Baculovirus expression of the maize mitochondrial protein URF13 confers insecticidal activity in cell cultures and larvae

(insect toxicity/*Bipolaris maydis* race T/cytoplasmic male sterility/*Phyllosticta maydis*/plant mitochondria)

KENNETH L. KORTH* AND CHARLES S. LEVINGS III†

Department of Genetics, Box 7614, North Carolina State University, Raleigh, NC 27695-7614

Contributed by Charles S. Levings III, January 13, 1993

ABSTRACT The URF13 protein, which is encoded by the mitochondrial gene *T-urf13*, is responsible for cytoplasmic male sterility and pathotoxin sensitivity in the Texas male-sterile cytoplasm (*cms-T*) of maize. Mitochondrial sensitivity to two host-specific fungal toxins (T toxins) is mediated by the interaction of URF13 and T toxins to form pores in the inner mitochondrial membrane. A carbamate insecticide, methomyl, mimics the effects of T toxins on isolated *cms-T* mitochondria. URF13 was expressed in *Spodoptera frugiperda* (fall armyworm) cells (Sf9) in culture and in *Trichoplusia ni* (cabbage looper) larvae with a baculovirus vector. In insect cells, URF13 forms oligomeric structures in the membrane and confers T toxin or methomyl sensitivity. Adding T toxin or methomyl to Sf9 cells producing URF13 causes permeabilization of plasma membranes. In addition, URF13 is toxic to insect cells grown in culture without T toxins or methomyl; even a T-toxin-insensitive mutant form of URF13 is lethal to cell cultures. Baculoviruses expressing URF13 are lethal to *T. ni* larvae, at times postinjection comparable to those obtained by injecting a baculovirus expressing an insect neurotoxin. This result suggests that URF13 could be useful as a biological control agent for insect pests. Our data indicate that URF13 has two independent mechanisms for toxicity, one that is mediated by T toxin and methomyl and one that is independent of these toxins. Similarly, male sterility and toxin sensitivity in *cms-T* maize may be due to independent mechanisms.

The *T-urf13* gene, which encodes the URF13 protein in the mitochondria of male-sterile maize carrying the Texas cytoplasm (*cms-T*), causes susceptibility to two fungal pathogens, *Bipolaris maydis* race T (formerly *Helminthosporium maydis* race T) and *Phyllosticta maydis*. Diseases caused by these pathogens, especially *B. maydis*, have curtailed large-scale use of *cms-T* maize for the production of hybrid seed. *cms-T* maize does not produce viable pollen or exert anthers (reviewed in ref. 1); the same protein, URF13, is also thought to be responsible for the male-sterile phenotype (reviewed in ref. 2).

Treating isolated *cms-T* maize mitochondria with host-specific toxins (T toxins) produced by *B. maydis* race T or *P. maydis* causes mitochondrial swelling, leakage of small molecules and ions, inhibition of malate-stimulated respiration, and uncoupling of oxidative phosphorylation. These effects are caused by pore formation in the inner mitochondrial membrane resulting from the interaction of URF13 and T toxin (reviewed in ref. 2). Similar events are observed in *Escherichia coli* expressing the cloned *T-urf13* gene; after exposure of these bacterial cells to T toxins, spheroplast swelling, inhibition of respiration, and ion leakage occur (3, 4). Methomyl {S-methyl-N-[(methylcarbamoyl)oxy]thioacetimidate}, the active ingredient in the DuPont insecticide

Lannate, mimics the effects of T toxins on isolated *cms-T* maize mitochondria (5) or *E. coli* expressing URF13 (3). Many mutations in *T-urf13*, including several at nucleotide positions encoding amino acid residue 39, render *E. coli* expressing the mutant URF13 insensitive to T toxins or methomyl (ref. 4; Mark E. Williams and Gerty C. Ward, personal communication).

URF13 has also been expressed in two heterologous eukaryotic systems. The *T-urf13* gene was expressed in *Saccharomyces cerevisiae* where the gene was modified to direct URF13 import into mitochondria (6, 7); in these cases URF13 accorded T toxin and methomyl sensitivity to the mitochondria. In contrast, when the URF13 protein was not modified to direct import into the mitochondria, it did not confer toxin sensitivity to whole yeast cells. URF13 conferred T toxin sensitivity to *Nicotiana tabacum*, however, when it was expressed in the cytoplasm without a mitochondrial targeting sequence (8).

Baculovirus vectors have provided a powerful technique for expressing large amounts of foreign proteins in insect cell cultures or larvae (reviewed in refs. 9 and 10). In addition, baculoviruses have been studied for use as biological control agents for insect pests; recently the effort has focused on decreasing the time required for these viruses to kill insects. Several groups have shown that baculoviruses expressing insect toxins kill lepidopteran larvae much more rapidly than wild-type baculoviruses (11–13).

To obtain large amounts of purified URF13 for structural studies and to assay the toxicity of URF13 in insects, we have cloned the *T-urf13* gene in a baculovirus expression system. URF13 expression was placed under control of the *Autographica californica* nuclear polyhedrosis virus (AcNPV) polyhedrin promoter. URF13 functions in this system by interacting with T toxin or methomyl to permeabilize the plasma membranes of insect cells. Also, URF13 has lethal effects when expressed in either invertebrate cell cultures or insect larvae, even without T toxin or methomyl.

MATERIALS AND METHODS

***T-urf13* Cloning.** DNA manipulations were carried out as described (14). A 2-kbp *Hind*III fragment from *cms-T* maize mitochondrial DNA (15) was ligated into pBluescript KS+ (Stratagene), and a *Bam*HI restriction site was created just upstream of the *T-urf13* open reading frame using site-directed mutagenesis (16). To create a T-toxin-insensitive form of URF13, nucleotides encoding residues 39 and 40 of URF13 were altered (16) to codons for glycine, from 5'-GAT-GAT-3' to 5'-GGT-GGT-3'. The alteration of residue 40 has

Abbreviations: AcNPV, *Autographica californica* nuclear polyhedrosis virus; mAb, monoclonal antibody; FDA, fluorescein diacetate; EGS, ethylene glycolbis(succinimidylsuccinate).

*Present address: Plant Biology Division, The Samuel Roberts Noble Foundation, P.O. Box 2180, Ardmore, OK 73402.

†To whom reprint requests should be addressed.

no known effect on T toxin or methomyl sensitivity conferred by URF13 in *E. coli*. The entire T-*urf13* open reading frame, included in 511-bp *Bam*HI/*Bgl* II fragments, was ligated into the *Bam*HI site of pAcYMI (provided by D. H. L. Bishop, Natural Environment Research Council Institute of Virology, Oxford) (17) to create constructs with the wild-type form of T-*urf13*, pAc13T, and the mutagenized form, pAc13.3940. In each case the T-*urf13* gene is downstream of the polyhedrin promoter of AcNPV. DNA sequencing (18) confirmed that the 5' insertion point was 5'...AAATA'CGGATC-CAATG...3', where A' is a remnant of the polyhedrin protein initiation codon, and ATG is the first codon of the T-*urf13* gene.

For expression in *E. coli*, the entire open reading frame of the T-*urf13* gene was ligated into an inducible expression vector, pKK223 (Pharmacia LKB); the construct has been designated pKK13T (Carl J. Braun, personal communication).

Baculovirus, Cell Culture, and Larvae Handling. pAc13T and pAc13.3940 were introduced along with AcNPV (provided by M. D. Summers, Texas A&M University) DNA into Sf21 cells via liposome-mediated transfection (19) or calcium phosphate precipitation (20). Recombinant viruses were selected by visual screening of cell monolayers for viral plaques not producing the polyhedrin protein (occlusion-negative) as described (20), except that 0.1% neutral red was added to plates.

Recombinant baculoviruses expressing β -galactosidase (BV β gal) were isolated using the same procedures (pAc360- β gal provided by M. D. Summers).

Isolation of AcNPV DNA, infections, and maintenance of cell cultures were performed as described (20). Viral infections were made with a multiplicity of infection of 5–10. All experiments with infected cells were carried out at 48 hr postinfection unless indicated otherwise. Antibiotics [gentamicin sulfate (50 μ g/ml) and amphotericin B (2.5 μ g/ml)] were used only during transfections and selection of recombinant viruses; they were not present during studies of URF13 function or toxicity. *Trichoplusia ni* larvae were maintained at 27°C as described (21).

Anti-URF13 Antibodies and Protein Handling. Production and characterization of anti-URF13 monoclonal antibody (mAb) have been described (22). SDS/PAGE was carried out on 16.5% acrylamide/Tris-tricine gels as described (23). Immunoblots were prepared as described (24) using a luminescent detection system (ECL; Amersham). Cross-linking was carried out with ethylene glycolbis(succinimidylsuccinate) (EGS) (Pierce) on whole cells (22).

For membrane preparation, Sf9 cells were suspended in phosphate-buffered saline (PBS; 10 mM Na₂PO₄/1.8 mM KH₂PO₄/0.8% NaCl/0.14% KCl, pH 7.2), 5 mM EDTA, 50 μ g of phenylmethylsulfonyl fluoride per ml, 2 mg of leupeptin per ml, and 1 mg of pepstatin per ml. The suspension was sonicated 4 $\times$ 20 s with a Fisher sonic dismembrator model 300 microtip at 35% power. Remaining whole cells and debris were removed by centrifugation at 8000 $\times$ g, and membranes were separated from soluble fractions by centrifugation at 150,000 $\times$ g. *T. ni* larvae were homogenized with a Teflon pestle in the same buffer as above with 0.01% phenylthiourea. Larval cell membranes were prepared the same as for Sf9 cultures, except that the 8000 $\times$ g spin was carried out three times.

URF13 Functional Studies. Fluorescein diacetate (FDA) in acetone (1 mg/ml) was diluted 1:4000 in PBS and added to cell suspensions at 1:3. Cells were suspended in PBS, incubated for 5 min with or without 8 mM methomyl (provided by DuPont) or with or without 780 ng of T toxin per ml (gift of H. W. Knoche and S. J. Danko, University of Nebraska), and then stained with FDA. Fluorescent cells were visualized

on a Nikon inverted microscope with a B-2A filter (excitation at 450–490 nm) and counted on a Neubauer hemacytometer.

Light absorbance of Sf9 cells was measured over time at 520 nm with constant stirring. Lannate, 12.3 μ l (1.3 M methomyl), was added (to 8 mM) to 2-ml suspensions of cells (2×10^7 cells per ml) in PBS. Alternatively, T toxin was added to 780 ng/ml to identical cell suspensions.

URF13 Toxicity Studies. Sf9 cells were counted on a Neubauer hemacytometer. Viable cells were identified by their failure to take up trypan blue, 0.04% final concentration in PBS.

Third to fourth instar *T. ni* larvae were injected with about 2×10^4 plaque-forming units in 1 μ l of complete medium. Those larvae not surviving the injection procedure were removed from the study. Larvae were counted as dead when they failed to respond to a slight prodding.

RESULTS

Expression of URF13 in Insect Cell Culture. Baculoviruses BV13T and BV13.3940, which contain T-*urf13* genes, were created via homologous recombination between pAc13T or pAc13.3940, respectively, and wild-type AcNPV DNA was created by cotransfection in insect cell cultures. BV13T contains the wild-type version of T-*urf13*, and BV13.3940 contains site-directed mutations at positions encoding amino acid residues 39 and 40, so that it is predicted to encode Gly-Gly instead of the wild-type Asp-Asp. Mutations at residue 39 render T-*urf13* incapable of conferring T toxin or methomyl sensitivity to *E. coli* (4).

BV13T- and BV13.3940-infected Sf9 cell extracts and cell membranes contain novel proteins of approximately 13 and 11 kDa compared with uninfected or AcNPV-infected cells, as determined by Coomassie blue staining of SDS/PAGE gels (Fig. 1). Scanning densitometry of infected cell extract lanes indicates that these two proteins together make up $\approx 2\%$ of the total stainable protein.

Infecting Sf9 cultures with viral isolates BV13T or BV13.3940 produces two membrane-bound proteins that are immunoreactive with a mAb specific for the carboxyl terminus (mAb-C) of URF13 (Fig. 2A). The higher molecular mass, immunoreactive protein in this blot migrates on SDS/PAGE gels at a position identical to that of URF13 from *cms-T* mitochondria (Fig. 2A). Antibodies that recognize URF13 do not react with proteins from uninfected cells, cells infected with wild-type AcNPV, or the soluble fraction obtained from BV13T- or BV13.3940-infected cells (Fig. 2A). Neither of the two URF13 species produced in infected cells can be removed from membranes by treatment with 0.1 M Na₂CO₃ (data not shown), indicating that they are both integral membrane proteins. Differential gel migration pre-

FIG. 1. Coomassie blue-stained SDS/polyacrylamide gel of uninfected or baculovirus-infected Sf9 cell extracts (60 μ g per lane) or membranes (50 μ g per lane). Positions and sizes (in kDa) of protein size standards are indicated. The abundant protein near the 29-kDa marker in AcNPV-infected cells is the polyhedrin protein. Asterisks indicate the location of novel proteins in BV13T- and BV13.3940-infected cells.

FIG. 2. SDS/PAGE immunoblots probed with the carboxyl-terminal-specific mAb-C. (A) Forty micrograms of *cms-T* mitochondrial protein, 40 μ g of Sf9 cell lysate, 40 μ g of AcNPV-infected Sf9 cell lysate, 30 μ g of soluble, and 20 μ g of membrane protein fractions from BV13T- and BV13.3940-infected Sf9 cells. (B) BV13T- and BV13.3940-infected cell lysates either untreated (-) or cross-linked (+) with EGS; *E. coli* cell lysates expressing URF13 either untreated (-) or cross-linked (+) with EGS as indicated. Values in the center indicate positions and sizes (in kDa) of protein size standards.

dicts that the lower molecular mass protein is about 2 kDa smaller than full-length URF13 (13 kDa). The smaller protein probably lacks amino acid residues largely from the amino terminus because it is reactive with the carboxyl-specific mAb-C. This same protein expression pattern was observed after infection with each of eight independent viral isolates.

URF13 forms multimeric structures in insect cell membranes, which have also been shown in membranes from *cms-T* mitochondria and *E. coli* cells expressing this protein (22). Treating BV13T- and BV13.3940-infected cells with a lysine-specific, hydrophobic, bifunctional crosslinker, EGS, gives rise to mAb-C reactive proteins that migrate on SDS/PAGE gels at positions predicted for URF13 homomultimers (Fig. 2B). The presence of multiple bands migrating near the putative dimers, trimers, and tetramers indicates that the smaller URF13 species produced in Sf9 cells also participates in forming multimers. The predominant multimeric URF13 species produced in Sf9 cells comigrates with mAb-C immunoreactive proteins from cross-linker-treated *E. coli* expressing the T-*urf13* gene product (Fig. 2B). A small amount of putative dimer is present in untreated *E. coli* and *cms-T* mitochondria (Fig. 2A and B); this form of URF13 often appears in protein immunoblots containing URF13 (22, 25).

Immunofluorescence studies of fixed Sf9 cells using mAb-C as a probe indicate that URF13 is found throughout

Table 1. Effect of methomyl (8 mM) or T toxin (780 ng/ml) on the integrity of Sf9 plasma membranes as measured by the number of virus-infected cells stained with a vital stain, FDA

Virus	Treatment	Cells per ml $\times 10^5$ *	$\Delta\%$ †
BV13T	None	3.00 $\pm$ 0.205	—
	Methomyl	0.675 $\pm$ 0.063	77.5
	T toxin	0.725 $\pm$ 0.063	75.8
BV β gal	None	3.10 $\pm$ 0.325	—
	Methomyl	3.20 $\pm$ 0.295	-3.2
	T toxin	2.95 $\pm$ 0.120	4.8
AcNPV	None	3.65 $\pm$ 0.235	—
	Methomyl	3.58 $\pm$ 0.110	1.9
	T toxin	3.60 $\pm$ 0.240	1.4
BV13.3940	None	3.10 $\pm$ 0.155	—
	Methomyl	2.95 $\pm$ 0.065	4.8
	T toxin	2.93 $\pm$ 0.125	5.5

*Mean ($\bar{x}$) $\pm$ SEM of four treatments.

† $\Delta\% = (1 - \bar{x}$ after treatment/ $\bar{x}$ with no treatment) $\times$ 100.

FIG. 3. Light absorbance of baculovirus-infected Sf9 cell suspensions measured over time at 520 nm. Arrows indicate addition of 12.3 μ l of 1.3 M methomyl.

BV13T-infected cells, whereas virtually no antibody binding occurs in cells infected with another recombinant virus, BV β gal (data not shown). In *cms-T* maize, URF13 is synthesized in the mitochondrial matrix and integrated into the inner mitochondrial membrane. Because mitochondrial targeting sequences were not included in BV13T or BV13.3940, URF13 was not expected to associate specifically with mitochondria in Sf9 cells.

Fungal Toxin and Methomyl Sensitivity Conferred by URF13 in Insect Cells. URF13 interacts with T toxin or methomyl to permeabilize membranes in maize mitochondria and *E. coli* cells. Sf9 cells expressing wild-type URF13 also show sensitivity to T toxin and methomyl. Treating BV13T-infected cells with methomyl or T toxin results in a >75% reduction in the number of cells that can be observed with the vital stain FDA (Table 1). The fluorescent, polar product of hydrolyzed FDA is retained only in cells with intact plasma membranes, whereas dead or dying cells rapidly leak the dye (26). Table 1 shows that a short incubation with T toxin or methomyl reduces the number of stained cells among cells producing wild-type URF13, probably by its effects on plasma membranes; in contrast, cells infected with BV13.3940, AcNPV, or BV β gal are virtually unaffected.

Suspensions of BV13T-infected Sf9 cells expressing wild-type URF13 show a significant decrease over time in light absorbance at 520 nm after adding methomyl to 8 mM (Fig. 3). No such change is observed in cells infected with BV13.3940 or BV β gal. The decrease in light scattering indicates a loss of membrane integrity, which is thought to be due to cell swelling. This technique has been used to show T-toxin sensitivity in *cms-T* mitochondria (27, 28) and *E. coli* spheroplasts expressing URF13 (3). This result provides further evidence that URF13 can act to permeabilize plasma membranes of methomyl-treated Sf9 cells. A similar decrease in absorbance was seen in Sf9 cells expressing wild-type URF13 after adding T toxin; no changes were observed in

FIG. 4. Effect of infection by various baculoviruses on cell viability of Sf9 cultures. Three repetitions were performed for each sample at each time point; error bars indicate SEM.

FIG. 5. SDS/PAGE immunoblot of BV13T- and BV13.3940-infected Sf9 cells (30 μ g per lane) grown for 48 hr in the absence (-) or presence (+) of 2 mM methomyl as indicated. The blot was probed with the URF13-carboxyl-terminal-specific mAb-C.

BV13.3940-infected cells or control group cells (data not shown).

Toxicity of URF13 in Cell Cultures and Insect Larvae. Cells expressing URF13 are dislodged from monolayer cultures much more easily than other viral-infected cells (unpublished observation). An explanation may be that overproduction of URF13 has detrimental effects on growth of Sf9 cells.

We measured the toxic effects of URF13 production in cell cultures by counting live cells at time intervals after infection. Baculoviral infection causes a rapid cessation of growth of insect cell cultures (29). Cells infected with BV13T or BV13.3940 die at a significantly faster rate than cells infected with either AcNPV (Fig. 4) or BV β gal (data not shown). The presence of 2 mM methomyl in these cultures did not cause a detectable difference, compared to cultures grown without methomyl, in the numbers of live cells over time (data not shown). In cultures grown with methomyl, however, the abundance of URF13 was significantly diminished, and the smaller URF13 species was virtually absent (Fig. 5). The

FIG. 6. (A) Effect of injection of various baculoviruses on the viability of third to fourth instar *T. ni* larvae, as measured in the percentage of larvae surviving over time. (B) SDS/PAGE immunoblot of BV13T-infected Sf9 cell lysate and BV13T-injected *T. ni* larvae at times after injection. The blot was probed with the URF13-carboxyl-terminal-specific mAb-C. Positions and sizes (in kDa) of protein size standards are indicated.

expression pattern of URF13 in BV13.3940-infected cultures was not affected by 2 mM methomyl.

URF13 has lethal effects when produced in insect larvae in the absence of T toxin or methomyl (Fig. 6A). One hundred percent of third to fourth instar *T. ni* larvae injected with BV13T or BV13.3940 is dead within 60 hr after injection. Larvae injected with either AcNPV or BV β gal live up to 106 hr or 100 hr after injection, respectively. Larvae injected with complete medium survive normally and undergo pupation (data not shown). URF13 apparently undergoes specific proteolysis in *T. ni* larvae. As in Sf9 cultures, a mAb-C-reactive protein of the same size as wild-type URF13 is localized in BV13T- and BV13.3940-infected larval membranes. Additional proteins of lower molecular mass are also recognized by mAb-C (Fig. 6B). Immunoreactive URF13 is detectable in larval samples beginning at 36 hr postinjection (Fig. 6B).

DISCUSSION

We have taken advantage of a baculovirus expression system to produce URF13, a maize mitochondrial protein involved in disease susceptibility and cytoplasmic male sterility. Two species of URF13 are produced in Sf9 cells infected with BV13T or BV13.3940. One species has a mobility on SDS/PAGE gels identical to that of URF13 from *cms-T* mitochondria; a smaller species of URF13 lacks a portion of the protein primarily from the amino terminus, which may result from a specific proteolytic cleavage of full-length URF13 (115 amino acid residues) *in vivo* or after cell lysis. Alternatively, the smaller protein might result from a late translational initiation event because an AUU codon can act as a translational initiation site in baculovirus vectors in insect cell cultures (30). The *T-urf13* gene has AUU codons at positions encoding amino acid residues 21 and 23. Initiation of translation at either of these sites is predicted to produce a protein approximately the size of the smaller URF13 species (1.9 or 2.2 kDa smaller than full-length URF13, respectively). This shortened version of URF13 is probably not functional because when amino acid residues 2–11 are deleted, URF13 no longer confers T toxin sensitivity (3).

URF13 is tightly bound to cell membranes in Sf9 and *T. ni* cells. Our studies show that wild-type URF13 in plasma membranes confers T toxin and methomyl sensitivity to insect cells grown in culture. URF13 forms oligomers in Sf9 membranes; this observation supports the proposal that URF13 causes T toxin and methomyl susceptibility as an oligomeric structure (22, 25, 31), although oligomer formation is not sufficient to cause susceptibility, because a T-toxin-insensitive form of URF13 also forms oligomers. Sensitivity of BV13T-infected Sf9 cells to T toxin or methomyl occurs at 780 ng/ml and 8 mM levels, respectively, which is comparable to the toxic levels in *E. coli* (3), *cms-T* maize mitochondria (5), and yeast mitochondria (6, 7). Lower (2 mM) concentrations of methomyl also have an effect on the level of wild-type URF13 accumulation in Sf9 cultures.

The presence of 2 mM methomyl in cultures infected with BV13T causes a significant decrease in the abundance of URF13, whereas it has no apparent effect on URF13 levels in cultures infected with BV13.3940. Methomyl in these cultures might select against cells that are producing functional URF13. Low levels of the smaller species of URF13 in BV13T-infected cultures may indicate that it accumulates only under conditions favoring high levels of URF13 expression.

Methomyl acts as an insect neurotoxin by inhibiting acetylcholine esterase (reviewed in ref. 32). Because concentrations of 2 mM or less have no detectable effect on the growth of uninfected Sf9 cultures, low levels of methomyl may be used in experiments involving insect cell cultures.

Several recent studies have shown that expression of known insect toxins via baculoviruses results in significantly more rapid death of insect larvae infected with these viruses than larvae infected with wild-type baculoviruses. In two studies the viruses were packaged in a polyhedron structure and administered orally (12, 13). Maeda *et al.* (11) expressed a scorpion peptide neurotoxin with a baculovirus and showed its lethal effects in silkworm (*Bombyx mori*) by injecting larvae with viral suspensions. In that study larvae showed the effects of the neurotoxin at 40–60 hr and died between 60 and 75 hr after injection. Using this same delivery system and similar amounts of virus, we have shown that URF13 has insecticidal effects in *T. ni* larvae comparable to that of a baculovirus expressing a well-characterized insect toxin. Larvae injected with BV13T all die within 60 hr. These results indicate that URF13 could be useful as a biological control agent against insects.

Attempts to produce an occluded form of BV13T for oral infection experiments were not successful (data not shown). Occluded forms of polyhedrin-negative recombinant viruses can sometimes be produced by coinfection of cultures with wild-type, polyhedrin-positive virus (33–35). Mixed infections of Sf9 cultures with BV13T and AcNPV were performed, with variation in respective multiplicity of infection and time between infections. When BV13T infection either preceded or occurred at the same time as AcNPV infection, few polyhedra were produced. When BV13T infection followed AcNPV infection, polyhedra were produced, but they contained only low levels of BV13T DNA, as determined by DNA blot hybridization analysis. The lethality associated with URF13 expression in Sf9 cultures may decrease the capacity of AcNPV-infected cells to produce polyhedra.

The mechanism of URF13 toxicity in insect cells was not determined; however, it is evident that URF13 is localized in membranes of Sf9 cells and *T. ni* larvae and that it interferes with normal cellular functions. Toxicity of URF13 in Sf9 cells or *T. ni* larvae seems unrelated to the capacity of URF13 to form membrane pores, because the T-toxin-insensitive form of URF13, encoded by BV13.3940, is as toxic as the wild-type URF13 molecule. Because URF13 synthesis occurs concomitant with other viral infection processes, the rapid onset of toxicity may result from synergistic effects. It is unclear whether toxicity is unique to URF13 or if it is a more general phenomenon expected *in vivo* with the overexpression of hydrophobic proteins. Many membrane-bound proteins have been expressed with baculoviruses (e.g., refs. 36–39), but these proteins have not been evaluated for toxicity.

Toxic effects of URF13 in insect cells may be related to the role that URF13 plays in causing male sterility in maize. URF13 might exert toxic effects in *cms-T* maize in a tissue-specific manner, causing abortion of the male gametes. The discovery that a T-toxin-insensitive form of URF13 is also toxic in insects suggests that Texas cytoplasmic male sterility and the related fungal susceptibility may be separable traits due to different properties of URF13.

We owe much thanks to Phillip Hartig and Mary Cardon (ManTech Environmental Technology) for their help with transfections and identification of recombinant plaques. Doug Anspaugh, Vasant Kalapur, and Chandana Majumder (North Carolina State University) graciously provided *T. ni* larvae, and Julie Dickson (Becton Dickinson) provided Sf9 cultures. We thank Cyril Kaspi and Jim Siedow (Duke University) for help with absorbance experiments, Jane Suddith for technical assistance, Suzanne Quick for editorial assistance, and members of our laboratory for reading the manuscript. This work was supported by a grant from the National Science

Foundation (C.S.L.) and by a fellowship from the North Carolina Biotechnology Center (K.L.K.).

1. Laughnan, J. R. & Gabay-Laughnan, S. (1983) *Annu. Rev. Genet.* **17**, 27–48.
2. Levings, C. S., III (1990) *Science* **250**, 942–947.
3. Dewey, R. E., Siedow, J. N., Timothy, D. H. & Levings, C. S., III (1988) *Science* **239**, 293–295.
4. Braun, C. J., Siedow, J. N., Williams, M. E. & Levings, C. S., III (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4435–4439.
5. Koeppe, D. E., Cox, J. K. & Malone, C. P. (1978) *Science* **201**, 1227–1229.
6. Glab, N., Wise, R. P., Pring, D. R., Jacq, C. & Slonimski, P. (1990) *Mol. Gen. Genet.* **223**, 24–32.
7. Huang, J., Lee, S.-H., Lin, C., Medici, R., Hack, E. & Myers, A. M. (1990) *EMBO J.* **9**, 339–347.
8. von Allmen, J.-M., Rottmann, W. H., Gengenbach, B. G., Harvey, A. J. & Lonsdale, D. M. (1991) *Mol. Gen. Genet.* **229**, 405–412.
9. Luckow, V. A. & Summers, M. D. (1988) *Bio/Technology* **6**, 47–55.
10. Luckow, V. A. (1990) in *Recombinant DNA Technology and Applications*, eds. Ho, C., Prokop, A. & Bajpai, R. (McGraw-Hill, New York), pp. 1–24.
11. Maeda, S., Volrath, S. L., Hanzlik, T. N., Harper, S. A., Majima, K., Maddox, D. W., Hammock, B. D. & Fowler, E. (1991) *Virology* **184**, 777–780.
12. Stewart, L. M. D., Hirst, M., Ferber, M. L., Merryweather, A. T., Cayley, P. J. & Possee, R. D. (1991) *Nature (London)* **352**, 85–88.
13. Tomalski, M. D. & Miller, L. K. (1991) *Nature (London)* **352**, 82–85.
14. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Plainview, NY).
15. Dewey, R. E., Levings, C. S., III, & Timothy, D. H. (1986) *Cell* **44**, 439–449.
16. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488–492.
17. Matsuura, Y., Possee, R. D., Overton, H. A. & Bishop, D. H. L. (1987) *J. Gen. Virol.* **68**, 1233–1250.
18. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
19. Hartig, P. C., Cardon, M. C. & Kawanishi, C. Y. (1991) *BioTechniques* **11**, 310–313.
20. Summers, M. D. & Smith, G. E. (1988) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures* (Tex. Agric. Exp. Stn., College Station, TX), Bull. No. 1555.
21. Roe, R. M., Hammond, A. M., Jr., & Sparks, T. C. (1982) *Ann. Entomol. Soc. Am.* **75**, 421–429.
22. Korth, K. L., Kaspi, C. I., Siedow, J. N. & Levings, C. S., III (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10865–10869.
23. Schagger, H. & von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379.
24. Harlow, E. & Lane, D. (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab., Plainview, NY).
25. Hack, E., Lin, C., Yang, H. & Horner, H. T. (1991) *Plant Physiol.* **95**, 861–870.
26. Rotman, B. & Papermaster, B. W. (1966) *Proc. Natl. Acad. Sci. USA* **55**, 134–141.
27. Miller, R. J. & Koeppe, D. E. (1971) *Science* **173**, 67–69.
28. Gengenbach, B. G., Miller, R. J., Koeppe, D. E. & Arntzen, C. J. (1973) *Can. J. Bot.* **51**, 2119–2125.
29. Volkman, L. E., Summers, M. D. & Hsieh, C.-H. (1976) *J. Virol.* **19**, 820–832.
30. Beames, B., Braunagel, S., Summers, M. D. & Lanford, R. E. (1991) *BioTechniques* **11**, 378–383.
31. Braun, C. J., Siedow, J. N. & Levings, C. S., III (1990) *Plant Cell* **2**, 153–161.
32. Baillie, A. C. & Wright, K. (1985) in *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*, eds. Kerkut, G. A. & Gilbert, L. I. (Pergamon, Oxford), Vol. 11, pp. 323–356.
33. Miller, D. W. (1988) in *Biotechnology for Crop Protection*, eds. Hedin, P. A., Menn, J. J. & Hollingworth, R. M. (Am. Chem. Soc., Washington, DC), pp. 405–421.
34. Price, P. M., Reichelderfer, C. F., Johansson, B. E., Kilbourne, E. D. & Acs, G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1453–1456.
35. Hamblin, M., van Beek, N. A. M., Hughes, P. R. & Wood, H. A. (1990) *Appl. Environ. Microbiol.* **56**, 3057–3062.
36. Greenfield, C., Patel, G., Clark, S., Jones, N. & Waterfield, M. D. (1988) *EMBO J.* **7**, 139–146.
37. Fafournoux, P., Ghysdael, J., Sardet, C. & Pouyssegur, J. (1991) *Biochemistry* **30**, 9510–9515.
38. Kartner, N., Hanrahan, J. W., Jensen, T. J., Naismith, A. L., Sun, S., Ackerley, C. A., Reyes, E. F., Tsui, L.-C., Rommens, J. M., Bear, C. E. & Riordan, J. R. (1991) *Cell* **64**, 681–691.
39. Smith, C. D., Hirayama, B. A. & Wright, E. M. (1992) *Biochim. Biophys. Acta* **1104**, 151–159.