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Virus-Encoded Toxin of *Ustilago maydis*: Two Polypeptides Are Essential for Activity

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Cells of *Ustilago maydis* containing double-stranded RNA viruses secrete a virus-encoded toxin to which other cells of the same species and related species are sensitive. Mutants affected in the expression of the KP6 toxin were characterized, and all were viral mutants. A temperature-sensitive nonkiller mutant indicated that the toxin consists of two polypeptides, 12.5K and 10K, that are essential for the toxic activity. The temperature-sensitive nonkiller mutant was affected in the expression of the 10K polypeptide, and its toxic activity was restored by the addition of the 10K polypeptide to its secreted inactive toxin. These results led to the reexamination of other mutants that were known to complement in vitro. Each was found to secrete one of the two polypeptides. Here we show for the first time that P6 toxin consists of two polypeptides that do not interact in solution, but both are essential for the toxic effect. Studies on the interaction between the two polypeptides indicated that there are no covalent or hydrogen bonds between the polypeptides. Toxin activity is not affected by the presence of 0.3 M NaCl in the toxin preparations and in the medium, suggesting that no electrostatic forces are involved in this interaction. Also, the two polypeptides do not share common antigenic determinants. The activity of the two polypeptides appears to be dependent on a sequential interaction with the target cell, and it is the 10K polypeptide that initiates the toxic effect. The similarity of the *U. maydis* virus-encoded toxin to that of *Saccharomyces cerevisiae* is discussed.

Viruses have been detected in more than 100 different fungal species. Among those characterized the vast majority are double-stranded RNA (dsRNA) viruses. In most fungi the persistent infection of these viruses has no discernable phenotype. In two systems, *Saccharomyces cerevisiae* and *Ustilago maydis*, the viruses encode a toxin that is secreted by these fungi and is effective against sensitive cells of the same species and closely related species (10, 14, 21, 22, 25). Systems in which a toxin is secreted are known as killer systems (for review, see references 5 and 27). In *U. maydis*, three different toxin specificities have been identified: KP1, KP4, and KP6. Each is associated with a different segmented dsRNA virus subtype known as P1, P4, and P6. Some of the P1 and P4 dsRNA segments have sequence homology, and the toxins they encode are similar in their mode of action. P6 dsRNAs have little homology to those of P1 and P4, and KP6 toxin has a different effect from those of KP1 and KP4 (7, 12).

The genome of the various types of *Ustilago* viruses consists of three to seven segments ranging from 6.2 to 0.36 kilobase pairs. On the basis of their size, they are referred to as H (heavy), M (medium), and L (light) segments. One specific M segment encodes the toxin in each viral subtype, whereas the viral capsid polypeptide is encoded by one or more H segments (2, 16). The L segment bears sequence homology to the M segment that encodes the toxin. The immunity to the toxin, at least in cells containing P1 virus, is associated with the L segment (16, 20).

The virus-encoded toxin of *U. maydis* has been characterized as a 10- to 12-kilodalton protein appearing as one band in 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gels and in isoelectric focusing gels (12, 19). So far all the induced and spontaneous nonkiller (NK phenotype) mutants derived from killer strains are virus associated. The NK phenotype showed a cytoplasmic mode of inheritance in mating experiments and in heterokaryon transfer experiments (13, 15, 17). Some of these were viral deletion mutants of the toxin-encoding M segment. Other viral mutants of P6 secreted an inactive protein with a mobility identical with that of KP6. Based on in vitro complementation of the secreted inactive molecules these mutants consisted of two groups. Interaction between the proteins of each of these two groups led to the restoration of the activity and precise specificity of KP6. Since only one protein was detected in SDS-polyacrylamide gels as the biologically active toxin, the complementation pattern suggested that the active molecule was a homomultimer. Complementation was thought to result from intracistronic complementation.

To gain an insight into the structure and expression of the toxin, temperature-sensitive nonkiller mutants (TSNK phenotype) were sought recently. The results obtained in the characterization of one of the recovered mutants suggested that KP6 consists of two different polypeptides that are essential for the toxic activity. The TSNK mutant was shown to be affected in one of these polypeptides. Reexamination of in vitro complementing mutants similar to those reported by Koltin and Kandel (15) provided additional support for the involvement of two polypeptides in the toxic activity. In addition, the results suggest that there is no interaction between the polypeptides in solution. The sequence of interactions between the polypeptides and the target cell is defined.

MATERIALS AND METHODS

Strains. The strains used are from the collection at Tel Aviv University (Table 1). All strains have been deposited in

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TABLE 1. Strains of *U. maydis*

Strain	Killer phenotype	Virus particle	dsRNA segment	Nuclear genotype[a]	Source or reference
75-1	K	P6	H1M2L	P6r (a2bK)	1
75-1 NK-3	NK	P6	H1M2L	P6r (a2bK)	This work
75-1 NK-13	NK	P6	H1M2L	P6r (a2bK)	This work
75-1 U1	NK	P6	H1	P6r (a2bK)	This work
3047-1	K	P6	H1M2L	P6r *ade1 nic3* (a1bA)	This work
18[b]	NK			P6s (a2bG)	15
ATCC 36990	K	P6	H1H2M1M2L	P6r *ade1 nic3* (a1bA)	15
ATCC 36991	K	P6	H1H2M1M2L	P6r *ade1 nic3* (a1bA)	15

[a] From left to right, nuclear gene for resistance, auxotrophic markers, and the mating type (in parentheses).
[b] The universal sensitive tester strain.

the American Type Culture Collection (Rockville, Md.). Mutants were induced in killer strain 3047-1, which was also used as the parental killer strain in genetic transfer experiments. Strain 75-1U1 with a deleted viral genome was obtained spontaneously from strain 75-1. 75-1U1 served as the recipient in the transmission of the viruses from strain 3047-1. The nonkiller mutant strain 75-1NK3 was obtained as a spontaneous mutant from killer strain 75-1. The mutant 75-1NK13 was obtained by cytoplasmic transfer of viruses from strain ATCC 36991 to 75-1U1. Thus, the entire study was conducted with an isogenic background of strain 75-1 containing either P6H1M2L wild-type virus that encodes an active toxin, P6H1 virus that does not encode the toxin, or P6H1M2L viruses that encode an inactive toxin. The in vitro complementation properties of the inactive toxin secreted by strains 75-1NK3 and 75-1NK13 are similar to those described earlier by Koltin and Kandel (16) for strains ATCC 36990 and ATCC 36991. These strains contained a more complex viral genome.

Strain 18 served as the universal sensitive strain to identify the toxic activity of cells and toxin fractions during purification procedures. No spontaneous nuclear resistant mutants were ever obtained from this strain.

Growth medium and isotopic labeling. Cells were grown in complete medium (24) containing 1% peptone, 1% yeast extract, and 2% glucose or in *Ustilago* minimal medium (24a) that was buffered to pH 7.0 with 0.05 M phosphate buffer. Agar (2%) was added when necessary. Buffering of the minimal medium was required since the pH of the unbuffered medium reached 2.5 within a short time. With buffered medium the yield of KP6 was similar to that obtained in complete medium, yet purification of the toxin from minimal medium was easier. The optimal growth temperature was 25°C. For growth of TS mutants the restrictive temperature was 34°C. This restrictive temperature was chosen after initial tests in which the effect of the temperature on cell growth and secretion of the toxin was determined. Heterokaryons were formed on solid double-strength *Ustilago* complete medium (4).

Labeled toxin was obtained by growing strain 75-1 to a density of 4×10^7 cells per ml in *Ustilago* minimal medium. Cells were transferred to minimal medium devoid of a sulfate source. ^{35}S (40 μCi/ml, Amersham International plc.) was added to the medium for 50 min. The supernatant of this culture was collected.

Mutagenesis and genetic procedures. A culture of strain 3047 was mutagenized with 3% ethyl methanesulfonate (Sigma Chemical Co., St. Louis, Mo.) to 50% survival as described previously (23). The survivors were dispensed into 20 tubes, and after growth for 24 to 36 h cells were spread on complete solid medium and grown at 25°C. TSNK mutants were screened by replica plating or spotting cells from individual colonies onto lawns of the sensitive strain 18 in duplicate. The plates of the indicator strain 18 were incubated at 25 and 34°C.

The procedure used to distinguish between nuclear host mutants and viral mutants was based on the classical test for cytoplasmic inheritance devised by Jinks (11). In this test compatible cells fuse on nonselective medium and form hyphae containing unfused nuclei of the parental strains with cytoplasmic constituents of both parents. Then a mycelial mat typical of a heterokaryon develops, and hyphal tips are isolated from the heterokaryon in an effort to dissociate between the two interacting strains. On synthetic medium this heterokaryon is unstable and dissociates to its yeastlike haploid parents. The transmission of cytoplasmic organelles and plasmids can be followed, since in the formation of the heterokaryons parental strains with the proper nutritional markers are used to allow a direct selection of one of the parental nuclear types among the descendants. Cytoplasmic characters can be defined if by cytoplasmic mixing certain characters can be transmitted from one strain to the other. Cytoplasmic inheritance was corroborated by the examination of the pattern of the viral dsRNA present in the recipient cells.

Characterization of the toxin. Two bioassays for testing the activity of the toxin were used. (i) Samples of toxin preparations were spotted on plates containing a known number of the sensitive cells in a 10-ml overlay of complete medium with 1% agar. The dilution endpoint of each preparation was determined by the size of the halos formed on the sensitive lawn. (ii) Cultures of the sensitive cells (optical density of 0.2 to 0.3 at 660 nm) were grown in volumes of up to 5 ml (in 25-ml flasks) and aerated vigorously. Toxin was added to the culture, and inhibition of growth was monitored by absorption at 660 nm.

Purification of the toxin from minimal medium. For most of the experiments the supernatant containing the toxin from the cultures of the wild-type and mutant cells was used. Partial purification of the toxin from minimal medium was performed on an ion-exchange column, CM Sephadex C-25, in 0.025 M acetate buffer (pH 5.5). The culture filtrate was passed through the column after adjustment of the pH to 5.5. The volume of the culture filtrate did not exceed the column volume by more than fourfold. The column was washed with the buffer, and the toxin was eluted with the same buffer containing either 0.5 M NaCl or a gradient ranging from 0.15 to 0.5 M NaCl. The proteins in the eluted fractions were monitored at 280 nm. Toxin was located by spotting 10 μl from each fraction of the eluent on a sensitive lawn. Active fractions were dialyzed and lyophilized. The second purification step involved size separation on Sephadex G-50. The

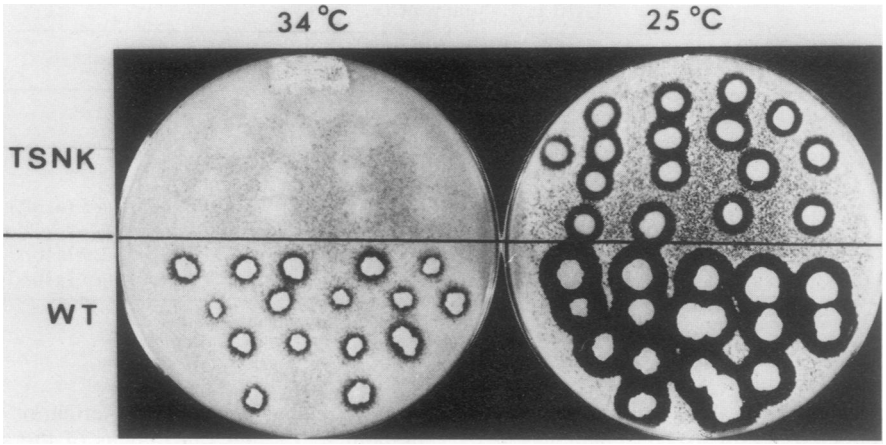

FIG. 1. Killing activity of the wild type (WT) and one of the TSNK mutants tested on a sensitive lawn incubated at 25 and 34°C.

toxin was loaded on the G-50 column as 1 to 2% of the column volume and eluted with 0.3 M NaCl in 0.05 M phosphate buffer. The purity of the toxin was examined electrophoretically in 18% polyacrylamide–SDS gel.

Preparation of immune sera and immunoprecipitation. Antiserum to toxin was prepared by immunization of BALB/c mice with 50 to 100 μg purified toxin (after CM and G-50 column elution) cross-linked with keyhole-limpet hemocyanin. A 1:10 dilution (in phosphate-buffered saline) of the immune sera was interacted with 150 μl of the ^{35}S-labeled supernatant at room temperature for 90 min. Goat anti-mouse F(ab)$_2$ (Bio-Yeda, Israel) was added to the complex. The mixture was incubated at room temperature for 90 min. *Staphylococcus aureus* cells were added and incubated for 1 h at room temperature. The entire complex was centrifuged and washed three times. The complex was separated by 18% SDS–polyacrylamide gel electrophoresis and autoradiographed.

Extraction of dsRNA. The extraction of the viral dsRNA was performed directly from the host cells, since the only dsRNAs found in these cells are the viral nucleic acids. The dsRNA was extracted as described earlier by Wigderson and Koltin (28) and purified by the method of Franklin (8) on a cellulose CF-11 column (Whatman, Inc., Clifton, N.J.).

Gel electrophoresis. Proteins were characterized by polyacrylamide gel electrophoresis as described by Thomas and Kornberg (26) and Davis (3). The dsRNA was characterized in 1% agarose slab gels or in 5% polyacrylamide gel electrophoresis and stained with ethidium bromide (1 μg/ml). The dsRNA gels were viewed with an Ultra-Violet Transilluminator and photographed with a Polaroid MP4 camera.

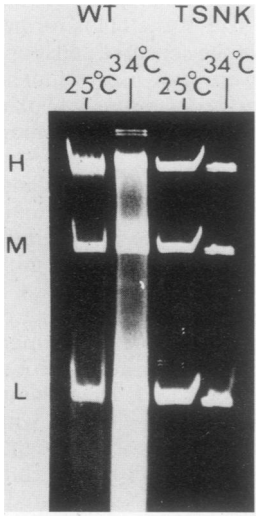

FIG. 2. Polyacrylamide (5%) gel electrophoresis of dsRNA extracted from *U. maydis* cells of strain 3047 (wild type [WT]) and the TSNK strain grown at 25 and 34°C.

RESULTS

Isolation of mutants. Mutants were induced in the killer strain 3047-1. Of 1,000 survivors tested for the killer phenotype at the permissive and the restrictive temperatures, 4 colonies displayed the killer phenotype at 25°C and no apparent killing at 34°C (Fig. 1). To verify that the lack of expression is not due to a temperature-sensitive mutation affecting cell division, the generation time of both the parental strain and the presumed mutants was determined at 25 and at 34°C; no difference was detected.

To determine whether the mutants were affected in nuclear genes or in viral genes, heterokaryon transfer experiments were performed. The expectation was that the temperature-sensitive phenotype would be expressed in the nonkiller recipient only if the mutation were in viral genes. If the mutation were in nuclear genes of the donor, the recipient should express the wild-type killer phenotype.

The heterokaryon transfer experiments were performed with the auxotrophic killer strain 3047-1 and with the compatible nonkiller prototrophic strain 75-1U1. Hyphal tips from the heterokaryon were plated on minimal medium to select the recipient prototroph. Of the developing yeastlike haploid colonies 0.2% displayed the killer phenotype, and all were like the mutant with the temperature-sensitive (TS) phenotype. Thus, the mutation is in the virus, and it is not a nuclear gene mutation of the host cell. The recipient with the TS phenotype was designated 75-1TB.

To determine whether the mutational lesion is related to the replication of the toxin-encoding segment of the viral dsRNA at the restrictive temperature, the dsRNA was extracted from the same number of cells of the mutant and the wild type was grown at 25 and at 34°C. There was no

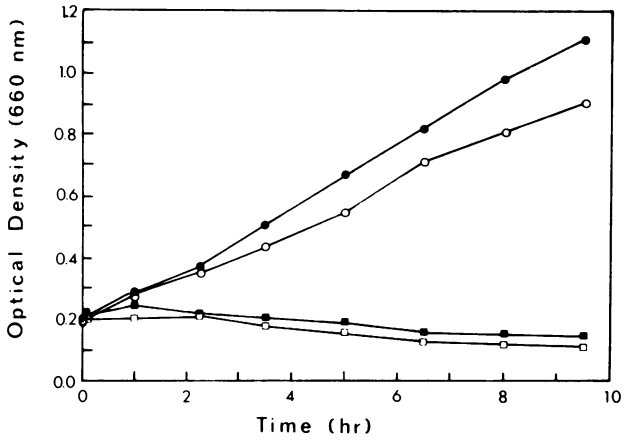

FIG. 3. Killing activity of the toxin from wild-type cells and the TSNK mutant grown at 25 and 34°C and tested at 25°C. The sensitive cells were grown for 9 h without (control) and in the presence of a 1:10 dilution of supernatant from the wild-type killer cells and from the TSNK mutant. Samples of 0.6 ml were withdrawn periodically for optical density determination. Symbols: ●, control; ○, wild type grown at 25°C; □, TSNK mutant grown at 25°C; ■, wild type grown at 25°C. The data for the TSNK mutant grown at 25°C were the same as for the control.

apparent difference between the viral dsRNA extracted from the mutant and the wild-type cells (Fig. 2). Both contained all three dsRNA segments typical of P6 virus, and there was no apparent loss of the toxin-coding segment M2. In addition, the virus-associated, RNA-dependent RNA polymerase activity that is normally associated with the fungal viruses was tested in virions obtained from 75-1TB cells (1). The level of enzyme activity did not vary significantly from the level found in the wild-type virions.

Characterization of the secreted toxins. The TSNK strain secreted a very low level of toxin at the restrictive temperature, as shown by concentrating the supernatant from cultures grown at 34°C. Furthermore, by a comparison of the relative activity of the toxin secreted by TSNK cells and by the wild type at the permissive temperature, it was evident that less active toxin was secreted by the TSNK mutant than the wild type. At the higher temperature the wild-type toxin (1:10 dilution) had the same effect as the toxin secreted at the permissive temperature, whereas the TSNK toxin (1:10 dilution) showed no killing activity (Fig. 3).

To characterize the toxins obtained from the TSNK mutant and the wild type, 18% polyacrylamide–0.09% bisacrylamide gel electrophoresis was performed to obtain better resolution of the low-molecular-weight polypeptides. Under these conditions two distinct bands were noticed for the first time at the position in which the toxin is usually detected as a single band in 12.5% polyacrylamide–0.33% bisacrylamide gel electrophoresis. The molecular weights of these polypeptides were 10,000 and 12,500 (10K and 12.5K polypeptides). These results suggested that the toxin may consist of two different polypeptides and not as suspected earlier as a homomultimer. The toxin secreted by 75-1TB at the permissive temperature showed mostly the 12.5K polypeptide (Fig. 4). Also, when the wild-type toxin was purified by ion-exchange chromatography, eluted with a salt gradient, and examined in 18% polyacrylamide–0.09% bisacrylamide gel electrophoresis in the presence of SDS the 12.5K polypeptide eluted earlier, followed by a region of overlap between 12.5K and the 10K polypeptides which was found alone in the last fractions (Fig. 5). The highest activity was detected in the early fractions, and low activity was present in fractions containing mostly the 10K polypeptide.

These findings led to reexamination of the polypeptides secreted by mutants 75-1NK3 and 75-1NK13 that were shown to secrete an inactive toxin that can be mutually complemented in vitro. These polypeptides were compared with those secreted by the wild-type strain 75-1 and the mutant 75-1TB. As a control the nonkiller strain 75-1U1 was also included in the test (the mutants and the wild type

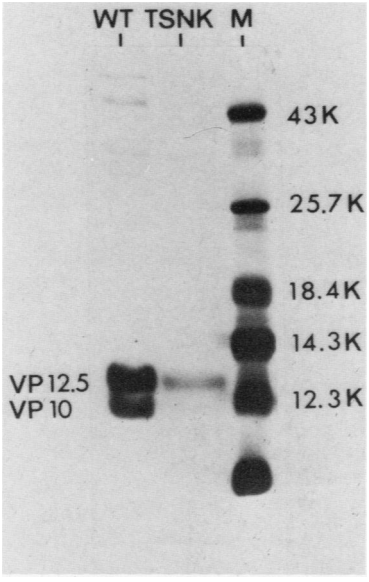

FIG. 4. Reducing SDS–18% polyacrylamide gel electrophoresis of toxins of the wild type (WT) and the TSNK mutant grown at 25°C and partially purified on carboxymethyl Sephadex as described in the text. Molecular mass markers (Bethesda Research Laboratories; low-molecular-mass markers).

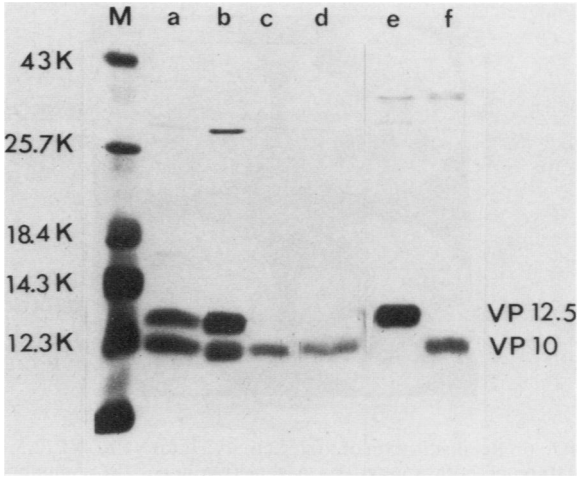

FIG. 5. Reducing SDS–18% polyacrylamide gel electrophoresis of toxin eluted from a carboxymethyl Sephadex column. Lanes: (M) molecular mass markers, (a) concentrated supernatant from culture of wild-type killer strain 75-1, (b through d) three fractions displaying toxic activity eluted by a 0.15 to 0.5 M NaCl gradient (maximal activity in the first fraction), (e) VP12.5 from strain 75-1NK3, (f) VP10 from strain 75-1NK13.

examined were all in an identical genetic background). All of the proteins were electrophoresed after purification on a carboxymethyl Sephadex column.

Cells containing the wild-type P6 virus secreted both 12.5K and 10K polypeptides (Fig. 4 and 5). Each one of the in vitro complementing mutants secreted one of the polypeptides; 75-1NK3 secreted the 12.5K polypeptide, and 75-1NK13 secreted the 10K polypeptide. Strain 75-1U1 secreted neither of the polypeptides (data not shown). The fact that the activity of the toxin could be restored by mixing the 10K and the 12.5K polypeptides showed that the toxic activity involves both polypeptides.

In vitro complementation of 10K and 12.5K polypeptides is demonstrated in Fig. 6. Control experiments showed that each of the polypeptides alone, even in large excess, had no effect. Furthermore, mutant 75-1TB, which produces limiting amounts of the 10K polypeptide, was complemented in vitro only by the addition of the 10K polypeptide. Thus, the contention that the toxin consists of two polypeptides is supported by (i) the absence of both polypeptides in the strain lacking the toxin-encoding segment, (ii) the enrichment of the two polypeptides simultaneous with the purification of the toxin and maximal toxin activity in those fractions containing the two polypeptides, (iii) the reconstitution of toxin activity by the 12.5K and 10K polypeptides, each secreted by one of the nonkiller complementing mutants, and (iv) the restoration of complete toxin activity by the addition of the 10K polypeptide to the polypeptides secreted by mutant 75-1TB. The 10K and 12.5K polypeptides will be referred to henceforth as VP10 and VP12.5, respectively.

Interaction between the two polypeptides. Examination of the behavior of the secreted polypeptides in denaturing gels under nonreducing conditions indicates that there are no covalent bonds between the two polypeptides. The active toxin migrated as two distinct polypeptides (Fig. 7A). In nondenaturing gels (15% polyacrylamide, pH 8.3) without SDS and mercaptoethanol, two distinct bands were distinguished in the purified toxin (Fig. 7B). VP12.5 migrated faster than VP10 under these conditions.

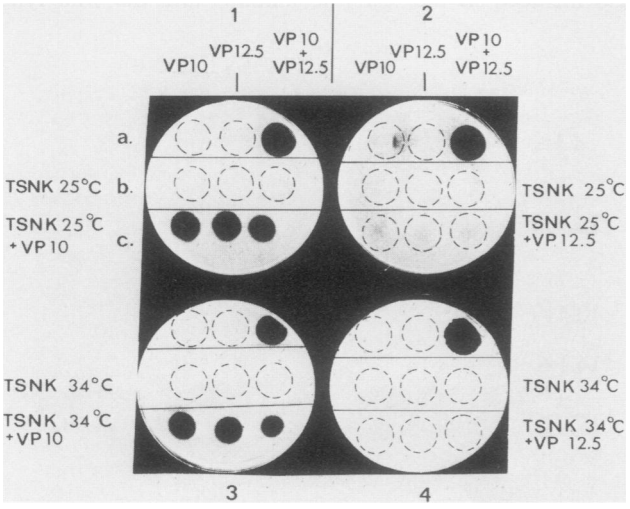

FIG. 6. Reconstitution of toxic activity. 1, (a) VP10, VP12.5, and a mixture of both spotted on a sensitive lawn; (b) a number of dilutions of the TSNK supernatant from 25°C spotted on the sensitive lawn; (c) the same as (b), but VP10 was added to each dilution. 2, The same as 1, but the supernatant of TSNK was from a culture grown at 34°C. 3, The same as 1, but in (c) VP12.5 was added to each of the dilutions of the supernatant from TSNK. 4, The same as 3, but supernatant of TSNK was from culture grown at 34°C.

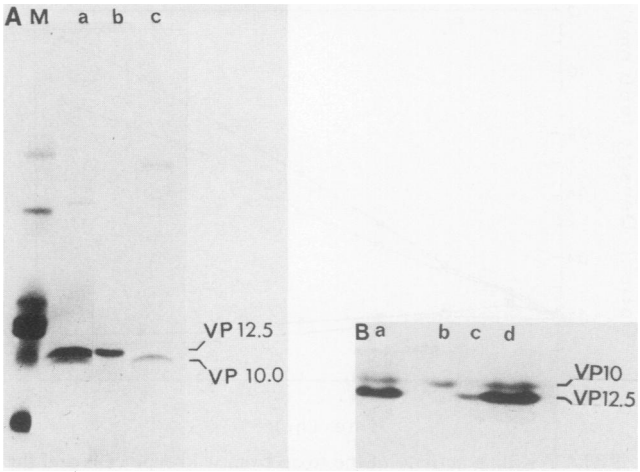

FIG. 7. Electrophoresis of the toxin and the polypeptides in denaturing and nondenaturing gels. A, Lanes: (M) low-molecular-mass markers, (a) partially purified toxin from strain 75-1, (b) partially purified VP12.5 from strain 75-1NK3, and (c) partially purified VP10 from strain 75-1NK13 run in SDS–18% polyacrylamide gel under nonreducing conditions. B, Lanes: (a and d) wild-type toxin, (b) VP10, and (c) VP12.5 run in nondenaturing 15% polyacrylamide gel.

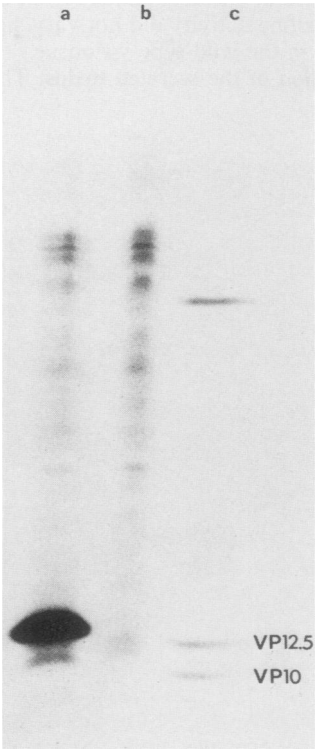

FIG. 8. Reaction of toxin with polyclonal antitoxin sera. Lanes: (a) reaction of toxin with immune sera, (b) reaction of toxin with preimmune sera, and (c) noninteracted supernatant from killer cells. For all tests the supernatant of a culture grown with ^{35}S was used.

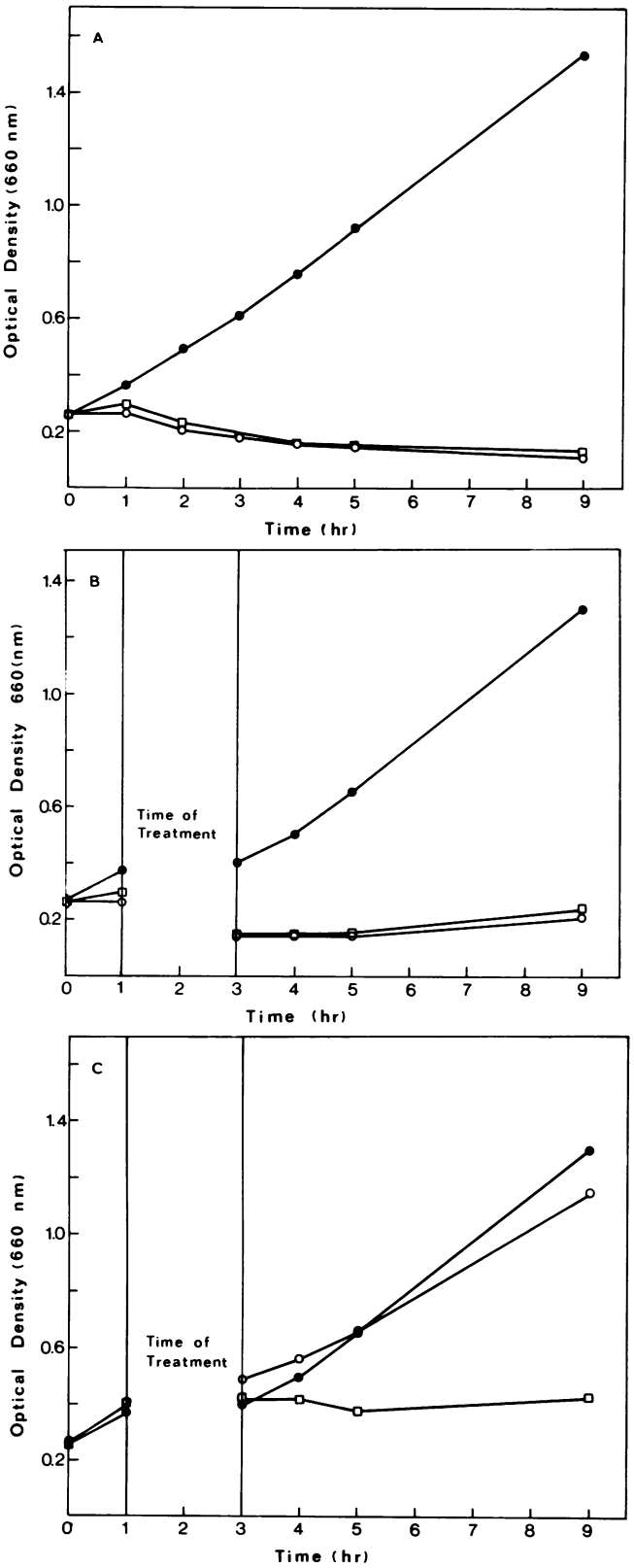

FIG. 9. Effect of KP6 and each of the polypeptides on the sensitive cells. A, Cell growth in the presence of supernatants containing KP6 from strain 75-1 and reconstituted toxin from the complementing 75-1NK3 and 75-1NK13 mutants. Symbols: ●, control (no toxin added); ○, wild-type toxin; □, reconstituted toxin. B, As in A, but toxin was removed after 1 h of exposure. C, Cell growth after exposure initially to supernatant containing one of the polypeptides; the cells were then washed as described in text and exposed to the second polypeptide. Symbols: ●, control (no toxin added); ○, VP12.5 and then VP10; □, VP10 and then VP12.5.

Heating of the purified toxin to 80°C for up to 10 min in the presence of 1% SDS did not affect the activity once the sample was cooled and the SDS was diluted to less than 0.03% (wt/vol). When each of the polypeptides was treated separately under the same conditions and then tested for its activity by adding the complementary polypeptide, the treated polypeptide showed no decline in activity. The cells were not sensitive to SDS alone below 0.05%.

In contrast to the insensitivity of the toxin and each of the polypeptides to SDS, mercaptoethanol did inactivate the toxin. The effect of 2.3 M mercaptoethanol was tested on the purified and concentrated toxin (dilution endpoint, 1:400) and each of the polypeptides (dilution endpoint, 1:128).

At room temperature VP10 was very sensitive to mercaptoethanol and was inactivated within 2 min, whereas VP12.5 was not affected by mercaptoethanol under the same conditions. However, incubation of the purified toxin or VP12.5 at 80°C in the presence of 2.3 M mercaptoethanol led to the inactivation of both within 2 min. The activity of the toxin was totally abolished, and that of VP12.5 was reduced from a dilution endpoint of 1:128 to ca. 1:10.

Considering the patterns of migration of the toxin in denaturing nonreducing gels and in native gels and the effect of SDS and mercaptoethanol, it appears that the polypeptides do not act as two subunits of KP6. The results indicate that there are no intermolecular S-S bonds but rather some intramolecular bonds.

Even an interaction between the polypeptides based on electrostatic bonds seems unlikely. The activity of the native toxin was the same in the presence of 0.3 M NaCl–0.01 M phosphate buffer as in the presence of 0.01 M phosphate buffer alone. If electrostatic forces were significant for the interaction between the two polypeptides it appears likely that the concentration of salt used would have hindered the charged sites needed for the interaction.

Further support that the two polypeptides are distinct and one is not derived from the other was obtained by immunoprecipitation of the toxin. ^{35}S-labeled toxin was reacted with polyclonal antitoxin antibodies. Only VP12.5 was immunoprecipitated (Fig. 8), indicating that VP10 and VP12.5 do not share common determinants and occur as separate units in solution.

Cooperative interaction between the polypeptides and the target cell. Cultures (5×10^6 cells in a 5-ml volume) of the sensitive strain were each exposed for 1 h at 25°C to supernatant from the wild-type killer strain, to the supernatant of each of the complementing nonkiller mutants, or to a mixture of equal volumes (2 ml) of the supernatants of both mutants. After 1 h of exposure the cells were washed in 10 ml of 0.01 M phosphate buffer (pH 7.0) and suspended in fresh medium, and their growth was monitored by absorbance at 660 nm.

The reaction of sensitive cells to the wild-type toxin and to the reconstituted toxin is shown in Fig. 9A. A decline in optical density was observed, indicating a decrease in cell size and lysis, which was confirmed also by microscopic examination. When the wild-type toxin or the reconstituted toxin was removed after 1 h of exposure, no significant increase in optical density was observed (Fig. 9B).

Exposure of the sensitive cells to the supernatant of each

of the complementing mutants (separately) had no effect on cell growth. However, when the cells were first exposed to the supernatant containing VP10 and then washed and exposed to VP12.5, total inhibition of growth was observed (Fig. 9C). When the sequence of exposure of the cells to the supernatants was reversed, no significant effect on cell growth was detected. Control experiments showed that VP12.5 was not degraded during this period, since the same killing effect was obtained by the addition of VP10 at any time up to 2 h after exposure.

DISCUSSION

Detection of the new viral mutant suggested that the *U. maydis* virus-encoded KP6 polypeptide consists of two components essential for its toxic activity. The identification of the impaired function in this mutant led to the reexamination of other viral mutants that were known to display in vitro complementation of the toxic activity. The results clearly indicate that KP6 can be resolved into two distinct polypeptides with no cross-reactivity. Each of the in vitro complementing mutants secretes one of the two polypeptides, and the new viral mutant secretes minute amounts of one of the two polypeptides. The interaction of the two polypeptides with the target cell suggests a sequential behavior initiated by VP10. This unit may act as a recognition component that interacts with a cell receptor, making the cells accessible to the catalytic unit VP12.5 (19). This polypeptide may act independently on the cells sensitized by VP10 or in cooperation with VP10 receptor complex. These aspects are currently under investigation.

The lack of intermolecular bonds between cooperating toxic polypeptides acting on a target cell is quite unique, and few examples for such an interaction are known in nature. A precedent for such cooperative phenomena is known in the crotoxin complex from the venom of the Brazilian rattlesnake (9). The crotoxin complex consists of a phospholipase A and an acidic peptide crotapotin. Phospholipase A is relatively nontoxic by itself, but the addition of crotapotin potentiates it to its high neurotoxicity. Likewise, each of the virus-encoded polypeptides is not toxic to the cells, but VP10 potentiates VP12.5. The nature of the interaction between the polypeptides and the sensitive cell and the molar ratio of the two components required for the toxic activity must be defined.

The only other fungal toxin encoded by dsRNA viruses that has been characterized in detail is found in the yeast *S. cerevisiae* (27). This toxin acts on the membrane of sensitive cells, leading to leakage of ions. The general organization of the yeast virus and the *U. maydis* virus is similar. Until recently the *S. cerevisiae* toxin that was considered as one polypeptide had been resolved into two subunits, alpha and beta, of a size range similar to that of the *U. maydis* polypeptides. However, intermolecular S-S bonds between the subunits were detected in the *S. cerevisiae* toxin, and dissociation of alpha and beta subunits leads to a loss of activity. Thus far, no distinction has been made between the recognition and catalytic components of this toxin. The differences in the intermolecular interactions of the *S. cerevisiae* and *U. maydis* toxins may be related to the molar ratios required by each toxin for activity. Nonetheless, the toxic activity bears some similarity, being highly species specific. In addition, the first step of interaction with the membranes may be similar.

The fungal toxins encoded by the dsRNA viruses bear a degree of similarity to the bacterial colicin in their species specificity and their dual function of recognition and catalysis (18). However, all bacteriocins examined thus far consist of one polypeptide chain. It is the bacterial protein toxins, like cholera and diphtheria toxins, that are not species specific (6). These toxins are more complex and bear a greater similarity in their organization to the virus-encoded toxins.

The precise role of each of the *U. maydis*-encoded polypeptides and their interaction with the target cell is currently under investigation.

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