusively whether differential accumulation of T-URF13 occurs.

ACKNOWLEDGMENTS

The microscopic aspects of this study were carried out in the Bessey Microscopy Facility. We thank Dr. Carol A. Hendrick for carrying out preliminary experiments, Dr. C. R. Stewart for helpful discussions, Sharon Thoma and Azadeh Khojasteh for excellent technical assistance, and Diane McDonald for maintenance of rabbits and help in preparing antisera to T-URF13. Antibodies to yeast Cyt oxidase subunit II were kindly provided by T. Mason, University of Massachusetts, to yeast F1-ATPase by G. Schatz, University of Basel, and to *Neurospora* outer membranes by C. A. Mannella, New York State Department of Health. The mitochondrial fractionation by osmotic shock was based on an unpublished procedure developed by M. Eilers, E. H., and C. J. Leaver at the University of Edinburgh.

LITERATURE CITED

1. **Anderson DJ, Blobel G** (1983) Immunoprecipitation of proteins from cell-free translations. Methods Enzymol **96:** 111–120
2. **Anonymous** (1984) Procedure for gel electrophoresis of low molecular weight proteins. Focus **6(3):** 5
3. **Barratt DHP, Flavell RB** (1975) Alterations in mitochondria associated with cytoplasmic and nuclear genes concerned with male sterility in maize. Theor Appl Genet **45:** 315–321
4. **Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC** (1984) A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Anal Biochem **136:** 175–179
5. **Braun CJ, Siedow JN, Williams ME, Levings III CS** (1989) Mutations in the maize mitochondrial T-*urf13* gene eliminate sensitivity to a fungal pathotoxin. Proc Natl Acad Sci USA **86:** 4435–4439
6. **Braun CJ, Siedow JN, Levings III CS** (1990) Fungal toxins bind to the URF13 protein in maize mitochondria and *Escherichia coli.* Plant Cell **2:** 153–161
7. **Bricker TM, Sherman LA** (1982) Triton X-114 phase-fractionation of maize thylakoid membranes in the investigation of thylakoid protein topology. FEBS Lett **149:** 197–202
8. **Capaldi RA, Gonzalez-Halphen D, Takamiya S** (1986) Sequence homologies and structural similarities between the polypeptides of yeast and beef heart cytochrome c oxidase. FEBS Lett **207:** 11–17
9. **Dewey RE, Levings III CS, Timothy DH** (1986) Novel recombinations in the maize mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell **44:** 439–449
10. **Dewey RE, Siedow JN, Timothy DH, Levings III CS** (1988) A 13-kilodalton maize mitochondrial protein in *E. coli* confers sensitivity to *Bipolaris maydis* toxin. Science **239:** 293–295
11. **Dewey RE, Timothy DH, Levings III CS** (1987) A mitochondrial protein associated with cytoplasmic male sterility in the T cytoplasm of maize. Proc Natl Acad Sci USA **84:** 5374–5378
12. **Douce R, Mannella CA, Bonner WD Jr** (1973) The external NADH dehydrogenases of intact plant mitochondria. Biochim Biophys Acta **292:** 105–116
13. **Eisenberg D, Schwarz E, Komaromy M, Wall R** (1984) Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J Mol Biol **179:** 125–142
14. **Forde BG, Leaver CJ** (1980) Nuclear and cytoplasmic genes controlling synthesis of variant mitochondrial polypeptides in male-sterile maize. Proc Natl Acad Sci USA **77:** 418–422
15. **Forde BG, Oliver RJC, Leaver CJ** (1978) Variation in mitochondrial translation products associated with male-sterile cytoplasms in maize. Proc Natl Acad Sci USA **75:** 3841–3845
16. **Fox TD, Leaver CJ** (1981) The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. Cell **26:** 315–323
17. **Fujiki Y, Fowler S, Shio H, Hubbard AL, Lazarow PB** (1982) Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes: comparison with endoplasmic reticulum and mitochondrial membranes. J Cell Biol **93:** 103–110
18. **Griffiths G, Hoppeler H** (1986) Quantitation in immunocytochemistry: correlation of immunogold labelling to absolute number of membrane antigens. J Histochem Cytochem **34:** 1389–1398
19. **Hashimoto F, Horigome T, Kanbayashi M, Yoshida K, Sugano H** (1983) An improved method for separation of low-molecular-weight polypeptides by electrophoresis in SDS-polyacrylamide gel. Anal Biochem **129:** 192–199
20. **Huang J, Lee S-H, Lin C, Medici R, Hack E, Myers AM** (1990) Expression in yeast of the T-URF 13 protein from Texas male-sterile mitochondria confers sensitivity to methomyl and to Texas-cytoplasm-specific fungal toxins. EMBO J **9:** 339–347
21. **Leaver CJ, Hack E, Forde BG** (1983) Protein synthesis by isolated plant mitochondria. Methods Enzymol **97:** 476–484
22. **Mannella CA** (1987) Isolation of the outer membrane of plant mitochondria. Methods Enzymol **148:** 453–464
23. **Nelson N** (1988) Structure, function, and evolution of proton-ATPases. Plant Physiol **86:** 1–3
24. **Newton KJ, Walbot V** (1985) Maize mitochondria synthesize organ-specific polypeptides. Proc Natl Acad Sci USA **82:** 6879–6883
25. **Neuhoff V, Arold N, Taube D, Ehrhardt W** (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis **9:** 255–262
26. **Pring DR, Lonsdale DM** (1989) Cytoplasmic male sterility and maternal inheritance of disease susceptibility in maize. Annu Rev Phytopathol **27:** 483–502
27. **Schmidt RJ, Myers AM, Gillham NW, Boynton JE** (1984) Immunological similarities between specific chloroplast ribosomal proteins from *Chlamydomonas reinhardtii* and ribosomal proteins from *Escherichia coli.* Mol Biol Evol **1:** 317–334
28. **Wise RP, Fliss AE, Pring DR, Gengenbach BG** (1987) *Urf13-T* of T cytoplasm maize mitochondria encodes a 13-kD polypeptide. Plant Mol Biol **9:** 121–128
29. **Young EG, Hanson MR** (1987) A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. Cell **50:** 41–49