The EMBO Journal vol.5 no.8 pp.1769–1774, 1986

Modulation of replication, aminoacylation and adenylation *in vitro* and infectivity *in vivo* of BMV RNAs containing deletions within the multifunctional 3′ end

Jozef J.Bujarski[1], Paul Ahlquist[1,2], Timothy C.Hall[1,4]*, Theo W.Dreher[1,4] and Paul Kaesberg[1,3]

[1]Biophysics Laboratory, [2]Department of Plant Pathology and [3]Department of Biochemistry, University of Wisconsin, Madison, WI 53706, USA
[4]Present address: Department of Biology, Texas A&M University, College Station, TX 77843–3258, USA

*To whom correspondence should be addressed

Communicated by L.Bosch

The genome of brome mosaic virus (BMV) is comprised of three (+) strand RNAs, each containing a similar, highly structured, 200 base long sequence at its 3′ end. A 134 base subset of this sequence contains signals directing interaction of the viral RNA with BMV RNA replicase, ATP,CTP:tRNA nucleotidyl transferase and aminoacyl tRNA synthetase. A series of mutants containing deletions within this region, previously constructed and tested *in vitro* for the effect on replication and aminoacylation activities, has now been assayed *in vitro* for adenylation function and *in vivo* for ability to replicate in isolated protoplasts and whole plants. These tests indicate that features of viral RNA recognized by BMV replicase overlap those directing adenylation, but are distinct from those directing aminoacylation. Consequently, the lethality of a deletion preferentially inhibiting aminoacylation suggests that this function may have an essential role contributing to viral replication *in vivo*. An RNA3 mutant bearing a 20-base deletion yielding normal levels of aminoacylation and enhanced levels of replicase template activity and adenylation *in vitro* was able to replicate in protoplasts and plants; however, its accumulation in protoplasts was reduced relative to wild-type. This suggests that additional functions affecting the replication and accumulation of viral RNA reside in the conserved 3′ sequence.

Key words: RNA virus/BMV/adenylation/aminoacylation/replication/infectious viral transcripts

Introduction

Brome mosaic virus (BMV) is a single-stranded RNA virus of cereal crops (Lane, 1981) whose replication mechanisms appear to have fundamental similarities to those of other groups of plant RNA viruses as well as the animal alphaviruses (Miller *et al.*, 1985; Haseloff *et al.*, 1984; Cornelissen *et al.*, 1984; Ahlquist *et al.*, 1985). Studies on the mechanism of BMV RNA replication have been considerably advanced by the isolation of an RNA-dependent RNA polymerase (replicase) from BMV-infected barley (Bujarski *et al.*, 1982; Miller and Hall, 1983). This enzyme is specific for BMV templates and is able to initiate *de novo* synthesis both of (−) strand RNA complementary to BMV RNAs 1, 2, 3 and 4 (Miller *et al.*, 1986) and of (+) strand subgenomic RNA4 *in vitro* (Miller *et al.*, 1985).

The three RNAs comprising the BMV genome share a conserved, highly structured 3′ terminal sequence ~200 bases long (Ahlquist *et al.*, 1981), the last 134 bases of which form a tRNA-like structure and are sufficient *in vitro* to direct adenylation with ATP,CTP:tRNA nucleotidyl transferase, tyrosylation with aminoacyl tRNA synthetase (Joshi *et al.*, 1983) and initiation of (−) strand viral RNA synthesis with BMV replicase (Miller *et al.*, 1986). Several distinct helical regions of the tRNA-like structure can be stacked to produce four major arms (Figure 1a), whose three-dimensional arrangement is thought to resemble the L-shaped conformation of tRNA (Rietveld *et al.*, 1983). Initiation of (−) strand synthesis occurs at the penultimate base of the (+) strand template (Miller *et al.*, 1986), requiring that the terminal A residue of mature BMV virion RNA be added by a subsequent template-independent reaction. This is supported by the observation that double-stranded replicative form RNAs isolated from infected plants lack 3′ terminal A (Miller *et al.*, 1986). Adenylation with ATP,CTP:tRNA nucleotidyl transferase (Deutscher, 1984), which is able to provide this terminal A, is thus likely to play a crucial role in viral RNA replication. In contrast, the function of aminoacylation has yet to be elucidated, but it may contribute to replication or stability of the viral RNA (Hall, 1979).

Isolation of cDNA clones to each of the BMV RNAs (Ahlquist and Janda, 1984), and the use of *in vitro* transcription systems, have allowed the synthesis of partial BMV RNA transcripts that are reactive in *in vitro* assays (Dreher *et al.*, 1984) and complete BMV RNA transcripts that are infectious to barley plants (Ahlquist *et al.*, 1984b) and protoplasts (French *et al.*, 1986). Recently, we described (Dreher *et al.*, 1984; Bujarski *et al.*, 1985) a series of modifications in the region of the tRNA-like structure of BMV RNA which differentially affected replication and aminoacylation functions *in vitro*, leading to the following generalizations: (i) substitutions and deletions within arm C (Figure 1a) which substantially reduce replicase template activity decreased aminoacylation only slightly; (ii) deletions within arm B led to substantial inhibition of aminoacylation; (iii) extensive deletions in arm D had little or no effect on aminoacylation and, in several cases, substantially increased the template activity of the transcribed RNA for replicase. Based on these findings, a series of mutants was chosen for analysis of adenylation functions *in vitro* and for infectivity *in vivo*. This revealed that deletion mutations causing loss of any of the activities tested *in vitro* are lethal. However, a deletion mutant with normal or enhanced levels of these activities is viable both in isolated protoplasts and in systemic infection of whole plants.

Results

The deletion mutants studied (Table I) were chosen to be representative of the range of functional modifications observed previously (Bujarski *et al.*, 1985). These were: X6, which shows a marked inhibition of aminoacylation; D2 and M5, which selectively inhibit replicase template activity; M4 which has normal aminoacylation activity and 2-fold enhanced replicase template activity; S1, which is inactive in aminoacylation and replication.

Adenylation of deletion mutants in vitro

The effects of the deletion mutants on adenylation *in vitro* are shown in Table I. Correctly terminated adenylation substrates which contained the last 201 bases of BMV RNA3, minus the

desired internal deletion, were synthesized by transcription *in vitro* from *Tth*111I-linearized plasmids as previously described (Dreher *et al.*, 1984). After removal of the 3′ terminal residue with snake venom phosphodiesterase, adenylation was carried out using [α-^{32}P]ATP, and the products were analyzed by denaturing gel electrophoresis and autoradiography (Figure 2). In general, the effect of the various deletions on adenylation paralleled their effect on template activity for BMV replicase. Thus, for both adenylation and replication, deletion M4 gave an ~2-fold enhancement of activity compared with a wild-type transcript, X6 gave an intermediate level of activity, D2 and M5 gave very low activity levels and S1 had no detectable activity. This reactivity profile is quite distinct from the effect of these deletions on aminoacylation (Table I).

Replication of RNA3 deletion mutants in protoplasts

Because the mutants described here showed lowered levels of specific viral functions *in vitro*, the phenotypes of viral RNAs bearing these deletions were examined *in vivo* as a means of gaining insight to the roles of these different processes in infection. Because the function of viral RNA aminoacylation is unknown, the behaviour *in vivo* of mutants such as deletion X6, which showed decreased aminoacylation activity *in vitro*, was of particular interest. Of similar interest were mutants showing enhanced activities *in vitro*, such as deletion M4, which is twice as active a template for adenylation and (−) strand synthesis as is wild-type, and which also has normal aminoacylation function (Table I). Therefore, the five deletions depicted in Figure 1b were substituted, as described in Materials and methods, for the wild-type sequence in BMV RNA3 clone pB3PM1 whose transcripts have been shown to be infectious *in vivo* (Ahlquist *et al.*, 1984b). In protoplasts, both BMV RNAs 1 and 2, but not 3, are required for viral RNA replication (French *et al.*, 1986; Kiberstis *et al.*, 1981). Because of this, the phenotypic effects of transcripts bearing mutations in RNA3 can be reliably assayed in protoplasts for *cis*-acting effects on viral replication and accumulation.

Transcripts from each variant, as well as transcripts from the unmodified pB3PM1 wild-type clone, were individually inoculated onto protoplasts in the presence of transcripts of both wild-type BMV RNAs 1 and 2. When assayed by gel electrophoresis of [^{3}H]uridine-labeled RNAs produced post-inoculation, repeated experiments showed that only transcripts from the wild-type and M4 RNA3 clones induced production

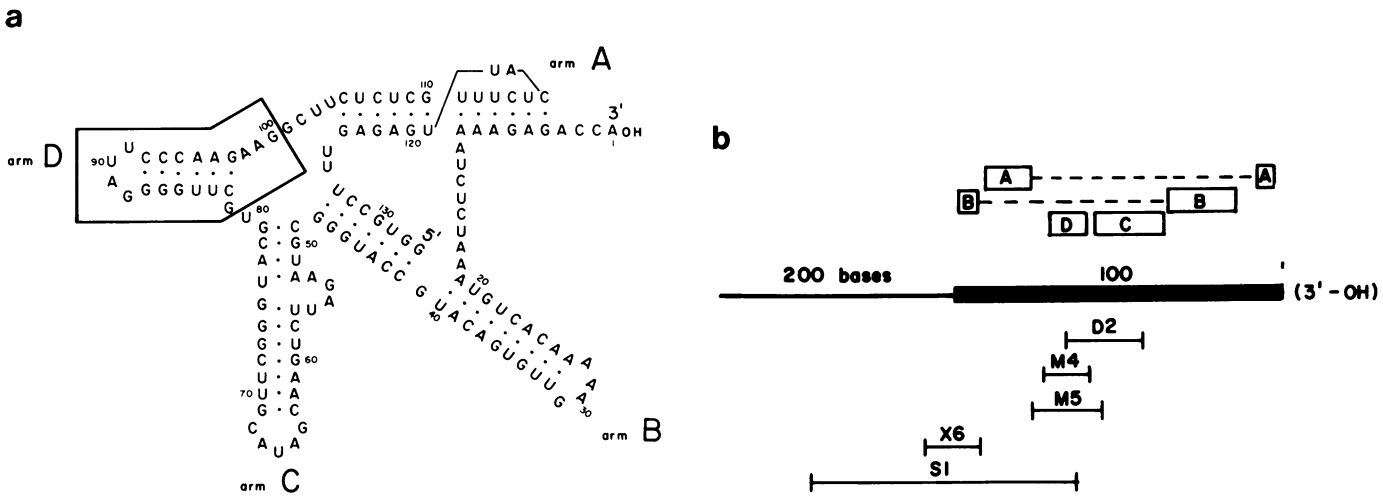

Fig. 1. (**a**) Tertiary arrangement for part of the secondary structure at the 3′ end of BMV RNA3 as proposed in Rietveld *et al.* (1983). The region deleted in the viable mutant M4 is outlined. (**b**) Map of deletions used in this study with respect to structural features in the 3′ terminal region of BMV RNA3. The sequence homology with BMV RNAs 1 and 2 extends over bases 1−193 from the 3′ end; additionally, a hairpin stem and loop centered at base 225 is conserved (Ahlquist *et al.*, 1984a). The 134-base 3′ region forming the core structure required for adenylation, aminoacylation and (−) strand RNA initiation *in vitro* is shown in the dark box; open boxes above the scale map the sequences contributing to arms A−D in Figure 1a. The labeled bars below the scale delimit bases removed in the indicated deletions.

Table I. Influence of various deletions in BMV RNA 3′ fragments on aminoacylation, adenylation and (−) strand synthesis *in vitro* and infectivity *in vivo*

Deletion name[a]	Bases removed	Arms affected[b]	Adenylation	(−) Strand initiation[c]	Amino-acylation[c]	Infectivity[d]
M4	81−100	D	250	200	100	+
X6	127−148	B	40	70	5	−
D2	58− 91	C,D	10	0	55	−
M5	76−104	C,D	2	5	72	−
S1	86−196	A,B,D	0	0	0	−

Values are given as a % of wild type activity. It is important to note that the aminoacylation data shown were obtained using transcripts synthesized *in vitro* (Dreher *et al.*, 1984) that contain a complete -CCA$_{OH}$ terminus. *In vivo*, 3′ structures such as those contained in M5 that cannot be adenylated, also cannot be aminoacylated, even though they may be recognized as substrates by the appropriate synthetase.

[a]From Bujarski *et al.* (1985), except for deletion S1 which is described in Miller *et al.* (1986) as plasmid pJM17.

[b]Refer to Figure 1a for definition of helical arms A−D in the 3′ terminal 133 bases of BMV RNA3.

[c]Activities for (−) strand synthesis and aminoacylation are the 30 min time points taken from Bujarski *et al.* (1985).

[d]See Figure 3 for evaluation of infectious (+) and non-infectious (−) mutants.

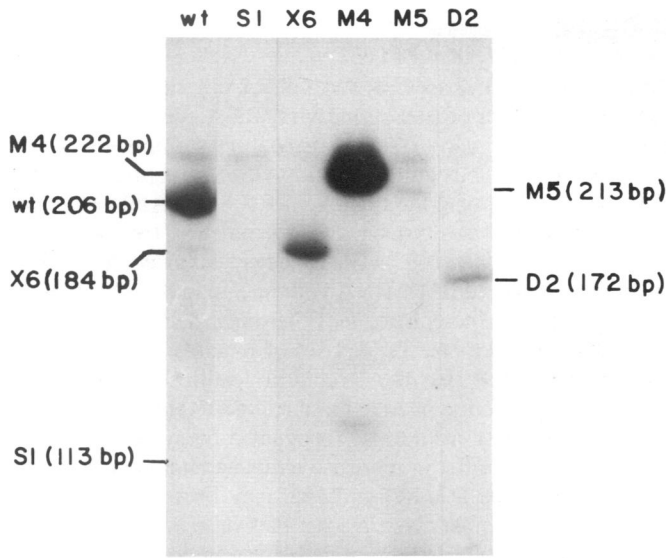

Fig. 2. Autoradiograph of a 6% polyacrylamide sequencing gel displaying pSP3HT transcripts (Dreher *et al.*, 1984) adenylated with [α-^{32}P]ATP and ATP,CTP:tRNA nucleotidyl transferase from *E. coli*. Deletions M4 and M5 migrated more slowly than wild type because the construction (in pSP62) contains 42 heterologous bases 5′ to the BMV sequence, compared with six bases in pSP64 used in all other constructions (Bujarski *et al.*, 1985). The expected positions for the adenylated products are indicated.

of progeny RNA bands corresponding to RNA3 and its subgenomic messenger, RNA4 (Figure 3a). All other deletions blocked detectable accumulation of RNAs 3 and 4. The RNA4 band induced by M4 transcripts migrated slightly more rapidly than did its wild-type counterpart, confirming retention of the M4 deletion in the progeny RNA. Densitometric analysis revealed that the normal molar excess of wild-type RNA3 to RNAs 1 and 2 was reduced 2-fold or more in the case of the M4 mutant (Figure 3a, compare lanes M4 and wild-type). This contrasts with the tests conducted *in vitro*, where the M4 deletion showed increased template activity for both replication and adenylation (Bujarski *et al.*, 1985; Table I).

Replication of the M4 deletion mutant in whole plants

BMV RNA replication in protoplasts, which does not require RNA3 (Kiberstis *et al.*, 1981; French *et al.*, 1986), clearly has less stringent constraints than does systemic infection of whole plants which does require RNA3. To see if the M4 derivative of RNA3 could support systemic BMV infection, barley plants were inoculated with *in vitro*-synthesized transcripts of wild-type RNAs 1 and 2 and the M4 derivative of RNA3. In several repeated experiments, plants so inoculated developed normal mosaic symptoms of BMV infection on a time scale similar to that of plants inoculated with all three wild-type components (Ahlquist *et al.*, 1984b). Virus was isolated from these symptomatic plants with a yield similar to that obtained from wild-type BMV infections. Parallel inoculations of barley plants with *in vitro* transcripts of wild-type RNAs 1 and 2 and each of the D2, M5,

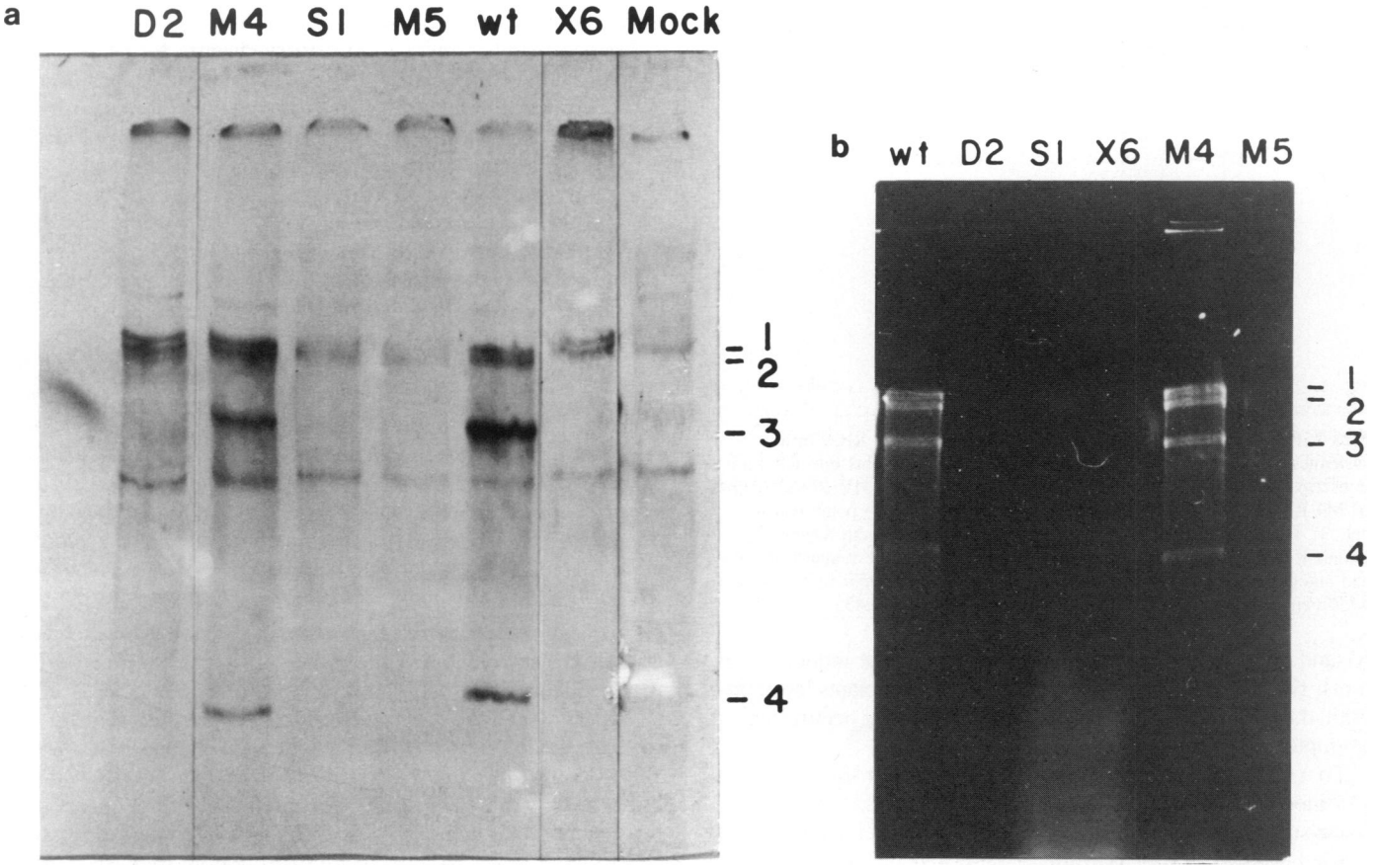

Fig. 3. Analysis of BMV RNA replication in (**a**) protoplasts and (**b**) whole plants. (**a**) RNA labeled with [^{3}H]uridine was extracted from protoplasts inoculated 24 h previously with transcripts of wild-type BMV RNA 1 and 2 and the indicated BMV RNA3 derivative. RNA was electrophoresed on a 2.4% polyacrylamide/0.5% agarose gel and fluorographed after treatment with EN^{3}HANCE (NEN). (**b**) Barley plants were inoculated with transcripts of wild-type BMV RNAs 1 and 2 and a selected BMV RNA3 derivative, grown for 2 weeks and harvested for virus isolation (Lane, 1974). RNA was extracted from isolated virions (Kiberstis *et al.*, 1981), electrophoresed on a 1% agarose gel, stained with ethidium bromide and photographed by ultraviolet illumination.

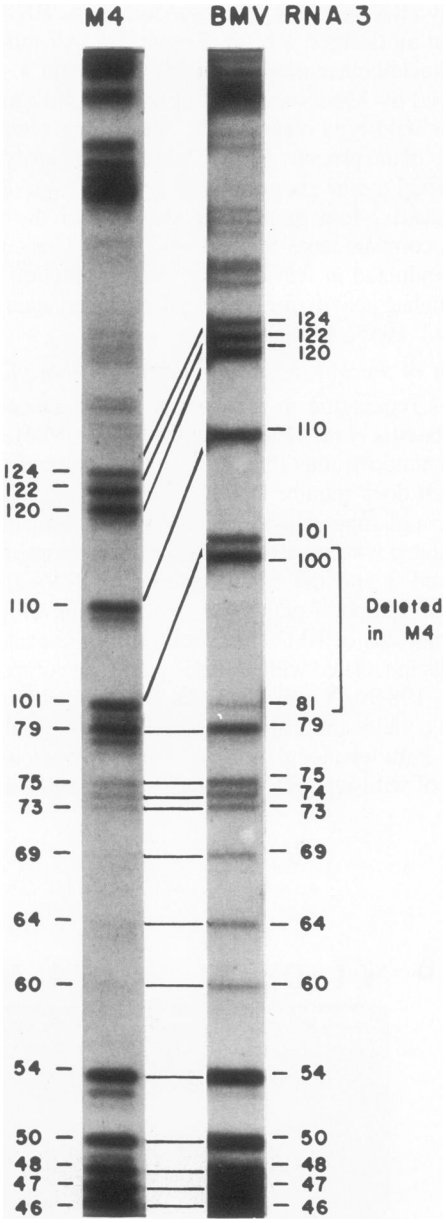

Fig. 4. Preservation of the M4 deletion in progeny virion RNA from systemically infected whole barley plants. RNA3 from plants infected with transcripts of BMV RNAs 1 and 2 and either wild-type BMV RNA3 (**right**) or M4 RNA3 (**left**) was isolated from a 1.2% low melting point agarose gel, 3′ ^{32}P-end-labeled (Barrell, 1971), partially digested with RNase T1 (Donis-Keller, 1980), fractionated on a 6% polyacrylamide sequencing gel and autoradiographed. G residues in both lanes are numbered to correspond with their position from the 3′ end of wild-type BMV RNA3.

S1 and X6 deletion derivatives of RNA3 did not induce symptoms. No detectable virus could be isolated from plants inoculated with these variants (Figure 3b), ruling out the occurrence of asymptomatic infections.

To verify that progeny RNA3 in the systemically infected M4-inoculated plants contained the M4 deletion, virion RNA3 from such plants was purified by preparative electrophoresis, labeled with [5′-^{32}P]pCp at the 3′ end, and the sequence of G residues determined by partial RNase T1 digestion (Figure 4). As shown, the results conform exactly to the sequence of the M4 deletion constructed in the cDNA clone used as a template for the original transcript inoculum, confirming that the M4 derivative of RNA3 is a fully viable deletion mutant.

Discussion

Adenylation of BMV RNAs

The last 132−137 bases of BMV RNA are sufficient *in vitro* to direct initiation of (−) strand viral RNA synthesis by BMV replicase, as well as (+) strand adenylation and aminoacylation. Mutants constructed within the tRNA-like structure have proven useful in mapping the regions of RNA involved in recognition by replicase and tyrosyl tRNA synthetase (Dreher *et al.*, 1984; Bujarski *et al.*, 1985). We have here extended this analysis to the interaction of BMV RNA with nucleotidyl transferase, an enzyme capable of completing the 3′ terminal -CCA in incomplete viral strands. From the limited set of mutants studied here, it can be concluded that the structural features recognized by nucleotidyl transferase overlap those recognized by replicase, and differ significantly from those recognized by synthetase (Table I). The rough parallel in replicase template and adenylation activities in mutants D2, M4 and M5, may reflect interaction of nucleotidyltransferase with at least part of arm C (Figure 1a), a region known to be important in the recognition of BMV RNA by replicase, but not involved in aminoacylation (Dreher *et al.*, 1984; Bujarski *et al.*, 1985). Although different regions are important in adenylation and aminoacylation (see M5, Table I), there is likely to be some overlap in the recognition sequences; both enzymes may be expected to interact with the acceptor stem and the 3′ end (arm A in Figure 1a) (Deutscher, 1984; Kao *et al.*, 1983), regions not probed in this study. Further, more discrete modifications to the tRNA-like structure are needed to precisely define and distinguish the regions recognized by nucleotidyl transferase, synthetase and replicase.

The specificity of aminoacyl tRNA synthetases for their cognate tRNAs shows that the 3′ tRNA-like structure must satisfy stringent structural requirements to direct aminoacylation of viral RNA. The probability that such requirements are maintained in response to a strict selection pressure has been considered persuasive evidence that aminoacylation is a required function of BMV RNA. Since recent analysis of (−) strand initiation confirmed the probable importance of adenylation in forming the mature 3′ end of BMV virion RNA (Miller *et al.*, 1986), it might be considered likely that a requirement for adenylation by tRNA nucleotidyl transferase selects for a sufficiently tRNA-like 3′ structure to allow aminoacylation. This is not the case, as the reactivity of various deletions in adenylation and aminoacylation display a lack of correlation (Table I); clearly, aminoacylatability cannot be indirectly maintained by selection for adenylatability. Rather, since non-adenylated BMV RNA functions well as a substrate for (−) strand synthesis (Miller *et al.*, 1986), adenylation itself may be preserved principally as part of the requirement for achieving an aminoacylatable 3′ end.

Ability of mutant sequences to replicate in vivo

Only one of the deletion mutants studied, deletion M4 which lacks arm D, proved viable in either protoplasts or plants. This verifies that the highly conserved, multifunctional 3′ region of BMV RNA is indeed vital for successful replication, as indicated by our replicase studies *in vitro* (Miller *et al.*, 1986; Dreher *et al.*, 1984; Bujarski *et al.*, 1985). Each of the non-viable mutants had low or no activity in one or more of the aminoacylation, adenylation or replication tests *in vitro*. The inviability of mutant X6, which is especially low in aminoacylation but retains 40% adenylation and 70% replication activity, does lend support to the contention that aminoacylation is essential *in vivo*, although one cannot rule out the possibility that other activities associated with the 3′ end and not studied in this work may be lost in the 21-base X6 deletion, and that inviability results from such loss. It is

noteworthy that no mutant incapable of adenylation (and consequently also of aminoacylation) is viable *in vivo* (mutants D2 and M5). However, an additional basis for the inviability of these latter mutants is presumably their poor replicase template activity. The general correlation between *in vitro* replicase template activity and *in vivo* replicatability (Table I) supports the validity of the *in vitro* BMV replicase assay.

The replicative competence of mutant M4 in both protoplasts and whole plant systemic infections is especially interesting, since it demonstrates that it is possible to alter the viral RNA sequence in informative ways, even in a crucial regulatory region. A natural structural precedent for M4 exists in the tRNA-like region of the RNAs of broad bean mottle virus (BBMV), a virus closely related to BMV. The aminoacylatable region of BBMV RNAs duplicates most of the features of the BMV RNA secondary structure but, like the M4 deletion, lacks arm D (Ahlquist *et al.*, 1981).

Mutant M4 showed high levels of all three *in vitro* activities including, interestingly, twice the replicase template activity of the wild-type. This replicative advantage relative to wild-type was not observed *in vivo*; in contrast, in protoplasts normal levels of RNAs 1 and 2 were observed, but only about half the normal level of RNA3, the only genomic RNA bearing the deletion. Also no apparent difference in relative levels of all RNA components would be seen in viruses isolated from M4 or wild-type-infected plants. The M4-type BMV RNA was proven to be unstable in prolonged infections, being repaired to the wild-type structure by recombination events (Bujarski and Kaesberg, 1986).

The reasons for the replicative disadvantage of M4 RNA3 are not obvious. If M4 RNA3 is indeed hyperactive in (−) strand synthesis *in vivo* as well as *in vitro*, this might result in a detrimental reduction in free (+) strand RNA3 due to polymerase binding or double-strand RNA3 formation. An additional explanation is that the M4 RNA3 deletion impairs a facet of performance of the BMV RNA 3′ end which is not directly characterized by the *in vitro* reactions discussed here. Possibilities discussed previously include initiation of BMV encapsidation, elongation factor binding, regulation of replication, and other viral RNA interactions (Hall, 1979; Ahlquist *et al.*, 1984a). There is no obvious impairment of M4 encapsidation since the profiles of total viral RNA from protoplasts and encapsidated viral RNA from whole plants are similar (see Figure 3). To distinguish among the above and other possibilities, further experiments combining the *in vitro* analysis of RNA sequences modified at discrete sites and their effects *in vivo* are needed to elucidate the role of the multifunctional 3′ end of BMV RNAs in infection processes.

Materials and methods

Chemicals and enzymes

Restriction enzymes were obtained from BRL, Boehringer-Mannheim, New England Biolabs and Promega-Biotec. DNA polymerase I (large fragment), calf intestinal phosphatase and snake venom phophodiesterase (from *Crotalus durissus*) were from Boehringer, T4 RNA ligase and S1 nuclease was from Pharmacia, T4 DNA ligase from BRL, *Escherichia coli* and SP6 RNA polymerases from Promega-Biotec and RNAse T1 from Sankyo. *E. coli* ATP,CTP:tRNA nucleotidyl transferase was a generous gift of Dr Henryk Cudny.

Transfer of deletions into pB3PM1 and production of transcripts

Because of the lack of convenient unique restriction sites, transferral of deletions into the full-length BMV3 clone pB3PM1 involved sequential steps. Initially, the 870-bp *Sal*−*Eco*RI 3′ terminal fragment of pB3PM1 (Ahlquist and Janda, 1984) was subcloned between the *Sal*I and *Eco*RI sites of M13mp18. The regenerated *Sal*I site, also recognized by *Hin*cII, was then removed by treatment with *Sal*I, S1 nuclease and T4 DNA ligase. The resulting vector had a single *Hin*cII site, and was a convenient recipient for the deletion-bearing BMV segments, which were subcloned as 120-bp *Hin*cII−*Kpn*I fragments from the pSP3HT plasmids (Bujarski *et al.*, 1985). Finally, the unique *Xba*I and *Eco*RI sites were used for transferral of each deletion clone 3′ end fragment into pB3PM1. Capped BMV RNAs from these pB3PM1 derivatives, as well as from clones of each full-length BMV RNA were prepared as described (Ahlquist and Janda, 1984; Ahlquist *et al.*, 1984b).

CTP,ATP:tRNA nucleotidyl transferase assay

^{3}H-Labelled transcripts from selected *Tth*111I-linearized pSP3HT plasmids were prepared as described (Dreher *et al.*, 1984), except that 10 U SP6 RNA polymerase were used per μg of DNA. After phenol extraction and ethanol precipitation, transcripts obtained from 5 μg of plasmid were digested with 0.03 μg of snake venom phosphodiesterase for 15 min at room temperature (Rietveld *et al.*, 1984), and the RNA recovered after phenol extraction by ethanol precipitation. Agarose gel electrophoresis of the recovered RNA revealed that 90% was full-length, hence no major degradation from contaminating endonuclease was encountered. The distribution of 3′ terminal nucleotides in such treated RNA was determined by 3′ end-labeling with [5′-^{32}P]pCp (NEN, 3 × 10^3 Ci/mmol) and RNA ligase (England *et al.*, 1980), followed by hydrolysis with 0.2 M NaOH for 1 h at 70°C and paper electrophoresis (Barrell, 1971). It was found that such phosphodiesterase digestion reduced the content of 3′ terminal A residues by 15−20% in all analysed transcripts; a parallel increase of terminal C residues was observed. Adenylation activity was assayed in reactions containing 40 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, 35 μM CTP and 10 μCi of [α-^{32}P]ATP (Amersham, 410 Ci/mmol), 0.1 U of *E. coli* nucleotidyl transferase and phosphodiesterase-treated RNA generated by transcription from 3 μg of plasmid. The mixture was incubated overnight at 0°C (Rietveld *et al.*, 1984). Products were analysed on 6% polyacrylamide sequencing gels. ^{32}P-containing RNA bands were located by autoradiography and adenylation activities were determined by scintillation counting both ^{3}H and ^{32}P in the excised bands (Dreher *et al.*, 1984).

BMV RNA replication in barley protoplasts

Barley mesophyl protoplasts were isolated from 6-day-old leaves and inoculated with BMV RNA transcripts as described (Loesch-Fries and Hall, 1980). After incubation for 24 h with 50 μCi of [^{3}H]uridine, RNA was isolated from protoplasts by phenol extraction in the presence of SDS and bentonite (Loesch-Fries and Hall, 1980) and analyzed by electrophoresis on 2.4% polyacrylamide−0.5% agarose gels followed by fluorography.

Infection of barley plants with transcripts

Ten primary leaves on 8-day-old barley plants were inoculated with viral transcripts generated from 5 μg of each selected DNA template exactly as described by Ahlquist *et al.* (1984b). After an additional two weeks, BMV was extracted from 10 g of secondary leaves (Lane *et al.*, 1974).

Acknowledgements

We thank Ola Dzianott-Bujarska for excellent technical assistance. This work was supported by N.I.H. grants AIO14666, AI15342 and a Research Career Award (AI21942) to P.K., by N.I.H. grant GM35072 to P.A. and by N.I.H. grants AI11572 and AI22354 to T.C.H.

References

Ahlquist,P., Dasgupta,R. and Kaesberg,P. (1981) *Cell*, **23**, 183−189.
Ahlquist,P., Dasgupta,R. and Kaesberg,P. (1984a) *J. Mol. Biol.*, **172**, 369−383.
Ahlquist,P., French,R., Janda,M. and Loesch-Fries,L.S. (1984b) *Proc. Natl. Acad. Sci. USA*, **81**, 7066−7070.
Ahlquist,P. and Janda,M. (1984) *Mol. Cell Biol.*, **4**, 2876−2882.
Ahlquist,P., Strauss,E.G., Rice,C.M., Strauss,J.H., Haseloff,J. and Zimmern,D. (1985) *J. Virol.*, **53**, 536−542.
Barrell,B.G. (1971) In Cantoni,G.I. and Davies,D.R. (eds), *Procedures in Nucleic Acids Research*. Harper and Row, New York, Vol. **2**, pp. 751−779.
Bujarski,J.J. and Kaesberg,P. (1986) *Nature*, **321**, 528−531.
Bujarski,J.J., Hardy,S.F., Miller,W.A. and Hall,T.C. (1982) *Virology*, **119**, 465−473.
Bujarski,J.J., Dreher,T.W. and Hall,T.C. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5636−5640.
Cornelissen,B.J.C., Brederode,F.T., Moormann,R.J.M. and Bol,J.F. (1984) *Nucleic Acids Res.*, **11**, 2881−2891.
Deutscher,M.P. (1984) In Jacob,S.T. (ed.), *Enzymes of Nucleic Acid Synthesis and Modification: Vol. 2 RNA Enzymes*. CRC Press, Boca Raton, pp. 159−183.
Donis-Keller,H. (1980) *Nucleic Acids Res.*, **8**, 3133−3142.
Dreher,T.W., Bujarski,J.J. and Hall,T.C. (1984) *Nature*, **311**, 171−175.
England,T.E., Bruce,A.G. and Uhlenbeck,O.C. (1980) *Methods Enzymol.*, **65**, 65−74.
French,R.C., Janda,M. and Ahlquist,P. (1986) *Science*, **231**, 1294−1297.
Hall,T.C. (1979) *Int. Rev. Cytol.*, **60**, 1−26.
Haseloff,J., Goelet,P., Zimmern,D., Ahlquist,P., Dasguta,R. and Kaesberg,P. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4358−4362.
Joshi,R.L., Joshi,S., Chapeville,F. and Haenni,A.L. (1983) *EMBO J.*, **2**, 1123−1127.

Kao,T.-H., Miller,D.L., Abo,M. and Ofengand,J. (1983) *J. Mol. Biol.*, **166**, 383–405.
Kiberstis,P.A., Loesch-Fries,L.S. and Hall,T.C. (1981) *Virology*, **112**, 804–808.
Lane,L. (1974) *Advances in Virus Research*, **19**, 151–220.
Lane,L. (1981) In Kurstak,E. (ed.), *Handbook of Plant Virus Infections and Comparative Diagnosis*. Elsevier/North Holland Biomedical Press, pp. 333–376.
Loesch-Fries,L.S. and Hall,T.C. (1980) *J. Gen. Virol.*, **47**, 323–332.
Miller,W.A. and Hall,T.C. (1983) *Virology*, **125**, 236–241.
Miller,W.A., Dreher,T.W. and Hall,T.C. (1985) *Nature*, **313**, 68–70.
Miller,W.A., Bujarski,J.J., Dreher,T.W. and Hall,T.C. (1986) *J. Mol. Biol.*, **187**, 537–546.
Rietveld,K., Pleij,W.A. and Bosch,L. (1983) *EMBO J.*, **2**, 1079–1085.
Rietveld,K., Linschooten,K., Pleij,W.A. and Bosch,L. (1984) *EMBO J.*, **3**, 2613–2619.

Received on 2 May 1986