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Multicomponent RNA plant virus infection derived from cloned viral cDNA

(brome mosaic virus/*in vitro* transcription/viral gene expression)

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ABSTRACT ***In vitro* transcripts from mixtures of appropriate brome mosaic virus (BMV) cDNA clones are infectious when inoculated onto barley plants. Infectivity depends on *in vitro* transcription and on the presence of transcripts from clones of all three BMV genetic components. Infectivity is destroyed by RNase after transcription, but it is insensitive to RNase before or to DNase after transcription. Virion RNAs from plants infected with cDNA transcripts hybridize to BMV-specific probes and coelectrophorese with virion RNAs propagated from conventional inoculum. Direct RNA sequencing shows that a deletion in the noncoding region of one infectious BMV clone is preserved in viral RNA from plants systemically infected with transcript mixtures representing that clone.**

Directly infectious cDNA forms have been constructed for three single-component RNA-based pathogens: bacteriophage Qβ, poliovirus, and potato spindle tuber viroid (1–3). However, the mechanisms by which such clones express their infectivity and the degree to which this expression is dependent on the biology of these particular pathogens is not understood. Moreover, no reports have been made of cDNA-initiated infections for multicomponent RNA viruses, which are particularly common in plant systems. Thus, for the vast majority of RNA viruses the ability to generate infections from cloned cDNA is lacking. This constitutes a serious limitation for RNA virology as it precludes recombinant DNA manipulations in broad classes of *in vivo* studies, which have proved extremely productive with DNA-based organisms. In principle, recombinant RNA techniques might be used to genetically manipulate such viruses, but the current power of recombinant RNA methods is not comparable to that of recombinant DNA (4).

We show here that appropriately constructed cDNA clones of brome mosaic virus (BMV), a multicomponent RNA plant virus, can direct infection of whole barley plants through a simple *in vitro* transcription procedure. Transcripts from functional complete cDNA clones of all three BMV genetic components are required for infection. A single-base deletion in one functional BMV cDNA clone is preserved in progeny RNA from infections derived from that clone. Unlike studies with single component RNA viruses, infectivity was not detected from untranscribed BMV cDNA clones. The system used to express BMV cDNA infectivity is likely to be applicable to many other RNA viruses.

MATERIALS AND METHODS

***In Vitro* Transcription.** Transcription reactions contained 25 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/150 mM NaCl/1 mM dithiothreitol/200 μM each rATP, rCTP, and rUTP/25 μM rGTP/500 μM m^7GpppG (P-L Biochemicals)/plasmid DNA (0.1 μg/μl)/*Escherichia coli* RNA polymerase (0.05 units/μl) (Promega Biotec, Madison, WI). Reactions were incubated 30 min at 37°C, by which time the rGTP was nearly exhausted. Additional rGTP was added to 25 μM and incubation continued a further 30 min. For uncapped transcripts, m7GpppG was deleted, rGTP was increased to 200 μM, the concentrations of DNA and polymerase were doubled, and incubation was carried out for 1 hr. Reactions were stopped by addition of EDTA to 10 mM and either diluted directly in inoculation buffer or phenol-extracted before nucleic acid recovery by ethanol precipitation. In most experiments, plasmids representing all three BMV components were pooled and cleaved at unique *Eco*RI sites 3 base pairs past the 3′ terminus of each BMV sequence before transcription.

Infectivity Testing. Seven-day-old barley seedlings (*Hordeum vulgare* L. cv. Morex) were dusted with carborundum powder and inoculated with either virion RNA or *in vitro* transcription mixes in 50 mM Tris PO_4, pH 8.0/250 mM NaCl/5 mM EDTA/ bentonite (5 mg/ml) (5); 15–30 plants in a single 13-cm-diameter pot were treated with the same inoculum, using 10–30 μl per plant. Plants were scored for the presence of mosaic symptoms 7–14 days after inoculation.

BMV Isolation. Fourteen days after inoculation, virus was isolated from barley plants as described (6), with the substitution of chloroform for carbon tetrachloride and a second polyethylene glycol precipitation for differential centrifugation. Viral RNA was isolated by phenol extraction and ethanol precipitation.

RESULTS

Infectivity Testing of BMV cDNA Clones and Their *in Vitro* Transcripts. BMV is a virus of grasses (7) whose genome is divided among three messenger-sense RNAs of 3.2, 2.8, and 2.1 kilobases (8, 9). Cloning of complete cDNA copies of all three BMV genetic components in a general transcription vector, pPM1, will be described elsewhere (37). DNA from such clones can be cleaved with *Eco*RI (Fig. 1) and transcribed *in vitro* in the presence of a synthetic cap structure (10) to produce complete RNA copies of the BMV components that have the same capped 5′ ends as authentic BMV RNAs, and an additional 6–7 nonviral nucleotides at their 3′ ends. These RNAs are active messengers in *in vitro* translation systems and direct production of proteins with the same electrophoretic mobility as those translated from authentic BMV RNAs.

To test the infectivity of these cloned DNAs and their transcripts, three plasmids, pB1PM18, pB2PM25, and pB3PM1, were selected. These and other complete BMV cDNA clones in pPM1 are named by the format pB*x*PM*y* where *x* = 1, 2, or 3 designates the BMV component cloned and *y* is an arbitrary isolate number. The selected clones thus contain cDNA copies of BMV RNAs 1, 2, and 3, respective-

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Abbreviation: BMV, brome mosaic virus.

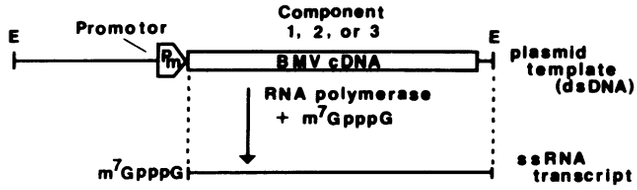

FIG. 1. Schematic representation of a template BMV cDNA clone in transcription vector pPM1 after linearization at the unique *Eco*RI site (E) and the capped runoff RNA transcript generated by RNA polymerase in the presence of m^7GpppG cap analog. Three such plasmids, each containing cDNA to one of the three BMV components, are needed for transcription of the total genome of BMV. The modified λ P_R promotor (P_M) allows RNA synthesis to initiate exactly at the 5′ terminus of each BMV sequence, and transcription continues to the end of the DNA template. ds, Double-stranded; ss, single-stranded.

ly, and represent, as a set, the complete BMV genome. For reasons clarified below, this set of plasmids and all *in vitro* or *in vivo* products arising from them are referred to as belonging to the Madison 1 or M1 strain of BMV, while the natural isolate of BMV propagated in our laboratory, from which the M1 cDNA was derived, is referred to by its usual designation of Russian strain. Mixtures of the *Eco*RI-cut M1 plasmids and their capped transcription products were inoculated onto barley plants in parallel with untranscribed DNA from the same plasmids. As judged by the production of normal viral symptoms, the transcribed plasmid mixture was infectious, while untranscribed plasmid mixture was not (Table 1).

The effects of various alterations to the transcription protocol were examined to more clearly characterize the infectious entity observed in plasmid transcription mixes. As shown in Table 2, infectivity requires transcription of clones representing all three BMV genetic components. Moreover, infectivity is sensitive to *Hin*fI before or to RNase A after transcription, but it is not significantly affected by RNase A before or *Hin*fI after transcription. *Hin*fI cleaves at 8 sites within pPM1 and at 15, 10, and 12 sites within BMV 1, 2, and 3 cDNAs, respectively. These results confirm that the observed infectivity arises from the *in vitro* transcripts rather than directly from their DNA templates. In addition, when plasmids are either not cut or are cut with *Pst* I before transcription (cleaving 2.7 kilobases rather than 7 bases downstream of the cDNA end), infection is not observed, suggesting that infectivity is affected by the structure of the transcript 3′ end. Finally, if the cap analog is omitted during *in vitro* transcription, no infection is detected, even if inoculum concentration is increased 20-fold.

Table 1. Comparison of infectivity of *Eco*RI-cut M1 plasmids, transcribed *Eco*RI-cut M1 plasmids, and Russian strain BMV virion RNAs over a range of inoculum concentrations

Pot no.	Inoculum, ng/μl	Plants with symptoms/total
	*Eco*RI-cut pB1PM18, pB2PM25, pB3PM1	
1	100	0/21
2	10	0/23
3	1	0/22
	Transcribed *Eco*RI-cut pB1PM18, pB2PM25, pB3PM1	
4	40	19/23
5	4	7/20
6	0.4	0/21
	Russian strain BMV virion RNA	
7	10	21/22
8	1	14/21
9	0.1	2/21
	Mock-inoculated	
10	0	0/22

In vitro transcription yields ≈3 BMV transcripts per plasmid (37). Total BMV transcript content of the inocula for pots 4–6 is thus ≈75, 7.5, and 0.75 ng/μl, respectively.

Infectivity of RNA transcribed *in vitro* from *Eco*RI-cut M1 plasmids was clearly lower than that of authentic BMV RNA. The number of infected plants produced from a given weight of *in vitro*-transcribed RNA was similar to that produced from 1/10th that weight of authentic BMV RNA (Table 1). The presence of the plasmid DNA template in the inoculum is not responsible for this effect, as addition of the same plasmid DNA to authentic BMV RNA does not affect its infectivity (Table 3).

Correlation of Symptomology with BMV Replication. Tables 1–3 report infectivity as scored by visual identification of characteristic BMV mosaic symptoms. To establish that such symptoms accurately reflect BMV replication, several molecular tests were applied. Nitrocellulose dot blots of total RNA (11) extracted from leaves of symptom-expressing and symptom-less plants inoculated with either authentic BMV RNA or *in vitro* BMV transcripts were probed with ^{32}P-labeled cloned BMV cDNA. In all cases, symptom-expressing leaves showed a positive hybridization response, and in all cases but one, symptomless leaves gave a negative response. The one exception was from a plant that had been inoculated with *in vitro* transcripts and showed no visible symptoms but gave a positive hybridization signal.

Virus isolated from plants infected with M1 cDNA transcripts is serologically identical to Russian strain BMV in double-diffusion tests with anti-BMV antisera (results not shown). Phenol extraction of M1 BMV releases four RNAs that comigrate with Russian strain virion RNAs, hybridize to BMV-specific DNA probes (Fig 2), and are highly infectious in subsequent inoculations (Table 3).

Propagation of a cDNA-Encoded Point Deletion in cDNA-Derived Progeny Virus. The above results show that symptom-expressing plants inoculated with *in vitro* M1 transcripts are infected with BMV. To obtain further independent evidence that these infections resulted from expression of the cloned BMV genes rather than from activation of a latent infection or some other source, selected regions of the M1 cDNA clones were sequenced (13–15) to identify deviations from the genomic RNA sequence of Russian strain BMV (8, 9). One such marker was identified in the BMV 2 cDNA of pB2PM25, where one of three consecutive guanine residues 73–75 bases from the 3′ end of Russian strain RNA2 is deleted. Direct RNA sequencing shows that this deletion is expressed in RNA2 from M1 progeny virus and verifies the distinctiveness of the M1 and Russian strains at this position (Fig. 3). M1 thus represents a genetically well-defined and distinguishable strain of BMV. Comigration of nucleotide bands below the deletion in Fig. 3, as well as examination of the extreme 3′ nucleotides on 20% sequencing gels, establishes that the 3′ end of RNA2 from M1 virus is coterminal with that of Russian RNA2. Similar 3′ sequencing of M1 viral RNAs 1 and 3 also reveals wild-type CCA (3′-OH) ends.

Characterization of a Nonfunctional BMV1 cDNA Clone. Table 3 includes infectivity tests with two clones outside of the M1 set. Transcripts of pB2PM24, like those of most pPM1/BMV genomic cDNA clones tested to date, support infection as part of a combination of clones representing all three BMV components. In similar experiments, transcripts from the BMV 1 clone pB1PM10 are nonfunctional. Restriction digests show that the cDNA in pB1PM10 contains no discernible insertions, deletions, or rearrangements and is

Table 2. Effect of plasmid deletion or other treatment on infectivity of transcribed M1 BMV clones

Pot no.	Inoculum	Plants with symptoms/total
1	pB1PM18, pB2PM25	0/29
2	pB1PM18, pB3PM1	0/29
3	pB2PM25, pB3PM1	0/25
4	pB1PM18, pB2PM25, pB3PM1	11/31
5	1.3 units of *Hin*fI per μg of DNA before transcription	0/28
6	RNase A (20 μg/ml) before transcription	13/30
7	1.3 units of *Hin*fI per μg of DNA after transcription	8/27
8	RNase A (20 μg/ml) after transcription	0/31
9	Plasmids cut with *Pst* I, not *Eco*RI	0/30
10	Plasmids not cut before transcription	0/29
11	100 ng/μl, transcribed without m7GpppG	0/16
12	Russian strain BMV RNA (10 ng/μl)	28/31
13	Mock-inoculated	0/30

Unless otherwise stated, each indicated plasmid was cut with *Eco*RI before transcription, transcribed in the presence of m7GpppG, and included in inoculum at 5 ng/μl along with its transcription products. DNA digestion and RNA intactness after *Hin*fI treatment as well as RNA digestion and DNA intactness after boiled RNase A treatment were verified by gel electrophoresis.

cloned in the correct orientation to produce positive strand *in vitro* transcripts. *In vitro* transcription of pB1PM10 produces normal yields of a BMV1-sized RNA. However, when translated in reticulocyte extracts, pB1PM10 transcripts fail to produce the expected 109-kDa BMV1a protein. Rather, they direct translation of a product with electrophoretic mobility similar to that of the 32-kDa BMV3a protein. DNA sequencing revealed that, while pB1PM10 contains the complete BMV1 5′ and 3′ cDNA ends and an unaltered 1a protein initiation codon, it bears a single base deletion at position 998 in the 1a coding sequence (9). The resultant frame shift would lead to premature termination and produce a translation product of the observed size. The biological inactivity of pB1PM10 may be due solely to this mutation. However, we have also identified a thymine to cytosine transition mutation at position 71 in pB1PM10 cDNA. This mutation and possibly others in unsequenced portions of the plasmid may also interfere with functional expression of the clone.

DISCUSSION

We have shown that multicomponent RNA plant virus infection can be derived solely from appropriately cloned viral cDNA by means of a simple transcription step. Although BMV has been used as a model system in the studies reported here, expression of viral cDNA through infectious *in vitro* transcripts should prove applicable to many other RNA viruses. The use of infectious cDNA transcripts may prove advantageous even for such RNA viruses as polio for which directly infectious cDNA forms can be constructed (2), because higher levels of infectivity may be achievable by transcription (see below). The vector pPM1 used here was designed as a general transcription vector to facilitate such applications (37), and it is likely that other vectors will prove useful as well.

Table 3. Infectivity tests of pB1PM10 and pB2PM24

Pot no.	Inoculum	Plants with symptoms/total
1	pB1PM10, pB2PM24, pB3PM1	0/16
2	pB1PM10, pB2PM25, pB3PM1	0/16
3	pB1PM18, pB2PM24, pB3PM1	11/16
4	pB1PM18, pB2PM25, pB3PM1	10/16
5	Russian strain BMV RNA (10 ng/μl)	19/21
6	Russian strain BMV RNA (1 ng/μl)	10/24
7	Russian strain BMV RNA (1 ng/μl) with *Eco*RI-cut M1 plasmid DNA (30 ng/μl)	13/26
8	M1 virion RNA (10 ng/μl)	23/26
9	Mock-inoculated	0/25

Inocula for pots 1–4 contained each indicated plasmid at 5 ng/μl along with its capped transcription products. An equimolar mixture of each of the M1 plasmids was used for pot 7.

Lack of Direct BMV cDNA Infectivity. Unlike DNA copies of some other RNA genomes (1–3), complete cDNA copies of the BMV genomic RNAs have not yet demonstrated direct infectivity. Since *in vitro* transcription of these clones

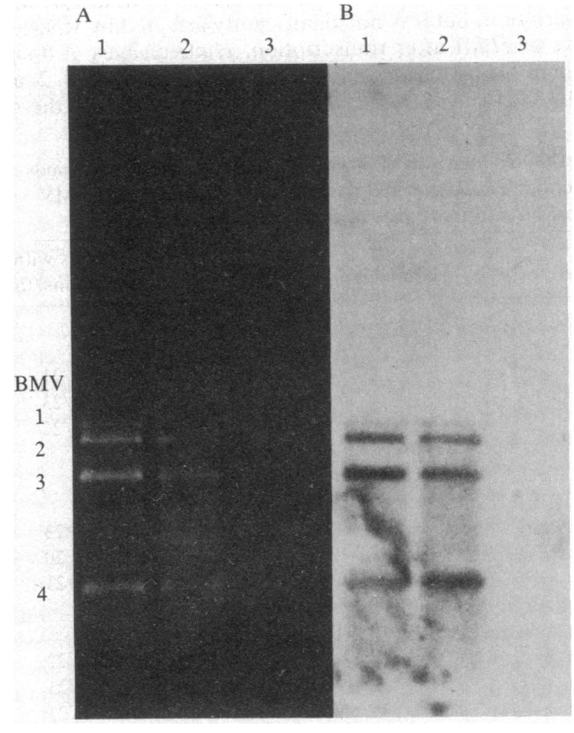

FIG. 2. (*A*) Ethidium bromide-stained 1% agarose gel after electrophoresis of virion RNAs isolated from Russian strain BMV (lane 1), Madison 1 strain BMV (lane 2), and black beetle virus (lane 3) (12). (*B*) Autoradiograph of an RNA blot of the gel in *A* after probing with a mixture of all three ^{32}P-labeled M1 cDNA clones.

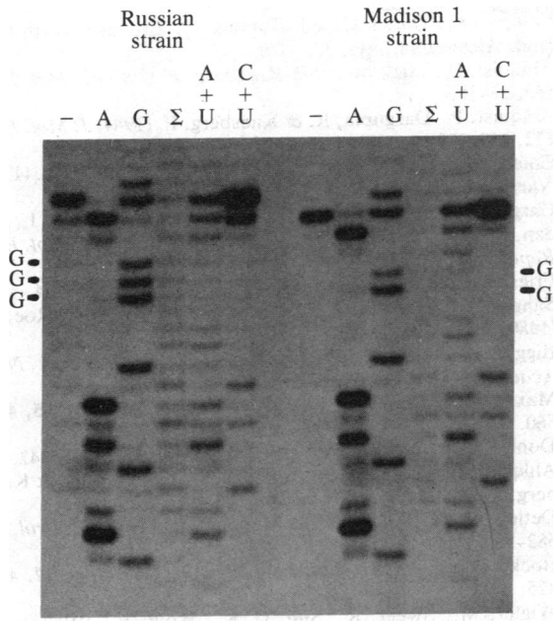

FIG 3. Direct sequencing results with RNA2 from Russian and Madison 1 strain BMV virions. Virion RNAs were isolated from 1.2% low melting point agarose gels (13) and the RNA2 fractions were 3′ ^{32}P-labeled (36) and sequenced (16, 17). The point of sequence variation between the two strains, occurring 75 bases from the 3′ end, is indicated.

can be carried out rapidly and conveniently to yield infectious products, this does not constitute a practical limitation. However, it poses important biological questions.

By comparison with infectious poliovirus cDNA, direct expression of BMV cDNA infectivity might be blocked at any of several stages. These blocks might be related to differences in biology of the host system, differences between the biology of polio and BMV, and/or differences in the construction of viral cDNA clones. Differences among host systems are likely to have major effects on viral cDNA infectivity. In general, the infectivity of animal virus RNA in mammalian cell cultures is considerably higher than the infectivity of plant virus RNA inoculated on whole plants. Poliovirus RNA, for example, yields well over 10^5 plaque-forming units (pfu)/μmol in cell culture, while BMV RNA yields only $\approx 10^2$ local lesions per μmol on appropriate plant hosts (18, 19). The infectivity of cloned polio cDNA has been reported to be $<10^2$ pfu/μmol (2), or 10^3 times lower than the infectivity of viral RNA. If the relative infectivity of cloned viral cDNA and RNA is similar for BMV, the lower sensitivity of whole plants to viral infection might account for failure to observe direct DNA expression.

In addition, differences between the biology of BMV and poliovirus may well play an important role. Although co-transformation of unlinked nuclear markers is a high-frequency event in mammalian systems (20, 21), expression of cloned viral cDNA may have very different requirements. Physical division of the BMV genome into three components requiring separate expression might seriously diminish infectivity of BMV cDNA compared to cDNA of single component viruses such as polio. Infectivity testing of cDNA to multicomponent animal and single component plant RNA viruses may illuminate this issue. Other features of BMV genome structure or replication could decrease BMV cDNA infectivity compared to polio. For example, if expression of poliovirus cDNA involves nuclear events, absence of 3′ poly(A) or presence of fortuitous splicing signals in BMV RNA might block the analogous expression route for BMV by interfering with stability or transport of viral transcripts. Finally, the observation that prokaryotic vector sequences can determine the infectivity of cloned animal virus DNA suggests that unrecognized features of BMV cDNA-containing plasmids could block their direct infectivity (22).

Factors Affecting Infectivity of *in Vitro* Transcripts. As noted from Table 1, the specific infectivity of capped *in vitro* BMV transcripts from *Eco*RI-cut plasmids is lower than that of virion RNA. Since plants infected from M1 transcripts develop full symptoms and yield highly infectious progeny virus, there do not appear to be functional defects in the M1 BMV cDNA sequences themselves. Lower specific infectivity is liable to result from structural differences between *in vitro* transcripts and virion RNAs, which include extra 3′ nucleotides and incomplete capping.

The BMV *in vitro* transcripts used here have heterogeneous 3′ ends bearing 6–7 additional nonviral nucleotides (37). It is not unlikely that this change would decrease RNA infectivity. The function(s) of the BMV RNA 3′ ends are not completely understood, but they are clearly important (23, 24). Additional 3′ nucleotides on the *in vitro* transcripts should block aminoacylation (25) and by this or other effects may influence the activity and/or stability of viral RNA. While *Eco*RI runoff transcripts have lowered infectivity, transcripts from uncut or *Pst* I-cut M1 plasmids have no detectable infectivity. Since each of these treatments will affect RNA polymerase recycling as well as add large blocks of sequence to the 3′ ends of the *in vitro* transcripts, unambiguous interpretation of these results is not possible. However, they are consistent with the possibility that major alterations to the BMV RNA 3′ ends diminish infectivity.

RNA sequencing shows that the 3′ ends of M1 progeny virus RNAs lack the additional 3′ nucleotides of their parent *in vitro* transcripts and have regained a wild-type CCA (3′-OH) end. This conversion may be accomplished by *in vivo* processing of the input *in vitro* transcript, by site-specific initiation of negative strand RNA synthesis, or by site-specific termination of positive strand synthesis. The second possibility is particularly likely, because it has recently been found that BMV RNA polymerase (26) initiates *in vitro* negative strand synthesis at the same site in both natural and 3′ extended BMV RNAs (W. A. Miller, personal communication).

Uncapped BMV *in vitro* transcripts are not detectably infectious at the sensitivity of current tests. Enhancement of transcript infectivity by capping would be expected, because studies in several laboratories show that capping not only affects translation efficiency (27) but drastically increases mRNA stability (28–30). These effects combine to give a 50-fold increase in *in vivo* interferon expression from capped mRNA over uncapped mRNA (10).

Although at further reduced rGTP concentrations capping might be more efficient (10), only 60% of the BMV transcripts are capped under the synthesis conditions described here (37). If effective delivery of all three components is the limiting factor in BMV RNA inoculations, the probability that a cell would receive capped transcripts of all three components is then only $(60\%)^3$ or 22% of the probability of receiving all three components. Since capping appears essential for detectable infectivity, the transcripts described here could then be no more than 22% as infectious as completely capped RNA.

Our sequencing of independent BMV cDNA clones for this and other studies has revealed many examples of single base sequence variations among clones. These mutations may arise both from preexisting heterogeneity in the viral RNA population (31–33) and from errors in reverse transcription (34). The examples cited above show that such mutations may yield either infectious or noninfectious phenotypes. Some mutations, such as the deletion in pB1PM10, may be recognizable as inactivating from sequence data.

However, most observed mutations to date are single base substitutions not subject to simple theoretical interpretation. Direct infectivity testing of viral cDNA clones, which also requires less labor than extensive sequencing, thus appears to be the most reliable way to identify functional viral sequences for further studies of structure, function, or expression. Viability of the deletion identified in pB2PM25 is also noteworthy. This deletion occurs within the 200-base region of extreme homology at the 3′ ends of all BMV RNAs (23) and within the region known to encode the BMV RNA polymerase recognition site (24). The effect of the deletion on the predicted secondary structure of this region would be to remove the terminal G-U base pair of a helical region and increase, by the resultant free uracil residue, the size of a bulge loop. The size of the analogous bulge loop is quite variable among related bromo- and cucomoviruses (23), which may reflect a degree of viral tolerance for sequence changes in this region.

The ability to generate virus infection from cloned cDNA represents a fundamental advance in the study of multicomponent RNA viruses. Unlike classical local lesion production, which may not resolve strain mixtures (35), cDNA expression allows the production of genetically well-defined RNA plant virus strains, such as the BMV M1 strain. More importantly, cDNA-dependent infection will allow the application of recombinant DNA technology to *in vivo* studies of multicomponent RNA viruses. Recombinant DNA methods should prove just as revolutionary for the study of these RNA viruses as they have for organisms with DNA genomes. In addition, the ability to manipulate multicomponent RNA viruses in cDNA form raises the prospect of using them as the basis for biological tools such as gene vectors.

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1. Taniguchi, T., Palmieri, M. & Weissmann, C. (1978) *Nature (London)* **274,** 223–228.
2. Racaniello, V. R. & Baltimore, D. (1981) *Science* **214,** 916–919.
3. Cress, D. E., Kiefer, M. C. & Owens, R. A. (1983) *Nucleic Acids Res.* **11,** 6821–6835.
4. Miele, E. A., Mills, D. R. & Kramer, F. R. (1983) *J. Mol. Biol.* **171,** 281–295.
5. Sarkar, S. (1967) in *Methods in Virology*, eds. Maramorosch, K. & Koprowski, H. (Academic, New York), Vol. 2, pp. 607–644.
6. Shih, D. S., Lane, L. & Kaesberg, P. (1972) *J. Mol. Biol.* **64,** 353–362.
7. Lane, L. (1981) in *Handbook of Plant Virus Infections and Comparative Diagnosis*, ed. Kurstak, E. (Elsevier/North-Holland, Amsterdam), pp. 333–376.
8. Ahlquist, P., Luckow, V. & Kaesberg, P. (1981) *J. Mol. Biol.* **153,** 23–38.
9. Ahlquist, P., Dasgupta, R. & Kaesberg, P. (1984) *J. Mol. Biol.* **172,** 369–383.
10. Contreras, R., Cheroutre, H., Degrave, W. & Fiers, W. (1982) *Nucleic Acids Res.* **10,** 6353–6362.
11. Garger, S. J., Turpen, T., Carrington, J. C., Morris, T. J., Jordan, R. L., Dodds, J. A. & Grill, L. K. (1983) *Plant Mol. Biol. Reporter* **1,** 21–25.
12. Friesen, P. & Rueckert, R. (1984) *J. Virol.* **49,** 116–124.
13. Sanger, F., Coulson, A., Barrell, B., Smith, A. & Roe, B. (1980) *J. Mol. Biol.* **143,** 161–178.
14. Biggin, M. D., Gibson, T. J. & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3963–3965.
15. Maxam, A. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
16. Donis-Keller, H. (1980) *Nucleic Acids Res.* **8,** 3133–3142.
17. Ahlquist, P., Dasgupta, R., Shih, D. S., Zimmern, D. & Kaesberg, P. (1979) *Nature (London)* **281,** 277–282.
18. Detjen, B. M., Lucas, J. & Wimmer, E. (1978) *J. Virol.* **27,** 582–586.
19. Bockstahler, L. E. & Kaesberg, P. (1965) *Virology* **27,** 418–425.
20. Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. & Axel, R. (1979) *Cell* **16,** 777–785.
21. Perucho, M., Hanahan, D. & Wigler, M. (1980) *Cell* **22,** 309–317.
22. Lusky, M. & Botchan, M. (1981) *Nature (London)* **293,** 79–81.
23. Ahlquist, P., Dasgupta, R. & Kaesberg, P. (1981) *Cell* **23,** 183–189.
24. Ahlquist, P., Bujarski, J. J., Kaesberg, P. & Hall, T. C. (1984) *Plant Mol. Biol.* **3,** 37–44.
25. Haenni, A. L., Joshi, A. & Chapeville, F. (1982) *Prog. Nucleic Acids Res. Mol. Biol.* **27,** 85–104.
26. Miller, W. A. & Hall, T. C. (1983) *Virology* **125,** 236–241.
27. Shih, D. S., Dasgupta, R. & Kaesberg, P. (1976) *J. Virol.* **19,** 736–742.
28. Furuichi, Y., LaFiandra, A. & Shatkin, A. J. (1977) *Nature (London)* **266,** 235–239.
29. Shimotohno, K., Kodama, Y., Hashimoto, J. & Miura, K.-I. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2734–2738.
30. Green, M., Maniatis, T. & Melton, D. (1983) *Cell* **32,** 681–694.
31. Domingo, E., Sabo, D., Taniguchi, T. & Weissmann, C. (1978) *Cell* **13,** 735–744.
32. Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S. & VandePol, S. (1982) *Science* **215,** 1577–1585.
33. Goelet, P., Lomonossoff, G. P., Butler, P. J. G., Akam, M. E., Gait, M. J. & Karn, J. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 5818–5822.
34. Fields, S. & Winter, G. (1981) *Gene* **15,** 207–214.
35. Garcia-Arenal, F., Palukaitis, P. & Zaitlin, M. (1984) *Virology* **132,** 131–137.
36. England, T. & Uhlenbeck, O. (1978) *Nature (London)* **275,** 560–561.
37. Ahlquist, P. & Janda, M. (1984) *Mol. Cell. Biol.* **4,** in press.