KILLER PHENOMENON IN *USTILAGO MAYDIS*: THE ORGANIZATION OF THE VIRAL GENOME*

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ABSTRACT

The double-stranded RNA content, the production of inactive killer protein, and the presence of virus-like particles were examined in induced nonkiller mutants and nonkiller progeny from a cross between a killer strain and a sensitive strain. A correlation between the loss of the 0.7×10^6 daltons dsRNA of the *Ustilago maydis* P6 virus and the lack of synthesis of the killer protein was established. *In vitro* and *in vivo* complementation between nonkiller strains provide additional support for the suggestion that the 0.7×10^6 daltons dsRNA is related to the killer function. The coding capacity of the various species of dsRNA is discussed in relation to their possible function.

INTERSTRAIN inhibition in *Ustilago maydis*, known as the killer phenomenon, was shown to result from the excretion of a proteinaceous inhibitory substance by some strains of Ustilago (HANKIN and PUHALLA 1971; KOLTIN and DAY 1975). Most strains are sensitive to this substance. Three killer specificities are known and the killer strain with one specificity is sensitive to the killer protein of a different specificity. The three specificities are known as P1, P4 and P6. Each killer is immune to the killer protein it produces. The immunity is inherited as a cytoplasmic character.

The excretion of the killer toxin is related to the presence of double-stranded RNA (dsRNA) virus-like particles (VLPs) in the cells of the killer strains (WOOD and BOZARTH 1973; DAY and ANAGNOSTAKIS 1973; KOLTIN and DAY 1976a). Strains without VLPs are sensitive, unless they carry a nuclear gene for resistance. Sensitive strains with dsRNA lacking VLPs and sensitive strains carrying VLPs with the entire viral genome were reported recently (KOLTIN and DAY 1976b; KOLTIN 1977). The latter are laboratory-induced nonkiller mutants.

The dsRNA recovered from the VLPs or from cells of killer strains is segmented. Three distinct dsRNA patterns are known, each typical of a distinct killer specificity (KOLTIN and DAY 1976a). Recently, studies were initiated to determine the organization of the viral genome in terms of the distribution of

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the information in the various segments, using mutants with partial genomes. The study of the genetic behavior and the dsRNA pattern of these nonkiller mutants (KOLTIN 1977) suggested that the information in the different dsRNA molecules is not redundant and that a specific molecule contains the information for viral replication and coat formation, whereas other molecules contain the information for the killer substance and immunity to the killer toxin.

An additional series of nonkiller mutants is described in the present paper. The results obtained with this series, together with the earlier reported findings (KOLTIN 1977), allow further assignments of viral functions to the different components of the viral genome. *In vitro* complementation data among nonkiller mutants also shed some light on the nature of the killer toxin.

MATERIALS AND METHODS

Strains: Nonkiller mutants were induced in an auxotrophic P6 killer strain (C-28-12). An adenine-requiring auxotrophic strain was obtained from a cross between a P6 killer and an auxotrophic sensitive strain. The induced nonkillers are designated NK-3, NK-5, NK-9, NK-12, NK-13, NK-15, NK-16 and NK-27. The nonkillers designated T-4, T-7, T-9 and T-11 were recovered as nonkiller progeny from a cross of the P6 killer No. 75 with the sensitive strain No. 7. Other nonkiller strains used in the study (NK-10 and NK-11) were those described by KOLTIN (1977).

Strains used in the genetic analysis were sensitive strain No. 12, P1 killer No. 1268, P4 killer No. 1407 and P6 killer No. 75. All the strains are from the collection of DR. P. R. DAY, The Connecticut Agricultural Experiment Station, New Haven, Conn.

Mutagenesis: The mutagenic treatment with N-methyl-N-nitro-N-nitrosoguanidine (NTG) was as described by KOLTIN (1977). Among a control sample of 534 untreated cells, no nonkillers were detected. Among 400 treated cells, eight independent nonkiller mutants were recovered. The mutant NK-16 was induced by UV irradiation.

Genetic analysis: Two types of genetic procedures were used; (a) formal crosses and (b) heterokaryon transfer experiments. The former is designed to test the effect of nuclear genes on the phenotype; the latter is designed to test the inheritance of certain phenotypes by cytoplasmic exchange.

The crosses, dissection of tetrads, and the isolation of spores were performed as described by STEVENS (1974) and PUHALLA (1968). Heterokaryons were formed between compatible strains on synthetic media, as described by DAY and ANAGNOSTAKIS (1973). The nonkiller parent always carried a nutritional requirement, whereas the strain that was to be tested for the acquisition of the cytoplasmic function was prototrophic. The unstable heterokaryon was dissociated on minimal medium, thus eliminating the auxotrophic parent. The prototrophic cells were then tested for specific phenotypic changes resulting from the cytoplasmic mixing in the absence of the exchange of nuclear information.

Identification of phenotypes as killers, nonkillers and sensitives was described by KOLTIN and DAY (1976a,b).

Analytical procedures: dsRNA was extracted following the procedure of DAY *et al.* (1977). The cells were disrupted in a Braun Homogenizer, as described by KOLTIN and DAY (1976a). Final purification of the dsRNA was performed on a cellulose CF11 column according to FRANKLIN (1966). Electrophoresis was performed in 5% polyacrylamide gels at 2 mA/gel for 2.5 to 3 hr. All other conditions of the electrophoresis as well as the isolation of the VLPs were as described by KOLTIN and DAY (1976a) and KOLTIN (1977).

The killer protein was recovered from the supernatant of cultures grown for 40 hr in liquid complete medium (STEVENS 1974) on a rotary shaker at 25°. The protein was concentrated 10-fold by lyophilization, followed by dialysis against 0.01 M phosphate buffer, pH 7.1. Samples

(100 μl) were electrophoresed on 10% SDS polyacrylamide gels according to the procedure of WEBER and OSBORN (1969). The gels were stained overnight with 0.2% Coomassie Blue in 10% trichloroacetic acid and destained in methanol:acetic acid: water (2:3:35 v/v). Polyacrylamide disc gel electrophoresis at pH 8.3 was performed according to the procedure of DAVIS (1964). Activity was eluted from sliced duplicate unstained gels.

RESULTS

The recovery of both nonkillers induced by mutagenesis and nonkillers derived as progeny from a cross between a killer strain and a sensitive strain permits the comparison of a spectrum of nonkillers from two sources and extension of the mapping of various viral functions. The comparison was primarily that of the pattern of dsRNA in the nonkillers and its relationship to the ability of nonkillers to produce killer protein and/or VLPs.

The dsRNA and the killer protein: The results (Table 1) indicate the presence of seven different variants of nonkillers, based on the dsRNA patterns. The spectrum of incomplete genomes ranges from NK-10 with a single component to the group NK-3, 5, 12, 13 and 15 containing all 5 dsRNA species found in the P6 killer parent, plus an additional component (0.06×10^6 daltons). The UV-induced mutant NK-16 lacks one species of dsRNA (0.7×10^6 daltons), but has 2 new species (0.4 and 0.06×10^6 daltons).

The pattern of dsRNA in nonkiller progeny obtained from the cross was not uniform. The four progeny represent three distinct patterns that do not overlap the patterns in the induced mutants. The pattern found in T-4, T-7 and T-10 can be interpreted as resulting from the loss of certain species of the P6 genome during zygote formation or during meiosis. The pattern displayed by T-11 is more complex and suggests the possibility of improper processing of the dsRNA resulting in the appearance of a new species (0.06×10^6 daltons). The incidence of this type of nonkiller among the nonkiller progeny is not known.

TABLE 1

dsRNA in killer P6 and in nonkiller mutants from killer P6

MW × 10^6 daltons	Parent	NK-3 NK-5 NK-12 NK-13 NK-15	NK-9 NK-11 NK-27	T-11	NK-16	T-7 T-10	T-4	NK-10
2.9	+	+	+	+	+	+	+	+
2.6	+	+	+	+	+	+		
0.7	+	+						
0.5	+	+	+	+	+	+	+	
0.4					+			
0.06		+		+	+			
0.05	+	+	+	+	+			
Killer protein	Active	Inactive	Not detected					

The patterns of dsRNA in the nonkillers indicate that nonkillers may contain the entire viral genome or partial genomes to varying extents. This result complicates the assignment of specific functions such as the killer function to specific dsRNA molecules. However, this difficulty can be overcome if the protein in its active and inactive form can be identified.

The development of a procedure for the purification of the killer protein and characterization of its behaviour on SDS polyacrylamide gels (KANDEL and KOLTIN, in preparation) provided a means to classify the nonkillers into two groups, those producing an inactive killer protein that migrates on the SDS gels at the same position as the normal killer protein, and a second group in which the protein is not produced at a level that can be detected on gels even after a 10-fold concentration of the culture supernatant (Figure 1). The first group includes mutants NK-3, 5, 12, 13, 15; the second group includes the mutants NK-9, 10, 11, 16 and 27, as well as the nonkiller progeny T-4, 7, 10 and 11.

Examination of the dsRNA patterns of the members of each of the two groups and the parental pattern indicates that all the nonproducers lack one common species of dsRNA, the 0.7×10^6 dalton component. All other species lacking in one of the nonkillers may be found in other nonkillers of the same group. Nonkillers that retained the 0.7×10^6 dalton dsRNA component appear to synthesize the killer protein in an inactive form. The results suggest that the 0.7×10^6 dalton dsRNA molecule contains information related to the synthesis of the killer

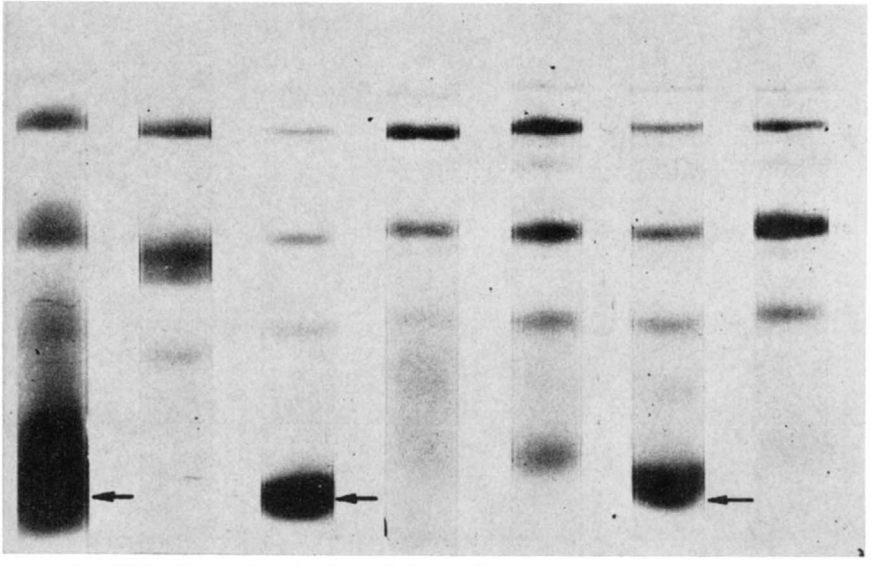

FIGURE 1.—SDS polyacrylamide disc gel electrophoresis of killer and nonkiller culture supernatants.

The arrow indicates the site of migration of active killer P6 toxin. From left to right: KP6-parent, NK-10, NK-15, T-10, NK-27, NK-12, NK-9. Positive protein bands were also identified in NK-3,5,13 and were absent in NK-11, NK-16, T-4 and T-7.

protein. This may be the information for the primary structure of the killer protein or a signal for its synthesis from either a second dsRNA component or the host genome.

The grouping of the nonkillers is further supported by *in vitro* complementation test. The concentrated supernatants of two nonkiller strains were mixed in equal volumes and added to an antibiotic test pad on a lawn of sensitive cells. Complementation between the inactive products of two nonkillers was detected as a zone of inhibition around the pad, whereas a ten-fold concentration of the products of nonkillers alone gave no killing (Figure 2). Similarly, "*in vitro*" complementation was tested with cells of the nonkillers by placing a suspension containing the mixture of two different nonkillers on a lawn of sensitive cells. The results from both methods were identical. *In vitro* complementation was detected in four combinations (Table 2). The mutant NK-13 complemented the mutants NK-3, 5, 12 and 15, showing the same specificity as the parent P6 killer. In all other combinations no indication of complementation was obtained.

Examination of the dsRNA pattern of the mutants in relation to the complementation results indicated that positive *in vitro* complementation occurs only among nonkillers possessing the 0.7×10^6 daltons dsRNA. Furthermore, the complementation is displayed only among the nonkillers that were earlier classi-

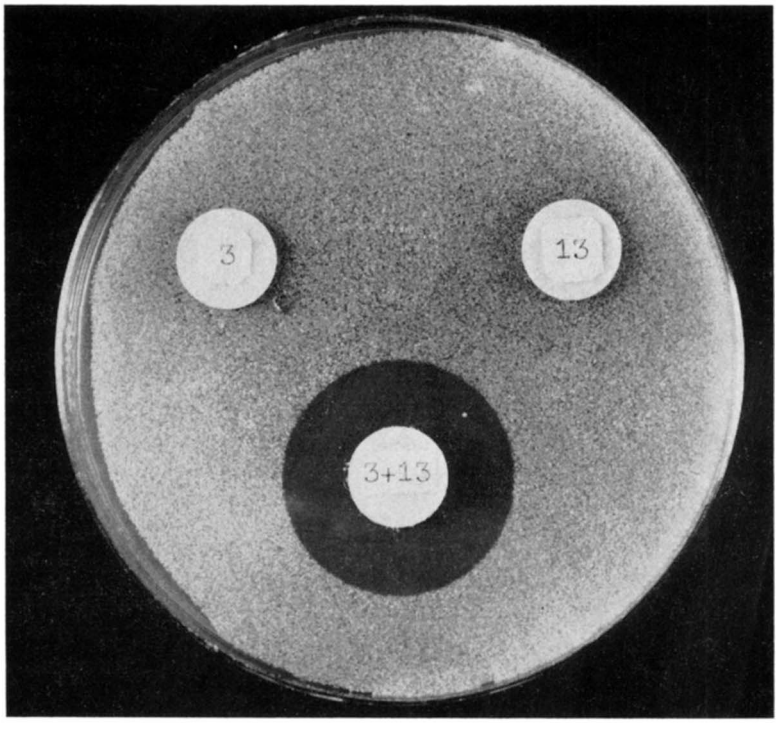

FIGURE 2.—*in-vitro* complementation between nonkiller mutants NK-3 and NK-13.

TABLE 2

In vitro *complementation of extracellular proteins from nonkiller strains of P6*

	Nonkillers													
	NK-3	NK-5	NK-12	NK-15	NK-13	NK-9	NK-11	NK-27	T-11	NK-16	T-7	T-10	T-4	NK-10
NK-3	—													
NK-5	—	—												
NK-12	—	—	—											
NK-15	—	—	—	—										
NK-13	+	+	+	+	—									
NK-9	—	—	—	—	—	—								
NK-11	—	—	—	—	—	—	—							
NK-27	—	—	—	—	—	—	—	—						
T-11	—	—	—	—	—	—	—	—	—					
NK-16	—	—	—	—	—	—	—	—	—	—				
T-7	—	—	—	—	—	—	—	—	—	—	—			
T-10	—	—	—	—	—	—	—	—	—	—	—	—		
T-4	—	—	—	—	—	—	—	—	—	—	—	—	—	
NK-10	—	—	—	—	—	—	—	—	—	—	—	—	—	—

"+" indicates positive complementation.

fied on the basis of the protein pattern as producers of an inactive killer protein. Thus, it appears that mutants NK-3, 5, 12, 13, 15 produce inactive killer protein of at least two types, one represented by NK-13 and the other by NK-3, 5, 12 and 15. The latter may all be identical.

Clones derived from heterokaryons formed between compatible cells were tested for killing activity by *in vivo* complementation. This was performed using nonkillers that did not display *in vitro* complementation, since *in vitro* complementation would lead to false positive results in the *in vivo* test. The results once more supported the contention that it is the 0.7×10^6 dalton dsRNA that is related directly to the killer function. *In vivo* complementation occurred between mutants NK-3, 5, 12, 15 and mutant NK-16. Also, mutant NK-3 was complemented by T-11. In each case, positive results were obtained when one of the two mutants in each complementation test contained the 0.7×10^6 daltons dsRNA. *In vivo* complementation was never detected in clones derived from 13 heterokaryons involving mutants NK-9, NK-10, NK-11, NK-27, T-4, T-7 and T-11. All these mutants lack the 0.7×10^6 daltons dsRNA.

The pattern of dsRNA in the killer clones derived from *in vivo* complementation was similar to the pattern found in P6 killer strains. The complementation can be visualized as resulting from either molecular reassortment or a situation similar to a helper virus system (LURIA and DARNELL 1967). In either case this is an intracellular phenomenon contrary to the *in vitro* complementation which is an extracellular phenomenon.

The *in vivo* complementation pattern also suggests that a lower molecular weight dsRNA may play some role in processing the killer information. The positive complementation between NK-3 and T-11 as well as the complementation between NK-16 and the mutants NK-3, 5, 12 and 15 suggest some involvement of the lowest molecular weight dsRNA, since the same group of mutants did not complement T-7 and NK-10. The difference between T-7 and NK-16 or T-11 is only in the low molecular weight dsRNA that T-7 lacks and NK-16 and T-11 possess. NK-11, which also possesses a low molecular weight dsRNA, gave only negative complementation. This mutant was derived following treatment with NTG and may possess a mutation in its lower molecular weight dsRNA which prevents complementation.

The pattern of *in vivo* complementation also distinguishes between NK-13 and the remaining mutants with a similar dsRNA pattern, namely, NK-3, 5, 12 and 15. Apparently, NK-13 harbors a more severe mutation in the inactive protein than the one found in the NK-3, 5, 12 and 15 group, since NK-13 cannot complement *in vivo* any nonkiller tested that lacks the 0.7×10^6 daltons dsRNA. The NK-3 group may be lacking only a final processing of the killer protein, a function that can be provided by nonkillers that carry the low molecular weight dsRNA. This difference is supported by other properties that distinguish between NK-13 and the NK-3, 5, 12 and 15 group. The two groups exhibit different migration patterns on polyacrylamide disc gels at pH 8.3. Inactive protein from the two groups can be eluted from the gels and identified by *in vitro* complementation. In addition, protein from members of the NK-3, 5, 12 and 15 group, but not NK-13, can be recovered with full activity, following elution from SDS polyacrylamide gels. Thus, NK-13 and the NK-3, 5, 12 and 15 group seem to be affected at two distinct sites.

The dsRNA and VLPs: In an earlier study including the mutants NK-10 and NK-11, it was suggested that the information related to the replication of the VLPs and the formation of coats is contained in the heaviest dsRNA component of the P6 genome (KOLTIN 1977). All the nonkillers included in this study also contain VLPs. In spite of the variation in the dsRNA patterns, the only common dsRNA species found in all the nonkillers derived from the P6 killer is the 2.9×10^6 dalton component, which is the heaviest species of dsRNA in the P6 genome. Thus, there is no deviation from the earlier findings.

Immunity to P6 killer: The loss or retention of immunity in the nonkiller mutants was tested earlier in mutants NK-10 and NK-11 (KOLTIN 1977). The test was extended to include NK-3, 5, 9, 11, 12, 13, 15 and NK-27. Since the P6 killer parent from which the mutants were derived carried a nuclear gene that confers resistance to the P6 killer protein, the test for immunity required the transfer of the dsRNA information into a sensitive strain by the heterokaryon transfer procedure or the analysis of formal crosses as described by KOLTIN (1977). Both procedures were followed in the current study and in both cases the indications are that immunity was lost along with the killer function. The dsRNA pattern from sensitive progeny derived from crosses of the nonkiller

mutants with a sensitive parent was determined. In addition, VLPs were sought in the same progeny. Without exception, the dsRNA molecules identified in the nonkiller mutants were also found in the sensitive progeny and these progeny contained VLPs. In addition, nonkillers containing the 0.7×10^6 dsRNA band retained the ability to produce an inactive protein while exhibiting sensitivity to the P6 killer and to their *in vitro* complement.

Of 11 mutants examined to date for the effect of the loss of the killer function on immunity to the killer protein only one is known to retain the immunity, NK-1 described by KOLTIN (1977). Although a specific assignment of the immunity function to a specific dsRNA molecule cannot be made at this stage, it seems that the two functions are intimately related and may be affected simultaneously. Perhaps both functions are contained in the same molecular species of dsRNA.

Genetics of the nonkiller mutants: With the exception of NK-16 all other induced mutants were crossed with a killer strain with P6 killer specificity and with a sensitive strain to determine whether or not the loss of killer expression was a result of a mutation in a nuclear gene that controls the maintenance and expression of the dsRNA and VLPs. Such genes have been reported in the yeast killer system (for review see LEMKE 1976; WICKNER 1976). If the loss of killer function were the result of nuclear gene mutations, hindering the expression of cytoplasmic viral information, segregation within tetrads was anticipated in the cross with a killer strain and the restoration of killer activity in half of the progeny of tetrads in crosses of nonkillers with sensitive strains.

In crosses of the nonkillers with the killer strain, the proportion of killer tetrads that were uniformly killers was only 30–50%. A similar proportion of uniformly nonkiller tetrads was detected in the crosses along with a few tetrads that displayed segregation of killers and nonkillers within a tetrad. Among the killers of the segregational tetrads, weak killers and strong killers were detected. This pattern of segregation is typical of the cytoplasmic suppression phenomenon described by KOLTIN and DAY (1976b) and in yeast by SOMERS (1973). This pattern of segregation cannot be related to a nuclear gene. Furthermore, in crosses with a sensitive strain lacking dsRNA and VLPs and known for its competence to become transformed to a killer, all the progeny were nonkillers. Therefore, the results provide no indication thus far for the involvement of any nuclear gene in the maintenance or expression of the killer function.

DISCUSSION

The information derived from studies with induced nonkiller mutants and nonkiller progeny from crosses provides additional indications that the genome of the dsRNA *Ustilago maydis* virus is complex. The segmented genome can be roughly divided into at least three functional segments. The heaviest dsRNA molecule, or "chromosome," is involved in replication and coat formation, one

of the medium size dsRNA molecules is involved in synthesis of killer protein, and the smaller dsRNA appears to be involved in processing of the killer protein. Thus, it is becoming increasingly clear that the segmented genome represents a group of molecules with unique information.

While little information is available concerning the structural and enzymic proteins required for VLP replication and coded for by the dsRNA, data have recently been obtained on some properties of the killer toxin (KANDEL and KOLTIN, in preparation). The toxin, which migrates as a single band on SDS polyacrylamide gels, has a molecular weight of approximately 9–11,000 daltons. The information for production of a molecule of this size could occur in a segment of dsRNA of a minimum molecular weight of $0.16 - 0.20 \times 10^6$ daltons. This eliminates the lower molecular weight dsRNA whose polypeptide product would be no greater than 30 amino acids, but fits well with our conclusions that the 0.7×10^6 dalton band is responsible for toxin synthesis. Although it has not been shown conclusively that the information in this band is responsible for the primary protein sequence, it appears likely that this is the case, since there has been no indication of involvement of nuclear genes.

In further support of this contention, we have been able to show that toxin is produced only in strains carrying the 0.7×10^6 dalton dsRNA. Mutants bearing this molecular weight species retain the ability to produce an inactive protein of similar size and are able to exhibit active killing through *in vitro* complementation. These mutants fall into two classes, NK-13 and the NK-3, 5, 12 and 15 group, which seem to be affected at two different sites or functions. This is suggested by the fact that only the latter group exhibits positive *in vivo* complementation, and the inactive protein of this group can be reactivated by SDS. It may be that NK-13 carries a mutation in the killer protein, whereas the NK-3, 5, 12 and 15 group is affected only in a processing step that leads to a conformation of the protein required for killing. *In vitro* complementation may, therefore, involve an interaction between two protein molecules with defective conformations that results in the formation of a functional active site, or an interaction between one molecule affected in its conformation and the other affected in the active site. A homomultimer may be the native state of the active killer protein.

The *in vivo* complementation between the NK-3 group and NK-16 and T-11 may result from the contribution of the appropriate information for final processing by NK-16 and T-11. This information would be ineffective in a nonkiller strain carrying a point or nonsense mutation in the 0.7×10^6 dsRNA.

Immunity appears to be closely related to killer activity. The immunity function may reside on the same "chromosome" as the killer function. This dsRNA molecule contains an additional coding capacity for a protein of molecular weight of approximately 30,000 daltons. Alternatively, the immunity may be part of the killer protein itself. This hypothesis is currently being tested by the selection of temperature-sensitive killer mutants.

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