Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8894–8897, November 1990
Microbiology

Systemic movement of an RNA plant virus determined by a point substitution in a 5′ leader sequence

(barley stripe mosaic virus/cDNA clones/viral genetics/*in vitro* mutagenesis/*in vitro* transcription)

I. T. D. PETTY*†, M. C. EDWARDS‡, AND A. O. JACKSON*§

*Department of Plant Pathology, University of California, Berkeley, CA 94720; and ‡U.S. Department of Agriculture–Agricultural Research Service, Northern Crop Science Laboratory, Fargo, ND 58105-5677

Communicated by Myron K. Brakke, August 27, 1990 (received for review July 6, 1990)

ABSTRACT **The ability of viruses to move through infected plants is an important determinant of host range and pathogenicity. We have investigated the genetic basis for the inability of the Type strain of barley stripe mosaic hordeivirus to undergo long-range systemic movement in the tobacco *Nicotiana benthamiana*. We show that, in this model system, a short open reading frame in the 5′ leader of the smallest viral genomic RNA prevents long-range vascular movement. As predicted by the ribosome scanning model, the leader open reading frame decreases the efficiency with which the 5′-proximal gene is translated *in vitro*. Thus, systemic pathogenicity in this system may be determined by the efficiency of translation of a viral gene *in vivo* and is not determined by the primary sequence of the encoded protein.**

The mechanisms by which plant viruses move from cell to cell and through the vasculature of infected plants are currently unknown. However, these processes generally require the interaction of specific viral gene products with the host and are an important constraint on viral host range and pathogenicity (1, 2). Although several plant viral proteins that are required for cell-to-cell movement have been identified (2), much less is known about those involved specifically in the potentiation of long-range vascular movement. The Type and ND18 strains of barley stripe mosaic hordeivirus (BSMV) (see ref. 3 for a review) accumulate to the same level in inoculated leaves of the tobacco species *Nicotiana benthamiana*, but only the ND18 strain is able to escape and spread systemically to the upper leaves (4). We have now used this model host–pathogen system to investigate the genetic basis for the difference in the ability of these two BSMV strains to undergo long-range vascular movement. The genome of each BSMV strain comprises three RNA components, which are designated α, β, and γ (5–7), and we show here by reassortment of the genomic RNAs and mutational analysis that a short open reading frame (ORF) encoded in the 5′ leader of RNA γ prevents systemic movement by the Type strain. This result suggests that, in this plant viral system, systemic movement may be determined by the efficiency of translation of a viral gene rather than the primary sequence of the protein it encodes.

MATERIALS AND METHODS

cDNA Clones and Mutagenesis. Full-length cDNA clones of the Type and ND18 strains of BSMV from which infectious *in vitro* transcripts can be derived have been described (4, 8). Deletion mutants lacking the viral γb gene (see Fig. 1) were prepared by digestion of full-length cloned cDNA with *Kpn* I and *Hpa* I, treatment with T4 DNA polymerase to render the ends flush, and recircularization of the large plasmid fragments according to standard procedures (9). Chimeric recombinants were constructed by exchanging 5′ and 3′ regions of γ cDNA at the conserved *Hin*cII and *Pst* I sites using γb deletion mutants of each strain. The 372-nucleotide (nt) direct repeat (4, 7) was removed from a full-length Type strain γ cDNA clone by digestion with *Nru* I and recircularization of the large plasmid fragment (see Fig. 1). This deletion mutant was then used to construct both the *Nru* I site cDNA chimeras and the oligonucleotide-directed mutations in the Type strain genetic background. *In vitro* transcription and plant inoculation with the mutant RNAs were carried out as described (4).

A full-length cDNA clone of RNA γ from the CV17 strain of BSMV was isolated in the transcription vector pBSMV(3′-T7) as described previously, except that the T7α Band-Aid oligonucleotide was used in place of T7γ (for details, see ref. 8). Sequencing of the cDNA clones using universal M13 primers was as described (8). Oligonucleotide-directed mutations were made by the procedure of Kunkel (10) as described (4) and confirmed by sequencing.¶

Analysis of Systemic Movement. The systemic movement of BSMV in *N. benthamiana* was assessed visually by the appearance of disease symptoms and by detection of viral coat protein antigen in extracts from inoculated and systemically infected leaves (or their equivalent in the case of nonmoving strains and mutants) 12 days after inoculation. Protein extracts prepared from *N. benthamiana* leaves were fractionated by electrophoresis in SDS/15% polyacrylamide gels and blotted onto nitrocellulose, and viral coat protein was detected with anti-BSMV antiserum as described (4).

***In Vitro* Translation.** For *in vitro* translation, capped *in vitro* transcripts of wild-type and mutant γ cDNA clones were prepared as described (4) and purified by precipitation from 3 M NaOAc at 0°C for 4 hr. *In vitro* translations were carried out in nuclease-treated rabbit reticulocyte lysates containing [^{3}H]leucine at an RNA concentration of 8 μg/ml (11) as recommended by the manufacturer (Promega). Equal volumes of the translation mixtures were fractionated by electrophoresis in SDS/7.5% polyacrylamide discontinuous gels (12), treated with EN^{3}HANCE (DuPont/New England Nuclear), dried, and fluorographed at −80°C overnight.

RESULTS

To map the viral genetic determinants of the systemic movement phenotype in *N. benthamiana*, we used infectious *in vitro* transcripts derived from full-length cDNA clones of the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BSMV, barley stripe mosaic virus; nt, nucleotide(s); ORF, open reading frame; TMV, tobacco mosaic virus.

†Present address: Department of Microbiology, North Carolina State University, Raleigh, NC 27695-7615.
§To whom reprint requests should be addressed.

¶The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M38631 (ND18), M38632 (Type), M38633 (CV17)].

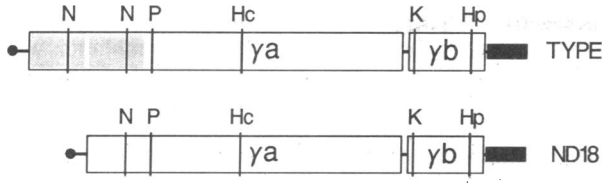

FIG. 1. Genetic organization of BSMV RNA γ and location of restriction sites used for cloning. The Type and ND18 strain γ RNAs are 3.2 and 2.8 kilobases long, respectively, the difference being due to a 372-nt direct repeat (shaded regions) in the former. The RNAs have 5′-methylguanosine caps (solid circles), 3′-terminal tRNA-like sequences common to all BSMV genomic RNAs (solid rectangles), and contain two genes (open rectangles) designated γa and γb (7). Restriction sites indicated were used to delete the γb gene (K, *Kpn* I; Hp, *Hpa* I) and to construct chimeric mutants (N, *Nru* I; P, *Pst* I; Hc, *Hin*cII).

Type and ND18 strains of BSMV (4, 8). Initially, each of the six possible heterologous combinations of ND18 and Type strain genomic RNAs were inoculated onto *N. benthamiana*. Only those pseudorecombinants that contained ND18 RNA γ were able to move systemically, indicating that this RNA carries a genetic determinant of the phenotype. The γ RNAs of the two strains are highly homologous (7), but that of the Type strain contains a 372-nt direct repeat that overlaps with the 5′-proximal γa cistron (Fig. 1). However, removal of the direct repeat from the Type strain RNA γ (by deletion of the *Nru* I fragment; Fig. 1) did not confer the ability to move systemically in *N. benthamiana* (data not shown). Expression of the 3′-proximal γb cistron (Fig. 1) is not required for the systemic infection of barley plants (13), and γb gene deletion mutants of the ND18 strain are also able to escape from the inoculated leaves of *N. benthamiana*, although their systemic movement is less effective than wild type (data not shown).

These results indicated the probable involvement of the ND18 γa gene in determining systemic pathogenicity to *N. benthamiana*. Accordingly, chimeric recombinant γ RNAs were constructed by exchanging 5′- and 3′-terminal cDNA fragments from the two strains using either the conserved *Hin*cII, *Pst* I, or *Nru* I restriction sites (Fig. 1). In each case, the chimera in which the 5′ portion of the RNA was derived from the ND18 strain was able to move systemically. Thus, the genetic determinant of the systemic movement phenotype lies within the 5′-terminal 325 nt of RNA γ bounded by the *Nru* I recognition sequence. Sequencing through this region revealed 5 nt substitutions between the two strains. One leads to an alanine to valine amino acid exchange in the γa protein, and four occur within the 5′ leader of the RNA (Fig. 2). When the amino acid substitution in the γa protein was introduced into the Type strain, the resulting mutant (U198) was indistinguishable in phenotype from the parental clone (Fig. 3). Therefore, the sequence differences in the 5′ leader determine the differential systemic movement phenotype of the two BSMV strains.

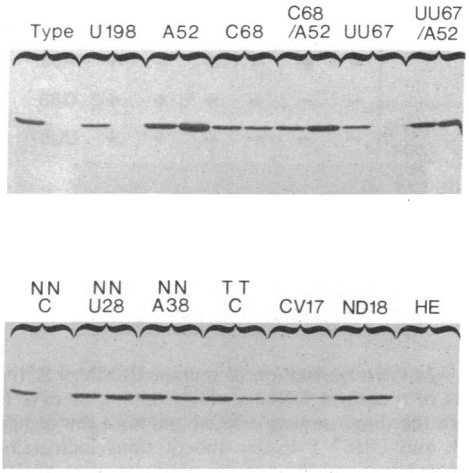

FIG. 3. Western blot analysis of systemic movement by BSMV mutants. Leaf protein extracts prepared from inoculated (left lane of each pair) and systemically infected leaves or their equivalent (right lane of each pair) were loaded in pairs, and the presence or absence of the BSMV coat protein was determined by Western blotting. (*Upper*) All inocula contained Type strain RNAs α and β plus wild-type (Type), U198, A52, C68, or UU67 mutant, or C68/A52 or UU67/A52 double mutant Type strain RNA γ as described in the text. All γ RNAs except the parental Type strain and mutants U198 and UU67 are able to direct virus spread to the uninoculated leaves. (*Lower*) Inocula contained ND18 (NN) or Type (TT) α and β RNAs plus wild-type (C), U28 mutant, or U28/A38 double mutant (labeled as A38) CV17 RNA γ as described in the text. Additional controls were inoculated with homologous combinations of CV17 or ND18 RNAs or mock-inoculated with no viral RNA (HE).

The Type strain leader sequence contains a short ORF upstream from the γa gene, which is not found in ND18 (Fig. 2). The importance of this element was assessed by constructing Type strain mutants in which the 5′-proximal AUG codon was replaced by AUA (A52), or the UGA termination codon of the leader ORF was replaced by CGA (C68) to make an in-frame fusion of this ORF to the γa gene. Both the A52 and C68 mutants were able to move systemically in *N. benthamiana*. Mutant A52 had the same aggressive systemic pathogenicity as ND18, whereas C68 induced an attenuated disease phenotype and had a reduced virus titer in the systemically infected leaves (data not shown). An additional mutant (UU67) was constructed by insertion of an extra uracil residue into the leader of mutant C68 between nt 67 and 68. This causes a frame-shift, which prevents read-through from the leader ORF into the γa gene. The UU67 mutation reverted the phenotype of C68 to that of the parental Type strain clone (Fig. 3), and both the C68 and UU67 mutants could be converted to full systemic pathogenicity by intro-

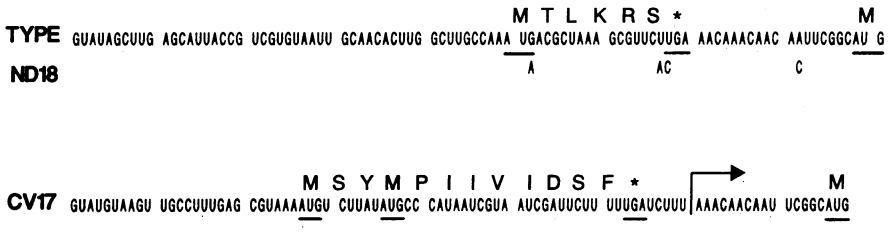

FIG. 2. The 5′-terminal sequences of RNA γ from the Type, ND18, and CV17 strains of BSMV. Nucleotide sequences shown begin at position 1 and extend to the initiation codon of the γa gene. Initiation codons (M) and in-frame termination codons (*) are underlined and the predicted translation products of the short ORFs are shown above the nucleotide sequence. The 4 nt at which the sequence of ND18 differs from that of Type are indicated below the complete sequence of the latter; the first and third correspond to the A52 and C68 substitutions referred to in the text. The angled arrow indicates the extent of α-specific sequence in CV17 (70 nt); the sequence AAACAACAA immediately following the arrow occurs in a similar location in both RNA α and RNA γ (5, 7).

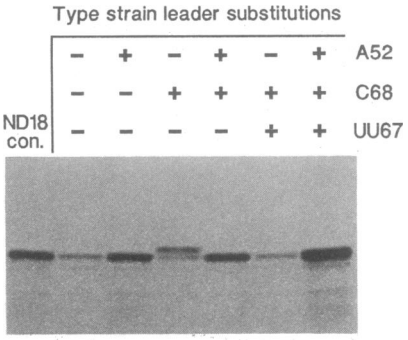

FIG. 4. *In vitro* translation of mutant BSMV γ RNAs. *In vitro* transcripts of a control ND18 γ cDNA clone, or of a Type strain clone, with the direct repeat deleted and with the combinations of A52, C68, and UU67 5′-leader substitutions indicated (+), were translated *in vitro*, and the protein products were analyzed by gel electrophoresis and fluorography. The predominant protein band in all lanes is the 74-kDa product of the γa gene, and the additional slower-migrating protein produced by translation of C68 is the product of the leader ORF-γa fusion gene in this mutant. The A52 substitution resulting in removal of the 5′-proximal AUG codon from the RNA increases the relative efficiency of translation of the γa gene in each case.

ducing an additional A52 mutation (Fig. 3; data not shown). Thus, a G to A transition at nt 52 in the 5′ leader of the Type strain RNA γ confers a full systemic movement phenotype, and furthermore this mutation (A52) is genetically dominant with respect to additional substitutions in the leader sequence (C68 and UU67).

The ribosome scanning model of eukaryotic translation predicts that the presence of a short ORF in the leader of an RNA will lead to decreased translation of a downstream gene (14, 15). *In vitro* translation of *in vitro* transcripts from wild-type and mutant cDNA clones confirmed that the leader ORF in the BSMV Type strain RNA γ affects the translation of the γa gene, as predicted by the ribosome scanning model (Fig. 4). However, we were unable to directly investigate this translational effect in infected *N. benthamiana* tissues because antibodies suitable for detection of the γa protein *in vivo* are not available.

To investigate the possible significance of the hexapeptide potentially encoded by the Type strain leader ORF, we exploited the observation that the γ-specific RNA of BSMV strain CV17 is a natural chimeric recombinant in which the 5′ leader is derived from RNA α (see Fig. 2). After inoculation of *N. benthamiana* with wild-type CV17 RNA γ, together with α and β RNAs from the ND18 strain, no systemic infection resulted (Fig. 3). However, mutation of either the first AUG (U28) or both AUG codons (U28/A38) present in the CV17 leader ORF permitted a systemic infection to occur (Fig. 3). As expected, the efficiency of γa protein translation from the RNAs was as follows: CV17 wild type < mutant U28 < mutant U28/A38 (data not shown).

Interestingly, although infections containing CV17 RNA γ are confined to the inoculated leaves of *N. benthamiana* when it is coinoculated with ND18 RNAs α and β, CV17 RNA γ can nevertheless confer the ability to move systemically when coinoculated with the α and β RNAs from either the Type strain or the homologous CV17 RNAs (Fig. 3).

DISCUSSION

Previous studies have indicated the importance of virus-encoded proteins in potentiating the movement of plant viruses from cell to cell and for their long-range spread through the vascular system (reviewed in ref. 2). Some plant viruses, such as brome mosaic virus, apparently require the assembly of virus particles for both types of movement process to occur (16). However, this is not the case in all plant virus systems. Tobacco mosaic virus (TMV) mutants, which can spread from cell to cell in the absence of virus particle assembly, have been known for some time (17), and both *in vitro* mutagenesis (18) and complementation experiments with transgenic plants (19) have implicated the TMV 30-kDa protein in potentiating the cell-to-cell movement process. *In vitro* mutagenesis studies have also demonstrated that both the coat protein and origin-of-assembly sequence are essential for efficient long-range systemic movement of TMV (20), suggesting that virus particles are required for this process. In contrast, a BSMV mutant that is unable to synthesize coat protein can infect *N. benthamiana* and is indistinguishable from the wild-type ND18 strain in both the timing and visual appearance of disease symptoms and in the accumulation of viral RNA in systemically infected leaves (21). Thus, BSMV differs significantly from TMV in not requiring virus particles for long-range movement.

The identification of a point substitution in the 5′ noncoding region of BSMV RNA γ as an important pathogenicity determinant is an interesting observation without direct precedent in plant virus systems. The hexapeptide encoded by the 5′ leader of the Type strain RNA γ is unlikely to contribute to the nonmoving phenotype of this strain since the unrelated leader sequence of the CV17 strain RNA γ confers the same phenotype. The 5′-proximal AUG codon is also unlikely to be a cis-acting sequence, which prevents systemic movement of the γ RNA *per se*, since the U28 mutation needs to be made only in the CV17 γ leader to allow movement, and not in the same sequence that is present in the 5′ leader of RNA α.

The nature of the sequence difference between the Type and ND18 strains of BSMV, and the properties of the *in vitro* constructed mutants, suggest a translational model in which the efficiency of γa gene translation determines the ability of BSMV to be a systemic pathogen of *N. benthamiana*. Short ORFs upstream of coding regions have been identified as mediators of translational down-regulation in simian virus 40 (22) and in the delayed early mRNAs of human cytomegalovirus (23). The latter example is particularly interesting because the short ORF alters the kinetics of protein accumulation *in vivo* (23). The relative efficiencies of γa protein translation *in vitro* from wild-type and mutant transcripts are consistent with a similar effect of the leader ORF of BSMV RNA γ. This model differs significantly from the previously characterized gene-for-gene type interactions (24), which are exhibited between TMV and the plant resistance genes *N′* in tobacco (25–28) and *Tm-1*/*Tm-2* in tomato (29, 30). In these cases, resistance-breaking mutations that allow systemic infection lead to amino acid substitutions in structural (*N′*) or nonstructural (*Tm-1*/*Tm-2*) TMV-encoded proteins.

Although specific virus-encoded proteins are required for systemic movement (2), the product of the BSMV γa gene is not a "movement protein" *per se*, since it contains an amino acid sequence motif associated with viral RNA polymerases (7), and its expression is essential for BSMV RNA replication *in vivo* (13). Interactions between the γa and the αa and/or the β gene products also appear to modulate the systemic movement phenotype of BSMV in *N. benthamiana*, because the systemic movement phenotype conferred by CV17 RNA γ depends on the source of RNAs α and β. Since the BSMV Type strain can move systemically in hosts other than *N. benthamiana* (4), interactions between host and viral factors must also be involved in determining the systemic movement phenotype. The generation of antibodies against the BSMV γa protein will allow the translational model we propose to be tested *in vivo* and should also facilitate investigation of the mechanism by which this protein influences long-range systemic movement.

We would like to thank Jim Onuffer for synthesizing the oligonu-

cleotides and Jack Morris for reviewing the manuscript. This research was supported by U.S. Department of Agriculture Grant 88-37263-3989 (A.O.J.).

1. Atabekov, J. G. & Dorokhov, Yu. L. (1984) *Adv. Virus Res.* **29,** 313–364.
2. Hull, R. (1989) *Annu. Rev. Phytopathol.* **27,** 213–240.
3. Jackson, A. O., Hunter, B. G. & Gustafson, G. D. (1989) *Annu. Rev. Phytopathol.* **27,** 95–121.
4. Petty, I. T. D., Hunter, B. G., Wei, N. & Jackson, A. O. (1989) *Virology* **171,** 342–349.
5. Gustafson, G., Armour, S. L., Gamboa, G. C., Burgett, S. G. & Shepherd, J. W. (1989) *Virology* **170,** 370–377.
6. Gustafson, G. & Armour, S. L. (1986) *Nucleic Acids Res.* **14,** 3895–3909.
7. Gustafson, G., Hunter, B., Hanau, R., Armour, S. L. & Jackson A. O. (1987) *Virology* **158,** 394–406.
8. Petty, I. T. D., Hunter, B. G. & Jackson, A. O. (1988) *Gene* **74,** 423–432.
9. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 1st Ed.
10. Kunkel, T. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
11. Dasso, M. C. & Jackson, R. J. (1989) *Nucleic Acids Res.* **17,** 3129–3144.
12. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
13. Petty, I. T. D., French, R., Jones, R. W. & Jackson, A. O. (1990) *EMBO J.,* in press.
14. Kozak, M. (1987) *Mol. Cell Biol.* **7,** 3438–3445.
15. Kozak, M. (1986) *Adv. Virus Res.* **31,** 229–292.
16. Sacher, R. & Ahlquist, P. (1989) *J. Virol.* **63,** 4545–4552.
17. Siegel, A., Zaitlin, M. & Sehgal, O. P. (1962) *Proc. Natl. Acad. Sci. USA* **48,** 1845–1851.
18. Meshi, T., Watanabe, Y., Saito, T., Sugimoto, A., Maeda, T. & Okada, Y. (1987) *EMBO J.* **6,** 2557–2563.
19. Deom, C. M., Oliver, M. J. & Beachy, R. N. (1987) *Science* **237,** 389–394.
20. Saito, T., Yamanaka, K. & Okada, Y. (1990) *Virology* **176,** 329–336.
21. Petty, I. T. D. & Jackson, A. O. (1990) *Virology,* in press.
22. Sedman, S. A., Good, P. J. & Mertz, J. E. (1989) *J. Virol.* **63,** 3884–3893.
23. Geballe, A. P. & Mocarski, E. S. (1988) *J. Virol.* **62,** 3334–3340.
24. Keen, N. T. & Staskawicz, B. (1988) *Annu. Rev. Microbiol.* **42,** 421–440.
25. Saito, T., Meshi, T., Takamatsu, N. & Okada, Y. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6074–6077.
26. Knorr, D. A. & Dawson, W. O. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 170–174.
27. Saito, T., Yamanaka, K., Watanabe, Y., Takamatsu, N., Meshi, T. & Okada, Y. (1989) *Virology* **173,** 11–20.
28. Culver, J. N. & Dawson, W. O. (1989) *Mol. Plant-Microbe Interact.* **2,** 209–213.
29. Meshi, T., Motoyoshi, F., Adachi, A., Watanabe, Y., Takamatsu, N. & Okada, Y. (1988) *EMBO J.* **7,** 1575–1581.
30. Meshi, T., Motoyoshi, F., Maeda, T., Yoshiwoka, S., Watanabe, H. & Okada, Y. (1989) *Plant Cell* **1,** 515–522.