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Genetic Complementation between Middle and Bottom Components of Two Strains of Radish Mosaic Virus

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SUMMARY

Kale virus (KV), turnip virus (HZ) and radish mosaic virus (RMV) are slightly related serologically to cowpea mosaic virus. Purified preparations comprize, top, middle and bottom components, of which only the last two contain nucleic acid. The nucleoprotein components of each virus were separated by centrifuging twice through a sucrose gradient in an MSE BXIV zonal rotor. Sometimes this was followed by centrifuging to equilibrium in a density gradient of rubidium bromide. Neither nucleoprotein component was infective alone, but infectivity was restored when each was mixed with the other component.

Mixtures in which the two components came from different viruses were infective only when they were made with KV and HZ. Serologically these two viruses are more closely related than either is to RMV. KV differed from HZ by two characters related to the coat protein. KV particles regularly formed aggregates of 12 particles and contained antigens that were not present in HZ. Single lesion isolates from mixtures of bottom component from one strain and middle component from the other did not form aggregates and lacked the antigens present in KV. Like cowpea mosaic virus, KV and HZ each have two coat proteins, and our results can be explained if each of the four virus components codes for a different coat protein. Therefore, the coat protein of the virus is coded by the nucleic acid of both middle and bottom components.

INTRODUCTION

A virus (KV) isolated from perennial kale (*Brassica oleracea* L) and another virus (HZ) isolated from turnip (*Brassica rapa* L) in Yugoslavia (Štefanac & Mamula, 1971) are both strains of radish mosaic virus (RMV) described in the U.S.A. (Campbell, 1964). They and the type strain (RMV) are slightly related serologically to cowpea mosaic virus and, like other members of this group, have top, middle and bottom components, only the last two of which contain nucleic acid. A characteristic of KV particles is that they form aggregates of 12 particles, similar in appearance to the aggregates formed by the satellite of tobacco necrosis virus (Kassanis & Woods, 1968). Preparations of middle or bottom component of cowpea mosaic virus are not infective alone, but virus infectivity is restored by mixing the two components (Bruening & Agrawal, 1967; Van Kammen & Van Griensven, 1970). Genetic complementation may also occur when middle and bottom components are taken from two different strains or mutants and, in some of these instances, it was possible to determine which component carried the genetic information for certain characters. For example, in one such mixture, the nucleic acid of the middle component determined the amount of the top component produced (Bruening, 1969; De Jager & Van Kammen, 1970)

and, in another, the type of local lesion and the middle to bottom component ratio were determined by the nucleic acid of the bottom component (Wood, 1972).

In the present paper, we confirm that both middle and bottom components of KV, HZ or RMV are needed to cause an infection. We also demonstrate genetic complementation between KV and HZ, which differ only slightly antigenically, and present evidence that the composition of the coat protein of KV and HZ is coded by both middle and bottom component nucleic acids.

METHODS

Virus isolates. KV was supplied by Dr J. A'Brook, HZ by Dr Z. Štefanac, RMV by Dr R. N. Campbell, and cowpea mosaic virus by Dr A. Van Kammen. KV, HZ and RMV were propagated in chinese cabbage (*Brassica pekinensis* Rupr.) and cowpea mosaic virus in French bean (*Phaseolus vulgaris* L.).

Infectivity tests. Infectivity was assayed in *Chenopodium quinoa* Willd. KV and HZ produced small chlorotic lesions 15 to 20 days after inoculation, while the larger lesions produced by RMV appeared one week after inoculation.

Virus purification. Young chinese cabbage plants were inoculated using carborundum and the infected leaves were collected 10 to 15 days later and crushed in a meat mincer. The sap was pressed through muslin and the pulp was extracted with a vol. of 0·1 M-EDTA, pH 7·7, equal to half the vol. of the sap. The sap and extract were combined and clarified by centrifuging. If the vol. of sap was large, the virus was condensed by precipitation with ammonium sulphate at 30 % saturation. The preparation was then treated with 1/4 vol. of chloroform–butanol mixture (50:50) and clarified by centrifuging. The virus was further purified by one cycle of high- and low-speed sedimentation. Such preparations were used for the separation of middle and bottom components by zonal sedimentation or further purified by two more cycles of high- and low-speed sedimentation.

Separation of components. Separation was made in an MSE BXIV zonal rotor with a capacity of 650 ml. An isokinetic gradient of 15 to 26 % (w/w) sucrose in 0·01 M-phosphate buffer, pH 7, was pumped into the rotor, while it was rotating at 2500 rev/min, using an MSE automatic variable gradient former. Four to 6 ml of virus at 5 mg/ml in 0·01 M-phosphate buffer, pH 7, with 5 % (w/w) sucrose was layered on top of the gradient, using a mechanical syringe driven at 2·5 ml/min, followed by an overlay of 130 ml of 0·01 M-phosphate buffer, pH 7, pumped with the gradient former. The rotor was then spun at 100000 ***g*** for 190 min at 15 to 20 °C. The contents of the rotor were displaced with 40 % (w/w) sucrose while the rotor was spinning at 2500 rev/min and 10 ml fractions were collected. The E_{254} of the effluent was monitored, using an ISCO Model 180 density-gradient fractionator. Several fractions from the trailing edge of the middle component zone were pooled, as were several fractions from the leading edge of the bottom component zone. Each component was dialysed for at least 24 h against 0·01 M-phosphate buffer and then concentrated by ultracentrifuging.

After two zonal separations, HZ bottom component was centrifuged to equilibrium in a density gradient of rubidium bromide. Rubidium bromide was added to the virus in 0·06 M-phosphate buffer, pH 7, to approximately 0·6 g/ml of virus solution and the preparation was centrifuged for 18 h at 44740 rev/min in a fixed partition cell in the analytical ultracentrifuge. The bottom component formed a thin band just below the partition. The upper compartment of the cell was emptied and washed several times before the solution in the bottom compartment was recovered.

Sucrose gradient sedimentation was used to separate aggregates of KV from unaggregated

virus. Density gradients from 10 to 40 % sucrose in water were made in 7·5 × 2·5 cm diam. centrifuge tubes. Two ml of virus (7 mg/ml) was layered on the gradient and the tubes were centrifuged for 2 h at 50000 ***g***. The zones were removed using an ISCO fractionator.

Determination of virus concentration. The virus concentration was determined spectrophotometrically using the extinction coefficient values of 8·1 for the unfractionated virus, 6·2 for middle and 10·0 for bottom component found by Van Kammen (1968).

Sedimentation coefficients. These were determined in a Spinco model E analytical ultracentrifuge using Schlieren optics and were calculated by the graphical method of Markham (1960). Normally, 0·1 mg/ml of virus was run in 0·01 M-phosphate buffer, pH 7, and the S value corrected for temperature.

Serological tests. An antiserum to KV was produced by injecting a rabbit intramuscularly with 1 ml of a virus preparation (7 mg/ml), emulsified with an equal vol. of Freund's complete adjuvant. Bleeding made after 6 weeks gave an antiserum with a titre of 1/640. Serological tests were made by double diffusion precipitation in gels containing 0·75 % Oxoid Ionagar no. 2 in 0·01 M-phosphate buffer, pH 7, with 0·02 % sodium azide.

Electron microscopy. The virus preparations were examined in a Siemens Elmiskop 1A after staining with 2 % sodium phosphotungstate.

RESULTS

General properties

KV often produced necrotic local lesions in chinese cabbage and always a severe systemic mottle, frequently accompanied by veinal necrosis. In turnip, it produced necrotic local lesions and severe systemic necroses sometimes forming line patterns; young plants were often killed. Symptoms varied considerably in severity, both among plants and with seasons, particularly in turnip, but the symptoms were usually more severe than those produced by HZ. The thermal inactivation point of KV was between 65 and 70 °C, similar to that described for HZ (Štefanac & Mamula, 1971). In gel diffusion tests, using KV antiserum, KV formed a small spur when placed in wells adjacent to HZ and a large spur when placed next to RMV. The serological difference between KV and cowpea mosaic virus was even greater in both gel diffusion tests and in tube tests. In tube tests, the antiserum to KV had an homologous titre of 1/640 and a titre against cowpea mosaic virus of 1/10.

Viruses of the cowpea mosaic group are transmitted by beetles of the families *Chrysomelidae* and *Curculionidae*. Campbell & Colt (1967) reported that RMV is inefficiently transmitted by *Phyllotreta* sp. We transmitted KV and RMV using *Phyllotreta consobrina* (Curt). With beetles given 5 days acquisition feed, we transmitted KV to 2/6 chinese cabbage and RMV to 2/8.

Electron microscopy

Purified preparations of KV always contain numerous aggregates each consisting of 12 particles (Fig. 1). The aggregates are icosahedra made up of two interlocking rings of five particles, with two particles placed one on either side of the formation. They are similar in appearance to the aggregates of the satellite of tobacco necrosis virus (Kassanis & Woods, 1968). Aggregates are seen in three different positions; usually they lie with a twofold axis vertical, less commonly with a threefold axis and rarely with a fivefold axis vertical. Paired aggregates share one particle and polymers of x aggregates contain $11x+1$ particles. Polymers lie with a twofold axis of each of the individual aggregates vertical. When a preparation containing numerous aggregates was sedimented in the analytical ultracentrifuge at 12600 rev/min, Schlieren optics showed four peaks with sedimentation coefficients of 500, 744,

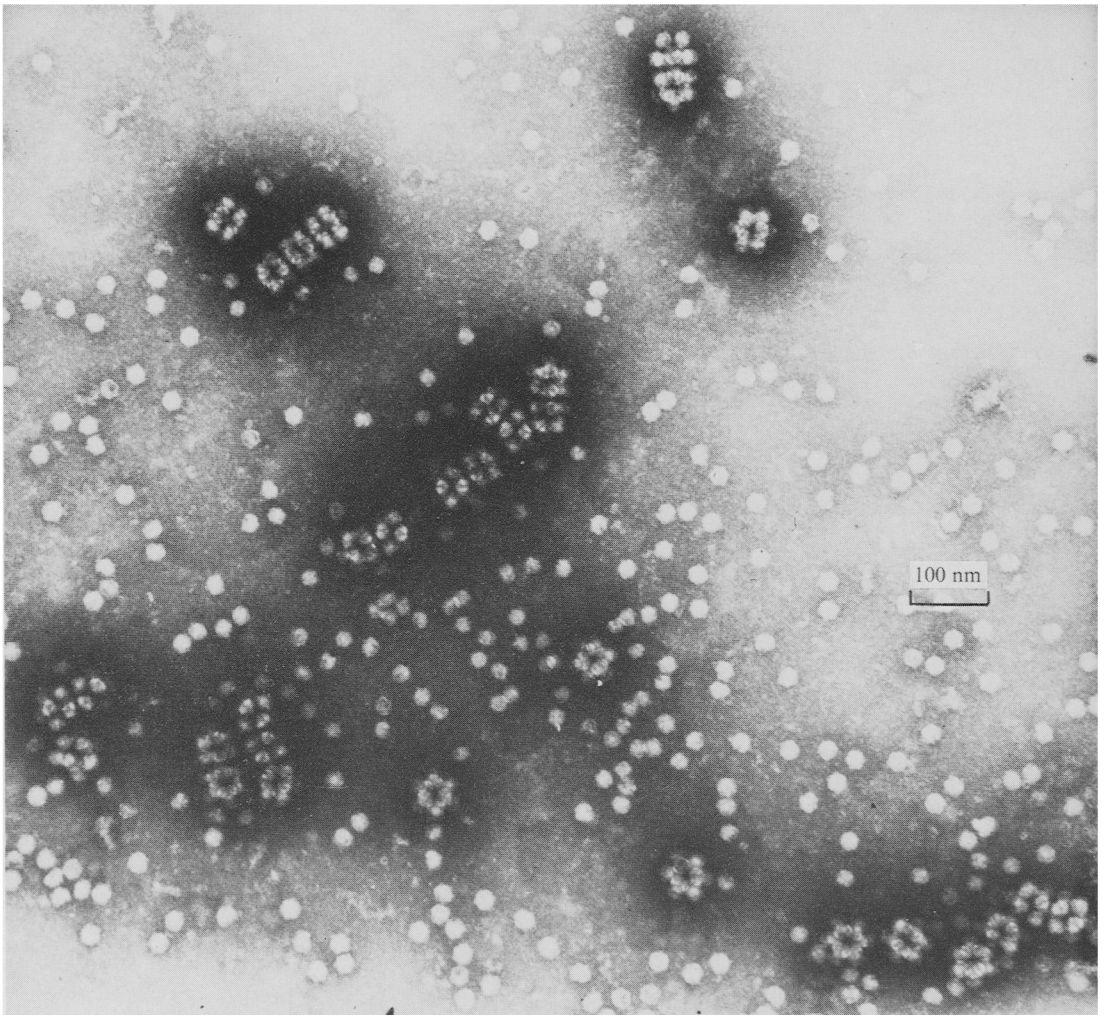

Fig. 1. Electron micrograph of a purified preparation of KV, showing aggregates.

920 and 1070 S, in addition to the peak corresponding to the unaggregated virus (Fig. 2). At this speed there was insufficient time for the unaggregated virus to separate into top, middle and bottom components. Usually, preparations of aggregated satellite virus showed three peaks corresponding to half aggregates, monomers and dimers (Kassanis & Woods, 1968). If the same relationship exists between the sedimentation coefficients of single particles and aggregates of KV, as found for satellite virus, then the four aggregate peaks obtained with KV in Fig. 2 are monomers, dimers, trimers and tetramers. Markham (1962) showed that the sedimentation coefficient of a dimer of an isometric virus is approximately $\sqrt{2}$ times the value for the monomer. The values 500, 744 and 1070 S form a series increasing by a factor of $\sqrt{2}$ and probably represent the monomers, dimers and tetramers. The value of 920 S, falling between the values for the dimer and tetramer, probably represents trimers.

Aggregates were not seen in extracted sap, but appeared during purification after the first sedimentation. After separating the unaggregated virus from the aggregates by sucrose

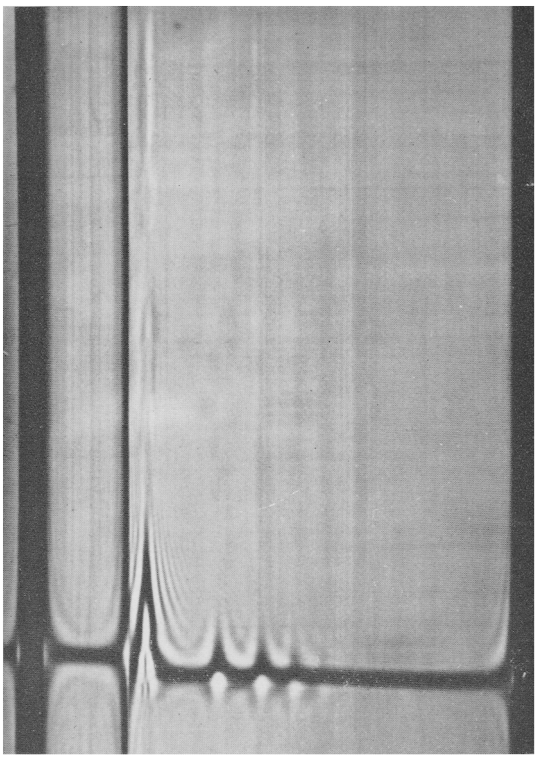

Fig. 2. Sedimentation pattern of a purified preparation of KV. The photograph was taken 6 min after a speed of 12590 rev/min had been reached with a Schlieren-bar angle of 45°.

gradient sedimentation, very few new aggregates formed when the preparation was kept for two months at 4 °C. When a preparation containing aggregates was kept, some broke up into individual particles. Attempts to break up the aggregates more quickly failed. Aggregates survived, for example, 7 h incubation with 0·1 M-urea or 0·5 % mercaptoethanol. Preparations of aggregates obtained by sucrose gradient sedimentation and largely free from unaggregated particles were highly infective, suggesting that aggregates consist of particles of both middle and bottom component. We also found that aggregates contain particles of all three components (White, Kassanis & Woods, 1973).

Relationship between the two nucleoproteins

When KV preparations were sedimented in the analytical ultracentrifuge at 29500 rev/min, the aggregates sedimented rapidly and the unaggregated virus formed three peaks corresponding to top, middle and bottom components. The sedimentation coefficients were 61, 103 and 125 S for both KV and HZ and 60, 99 and 123 for RMV. U.v. optics showed that only the two heavier components of each strain contain nucleic acid.

After one zonal sedimentation, preparations of the middle components of all three strains were non-infective. The bottom components of KV and RMV were subjected to a second zonal sedimentation and non-infective preparations were obtained. By contrast, the bottom component of HZ was somewhat infective, even after a second zonal sedimentation, but its infectivity was reduced a little by centrifuging it to equilibrium in a density gradient of rubidium bromide. The results of the infectivity tests with KV and RMV in Table 1 show

Table 1. *Infectivity of the virus components separately and in mixtures*

	KV			RMV		
Experiments	1	2	3	1	2	3
Middle component	0 (75)*	0 (25)	0 (25)	10 (4)	2 (1)	0 (5)
Bottom component	0 (75)	0 (75)	2 (225)	4 (0·1)	5 (1)	45 (5)
Mixture of the two at the same final concentration	225	175	246	236	383	1283

Figures in parentheses denote concentration of virus in μg/ml.
* Total number of lesions from 8 half-leaves.

that, with each strain, middle and bottom components are not infective, but infectivity is restored when the two are mixed. That two components are needed to cause infection was also suggested by the steepness of the dilution curve of purified KV inoculated in *Chenopodium quinoa*. Mr R. Jones, of the Statistical Department at Rothamsted, found that the dilution curve fitted a theoretical two-hit curve very well, but could not be fitted to a one-hit curve.

Phenol extracts from leaves inoculated with a single component of KV

Tobacco rattle virus has a two-component system consisting of a long rod-shaped particle and a short one. Both are needed for the production of complete virus particles and it was shown that the nucleic acid of the short particles codes for virus coat protein (Lister, 1966). Long particles are infective alone, but in such infections the virus nucleic acid remains uncoated. To find if something similar happens with KV when only one of the nucleoproteins is inoculated, some plants of *Chenopodium quinoa* were inoculated with middle, and others with bottom, component. Two weeks later, phenol extracts were made from the inoculated but symptomless leaves. Each extract was mixed with the nucleoprotein of the other component and inoculated to *C. quinoa*. No local lesions appeared, suggesting that, when middle or bottom component is inoculated alone, virus nucleic acid does not replicate.

Mixing components from different strains

Middle or bottom components of KV were not infective when mixed with the opposite component of RMV. By contrast, mixtures having one component from KV and the other from HZ were infective. The HZ bottom component preparation was not as pure as the others, but nevertheless the large increase in infectivity obtained by adding KV middle component leaves no doubt that genetic complementation occurred (Table 2).

The two strains differ qualitatively in two characters which reflect differences in the coat proteins – KV has an antigen that HZ lacks, and KV, but not HZ, forms aggregates. To find which of the two components codes for these two characters, we isolated single lesions from the two combinations of middle and bottom component of the two strains and inoculated them to very young turnip seedlings. The virus isolates obtained were propagated in chinese cabbage, purified and examined in the electron microscope and in gel diffusion tests. Of 11 isolates from the mixture of KV bottom and HZ middle examined, 10 resembled HZ in not forming aggregates and lacking the KV-specific antigen. The remaining isolate formed a few aggregates, but gel tests did not detect the KV antigen, and we think that this isolate was contaminated with KV middle component.

We examined 21 isolates from the mixture of HZ bottom component and KV middle component. One isolate formed numerous aggregates and did not differ antigenically from

Table 2. *Infectivity of the virus components of two strains separately and in mixtures*

Inoculum	Total no. of lesions from 7 half-leaves
Middle component of HZ at 20 μg/ml	0
Bottom component of KV at 20 μg/ml	1
Mixture containing 20 μg/ml of each component	345
Middle component of KV at 25 μg/ml	0
Bottom component of HZ at 25 μg/ml	29
Mixture containing 25 μg/ml of each component	111

KV. It was probably a KV contaminant. The remaining 20 isolates behaved like HZ in not forming aggregates and lacking the KV-specific antigen. The infectivity of the mixture of KV middle and HZ bottom (Table 2) suggests that at least 1/4 of the isolates from this mixture would be expected to be pure HZ because about 30 of the 111 lesions would be caused by HZ middle and bottom components. Consequently, about 15 isolates would be expected to have originated from mixed infections. However, all isolates behaved like HZ.

The results show that each coat protein character is determined by both middle and bottom component. If middle or bottom component of KV is replaced by the complementary component of HZ, the protein is changed so that aggregates are not formed and the KV-specific antigen is lacking. These two characters of the coat protein of KV are constant because, of 14 single lesion isolates of KV investigated, 13 formed numerous aggregates and were serologically identical to KV. The other did not form aggregates, but when examined in the analytical ultracentrifuge was found to contain a large amount of top component. Both KV and HZ have a small amount of top component, so it is probable that this isolate was a defective mutant of KV.

DISCUSSION

The three viruses are slightly related serologically to cowpea mosaic virus, which has been studied extensively. The interpretation of the relationship between the two nucleoprotein components of cowpea mosaic virus depended on the purity of the preparations. Agrawal (1964) obtained a middle component that was more infective than the bottom. By contrast, Van Kammen (1967) found that the bottom component only was infective alone and adding middle component did not increase its infectivity. Bruening & Agrawal (1967) found the mixture of the two components was eight times more infective than would be expected from the infectivity of each component separately. Finally, Van Kammen (1968) and Van Kammen & Van Griensven (1970), using pure preparations, showed that neither component alone is infective, but both together are. We have shown this to be true for KV and RMV.

Several viruses are known to have their genome divided between a number of components so that each component codes for specific functions. For example, with tobacco rattle, alfalfa mosaic and raspberry ringspot viruses, the virus protein is coded by only one of the components (Lister, 1966; Van Vloten-Doting, Dingjan-Versteegh & Jaspers, 1970; Harrison, Murant & Mayo, 1972). The formation of excess top component, the middle-to-bottom component ratio and the type of local lesion of cowpea mosaic virus are also coded by one component (Bruening, 1969; De Jagar & Van Kammen, 1970; Wood, 1972). By contrast, our results with KV and HZ show that the type of coat protein depends on both components. This is the first time that the coat protein of a virus with a divided genome has been found to be coded by two components. As KV and HZ belong to the cowpea

mosaic virus group, it is likely that a similar situation exists with other viruses of this group. The explanation probably lies in the fact that these viruses have two coat proteins. Cowpea mosaic virus has two proteins with mol. wt. of 22000 and 42000 present in the virus coat in equimolar amounts (Wu & Bruening, 1971; Geelen, Van Kammen & Verduin, 1972). Preliminary tests at Rothamsted showed that KV, HZ and RMV also have two proteins. If this is confirmed, the complementation experiments suggest that each component codes for a different protein. On this assumption, the mixed isolates would contain one protein from each of the two parent strains and would, therefore, differ antigenically from them. In our serological tests we used KV antiserum and compared the reaction of mixed isolates only with that of KV. We found that each of them lacked an antigen present in KV, but these tests would not have detected the presence, in the mixed isolates, of a protein not found in HZ. The absence of aggregates in all the mixed isolates can be explained if both KV proteins are necessary for aggregation and each mixed isolate has only one or the other of them. The results also show that both KV proteins contribute to KV antigenicity and suggest that both virus proteins are present on the surface of the particle.

KV is, in many respects, very similar to HZ, but we found great difficulty in producing pure preparations of the bottom component of HZ, as measured by residual infectivity, although we used the same techniques. There was no evidence of the formation of dimers containing middle and bottom component, as the runs in rubidium bromide gave only two zones.

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