VIRUS-LIKE PARTICLES IN *USTILAGO MAYDIS*: MUTANTS WITH PARTIAL GENOMES[1]

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ABSTRACT

Mutants with partial genomes for the virus-like particles of *U. maydis* were recovered following treatment with nitrosoguanidine. Examination of the properties retained by progeny of genetic crosses indicates that the 2.9×10^6 dalton component of double-stranded RNA contains the information for capsid formation and dsRNA replication. Other components appear to contain the information for killer function and immunity to killer. The use of such mutants for studies on the evolution of viruses with segmented genomes is discussed.

THREE strains of *Ustilago maydis* inhibit many other strains of the same species (PUHALLA 1968; PUHALLA, unpublished). The three strains are referred to as killers, and since each is sensitive to the other two, three killer specificities can be identified, designated P1, P4 and P6.

The killer phenomenon is related to the presence of double-stranded RNA (dsRNA) and associated virus-like particles (VLPs) in the cells. Such particles are absent in sensitive strains (WOOD and BOZARTH 1973; DAY and ANAGNOSTAKIS 1973; KOLTIN and DAY 1976a). Killer substance is a proteinaceous material that is excreted into the culture medium (HANKIN and PUHALLA 1971; KOLTIN and DAY 1975). Each killer strain is immune to its own inhibitory substance and transmits the killer character, together with immunity, through cytoplasmic inheritance. The interrelationship of killer function, immunity, and sensitivity, and the presence of dsRNA and VLPs is detailed in Table 1.

The dsRNA contained in the VLPs appears segmented when examined by polyacrylamide gel electrophoresis, and each killer specificity has a distinct pattern of dsRNA components (KOLTIN and DAY 1976a). Neither the distribution of genetic information in the various species of dsRNA nor the role of nuclear genes in the replication, maintenance and expression of the killer function is known. The isolation and characterization of mutants of the host that have lost the killer function could serve to identify nuclear genes that affect the cytoplasmic determinants, as well as to detect strains carrying partial genomes of dsRNA. Such mutants could make possible the assignment of functions, such as replication of dsRNA, VLP coat formation, killer function and immunity, to the various

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TABLE 1

The relation between the presence of dsRNA and VLPs and killer function, immunity and sensitivity (based on KOLTIN *and* DAY *1976a,b)*

Phenotype	VLP	dsRNA	Designation	Remarks
Killer	+*	+	P1	Kills or inhibits sensitive strains.
Killer	+	+	P4	Each killer is sensitive to the two
Killer	+	+	P6	other killers.
Sensitive	—*	—	P2	
Sensitive	—	+	P2	Suppressive to certain killer and immune strains.
Immune	+	+	P3(1)	Each is immune to the killer specificity
Immune	+	+	P3(4)	shown in parenthesis. Lacks some
Immune	+	+	P3(6)	of the dsRNA found in killers.

* + present; — absent.

segments of the dsRNA genome. Suppression of killer phenotype in sensitive strains of *U. maydis* by a dsRNA not packaged in VLPs was recently reported (KOLTIN and DAY 1976b). Mutants with incomplete dsRNA genomes may throw light on the determination of suppressor specificity. This paper describes three mutants of P6 that have lost killer function. Two of these mutants have incomplete genomes and thus provide an opportunity for assigning genetic functions to specific dsRNA components. A similar approach was used recently for reovirus, a dsRNA mammalian virus with a segmented genome (SPANDIDOS and GRAHAM 1975a,b; SPANDIDOS, KRYSTAL and GRAHAM 1976).

MATERIALS AND METHODS

Strains: The strains of *U. maydis* used were from the collection of DR. P. R. DAY, The Connecticut Agricultural Experiment Station, New Haven, Conn. The mutants were isolated from a P6 killer strain (No. 75). Other killer strains used were a P6 killer (No. 1371), P4 killer (No. 77) and P1 killer (No. 28). The sensitive strain used was No. 5.

Mutagenesis: Log phase cells of strain 75 were treated with 10μg/ml of N-methyl-N-nitro-N-nitrosoguanidine (NTG) in 0.2M citrate buffer pH 5.0 for 27–33 min. The survival after exposure for 33 minutes was $< 1.0\%$. The survivors after 27, 30 and 33 minutes were tested for killer activity. Nonkillers were retained for characterization.

Analytical procedures: Media, crosses, dissection of tetrads and isolation of spores were described by STEVENS (1974). Identification of phenotypes as killers, nonkillers and sensitives was described in KOLTIN and DAY (1976a,b). Extraction of dsRNA followed VODKIN, KATTERMAN and FINK (1974), the conditions for electrophoresis and isolation of VLPs were as described in KOLTIN and DAY (1976a). The electrophoretic runs were adjusted to detect low molecular weight components within the range of 0.01×10^6 daltons. To improve the recovery of VLPs, sucrose gradient fractions showing significant absorption at 260 nm were pooled and pelleted at 100,000*g* for 150 min in a Type 40 rotor.

RESULTS

Seven nonkillers were detected among 389 survivors from NTG treatment. Normally, during mitotic divisions, the loss of the killer function occurs very rarely (DAY and ANAGNOSTAKIS 1973); in a control sample of 1000 untreated

cells, no nonkillers were detected (KOLTIN and DAY, 1976b; KOLTIN and LEVINE, unpublished). To determine whether the loss of killer function was associated with loss of dsRNA and to see if the nonkillers represented a single repeat or a range of mutations, the dsRNA from each nonkiller was extracted and characterized by electrophoresis in 5% polyacrylamide gel.

The nonkillers retained either all or part of the dsRNA (Figure 1). Three patterns were identified. One nonkiller retained all the components known to occur in a normal P6 killer (2.9, 2.6, 0.7, 0.5 $\times$ 10^6 daltons) as reported by KOLTIN and DAY (1976a). An additional component (0.05 $\times$ 10^6 daltons) found recently both by KOLTIN and by DAY (personal communication) in the P6 killer was also found in the nonkiller that retained all other components. Four of the nonkillers had a second pattern in which the 0.7 and 0.05 $\times$ 10^6 daltons dsRNA components were missing. Thus, these nonkillers retained three of the five molecular components. The third pattern, displayed by two nonkillers, resulted from the loss of four of the five molecular components of the wild type, only the heaviest component (2.9 $\times$ 10^6 daltons) was retained.

In the first type of nonkiller the loss of the killer function may result from a point mutation or a very small deletion that did not affect the molecular weight of any of the five components. However, the other two patterns clearly demonstrate the loss of part of the genetic information associated with the killer function.

To see if the loss of killer function and specific dsRNA components is caused by nuclear genes, a representative of each pattern of dsRNA among the nonkillers was crossed to a P6 killer. The nonkillers used will be referred to henceforth as NK-1, NK-11 and NK-10, each representing the three dsRNA patterns

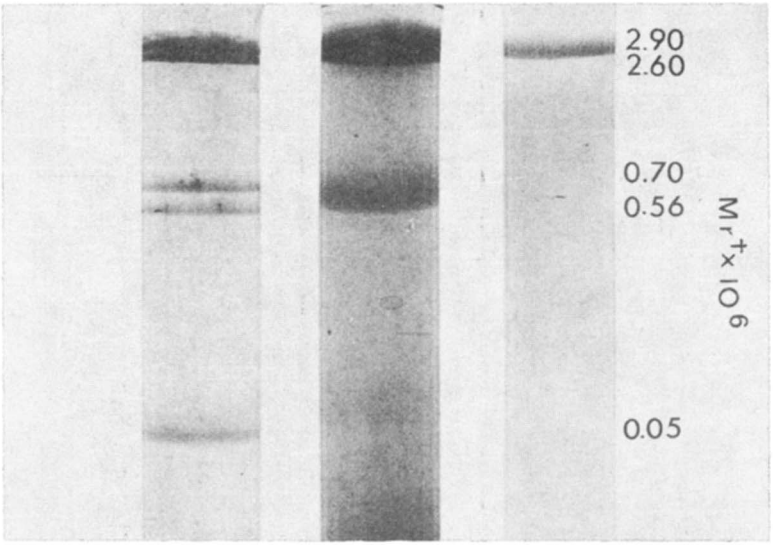


FIGURE 1.—Double-stranded RNA patterns of the mutants NK-1 (identical with the P6 progenitor). NK-11 and NK-10 (from left to right).

† Mr, molecular weight.

in the order described above. The segregation of a nuclear gene affecting the killer function would lead to the segregation of the killer phenotype within tetrads. Otherwise, the tetrads would be uniformly killers or uniformly nonkillers. Due to difficulties in spore germination in one cross, results were obtained in only two of the three crosses. In none was there any indication of segregation of a nuclear gene (Table 2). Further crosses of the mutant NK-1, which did not provide any information in the cross with P6, with other killers failed to indicate segregation of a nuclear gene affecting the killer function.

The role of various dsRNA molecules in functions other than killer can be examined with mutants having partial genomes. VLPs were sought and found in all of the nonkillers. No deviations in size or shape from the description provided by WOOD and BOZARTH (1973), for particles of P1, were noticed. Thus, even the mutant with a single dsRNA component maintains and replicates the nucleic acid and the capsid of the VLP.

A direct test for retention of immunity could not be performed because the progenitor killer strain possessed a nuclear gene for resistance to the P6 killer. Therefore, a representative of each group of mutants, classified on the basis of the dsRNA pattern, was crossed to a compatible sensitive strain (devoid of dsRNA).

The recovery of a high proportion of sensitive progeny would indicate the absence of the immunity factor in the mutant. The cross of each of the two mutants with the partial genomes, NK-10 and NK-11, with the sensitive strain yielded nearly 50% sensitive progeny. When mutant NK-1, which retained all the components of dsRNA, was crossed with the sensitive strain, it yielded 10 tetrads without a single sensitive progeny; and, among 76 random meiotic products, none were sensitive.

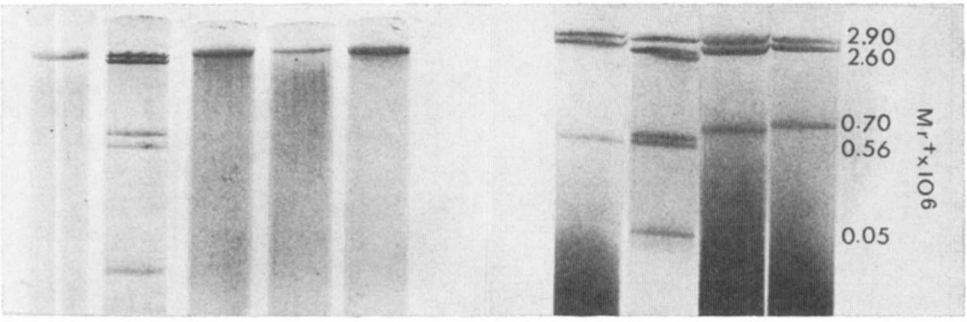
Sensitive progeny from different tetrads were examined for the presence of dsRNA and VLPs. Of fifteen sensitive progeny from fourteen tetrads examined, only one strain contained no dsRNA. All others contained dsRNA components

TABLE 2

Crosses of nonkiller mutants with killer strains

Cross	Random progeny K	NK	Total	Tetrads K	NK	Total
NK-1 × P1	0	261	261	—	—	—
P4	64	0	64	13	0	13
P6	—	—	—	—	—	—
NK-10 × P1	38	72	110	0	6	12*
P4	308	19	327	14	1	15
P6	—	—	—	19	2	21
NK-11 × P1	0	289	289	—	—	—
P4	98	0	98	—	—	—
P6	—	—	—	22	0	22

* Six tetrads contained killer and nonkiller progeny (segregational tetrads).



(a) (b)

FIGURE 2.—From left to right:

(a) NK-10, P6 killer progenitor of NK-10, progeny from a cross between NK-10 and a sensitive strain;

(b) NK-11, the P6 progenitor of NK-11 and progeny from a cross of NK-11 with a sensitive strain.

†Mr, molecular weight.

identical with those found in the parental nonkiller strain (Figure 2). In every case in which dsRNA was detected, VLPs were also found. It thus appears that the mutant that retained all the dsRNA components also retained the immunity function, and that mutants with partial genomes lost this function. These results suggest that the immunity function, at least in part, must reside in components other than 2.9, 2.6 and 0.5×10^6 dalltons. In other words, immunity should be associated with the 0.7 and 0.05×10^6 daltons components. However, the information for dsRNA replication and capsid formation should reside in the 2.9×10^6 daltons component.

Two additional aspects may involve interaction with, or recognition of, nucleic acids of the different VLPs, *i.e.*, the exclusion phenomenon (DELBRUCK 1945; MATTHEWS 1970; REEVES 1972), and the suppression of killer phenotypes (KOLTIN and DAY 1976b). Various exclusion interactions are known to occur in crosses between different killer strains. In a cross between the P6 killer and the P1 killer, the killing activity of both killers is excluded, whereas in a cross between P4 and P6, the latter is excluded and all the progeny resulting from this cross are of the P4 phenotype (KOLTIN and DAY 1976a). The results of crosses between the nonkillers and P1 and P4 can provide some insight into the recognition phenomenon that leads to exclusion. Similarly, the phenomenon of cytoplasmic suppression can be approached. The suppression effect is displayed by a few sensitive strains, some of which carry dsRNA that is not packaged into coats. Crosses between such strains and killer strains result in dilution of the killer effect in the progeny up to the total loss of the killer character during mitotic replication (KOLTIN and DAY 1976b). The effect is discernible in tetrad analysis since a significant proportion of the tetrads show segregation for the killer phenotype within the tetrad and instability within the killer progeny, an unexpected pattern for the cytoplasmic inheritance of the killer character. In addition, the proportion of killer progeny is far below the expected range for a

cytoplasmic trait; in the case of killer, the proportion is greater than 80% among numerous crosses between killer strains and sensitive strains devoid of any suppressive effect.

The three mutants NK-1, NK-10 and NK-11 were crossed to a P1 killer, a P4 killer, and a P6 killer and the results are shown in Table 2. Only in the cross between P1 and NK-10 were progeny with the P1 killer specificity recovered. Thus, exclusion was demonstrated using NK-1 and NK-11 and was absent when NK-10 was used. The mutant NK-1 is indistinguishable from the wild-type P6 with respect to the dsRNA and also with respect to the exclusion phenomenon. The mutant NK-11 with its three dsRNA components also excludes P1; but NK-10, which retained only the 2.9×10^6 daltons components, does not exclude P1. This latter component is shared by NK-10 and NK-11. Therefore, the only two other species that may directly be involved in the exclusion are the 2.6 and 0.5×10^6 daltons components, found in both NK-1 and NK-11.

The exclusion relations of P4 and the mutants were examined on a lawn of sensitive cells carrying nuclear resistance genes to either P4 or P6. The killer progeny recovered always had the P4 killer specificity, which was verified by extraction of dsRNA from a few killer progeny. The proportion of nonkillers recovered from these crosses was very low. However, nonkiller progeny were recovered from the cross between a P4 killer and NK-10. The dsRNA of progenies from two different tetrads was identical with the pattern found in strains immune to the P4 killer protein, P3(4). The 2.9×10^6 daltons component typical of NK-10 was absent in both. It is therefore conceivable that the exclusion relations between P4 and P6 involves the recognition of different components from those recognized in the interaction between P1 and P6. As pointed out earlier, the outcome of the interactions between the killers differ, depending on the killers involved. Extension of these studies to other mutants with partial genomes may reveal the nature of the exclusion phenomenon.

The mutants with partial genomes failed to display any suppressive effect on P4 and P6. The tetrads from such crosses displayed a high frequency of killer

TABLE 3

Functions retained in the nonkiller mutants

Function	Mutant NK-1	NK-10	NK-11
Killer	—	—	—
Immunity	+	—	—
dsRNA replication	+	+	+
Capsid	+	+	+
Exclusion			
of P1	+	—	+
by P4	?	+	?
Suppression			
of P1	?	+	?
P4	—	—	—
P6	—	—	—

tetrads, and great uniformity and stability. Only in the cross between NK-10 and P1 was a low frequency of killer progeny found in a random sample of progeny. Segregational tetrads occurred in a high frequency. The killer progeny were unstable and within four weeks lost the killer function, as well as various dsRNA components of the P1 killer. Thus, the retention of only the one species of dsRNA of P6 is sufficient to cause suppression, but not mutual exclusion. This suggests that suppression and mutual exclusion may be interrelated, both involving some form of recognition of dsRNA.

DISCUSSION

Analysis of the functions retained by nonkiller mutants (Table 3) and characterization of their dsRNA species make possible the assignment of specific functions to specific dsRNA components. The 2.9×10^6 dalton component is essential for replication and capsid formation of the P6 VLP. Other components, such as the 0.7×10^6 daltons, may be involved in the killing function and in immunity. Probably all the species contain information involved in the exclusion phenomenon. Furthermore, the results suggest that the information in the various components of dsRNA is not merely derived by breakage of the 2.9×10^6 dalton component, *i.e.*, the different components are not redundant.

It is evident that an organism can harbor a VLP which can replicate but which lacks information for other functions. A similar phenomenon has been reported in yeast by ADLER, WOOD and BOZARTH (1976). However, the rarity of these defective forms suggests that the forms with a complete genome have a selective advantage. The necessity for at least killer and immunity functions to reside together is easily rationalized.

The Ustilago killer phenomenon parallels in many respects the killer system described in yeast. However, in yeast, a number of nuclear genes have been described which are involved in the maintenance and expression of the killer (SOMERS and BEVAN 1969; NESTEROVA and ZEKNOV 1973; WICKNER 1974, 1976; WICKNER and LEIBOWITZ 1976). In Ustilago no nuclear genes have yet been detected, although the analysis of tetrads reported here would be one way to detect them. Further studies already in progress with nonkillers should resolve this question.

Viruses with multipartite dsRNA genomes are found in animals and higher plants as well as in fungi (WOOD 1973; JASPERS 1974). The VLPs described here which contain incomplete genomes provide ideal material for laboratory construction, by complementation, of VLP's with new specificities. This approach may offer insight into the mode of evolution of VLPs with segmented genomes.

LITERATURE CITED

ADLER, J., H. A. WOOD and R. F. BOZARTH, 1976 Virus-like particles from killer, neutral and sensitive strains of *Saccharomyces cerevisiae*. J. Virol. **17**: 472–476.

DAY, P. R. and S. L. ANAGNOSTAKIS, 1973 The killer system in *Ustilago maydis*: Heterokaryon transfer and loss of determinants. Phytopathology **63**: 1017–1018.

DELBRUCK, M., 1945 Interference between bacterial viruses. III. The mutual exclusion effect and the depressor effect. J. Bacteriol. **50**: 151–170.

HANKIN, L. and J. E. PUHALLA, 1971 Nature of a factor causing interstrain lethality in *Ustilago maydis*. Phytopathology **61**: 50–53.

JASPERS, E. M. J., 1974 Plant viruses with a multipartite genome. Adv. Virus Res. **19**: 37–149.

KOLTIN, Y. and P. R. DAY, 1975 Specificity of *Ustilago maydis* killer proteins. App. Microbiol. **30**: 694–696. ——, 1976a. Inheritance of killer phenotypes and double stranded RNA in *Ustilago maydis*. Proc. Natl. Acad. Sci. USA **73**: 594–598. ——, 1976b. Suppression of the killer phenotype in *Ustilago maydis*. Genetics **82**: 629–637.

MATTHEWS, R. E. F., 1970 pp. 399–402. In *Plant Virology*. Academic Press, New York.

NESTEROVA, G. F. and A. M. ZEKNOV, 1973 Genes controlling "killer" character in *Saccharomyces cerevisiae*. Vestnik Leningradovska Universiteta **21**: 147–149.

PUHALLA, J. E., 1968 Compatibility reactions on solid medium and interstrain inhibition in *Ustilago maydis*. Genetics **60**: 461–475.

REEVES, P., 1972 pp. 42–43. In: *The Bacteriocins*. Springer-Verlag, New York.

SOMERS, J. and E. A. BEVAN, 1969 The inheritance of the killer character in yeast. Genet. Res. **13**: 71–83.

SPANDIDOS, D. A. and A. F. GRAHAM, 1975a Complementation of defective reovirus by its mutants. J. Virol. **15**: 954–963. ——, 1975b Complementation between temperature sensitive and deletion mutants of reovirus. J. Virol. **16**: 1444–1452.

SPANDIDOS, D. A., G. KRYSTAL and A. F. GRAHAM, 1976 Regulated transcription of the genomes of defective virions and temperature-sensitive mutants of reovirus. J. Virol. **18**: 7–19.

STEVENS, R. B. (editor), 1974 p. 506–524. In: *Mycology Guidebook*. University of Washington Press, Seattle and London.

VODKIN, M., F. KATTERMAN and G. A. FINK, 1974 Yeast killer mutants with altered double stranded ribonucleic acid. J. Bacteriol. **117**: 681–686.

WICKNER, R. B., 1974 Chromosomal and nonchromosomal mutations affecting the killer character of *Saccharomyces cerevisiae*. Genetics **76**: 423–432. ——, 1976 Mutants of the killer plasmid of *Saccharomyces cerevisiae* dependent on chromosomal diploidy for expression and maintenance. Genetics **82**: 273–285.

WICKNER, R. B. and M. J. LEIBOWITZ, 1976 Two chromosomal genes required for killing expression in killer strains of *Saccharomyces cerevisiae*. Genetics **82**: 429–442.

WOOD, H. A., 1973 Viruses with double-stranded RNA genomes. J. Gen. Virol. **20**: 61–85.

WOOD, H. A. and R. F. BOZARTH, 1973 Heterokaryon transfer of virus-like particles associated with a cytoplasmically inherited determinant in *Ustilago maydis*. Phytopathology **63**: 1019–1021.

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