Description and Complementation Analysis of 13 Temperature-sensitive Mutants of Alfalfa Mosaic Virus

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SUMMARY

Thirteen thermosensitive (ts) mutants of alfalfa mosaic virus (AMV), a virus with a tripartite genome, are described. Eight of these mutants were spontaneous, one was induced with HNO₂ and four were induced by u.v. irradiation of one purified component. Using supplementation tests six ts mutations were located on top component b (Tb), three on middle component (M) and four on bottom component (B). Complementation tests with mutants with ts defects on the same component showed that none of the ts mutants on Tb could complement each other; the three ts mutants on M could be subdivided into two complementation groups, while only one pair of mutants showed complementation from the four ts mutants on B. It was demonstrated that the coat proteins from the six ts mutants on Tb were not able to activate the AMV genome at 30 °C in tobacco.

INTRODUCTION

For many plant viruses the genetic information is combined in a nucleic acid of mol. wt. 2 × 10⁶ to 3 × 10⁶. The only virus-coded protein of known function is the coat protein which accounts for only a small part of the genetic information present. There are indications that other virus information plays a role in the type of symptoms produced by the host (Kado & Knight, 1966; Dingjan-Versteegh et al. 1972; Bancroft & Lane, 1973; Robinson, 1973, 1977; Habili & Francki, 1974; Lane, 1974; Marchoux et al. 1974) and in the transport of the virus through the host (Dingjan-Versteegh et al. 1972; Nishiguchi et al. 1978). Furthermore it has been assumed that the virus RNA also encodes an RNA polymerase (subunit). However, up to now no biochemical evidence for the latter has been found (see Linthorst et al. 1280). Recently, Dawson & White (1978, 1979) reported the isolation of mutants from tobacco mosaic virus (TMV) in which the synthesis of single-stranded RNA or of both single and double-stranded RNA was thermosensitive.

In order to obtain more information about the number and the nature of processes in which virus-coded proteins are involved we searched for temperature-sensitive (ts) mutants of alfalfa mosaic virus (AMV). We have chosen ts mutants since they are conditional lethal mutants which can, at least theoretically, be obtained for every gene product with an indispensable function for virus production. AMV is suitable for such a study since its genome consists of three RNA species [RNA 1: 1.1 × 10⁶, RNA 2: 0.8 × 10⁶ and RNA 3: 0.7 × 10⁶ (Heijtink et al. 1977)] encapsidated separately into nucleoproteins [called bottom (B), middle (M) and top component b (Tb), respectively], which can be purified by centrifugation. Mutations can be assigned to one of the components by a supplementation test, or alternatively purified components can be treated separately with a mutagen. By studying...
Table 1. Biological activity of purified wild-type components

<table>
<thead>
<tr>
<th>Inoculum (component)</th>
<th>Infectivity* 1976†</th>
<th>Infectivity* 1979‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tb</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B+M</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B+Tb</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>M+Tb</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B+M+Tb</td>
<td>78</td>
<td>68</td>
</tr>
</tbody>
</table>

* Solutions were assayed in an incomplete block. Lesion number is the mean value of seven half leaves.
† Final concentration of each component 1 μg/ml.
‡ Final concentration of each component 12 μg/ml.

combinations of mutants with a ts defect on the same RNA species the number of complementation groups per RNA species can be determined.

METHODS

Virus culture and isolation. All virus isolates were grown in plants of *Nicotiana tabacum* L. var. ‘Samsun NN’, cultivated in a greenhouse, average temperature 22 °C. Material of AMV 425 (wild-type, wt) and of mutants was isolated as described previously (Van Vloten-Doting & Jaspars, 1972). Virus was always stored and handled at 4 °C in 0.01 M-sodium phosphate, pH 7.0, containing 1 mM-EDTA and 1 mM-NaNa (PEN buffer) unless stated otherwise.

Separation of virus components. The wt components were purified by zonal centrifugation as described by Bol *et al.* (1971). Appropriate fractions of the last zonal centrifugation run were used without removal of sucrose. Mixtures of two components were not or were only slightly infectious; upon addition of the third component the infectivity increased considerably (Table 1). For all experiments described the same preparations of B, M and Tb were used. The experiments were performed over a 3 year period during which the specific infectivity of the components decreased to less than one-tenth and consequently the concentration used had to be increased (Table 1).

Infectivity assay, serology, single lesion transfer and extraction of virus RNA. The methods used were those described for infectivity assay and serology (Van Vloten-Doting & Jaspars, 1967), single lesion transfer (Van Vloten-Doting *et al.* 1968) and extraction of RNA (Bol & Van Vloten-Doting, 1973).

Preparation of virus coat protein. Protein was always prepared directly before use by dissociation of nucleoprotein with MgCl₂ (final concentration 5 mg/ml and 0.5 M, respectively; Kruseman *et al.* 1971). The RNA was removed by centrifugation and the supernatant containing the protein was diluted 100-fold with distilled water.

Conservation of mutant stock. Systemically infected leaves of tobacco plants infected with a mutant isolate, which had been passed through at least three serial single lesion transfers, were dried over CaCl₂ at 4 °C and stored at −20 °C (Bos, 1969).

Determination of ts character. For all mutants the virus production ratio was determined in the following way. Leaves from three to five tobacco plants were inoculated with a homogenate of a systemically infected leaf. Directly after inoculation 10 discs (diam. 1.2 cm) were punched out. The discs were placed in an Erlenmeyer flask (50 ml) containing 15 ml 0.01 M-Na₂HPO₄, pH 7.0, pre-incubated in a waterbath at 30 °C. From the same
leaves 10 more discs were punched out and placed in an Erlenmeyer flask with buffer in a waterbath at 23 °C. The discs were incubated at the indicated temperature for 4 or 5 days under continuous light (3000 lux, TL number 33). To enable a reliable estimate of the virus produced in the discs, these were homogenized with 2 ml PEN buffer and four serial dilutions of the homogenate (according to the virus production expected) were assayed on bean (*Phaseolus vulgaris* L. var 'Berna'). Corresponding dilutions of homogenates incubated at 30 and 23 °C were always compared on half leaves of the same plants. Only tests in which the number of lesions induced by the sample incubated at 23 °C was between 100 and 300 were taken into account. Wt AMV had a virus production ratio of 60 to 80%; mutants suitable for further study had a virus production ratio of < 1%.

**Supplementation test.** Mutations were assigned to virus components using a modified supplementation test (Bancroft & Lane, 1973; De Jager, 1976). A systemically infected leaf was homogenized in PEN buffer and divided into four samples. To three samples an equal volume of the three purified wt components (at a suitable concentration) was added separately and to the fourth sample an equal volume of PEN buffer was added. Each combination was inoculated on to tobacco plants and the virus production ratio was determined.

**Complementation test.** In the literature on *ts* mutants of animal or bacterial viruses, complementation is usually expressed as the complementation index (that is, yield of mixed infection divided by the sum of yields of the single infections at the restrictive temperature, e.g. Eckhart, 1977). Due to the variation in susceptibility of individual tobacco plants for virus infection the complementation index would not be a reliable parameter for the complementation of plant virus mutants. We have used the virus production ratio described above. Tobacco plants were infected with a mixture of two nucleoprotein preparations or with a mixture of two homogenates of leaves systemically infected with different mutants and the virus production ratio was determined. The ratio was also measured for the individual mutants. For these experiments, high concentrations of virus were used to enhance the possibility that cells were indeed inoculated with mixtures of mutants.

**RESULTS**

**Induction of mutants**

Preliminary attempts to mutate AMV nucleoprotein with *N*-methyl-*N'*-nitro-nitroso-guanidine (Singer & Fraenkel-Conrat, 1967) failed, since the treatment resulted in inactivation of the virus without the production of mutants. This is in contrast to the results of Hartmann *et al.* (1976) who were able to obtain mutants of AMV strain S with this mutagen. Treatment with HNO$_2$ is unsuitable for AMV nucleoprotein, as at the pH required for this treatment AMV nucleoprotein precipitates (Hull, 1969). Treatment of AMV RNA with HNO$_2$ (Gierer & Mundry, 1958) did induce some aberrant symptoms (8 out of 36) and one mutant proved to be thermosensitive. However, this method did not seem very attractive since it would require the purification of large amounts of each of the AMV RNA species.

We had noticed that tobacco plants systemically infected with AMV and kept under continuous light from high pressure mercury lamps (Philips HLRG 400 W), frequently gave yellow or green spots which were distinct from the normal faint chlorotic symptoms; stable isolates were made from these distinct symptoms and a high proportion (13 out of 25) of them were found to be thermosensitive. Since this type of lamp is known to produce a slight leakage of u.v. light it seemed possible that the mutations may have been induced by u.v. light. Therefore we tried u.v. irradiation of purified nucleoprotein components. In Fig. 1 the loss of infectivity upon u.v. irradiation of Tb (assayed in the presence of untreated B + M) is shown. The Tb samples irradiated for 9 to 15 min (shaded area) were inoculated,
Fig. 1. Loss of infectivity upon irradiation of purified Tb (14 µg/ml) assayed in the presence of untreated B (5 µg/ml) and M (2 µg/ml). U.v. irradiation was performed at room temperature in 1 cm quartz cuvettes at an intensity of 1.15 J/m²/s. Lesion numbers are mean values of seven half leaves.

Table 2. Localization of the ts defect by the supplementation test*

<table>
<thead>
<tr>
<th>Mutant alone</th>
<th>Mutant + wt Tb</th>
<th>Mutant + wt M</th>
<th>Mutant + wt B</th>
<th>ts defect on:</th>
<th>Mutant name†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>93</td>
<td>0</td>
<td>2</td>
<td>Tb</td>
<td>Tbs 7(uv)</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>13</td>
<td>0</td>
<td>M</td>
<td>Mts 1(ni)</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>18</td>
<td>3</td>
<td>M</td>
<td>Mts 2(s)</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>19</td>
<td>2</td>
<td>M</td>
<td>Mts 3(s)</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>1</td>
<td>18</td>
<td>B</td>
<td>Bts 4(s)</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

* Either mutant alone or mutant plus separated wt component inoculated on to tobacco and assayed on bean as described in Methods.

Values represent virus production at 30 °C x 100.

† Mutants either spontaneous (s) or produced by HNO₂ (ni) or u.v. (uv) treatment.

in the presence of untreated B + M, on to tobacco. Three out of five isolates (picked at random and carried through three single lesion transfers) were thermosensitive. This proportion is relatively high and it is possible that one or two of these mutants carry more than one ts mutation. In a control experiment with non-irradiated virus, 49 isolates were assayed and none was thermosensitive.

The possibility of inducing in vitro mutations in the AMV genome with u.v. light does not prove that u.v. light was responsible for the induction of AMV mutants in infected tobacco plants. Therefore the latter class of mutants will be designated spontaneous mutants (s).

Supplementation tests

To assign the ts mutation of the spontaneous mutants to one particular component, supplementation tests were performed; the tests were repeated at least twice. Representative results with six mutants are listed in Table 2. Mutants are named after the component in which they carry a ts defect followed by an arabic number and then the type of mutagen (s, spontaneous; ni, HNO₂ and uv, u.v. induced). A small amount of growth at 30 °C with
Complementation between ts mutants of AMV

Mts mutants in the presence of wt Tb or B could be expected since the wt Tb and B preparations were slightly contaminated with M (Table 1). Likewise it is understandable that Bts mutants can produce a low amount of virus at 30 °C in the presence of wt M, but the growth of Bts mutant with wt Tb was not expected and was found only once.

For most mutants the results were clear cut. However, one of the mutants failed to grow at 30 °C when tested in combination with each of the three wt components. It is possible that this isolate carries a ts mutation in more than one component and therefore it was not used for the present study. From the fact that only one out of a total of thirteen spontaneous mutants appeared to carry two or more ts mutations located on different RNA species, we can deduce that it is unlikely that one of the other spontaneous mutants carries more than one ts mutation in the same RNA strand.

The supplementation test was also used to check if the u.v.-induced mutants indeed carried a ts defect in the irradiated component.

The results obtained with all three Mts mutants are shown in Table 2. Since the assignment of the ts mutations to Tb or B by the supplementation test was confirmed by the results of the complementation tests, only one example of a Tbts and a Bts mutant is given.

Stability of mutants

All mutants except Bts 1(uv) were stable in dried infected leaves stored at -20 °C. For Bts 1(uv) we found that leaves stored at -20 °C for more than 4 years no longer contained thermosensitive virus, while material which had been kept in culture was still thermosensitive. Apparently the original dried material contained, besides the mutant, a very small amount of wt or revertant virus, which 'survived' while the mutant was inactivated. A similar observation has been made with mutant N3 from cowpea mosaic virus (C. P. De Jager, personal communication).

All mutants except Tbts 6(s) were stable during subculturing in tobacco. The virus production ratio of Tbts 6(s) was about 2% in initial experiments, but in later experiments this value increased to about 15%. Purified nucleoprotein from Tbts 6 (from one of the later experiments) showed a loss of its thermosensitive character during storage at 4 °C. These results suggest that during subculturing this mutant became contaminated with a revertant or with wt; the contaminating virus multiplied faster and was more stable at 4 °C than mutant Tbts 6(s).

Complementation tests

To gain information about the processes in which the product(s) of each RNA species is (are) involved, we performed complementation tests with mutants carrying a ts mutation in the same RNA species.

The percentage of growth found at 30 °C for the different combinations of mutants varied from experiment to experiment. However, we found consistently that certain combinations of mutants were able to grow at 30 °C in contrast to others. The variability in the actual percentage of complementation could be due to variations in the concentration of the mutants in the leaf homogenates used as inoculum. The use of purified nucleoprotein preparations at concentrations inversely proportional to their specific infectivity did not improve the reproducibility of the complementation test, probably because some mutant preparations lost their infectivity much faster than others.

Table 3(a) shows that all six Tbts mutants fall into one complementation group. The three Mts mutants fall into two complementation groups: group I is formed by Mts 1(ni) and Group II by Mts 2(s) and Mts 3(s) (Table 3b). Bts 1(uv) and Bts 4(s) belong to different complementation groups while Bts 2(s) and Bts 3(s) show no significant complementation with any of the Bts mutants (Table 3c). The lack of complementation between Bts 2 and Bts 3 and with the other Bts mutants will be discussed later. However, we would like to
Table 3. **Complementation analysis of ts mutants of AMV**

(a) Tbts mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>1(s)</th>
<th>2(s)</th>
<th>3(s)</th>
<th>4(uv)</th>
<th>5(uv)</th>
<th>7(uv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(s)</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>2(s)</td>
<td></td>
<td></td>
<td>o</td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>3(s)</td>
<td></td>
<td></td>
<td></td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>4(uv)</td>
<td></td>
<td></td>
<td></td>
<td>o</td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>5(uv)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>o</td>
<td>o</td>
</tr>
<tr>
<td>7(uv)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>o</td>
</tr>
</tbody>
</table>

(b) Mts mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>1(ni)</th>
<th>2(s)</th>
<th>3(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(ni)</td>
<td>1</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>2(s)</td>
<td></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3(s)</td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

(c) Bts mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>1(uv)</th>
<th>2(s)</th>
<th>3(s)</th>
<th>4(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(uv)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>76</td>
</tr>
<tr>
<td>2(s)</td>
<td></td>
<td>o</td>
<td>o</td>
<td>0</td>
</tr>
<tr>
<td>3(s)</td>
<td></td>
<td></td>
<td>o</td>
<td>0</td>
</tr>
<tr>
<td>4(s)</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

* Mutants either spontaneous (s) or produced by HNO₃ (ni) or u.v. (uv) treatment

Values represent \( \frac{\text{virus production at 30°C}}{\text{virus production at 23°C}} \times 100 \)

stress at this point that the assignment of the ts mutations to the B component is confirmed by this finding. If, for example, the complementation found between Bts 4 and Bts 1 was due to the fact that the ts mutation of Bts 4 was not located on the B component, this mutant would be able to complement all Bts mutants, since the mixture would contain all three wt components. The same reasoning holds true for the Tbts mutants.

**A ts coat protein?**

The genome of AMV is only infectious in the presence of the coat protein (Bol et al. 1971). This offers the possibility of investigating whether the activity of the coat protein of any of the ts mutants is thermosensitive. From genetic work (Dingjan-Versteegh et al. 1972), RNA hybridization studies (Bol et al. 1975) and oligonucleotide mapping (Pinck & Fauquet, 1975), we know that the information for the coat protein is located on the RNA in Tb (RNA 3). Coat protein from each of the Tbts mutants and from wt virus was pre-incubated for 5 min at 30°C and inoculated simultaneously with a mixture of wt RNAs 1, 2 and 3 on to tobacco plants kept at 30°C. From the inoculated tobacco leaves (still at 30°C) discs were punched out and the virus production at 30 and 23°C was measured in the usual way. Table 4 shows that virus production at 30°C is induced by wt RNA in the presence of wt coat protein, but not in the presence of any of the mutant coat proteins. From the fact that virus production occurs in the discs shifted to 23°C, we can conclude that the activity of the coat protein was not lost irreversibly. These results are consistent with the idea that the coat protein from all six mutants is altered such that it cannot activate the AMV genome at 30°C.
Complementation between ts mutants of AMV

Table 4. Thermosensitivity of mutant coat proteins

<table>
<thead>
<tr>
<th></th>
<th>Virus production in tobacco leaf discs*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. I</td>
</tr>
<tr>
<td></td>
<td>23 °C</td>
</tr>
<tr>
<td>+ coat protein‡ from:</td>
<td></td>
</tr>
<tr>
<td>Wt RNA 1 + 2 + 3† + coat protein‡ from:</td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>423</td>
</tr>
<tr>
<td>Tbs 1(s)</td>
<td>243</td>
</tr>
<tr>
<td>Tbs 2(s)</td>
<td>150</td>
</tr>
<tr>
<td>Tbs 3(s)</td>
<td>85</td>
</tr>
<tr>
<td>Tbs 4(uv)</td>
<td>86</td>
</tr>
<tr>
<td>Tbs 5(uv)</td>
<td>291</td>
</tr>
<tr>
<td>Tbs 7(uv)</td>
<td>438</td>
</tr>
</tbody>
</table>

* Assayed after 4 days on bean leaves. Lesion number is the average number of lesions produced when the 10 discs were homogenized in 6 ml PEN buffer.
† 40 µg/ml; the mixture of RNAs itself was not infectious.
‡ 40 µg/ml; mutants either spontaneous (s) or produced by u.v. (uv) treatment.

Trypsin peptide maps from Tbs 7(uv) revealed a small difference between wt AMV and the mutant (results not shown). Tbs 1(s) has been shown to have at least two amino acid substitutions (Kraal, 1975). None of the mutants could be distinguished from wt AMV using antiserum raised against wt virus (results not shown).

Phenotype of mutants

Three of the four u.v.-induced mutants caused symptoms in both tobacco and bean that were very similar or identical to those caused by wt AMV. Tbs 7(uv) caused chlorotic symptoms with a necrotic boundary in the infected leaves of tobacco; the symptoms in the systemically infected leaves were also more necrotic than those of the wildtype (Fig. 2). The symptoms caused by Tbs 7(uv) in bean were normal. It is possible that the alteration in the coat protein of Tbs 7 is responsible for the altered symptoms; however, it is also possible that the RNA 3 of Tbs 7 carries a second (non)-ts mutation.

As far as the spontaneous mutants and the HNO₃ mutant are concerned, a description of the symptoms is probably irrelevant, because it is possible that they carry, besides a mutation on the indicated component, other mutations on one or both of the other components.

Effect of host on ts character

All thirteen mutants which displayed a ts character on tobacco were assayed on cowpea. Unfortunately it was impossible to do the experiments in the same way as with tobacco, because discs from cowpea leaves did not survive the incubation in phosphate buffer. We compared virus production in cowpea plants at 30 °C with that in plants at 23 °C. Wt virus induces local lesions in cowpea leaves, both lesion size and lesion number decreasing with increase in temperature. The amount of virus present in the local lesions is very small. Furthermore, virus production of wt at 30 °C was less than 10% of that at 23 °C and therefore it did not seem worthwhile to compare the virus production of the mutants at 23 and 30 °C in this way.

Based on their ability to induce lesions on cowpea leaves none of the mutants could be classified as ts on cowpea. This accords with the fact that all mutants were able to multiply equally in cowpea protoplasts at both 23 and 29 °C (H. J. M. Linthorst, personal communication).

Similar problems were encountered when bean (Phaseolus vulgaris L. var. ‘Berna’) was used as host. Wt virus induced local lesions in this host and again the amount of virus
present appeared to be very low. All mutants except $Tbts_1$, $Tbts_7$, $Mts_3$ and $Bts_4$ were able to induce local lesions in bean at the non-permissive temperature.

**DISCUSSION**

Temperature-sensitive mutants have proved to be of great value in the study of bacterial and animal viruses. No systematic studies with thermosensitive mutants of plant viruses have been reported so far. This is due partly to the problem of inducing stable $ts$ mutants, partly to the fact that this type of work is relatively difficult with plants (due to the variation in susceptibility of individual plants) and partly because there is no quick selection method. For AMV the first problem seems to be solved by the finding that u.v. irradiation yields a high percentage of stable $ts$ mutants and the second problem is solved for tobacco plants by incubating discs of the same leaves at different temperatures; the third problem is not yet solved. In an attempt to find a suitable selection procedure for $ts$ mutants of TMV Dawson & Jones (1976) produced only 25 mutants out of 225 isolates. We found that from 25 isolates of AMV with deviating symptoms, 13 were thermosensitive.

It was not expected that all six $Tbts$ mutants would fall into one complementation group. Previous studies have shown that the RNA in $Tb$ (RNA 3) contains the genetic information for the coat protein (Dingjan-Versteegh et al. 1972) and for a 35000 protein (Mohier et al. 1976; Van Vloten-Doting, 1976; Rutgers, 1977). As the activity of the coat protein from all six mutants is lost at 30 °C, it follows that each carries a $ts$ mutation in the coat protein cistron. Possible explanations for the absence of mutants with a $ts$ 35000 protein are that
Complementation between ts mutants of AMV

Mutations in this protein are nearly always lethal and only very seldom ts, or that in this protein mutations can be easily tolerated without rendering the protein ts.

The availability of mutants coding for proteins which can be switched 'on and off' in their ability to activate the AMV genome may help to elucidate the precise role played by the coat protein during the infection cycle. Amino acid sequence data of the coat protein of Tbs mutants should yield information about the location and nature of the active site.

We found two complementation groups on M, while in vitro studies have shown that RNA 2 (the RNA inside M) can be translated into one large protein corresponding to the total amount of genetic information present in the RNA (Mohier et al. 1975; Van Tol & Van Vloten-Doting, 1979).

The situation with B is more complicated than with M, since, besides two complementation groups, there are also two mutants which fail to complement any of the Bts mutants. This phenomenon is not due to interference (Cooper, 1977), since both mutants could be supplemented with wildtype B. As Bts 2(s) and Bts 3(s) are spontaneous mutants, it is unlikely that they are double ts mutants. In vitro translation studies have shown that RNA 1 (the RNA inside B) can be translated into one large protein (Mohier et al. 1975; Van Tol & Van Vloten-Doting, 1979).

Among the possible explanations which could reconcile the results from the in vitro translation studies with the genetic data are: (1) The large primary translation product is processed into smaller functional proteins (Cooper, 1977). In Bts 2 and/or Bts 3 the processing is either impaired or incorrect. Alternatively the primary product of RNA 1 is processed into more functional units and Bts 2 and Bts 3 represent a third (or a third and a fourth) complementation group. However, the protein(s) coded for by Bts 2 and/or Bts 3 is (are) cis-acting protein(s) (Spandios & Graham, 1975a, b). (2) In vivo subgenomic mRNA(s) is (are) generated from the genomic RNA [these smaller RNA(s) may be read in the same or in a different reading frame]. (3) The protein functions as a multimer. Subunits with defects in different regions of the sequence may be able to compensate each other, restoring some of the activity of the multimer by intracistronic complementation (Crick & Orgel, 1964). The proteins encoded by Bts 2(s) and/or Bts(s) are so distorted that no activity can be restored by association with other subunits. This explanation is not very likely for the Bts mutants since the complementation between Bts 1 and Bts 4 is efficient, while the efficiency of this kind of intracistronic complementation is low (Jamieson & Subak-Sharpe, 1974; Williams et al. 1974). (4) The protein has two 'independent' functional domains which can be mutated separately (Spoerel et al. 1979). The mutation of Bts 2(s) and/or Bts 3(s) is located such that the two functional domains represented by Bts 1(uv) and Bts 4(s) are both distorted. Attempts to demonstrate that some of the mutants induce in tobacco a replicase which is thermosensitive in vitro have been unsuccessful (see Linthorst et al. 1980).

It was unexpected and disappointing that none of the thirteen mutants which were ts on tobacco were ts on cowpea leaves (or in cowpea protoplasts assayed one degree below the non-permissive temperature). It has been reported previously that ts mutants of plant viruses behave differently in different hosts: for example tobacco rattle virus (Robinson, 1973), tobacco mosaic virus (Dawson & Jones, 1976) and cowpea chlorotic mottle virus (Bancroft et al. 1972). The lack of ts activity on cowpea of the Tbs mutants is not in conflict with the results obtained with tobacco (Table 4). In the latter experiments the coat protein was pre-treated for 5 min at 30 °C in the absence of RNA. In the nucleoprotein particle the protein remains active, probably because the RNA keeps the protein in the correct conformation. This 'protected' protein is apparently sufficient to start the infection. However, the virus replication will stop later since the newly synthesized protein is inactive. The small amount of virus produced in the inoculated cells will be below the detection level in the
tobacco experiments, but it may be sufficient to start lesion formation. The results with the \textit{Bts} and \textit{Mts} mutants can be explained by assuming that the altered proteins are required for systemic infection but not for local lesion assay. However, this explanation is not very attractive since the seven mutants described represent at least four complementation groups and it is very unlikely that none of these functions is required for local lesion induction. Neither is it very probable that the mutants of these four complementation groups all code for virus proteins involved in the transport of the virus (Nishiguchi et al. 1978). It seems more likely that at least some of these functions are performed by virus-coded proteins in close interaction with host-coded proteins, and that the difference between tobacco and cowpea suggests that this interaction (for at least some of these functions) is more sensitive in tobacco.

The present results represent the first unambiguous proof of complementation between mutants of plant viruses which carry a mutation on the same RNA. De Jager (1978, 1979) has reported that a mixture of the two mutants \textit{N123} × \textit{N142} of cowpea mosaic virus middle component induces wild-type symptoms, which each of the mutants alone induces another type of symptom. This has been interpreted as complementation between the two mutants. However, since the basis of symptom induction is unknown it cannot be excluded that the induction of wild-type symptoms by the mixture of the mutants is due to some other phenomenon.

The number of \textit{ts} mutants described in this study is rather small and it is quite possible that further work could reveal the presence of more complementation groups within the AMV genome.

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Complementation between ts mutants of AMV


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