Point mutation in the 30-K open reading frame of TMV implicated in temperature-sensitive assembly and local lesion spreading of mutant Ni 2519

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Tobacco mosaic virus mutant Ni 2519 forms local lesions on tobacco cultivars carrying the N gene which, unlike wild-type lesions, do not enlarge at elevated temperature. This may reflect temperature sensitivity of a viral gene product required for cell to cell spreading of infectivity. Ni 2519 also carries an unselected *cis*-dominant lesion in viral assembly. Peptide mapping of *in vitro* translation products of Ni 2519 RNA reveals at least one, and possibly two changes in p30 and p19, two products of the 30-K open reading frame, compared with its parental strain A14. An A to G transition at position 5332 in Ni 2519 RNA accounts for the altered mobility of the major variable peptide. The corresponding A14 peptide itself differs from the wild-type due to another A to G transition at residue 5329. These residues are close to the viral assembly origin. A revertant virus population which could assemble at the restrictive temperature regained the wild-type sequence in place of the point mutation specific to Ni 2519 at position 5332, and formed wild-type local lesions as efficiently as the parental strain. This result implicates mutation of residue 5332 in the temperature sensitivity of viral assembly (by altering the structure of the RNA close to the assembly origin) and/or local lesion spreading (via a radical Arg to Gly substitution in p30 or its derivatives). The mutation occurs in a position where the predicted amino acid sequence shows homology with a group of proteins encoded by yeast mitochondrial introns.

Key words: temperature-sensitive mutant/tobacco mosaic virus/host-controlled localisation/viral intercellular movement/virus assembly

Introduction

Tobacco mosaic virus (TMV) coat protein mutants contributed to the elucidation of the genetic code (Wittmann, 1962; Tsugita, 1962), but limited use has been made of genetic methods in the study of the virus itself. This situation reflects difficulties in selection and maintenance of stable mutants, and the lack of precise and systematic genetic mapping methods for viruses with RNA genomes. By now, however, mutants with a variety of phenotypes have been isolated, although knowledge of their molecular biology is still limited. Mapping the genetic lesions in these mutants by direct sequencing is now becoming feasible, even for point mutants, although distinguishing which mutations are relevant is a special problem since there is a degree of pre-existing sequence heterogeneity in many plant virus populations. Given the large body of information now available about the coding capacity of the TMV genome, which has recently been se-

quenced in its entirety (Goelet *et al.*, 1982) such studies should assist in understanding the functions specified by the genome. Here we show that the complex temperature-sensitive phenotype of a TMV mutant known as Ni 2519 (originally isolated by Jockusch) is associated with a point mutation close to the viral assembly origin within the 30-K open reading frame (ORF).

Ni 2519 is a nitrous acid-induced mutant selected as temperature sensitive for local lesion spreading on hosts such as *Nicotiana tabacum* cv Xanthi nc which respond to TMV infection with a localised necrosis. (For a recent review of the local lesion response and its dependence on the host N gene see Sela, 1981.) Ni 2519 lesions expand at 23°C, but not at 33°C, unlike lesions formed by the wild-type virus (Jockusch, 1966; 1968). While the mutant phenotype is clear-cut, the local lesion response itself is notoriously variable, and Peters and Murphy (1975) found that a proportion of wild-type lesions may resemble mutant lesions, depending partly on the conditions under which the plants were grown. They suggested that small lesion formation might have multiple causes, and it is not clear how many factors are at work. However, of the two isolates they studied, one (II-27), like Ni 2519, gave reproducibly smaller lesions even at 23°C, and was temperature sensitive, always forming lesions lacking the halo surrounding wild-type lesions. This clearly indicates an element of viral genetic control, and these properties may characterise a class of small lesion mutants with similar underlying defects. One other well characterised TMV mutant (LS₁, a spontaneous mutant of a tomato strain of TMV) has this phenotype (Nishiguchi *et al.*, 1978) and shows many parallels with Ni 2519. Growth of both these mutants in single cells or protoplasts seems normal (Bosch and Jockusch, 1972; Nishiguchi *et al.*, 1978), although in Ni 2519 the results are complicated by an unselected defect in assembly (see below). Jockusch suggested that Ni 2519 was deficient in cell to cell spreading of infectivity (which is known not to be affected in coat protein mutants (Siegel *et al.*, 1962). There is direct experimental evidence for a defect in intercellular movement in LS₁ (Nishiguchi *et al.*, 1978, 1980).

Ni 2519 is a complex mutant because it carries two additional deficiencies. Virus assembly is defective at the restrictive temperature (Bosch and Jockusch, 1972; Taliansky *et al.*, 1982a), although Ni 2519 coat protein is indistinguishable by amino acid analysis of tryptic peptides from that of A14, its parental strain (Wittmann *et al.*, 1965), and is functional in complementation (Taliansky *et al.*, 1982b). Unlike coat protein mutants the defective assembly of Ni 2519 cannot be complemented, that is, the defect is dominant in *cis* (Taliansky *et al.*, 1982b). Taliansky *et al.* (1982a) and Kaplan *et al.* (1982) have shown that Ni 2519 assembly also fails *in vitro* at 33°C, probably due to a temperature-sensitive RNA substrate, which could explain the dominant lesion in assembly.

Taliansky *et al.* (1982b) also reported that Ni 2519 forms local lesions rather than initiating a systemic infection on hosts such as *N. sylvestris*, which carry an allele of the major

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host determinant of resistance and localisation (sometimes known as the N' gene) which cannot localise infections by the wild-type virus (Sela, 1981). This characteristic is expressed at the permissive temperature; it is not yet clear whether it is related to temperature sensitivity in lesion spreading (see Discussion).

Both these unselected deficiencies directed attention to the same region of the RNA molecule. We suspected that a likely location for a mutation affecting assembly in a *cis*-dominant fashion was within the assembly origin, which lies ~1 kb from the 3' end of TMV RNA within an open reading frame (the 30-K ORF) (Zimmern and Wilson, 1976; Guilley *et al.*, 1979; Goelet *et al.*, 1982). This ORF is also within the region where mutations resulting in local lesion formation on *N. sylvestris* were mapped by Kado and Knight (1966), when their data are corrected for an error in the orientation of the map (Wilson *et al.*, 1976). Here we present evidence based on peptide mapping, nucleotide sequencing and reversion analysis that an A to G transition close to the assembly origin of Ni 2519 is associated with one or both temperature-sensitive defects. This mutation could have a dual effect, both altering the conformation of the assembly origin and resulting in a radical amino acid substitution in the encoded protein, p30.

Results

Screening for mutations using tryptic peptide mapping

To estimate the number and location of mutations in the Ni 2519 genome, we initially screened polypeptides synthesised *in vitro* on templates of viral RNA from Ni 2519, its parental strain A 14, and wild-type TMV (the U₁ stock) for alterations detectable by tryptic peptide mapping. (A14, a spontaneous mutant of the wild-type, has a single amino acid substitution in the coat protein (Wittmann, 1962) which is slightly more thermostable as a result (Jockusch, 1968); it forms wild-type local lesions and infects *N. sylvestris* systemically. Strain derivations and interrelationships are listed in Table I.) All the known proteins encoded by the TMV genome (see Figure 1 for map locations) can be made by *in vitro* translation of RNA extracted from virus particles except for the coat protein, but Ni 2519 coat protein has already been shown to yield tryptic peptides indistinguishable from those of the parental strain A14 (Wittmann *et al.*, 1965).

We could find no detectable changes on [³⁵S]methionine-labelled tryptic peptide maps of Ni 2519 p126 or p30 compared with those of its parental strain, A14 (data not shown). However, the p126 of U₁ TMV was sufficiently different from the other two to be distinguishable by a slight difference in electrophoretic mobility on a 7.5% SDS-polyacrylamide gel (Figure 1) and by several differences on [³⁵S]methionine-labelled peptide maps (not shown). This is perhaps surprising since both U₁ and A14 derive from the same Wisconsin isolate (Johnson, 1926, see Table I). However, A14 was selected from a wild-type stock (known as *vulgare*) which had been maintained separately at Tübingen since 1937, while the U₁ stock was independently derived from the original isolate in 1951 (Table I). Cumulative differences may have arisen in the sequences of the respective p126 genes during these intervals of serial passage; alternatively the variant may have been selected when A14 was isolated. TMV *vulgare* was not immediately available for comparison so we cannot decide between these possibilities.

Fig. 1. (a) Translation products of TMV strains U₁ (RNA purified from long rods, a gift of Milt Zaitlin), A14 and Ni 2519 (both RNA from unfractionated virus particles), displayed on a 7.5% acrylamide SDS gel. Products mentioned in the text are indicated. (b) Map locations of products shown in (a). p183 is an amber readthrough product of p126 extending from nucleotide 69 to nucleotide 4916. p30 is encoded by residues 4903 to 5706, while p19 is made *in vitro* in the same 30-K reading frame as p30 starting at the internal methionine residue encoded by nucleotides 5191–5193. O_a is the assembly origin. CP is the coat protein (absent from the translation in (a) since its mRNA is not packaged).

Since methionine-labelled tryptic peptides only represent a proportion of the polypeptide sequence, a ¹⁴C-labelled amino acid mixture which was expected to label all the tryptic peptides was used to screen the products of A14 and Ni 2519 translation in more detail. We could not detect any reproducible strain-specific differences when p126 maps were compared, although the resolution of the method is approaching saturation in maps of this complexity (Figure 2a and b), and

Table I. Summary of strains and mutants

Strain	Reference	Derived from	RNA		p30		Assembly and lesion phenotype
			Posn:5329 ^b	5332	Posn:143 ^b	144	
Type tobacco isolate ^a	Johnson, 1926	—	A	A	Ile	Arg	wt
U ₁ ^a	Singer <i>et al.</i> , 1951	Type	A	A	Ile	Arg	wt
A14	Wittman, 1962	Type Tubingen stock ^a 'Strain vulgare' (Spontaneous mutant)	G	A	Val	Arg	wt
Ni2519	Jockusch, 1966	A14 (Nitrous acid mutant)	G	G	Val	Gly	Assembly and local lesion spreading defective at 33°C
Ni2519 Rev.	This work	Ni2519 (selection, no mutagenesis)	G	A	Val	Arg	wt
Type Heidelberg stock ^a	Zimmern, 1977	Type (Cambridge stock)	G	A	Val	Arg	Assembly normal, lesion phenotype not checked

^aThe original isolate of Johnson was a mixture, containing ~10–15% of strain U₂ (Johnson, 1947; Singer *et al.*, 1951). The major component was purified independently in various laboratories, resulting in the sublines U₁ and vulgare. In other cases differences have clearly arisen over long periods of serial passage (here denoted 'X' stock, where X is the name of the laboratory). Most of the lineages can be reconstructed in outline, but not in detail.

^bNumbering throughout this paper is based on the RNA sequence of the Cambridge wild-type stock (Goelet *et al.*, 1982) which is assumed to represent the type isolate, from which it derives via W.M.Stanley and H.Fraenkel-Conrat.

there were two areas where replicate maps varied slightly in appearance. In the position marked A in Figures 2a and b, two spots are resolved on some maps where only one occurs on others. This variation is not correlated with the strain supplying the template RNA. In the second area there are two peptides (arrowed B) whose mobilities are slightly variable relative to the surrounding peptides and each other. Neither peptide shows a consistent difference in mobility correlated either with the virus strain or with the number of spots at position A. Furthermore, when a mixture of the A14 and Ni 2519 p126 proteins was digested and analysed, no unique peptide could be resolved at either position (data not shown). We concluded that no strain-specific differences could be detected between the p126s of A14 and Ni 2519 using this technique.

On comparing tryptic digests of ¹⁴C-labelled p30 of A14 and Ni 2519, we observed that the maps were nearly identical, but that each digest had a unique peptide in one particular region of the map (arrowed X and X' in Figures 2d and e). The map of strain U₁ p30 lacked both these peptides, but instead had its own unique peptide (X'', see below). The differences observed in mobility of this peptide from strain to strain were confirmed in digests of mixtures of translation products of pairwise combinations of strains, where all three peptides could be resolved from each other (data not shown). It seemed likely that these peptides were related by amino acid substitutions. No other differences were visible on the maps of A14 and Ni 2519 p30s, although the U₁ map had additional differences in some peptides, all of which were present in minor yields.

p30 is only one of a family of C-coterminal proteins made *in vitro* from the 30-K ORF on TMV RNA; at least two smaller proteins exist whose N termini map at methionine residues within the 30-K ORF (Hunter *et al.*, 1983). In order to map the unique tryptic peptides more precisely, we prepared ¹⁴C-labelled tryptic peptide maps of a smaller member of the family, p19 (formerly known as 23 K from its apparent SDS gel mobility), from all three viruses (Figures 2g–i). Peptides X, X' and X'' were present with mobilities characteristic of each strain on all three maps among the digestion products of p19, showing that they were each encoded in the C-terminal two-thirds of the 30-K ORF, between nucleotides 5191 and 5706, inclusive, on TMV RNA (Figure 1). These maps also revealed that a minor spot (arrowed, Y)

was missing from the digest of Ni 2519 p19 compared with that of A14 (see below), although it was present on both p30 maps. This peptide was also missing from the maps of both p30 and p19 of strain U₁, with the coordinate appearance of another peptide of slightly reduced mobility in both dimensions (denoted Y'), suggesting that peptide Y derives from a region of p30 whose sequence differs slightly between strain U₁ and the other two strains.

Identification of a point mutation close to the assembly origin

The unique internal assembly origin of wild-type TMV lies within the RNA sequence encoding protein p19. Ni 2519 is defective in assembly, and a mutation causing an amino acid substitution in p19 might also affect assembly by altering the base sequence in this region. Preliminary experiments had shown that the assembly origin loop could be used as a priming site for determination of the 5' half of the sequence encoding p19 using the RNA dideoxy method. Accordingly a specially synthesised pentadecanucleotide primer (dGTCTTCTTCAGCAAC) complementary to the assembly origin loop was used to examine the nucleotide sequences of U₁, A14 and Ni 2519 RNAs for base changes between the assembly origin and the N terminus of p19 (residues 5191–5457). The U₁ RNA sequence proved to be identical to the reference wild-type sequence in this region. The sequence of A14 showed a single A to G transition at base 5329 (Figure 3). This change has previously been observed to occur spontaneously in a wild-type TMV stock (Zimmern, 1977: see Table I), and there is no evidence that this materially affects the properties of the virus. It would cause a conservative substitution in the amino acid sequence of p30 from Ile to Val. The sequence of Ni 2519 showed the A to G change of A14, and an additional A to G change three bases away at position 5332 (Figure 3). An A to G change is consistent with the mechanism of mutagenesis of nitrous acid, the mutagen used to make Ni 2519. Furthermore, this base change would result in both a radical amino acid substitution of Gly for Arg in the amino acid sequence of p30 and related proteins at position 144, immediately adjacent to the first Val to Ile substitution, and an alteration in the RNA structure close to the assembly origin (see accompanying paper). These observations (summarised in Table I) suggested that the functional defect in Ni 2519 might be due to a nitrous acid-induced point

Fig. 2. Tryptic peptide maps of: (top) p126 and (middle) p30 translated from (a and d) A14 RNA, (b and c) Ni 2519 RNA, and (c and f) Ni 2519 revertant RNA. (Bottom) p19 translated from (g) U₁ RNA, (h) A14 RNA and (i) Ni2519 RNA. All the proteins were labelled with a [¹⁴C]amino acid mixture. 1st dimension: pH 4.7 electrophoresis. 2nd dimension: ascending chromatography. A, B, X, X', X'', Y and Y' are peptides mentioned in the text. U₁ p19 was a product of RNA extracted from viral particles partly stripped with alkali (Hunter *et al.*, 1983), all the other proteins were products of unfractionated viral RNA.

Fig. 3. Sequencing gel of part of the p19 coding sequence in U₁, A14, Ni 2519 and Ni 2519 revertant RNAs. The nucleotide sequence spanning tryptic peptide X" is shown below the gel, with the predicted amino acid sequence above it. The positions of the mutations in the peptide X" coding sequence at residues 5329 and 5332 of A14 and Ni 2519 respectively are marked on the gel and shown below the RNA sequence. The predicted amino acid substitutions are shown above the peptide sequence.

mutation at position 5332.

The sequential substitutions can also account for the relative mobilities of tryptic peptides X, X' and X" of p30 and p19 in A14, Ni 2519 and U₁. Mobilities in both dimensions calculated for the tryptic peptide spanning the sites of mutation agree well with those observed in all three cases. Val runs slower than Ile in our chromatography system, accounting for the reduced second dimension mobilities of the A14 peptide compared with that of U₁ (Figures 2g and h). The Ni 2519 Arg to Gly mutation lengthens this tryptic peptide by three amino acids (Asn-Val-Lys) by abolishing a trypsin cleavage site. Since this increases the mass without altering the charge the corresponding peptide is retarded in the electrophoretic dimension compared with those of U₁ and A14 which have identical charge and length and migrate to identical positions upon electrophoresis. The additional amino acids also reduce the hydrophobicity of the peptide with a consequent reduction in its chromatographic mobility (Figure 2d, e, g-i). The fusion of two tryptic peptides might also at first sight be thought to account for the disappearance of spot

Y from the map of Ni 2519 p19 (Figure 21). However, peptide Y is absent from the map of strain U₁ p19 (Figure 2g) although Asn-Val-Lys is expected from the RNA sequence to be present in the digest. Furthermore, a second Asn-Val-Lys tripeptide is expected to be derived from amino acid residues 238-240 of p30 according to nucleotide sequences derived for three different wild-type stocks of TMV (Guilley *et al.*, 1979; Goelet *et al.*, 1982; Meshi *et al.*, 1982). Consequently, the spot corresponding to Asn-Val-Lys should not disappear altogether from the digest of Ni 2519 p19 unless A14 and Ni 2519 differ from the sequenced isolates in that region. On both these grounds it is unlikely that peptide Y is absent because it is fused to peptide X. Since a peptide with the same mobility as spot Y appears in the digest of Ni 2519 p30 (Figure 2e), spot Y probably represents a peptide recovered in variable yield. Alternatively, there may be another mutation in Ni 2519 p19 that alters the C-terminal copy of an internally duplicated sequence in p30, of which there are several, although if this is so both copies of the duplicated sequence must differ from those in strain U₁.

Fig. 4. Local lesion assay on Xanthi tobacco of A14, Ni 2519 and Ni 2519 revertant. Virus strains tested on opposite half leaves are shown on the figure. Arrowheads on the enlargements indicate Ni 2519 lesions. Enlarged area in top right panel is boxed on leaf at top left to indicate relative size.

Reversion analysis

Tryptic peptide mapping, while useful for screening purposes, cannot detect changes in untranslated regions of the genome, or in redundant positions of the genetic code. Nor did we examine maps of p183, because in our hands incorporation into this protein using a [^{14}C] amino acid mixture for labelling was insufficient for peptide mapping, while the resolution of the method is already approaching its limits when applied to a protein the size of p126. The total number of mutations in Ni 2519 cannot therefore be established by this method. Accordingly, we returned to genetic methods to establish whether or not the A to G transition at position 5332 was related to the phenotype of Ni 2519.

A twice passaged Ni 2519 inoculum was used to initiate a systemic infection on *N. tabacum* cv Sansum, and the plants were grown at the restrictive temperature of 33°C. (Ni 2519 generates ~0.5–1% wild-type progeny per systemic passage at the permissive temperature (Jockusch, 1968).) After 9 days the plants were processed to isolate any mature virus present. Since the assembly defect is *cis*-dominant this selects directly for its reversion. No direct selection for local lesion phenotype was used, but since the progeny were from a systemic infection revertants for any underlying deficiency in cell to cell spreading would probably also be selected. Since packaging is not obligatory for cell to cell spreading (Siegel *et al.*, 1962), however, this selection is independent of selection for packaging. If these defects were due to two separate mutations one

would expect that only double revertants would be cured of both deficiencies, while some proportion of the singly revertant viral genomes that were still defective in cell to cell spread might be rescued by complementation. If both defects were due to one mutation, on the other hand, selection for ability to assemble should cause co-reversion of the defect in local lesion spreading whatever its basis.

In fact, progeny virus could be recovered from the infection at 33°C, and these gave lesions of wild-type morphology at a frequency of 88%: identical, within experimental variation ($\pm 8\%$), to that of A14 (90.5%) when assayed for temperature sensitivity of local lesion spreading on *N. tabacum* cv Xanthi nc (Figure 4). Sequence analysis showed that this phenotypic reversion was accompanied by reversion of the G residue at position 5332 to an A residue (Figure 3), while the G at position 5329 was unaltered, giving a sequence identical to that of the parental strain A14. Consistent with this, the peptide map of p30 translated from revertant RNA showed a mobility for peptide X identical to that of A14 (Figure 2f). The p126 map was similar to previous ones (Figure 2c). At least one other change was also observed on the p30 fingerprint compared with those of Ni 2519 and A14 (arrowed in Figure 2f); we do not yet know if this represents an additional sequence change. Further nucleotide sequencing is being undertaken to attempt to define any such change, and to see if the absence of peptide Y from the map of Ni 2519 p19 reflects a genuine second mutation.

Discussion

Our results demonstrate at least one and possibly two alterations in the 30-K ORF of TMV mutant Ni 2519 compared with its parental strain A14 detectable by tryptic peptide mapping. One of these, due to an A to G base change at position 5332, is implicated in one or both of the mutant's temperature-sensitive defects. Since this mutation lies close to the assembly origin, and since reversion resulted from selection applied directly to a *cis*-dominant defect, it seems probable that the temperature-sensitive assembly of Ni 2519 is related to this lesion. A possible mechanism for this deficiency involving a temperature-sensitive RNA structure is considered in more detail in the accompanying paper. Since progeny resulting from this selection were also able to form wild-type local lesions with high efficiency, this same mutation may also cause the selected defect in local lesion spreading, presumably as a result of the radical substitution of glycine for arginine in (a) translation product(s). The idea that both defects result from a single mutation economically explains how a mutant selected for lesion morphology showed an unselected assembly deficiency, while revertants selected for competence to assemble also reverted in lesion morphology. However, we still cannot entirely rule out the possibility that a second mutation is responsible for the defect in lesion spreading, since ability to spread systemically would also have been favoured in the selection for revertants. Nor do we know yet whether there are any other changes in the 30-K ORF of Ni 2519 or its revertant, but the existence of other changes would not affect our main conclusions: that structural alterations in the 30-K ORF and its products are strongly implicated in both defects of Ni 2519, and that the forward and backward mutation of both phenotypic markers correlate perfectly with purine transitions at position 5332.

The amino acid sequence of p30 includes a block of 128 amino acids (residues 69–196) which resemble a conserved sequence block in a group of proteins encoded by yeast mitochondrial introns (Hensgens *et al.*, 1983; Zimmermann, 1983). The sequence Asn-Ile-Arg-Asn (residues 142–145 of p30) surrounding the site of mutation in Ni 2519 is identical to the corresponding sequence in the protein encoded by the fourth intron of the yeast apocytochrome *b* gene. It is not yet clear if this structural resemblance reflects common or related functions, although one possibility is that these residues form part of an RNA-binding domain. However, if the proposed alignment is correct, conservation around the position corresponding to residue 144 of p30 strongly supports the genetic evidence for an important structural role for this part of the protein.

What is the underlying defect in Ni 2519?

While mapping mutations in Ni 2519 RNA is a necessary step towards a functional analysis, our experiments did not directly address the underlying causes of the temperature-sensitive spreading of lesions. Previous work suggests two possibilities: first that the mutant is unable to move from cell to cell at high temperature, and second that some function involved in induction of the local lesion response has been altered.

The tomato strain local lesion spreading mutant LS₁ has been shown directly to be defective in cell to cell spreading of infectivity by Nishiguchi *et al.* (1978, 1980). In an independent study Leonard and Zaitlin (1982) have presented evidence that methionine-labelled tryptic peptide maps of

protein p30 are slightly different in LS₁ and its parental strain. Our results confirm this correlation, and provide evidence for a functional connection. If we can take results with both mutants together, they suggest that a product of the 30-K ORF is involved in cell to cell spread of infectivity within the infected plant. This process is known not to require coat protein (Siegel *et al.*, 1962), and is thought to occur via the membrane channels (plasmodesmata) that perforate plant cell walls and connect adjacent cells. It is not yet clear how a viral gene product might be involved in this process. Viroid RNAs seem to be transported from cell to cell entirely by host mechanisms without any need for an exogenous gene product.

A second possibility depends on the idea that the spreading of visible necrotic lesions on the leaf reflects the diffusion of some substance induced by the virus infection in addition to the enlargement of the centre of infection. Some viral function involved in triggering the host response may be altered in Ni 2519 since hosts carrying the N' allele of the major host gene for localisation and resistance such as *N. sylvestris*, which localise only some TMV strains, are reported to produce local lesions on infection with Ni 2519 (Taliensky *et al.*, 1982b), rather than the systemic infection seen with its parental strain A14. The region of TMV RNA whose mutation leads to local lesion formation on *N. sylvestris* was roughly mapped by Kado and Knight (1966), and includes the 30-K ORF. Therefore, it is possible that this defect is due to the point mutation at position 5332, and that altered induction of the host response causes temperature sensitivity of local lesion spreading by a different mechanism to that seen in LS₁. It is also possible that the processes of cell to cell spreading and local lesion induction are fundamentally linked in some way. While more biochemical work is obviously needed to clarify interpretation of the genetic data, these mutants should be useful for dissecting the induction of host-controlled localisation and/or the mechanism of viral spreading from cell to cell.

Materials and methods

Viruses

Strain derivations are listed in Table I. Strain U₁ was obtained from M. Zaitlin, and A14 from K.W. Mundry via Jo Butler. Both were used without further passaging. Ni 2519 was a gift from Harald Jockusch. The inoculum was amplified through two short passages of systemic growth (3 days and 5 days, respectively) and assayed for retention of temperature sensitivity of local lesion spreading. No wild-type lesions were observed.

Revertants of Ni 2519 were selected by inoculating seven Sansum tobacco plants with a total of 1.2 ml of 0.5 mg/ml Ni 2519 in 40 mM sodium phosphate buffer, pH 7, followed by growth at 33°C for 9 days. Virus was extracted from uninoculated upper leaves.

Local lesion assay and temperature shift

Two or three pairs of opposite half leaves of greenhouse grown *N. tabacum* cv Xanthi nc (6–9 weeks old) were inoculated with purified virus (1–30 µg/ml) or RNA (60–120 µg/ml) using a 1:1 mixture of Carborundum and Celite and a cotton bud. The plants were then transferred either to a 20°C room or to a growth chamber at 23°C under continuous light. Lesions appeared after 2.5–5 days. For temperature shift assays (q.v. Jockusch, 1966; Peters and Murphy, 1975; Nishiguchi *et al.*, 1978) plants were then maintained at 32–33°C/90% relative humidity/23.5 h light at 5000 lux for 24 h in a Fitotron 600H growth chamber, followed by a return to 23°C for 18–24 h. Ni 2519 lesions were scored using a dissecting microscope at 6x magnification.

Virus and RNA preparation

Virus was purified by PEG precipitation (Leberman, 1966) followed in some cases by CsCl density gradient centrifugation and RNA extraction as described (Zimmermann, 1975).

In vitro translation and peptide mapping

Reticulocyte lysate conditions and peptide mapping protocol were described previously (Pelham and Jackson, 1976; Hunter *et al.*, 1983 and references therein). The [¹⁴C]amino acid mixture (NEN catalogue no. NEC 445, 54 mCi/matom, containing all the amino acids except Gln, Asn, Met, Cys and Trp) was used at 100 μ Ci/ml in a lysate which had not been supplemented with unlabelled amino acids.

RNA sequencing

The dideoxy method (Sanger *et al.*, 1977) modified for reverse transcription of an RNA template was used as previously described (Zimmern and Kaesberg, 1978), except that all reaction mixtures (4 μ l) contained 20 μ M dCTP and 10 μ M the remaining three triphosphates. This eliminates pile ups due to the lower concentration of labelled triphosphate and renders a chase unnecessary. 2.5 μ M additional labelled dATP or dGTP (Amersham, 400 Ci/mmol) was added to the standard mixture. Dideoxy triphosphates (PL Biochemicals) were titrated before use, actual concentrations were: ddATP 2 μ M, ddCTP 0.3 μ M, ddGTP and ddTTP 4 μ M. Reactions were analysed on 6% acrylamide 8 M urea thin gels (Sanger and Coulson, 1978).

Primer synthesis

The phosphotriester method on a polyamide support was used as described (Gait *et al.*, 1982). Protected monomers and MSNT were from Cruachan Chemicals.

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