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Intercistronic As Well As Terminal Sequences Are Required for Efficient Amplification of Brome Mosaic Virus RNA3

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The genome of brome mosaic virus (BMV) is divided among messenger polarity RNA1, RNA2, and RNA3 (3.2, 2.9, and 2.1 kilobases, respectively). *cis*-Acting sequences required for BMV RNA amplification were investigated with RNA3. By using expressible cDNA clones, deletions were constructed throughout RNA3 and tested in barley protoplasts coinoculated with RNA1 and RNA2. In contrast to requirements for 5′- and 3′-terminal noncoding sequences, either of the two RNA3 coding regions can be deleted individually and both can be simultaneously inactivated by N-terminal frameshift mutations without significantly interfering with amplification of RNA3 or production of its subgenomic mRNA. However, simultaneous major deletions in both coding regions greatly attenuate RNA3 accumulation. RNA3 levels can be largely restored by insertion of a heterologous, nonviral sequence in such mutants, suggesting that RNA3 requires physical separation of its terminal domains or a minimum overall size for normal replication or stability. Unexpectedly, deletions in a 150-base segment of the intercistronic noncoding region drastically reduce RNA3 accumulation. This segment contains a sequence element homologous to sequences found near the 5′ ends of BMV RNA1 and RNA2 and in analogous positions in the three genomic RNAs of the related cucumber mosaic virus, suggesting a possible role in plus-strand synthesis.

Brome mosaic virus (BMV) is an isometric plant virus (28) whose 8.2-kilobase genome is divided among three messenger polarity RNAs, designated as genomic RNA1, RNA2, and RNA3 (3, 6). BMV is related by nonstructural protein homologies and common features of RNA replication to several other plant virus groups and to at least one major animal virus group, the alphaviruses (7, 16, 24). During infection by these viruses, efficient RNA-directed amplification of viral but not cellular RNAs occurs. Similar specificity has been observed in vitro with an RNA-dependent RNA polymerase extract from BMV-infected cells. This preparation synthesizes RNA complementary to input BMV plus-strand RNA but not to other viral or cellular RNAs (12, 35). The domain of RNA responsible for this specificity in vitro has been localized to the 3′-proximal 134 bases of the plus-strand template (11, 33). Specific *cis*-acting sequences within the viral template are thus required for at least one and possibly several steps in successful viral RNA amplification. In general, however, the study of such sequences has been hindered by difficulties both in engineering changes in RNA genomes and in propagation of strains with *cis*-acting lethal mutations.

For a small number of RNA viruses, including poliovirus and BMV, complete viral cDNA clones have been constructed which can be used to initiate infection, allowing engineering of the viral RNA genome by recombinant DNA techniques (4, 37). While all three BMV RNAs are required for systemic infection of whole plants, the two largest RNAs (RNA1 and RNA2) direct their own replication in isolated barley protoplasts in the absence of the smallest genomic RNA, RNA3 (18, 26). Because of this convenient division of function, a wide variety of changes can be engineered into RNA3 without inhibiting its replication in BMV-infected protoplasts. This flexibility is sufficient to allow foreign genes inserted in RNA3 to be replicated and efficiently expressed in BMV-derived RNA-based expression vectors (18).

Accordingly, we have used a previously described BMV cDNA expression system (5) to construct partial deletions throughout RNA3 and have tested these deletion derivatives for productive replication and subgenomic mRNA production in barley protoplasts coinoculated with RNA1 and RNA2. The results show that while deletion of either of the two RNA3 coding regions has little or no effect on RNA3 accumulation, deletions overlapping a 150-base subset of the RNA3 central intercistronic region reduce RNA3 accumulation over 100-fold. The amount of 3′ noncoding sequence required in vivo for efficient RNA3 accumulation (over 162 bases) is greater than that required in vitro for minus-strand initiation (11, 33). Moreover, although both genes are individually dispensable, deletion of substantial portions of the 3a gene in combination with total deletion of the coat gene does inhibit RNA3 accumulation. Such inhibition can be relieved, however, by insertion of heterologous sequences.

MATERIALS AND METHODS

BMV cDNA clones. Plasmids pB1PM18, pB2PM25, and pB3PM1 contain cDNAs of BMV RNA1, RNA2, and RNA3 (5). Plasmids pB1TP1, pB2TP3, and pB3TP7 were constructed from the above, respectively, by replacing the modified lambda p_R promoter of each starting clone with a bacteriophage T7 promoter, kindly supplied by J. Dunn (M. Janda, R. French, and P. Ahlquist, Virology, in press). Transcription of these plasmids by T7 RNA polymerase yields RNAs containing one additional 5′ nonviral G residue. Insertion mutant CA81 has the same viral cDNA structure as that of the previously described pB3CA42 (18) but is similarly transcribed from the T7 promoter. Plasmid pB3PM1 (5), which was used as the basis for constructing most of the RNA3 deletion mutants, is 5,670 bases long and has unique *Pst*I and *Eco*RI sites 858 bases 5′ and 3 bases 3′ to the RNA3 cDNA sequences, respectively. Plasmid pB3C49 was con-

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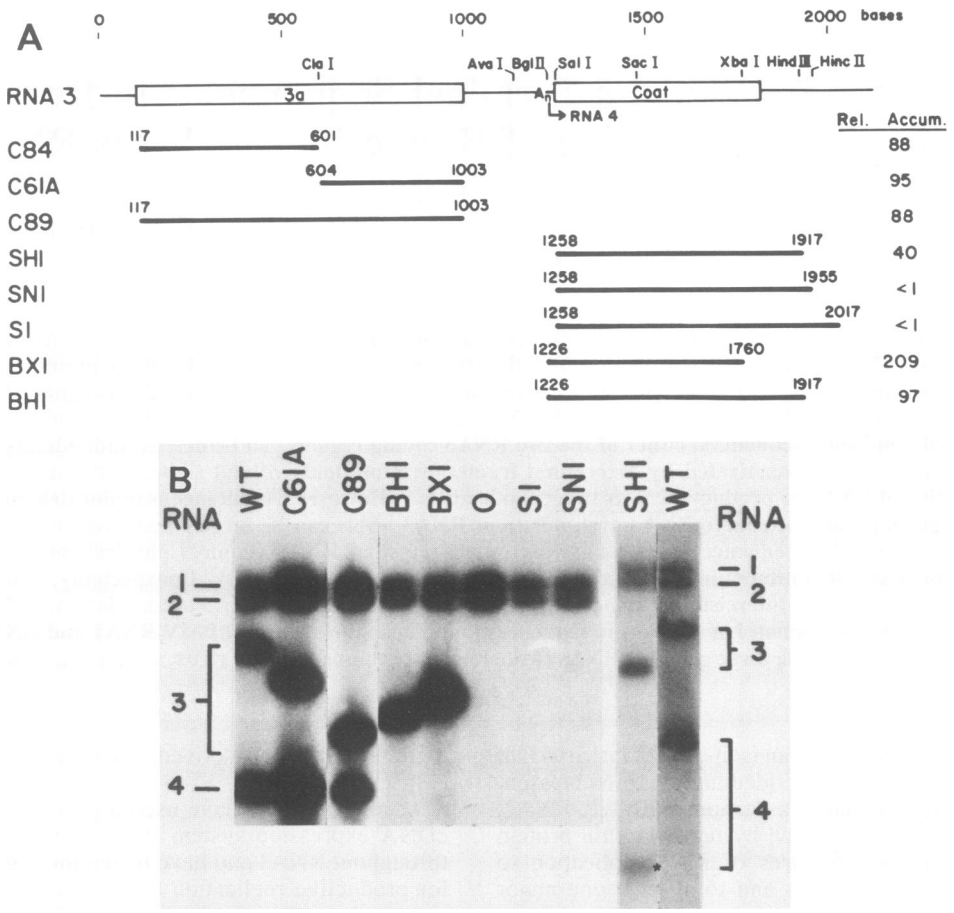

FIG. 1. Deletions in RNA3 coding regions and the 3′ noncoding region. (A) Schematic representation of individual deletions discussed in the text. The extent of deleted sequences for each mutant is represented by a solid line. Positions of the deletion endpoints are given with respect to the RNA3 sequence (6). Numbers to the right of the maps represent the extent to which each mutant RNA3 accumulated during infection relative to the level of RNA1 accumulation, expressed as a percentage of the RNA3/RNA1 ratio for wild type. The top of the diagram is a map of BMV RNA3 with noncoding regions shown as a single line and the 3a protein (bases 92 to 1003) and coat protein (bases 1251 to 1820) cistrons shown as boxed regions. The start of subgenomic RNA4 sequences on RNA3 (base 1242) is shown by a bent arrow, and the internal oligo(rA) sequence (starting at base 1200) is designated as A_n. The locations of relevant restriction sites in RNA3 cDNA are shown above the map. (B) Examples of RNA blot hybridization analysis of BMV RNA progeny from inoculated protoplasts. Barley protoplasts (30) were inoculated with in vitro transcripts from BMV RNA1 and RNA2 cDNA clones (pB1TP3 and pB2TP1) plus the indicated RNA3 deletion mutant clone transcripts. Lanes WT were inoculated with transcripts of the parental wild-type RNA3 cDNA clone (pB3PM1), while lane 0 received no RNA3. After incubation of inoculated protoplasts at 24°C for 20 h, total nucleic acids were extracted and separated on a 1% agarose gel before transfer to a Zetaprobe (Bio-Rad) membrane. The membrane was then probed with ^{32}P-labeled RNA (31) complementary to the 3′-terminal 200 bases of BMV RNA. The positions of wild-type BMV RNA1, RNA2, and RNA4 are indicated at the left, and the bracket encompasses the positions of wild-type RNA3 and the various deletion derivatives. An asterisk has been placed to the right of the small subgenomic RNA4 produced by mutant SH1.

structed by cleaving pB3PM1 with *Cla*I (Fig. 1), repairing the *Cla*I ends with Klenow DNA polymerase, and recircularizing the plasmid with T4 DNA ligase after insertion of a single 12-base *Bam*HI linker (New England BioLabs, Inc.).

Deletion constructions. *Cla*I-linearized pB3PM1 was digested with BAL 31, and the ends were repaired by treatment with T4 DNA polymerase. After ligation to *Bam*HI linker [d(CGCGGATCCGCG); New England BioLabs], a portion of the DNA was digested with *Pst*I and *Bam*HI, and the smaller resulting fragment collection was ligated with the large *Pst*I-*Bam*HI fragment of pB3C49. The remaining BAL 31-digested, linker-ligated DNA was cleaved with *Eco*RI and *Bam*HI, and the smaller fragment was ligated with the large *Eco*RI-*Bam*HI fragment of pB3C49. BAL 31 deletion endpoints were determined by dideoxy sequencing of appropriate M13 subclones (8), and a set of clones, including C61A, C63A, C65A, C67A, and C84 (Fig. 1 and 2), was selected for further study. Mutant BC1 (Fig. 2) was similarly constructed, except that the BAL 31-digested DNA was first subcloned into M13mp18 and sequenced. The small *Eco*RI-*Bam*HI fragment from this M13 subclone was then inserted between the *Eco*RI and *Cla*I sites of pB3TP7 after first repairing the noncompatible *Bam*HI and *Cla*I ends with Klenow DNA polymerase. Deletion C89 was constructed by ligating the small *Pst*I-*Bam*HI fragment of C84 with the large *Pst*I-*Bam*HI fragment of C61A. Mutant C97 was similarly constructed from other *Bam*HI-flanked deletion endpoints in pB3TP7.

Mutants BB1, BB2, and BB3 were constructed by BAL 31 digestion of *Bgl*II-linearized pB3TP7. After repair of the ends with T4 DNA polymerase, the DNA was cleaved at the *Pst*I site 5′ to the inserted BMV cDNA sequences and ligated between the *Pst*I and *Sma*I sites of M13mp19, and the BAL 31 digestion endpoints in the resulting recombinant

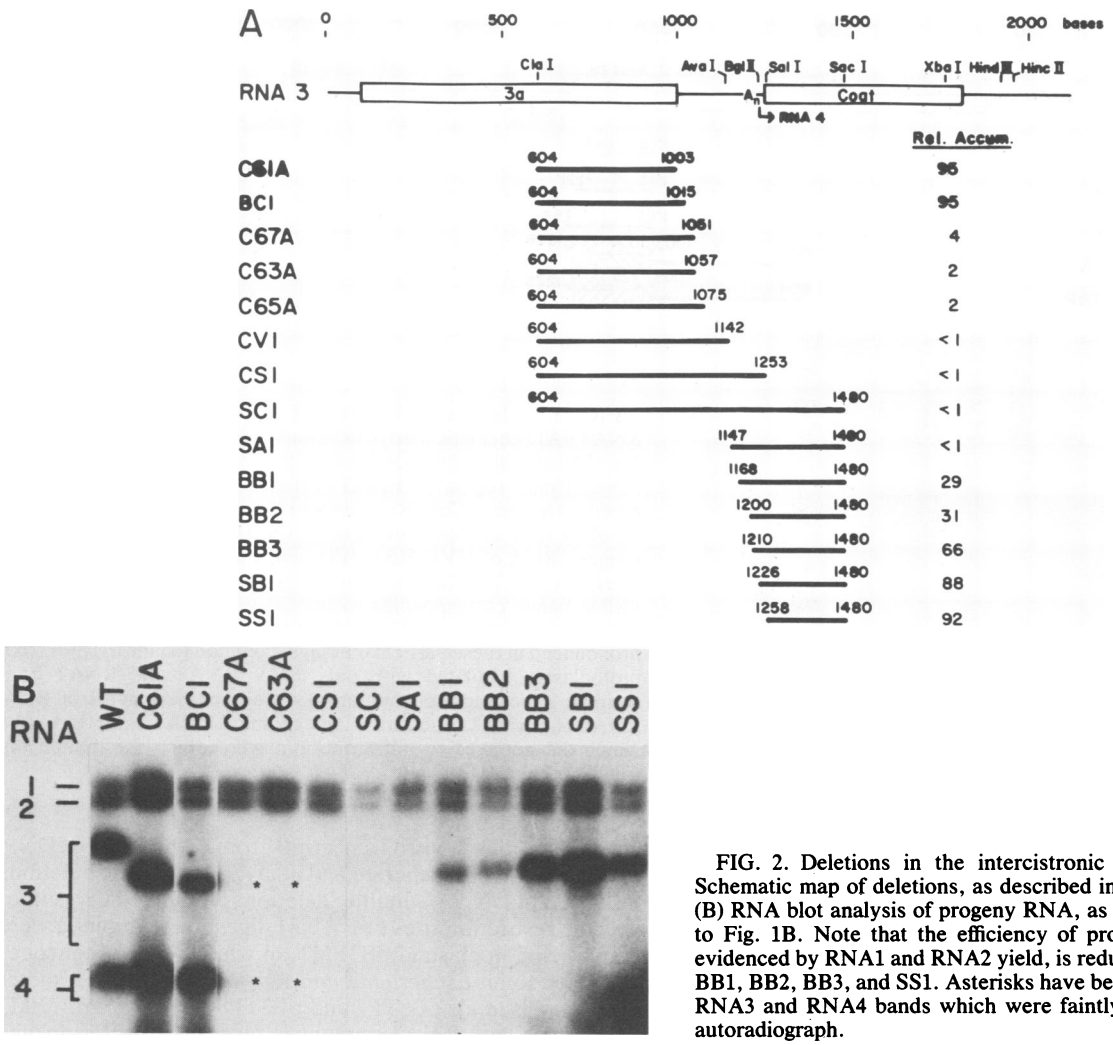

FIG. 2. Deletions in the intercistronic region of RNA3. (A) Schematic map of deletions, as described in the legend to Fig. 1A. (B) RNA blot analysis of progeny RNA, as described in the legend to Fig. 1B. Note that the efficiency of protoplast inoculation, as evidenced by RNA1 and RNA2 yield, is reduced in lanes SC1, SA1, BB1, BB2, BB3, and SS1. Asterisks have been placed to the right of RNA3 and RNA4 bands which were faintly visible in the original autoradiograph.

phage were determined by dideoxy sequencing (8). Selected M13 clones were then digested with *Pst*I and *Sac*I, and the resulting small fragment was ligated with the large *Pst*I-*Sac*I fragment of pB3TP7.

Mutants SH1, SN1, BX1, BH1, CV1, CS1, SC1, SA1, SB1, SS1, HV1, HB1, and HS1 were constructed by deleting regions between restriction sites mapped in Fig. 1A, 2A, and 3A. In each case, plasmid pB3PM1 or pB3TP7 was digested with the appropriate restriction enzymes, the resulting ends were blunted with T4 or Klenow DNA polymerase, and the larger plasmid fragment was recircularized with T4 DNA ligase.

Discontinuous deletions in C94, C95, C96, and C98 were constructed by combining 3a gene deletions with coat gene deletions via the intercistronic *Ava*I site (Fig. 1A and 4A). Mutant C99 (Fig. 4) was similarly constructed from CA81 and C97, and RS9 was constructed by similarly combining two RNA3 single frameshift mutants (kindly provided by R. Sacher), one with the insertion of d(TCGA) after the coat gene initiation codon (RNA3 base 1253) and the other with the substitution of d(GGGATCC) between RNA3 bases 100 and 110 (resulting in a net loss of two bases).

In vitro transcription. RNA transcripts from *Eco*RI-linearized plasmids containing the modified lambda p_R promoter were obtained as previously described (4). Plasmids containing the bacteriophage T7 promoter were linearized with *Eco*RI (*Pst*I for CA81 and C99) and transcribed by T7 RNA polymerase (Bethesda Research Laboratories, Inc.) by using the reaction conditions described by Melton et al. (31), except that the rGTP concentration was reduced to 75 μM, and either cap analog GpppG (Pharmacia, Inc.) or m^7GpppG (New England BioLabs) was added to 500 μM. Yields of full-length transcripts were assessed by agarose electrophoresis of a portion of each transcription reaction.

Protoplast inoculations. Barley protoplasts were prepared as described previously (30). Protoplasts were inoculated with in vitro transcripts of *Eco*RI-linearized BMV cDNA plasmids by a previously described polyethylene glycol procedure (18, 40). For each test, approximately 10^5 protoplasts were inoculated with about 1 μg each of BMV RNA1 and RNA2 transcripts from a common stock plus approximately 1 μg of transcript from the appropriate mutant BMV RNA3 clone. Inoculated protoplasts were incubated for 20 h at 24°C under continuous light. An average of four replicate experiments was done with each mutant.

RNA analysis. Total nucleic acids were extracted from protoplasts 20 h after inoculation as described previously (30) followed by electrophoresis on 1% agarose. The nucleic acids were electrophoretically transferred to Zetaprobe membranes (Bio-Rad Laboratories), and BMV-specific RNAs were detected by hybridization to suitable ^{32}P-labeled probes. The hybridization probe for plus-strand

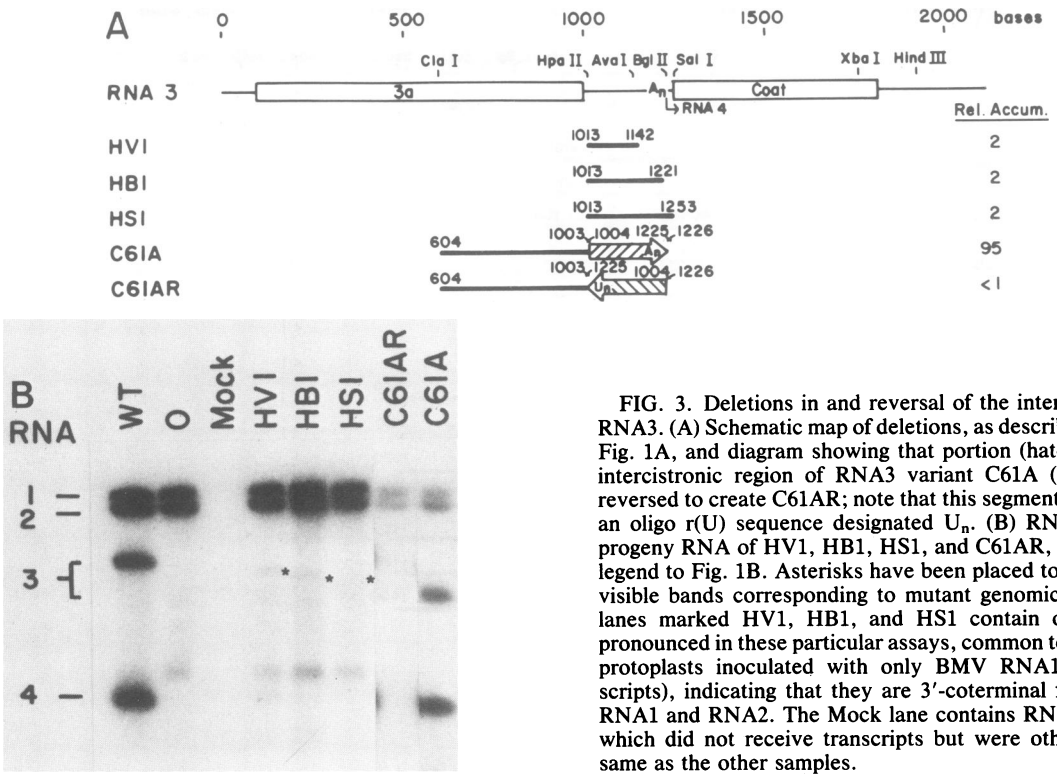

FIG. 3. Deletions in and reversal of the intercistronic region of RNA3. (A) Schematic map of deletions, as described in the legend to Fig. 1A, and diagram showing that portion (hatched arrow) of the intercistronic region of RNA3 variant C61A (Fig. 1A) that was reversed to create C61AR; note that this segment therefore contains an oligo r(U) sequence designated U_n. (B) RNA blot analysis of progeny RNA of HV1, HB1, HS1, and C61AR, as described in the legend to Fig. 1B. Asterisks have been placed to the right of faintly visible bands corresponding to mutant genomic RNAs. Note that lanes marked HV1, HB1, and HS1 contain other bands, more pronounced in these particular assays, common to lane 0 (RNA from protoplasts inoculated with only BMV RNA1 and RNA2 transcripts), indicating that they are 3'-coterminal fragments of BMV RNA1 and RNA2. The Mock lane contains RNA from protoplasts which did not receive transcripts but were otherwise treated the same as the other samples.

BMV RNA1 to RNA4 was an SP6 polymerase in vitro transcript of the 200-base *Hin*dIII-*Eco*RI fragment of 3'-terminal BMV RNA3 cDNA sequences from pB3PM1, cloned in transcription plasmid pGEM2 (Promega). The hybridization probe for minus-strand BMV RNA3 sequences was an SP6 polymerase in vitro transcript from full-length BMV RNA3 cDNA sequences cloned in the *Hin*dIII-*Eco*RI sites of transcription plasmid pSP64 (31). The amount of RNA3 produced during infection was quantitated by densitometry of autoradiographs and normalized for both inoculation efficiency and protoplast viability by determining the ratio of RNA3 production relative to the production of wild-type RNA1 in each infection. As judged by densitometry of known amounts of radioactivity, such quantitation was linear over a 10-fold range of signal strength but may overestimate the amounts of poorly accumulating RNA3 variants (1 to 10% of the wild-type level) resulting from film saturation of RNA1 bands after necessarily longer exposures. Some of the quantitative data obtained by densitometry was confirmed by direct scintillation counting of radioactive bands excised from hybridization filters.

RESULTS

The smallest BMV genomic RNA, RNA3, contains coding regions for both the 3a and coat protein genes, separated by a 250-base intercistronic noncoding region (6) (Fig. 1). The coat gene is translated from a subgenomic RNA4 (28) which is produced by internal initiation of viral transcription on the minus-strand RNA3 template (34). BMV RNA1 and RNA2 are competent for viral RNA replication in the absence of RNA3 (18, 26), suggesting that RNA3 provides no *trans*-acting functions, but only *cis*-acting regulatory sequences, for its own replication. To determine which portions of RNA3 contribute to its productive replication in infected cells, infectious in vitro transcripts from cloned BMV cDNA (4, 5) were used to generate and analyze systematic deletions in RNA3 cDNA. Suitable deletions, bounded by either specific restriction sites or BAL 31 digestion endpoints, were constructed in plasmid pB3PM1, in which RNA3 sequences are fused to an *Escherichia coli* RNA polymerase promoter (5), or in plasmids pB3TP7 and pB3TP8, which are pB3PM1 derivatives with RNA3 sequences fused to a bacteriophage T7 promoter (Janda et al., in press). In vitro transcription of such clones produced deleted versions of RNA3 which were tested for their ability to be replicated in barley protoplasts coinoculated with RNA1 and RNA2 cDNA transcripts. Net viral RNA synthesis in infected cells was assayed after gel electrophoresis by hybridization with an RNA probe complementary to the conserved 3'-terminal 200 bases of BMV genomic and subgenomic RNAs (2). Protoplasts inoculated with only RNA1 and RNA2 transcripts or with all three wild-type genomic transcripts served as controls. Such hybridization assays allowed detection of RNA3 at levels several-hundred-fold lower than that produced during wild-type infection, and the extent of RNA1 and RNA2 replication served as a convenient internal standard to assess the efficiency of each inoculation and the ability of each protoplast sample to support viral RNA replication. Amplification of each RNA3 variant was quantitated by densitometry of autoradiographs, and the results were expressed as percent accumulation relative to wild-type RNA3 after normalization for inoculation efficiency, as measured by the amount of RNA1 produced in the infection.

Deletions in the 3a gene. Deletions of 100 to 500 bases were made in either direction from a central *Cla*I site in the 3a gene and tested for progeny accumulation in protoplasts (Fig. 1). Multiple deletions in the 3a gene were tested, showing that the entire gene or any portion thereof can be deleted with little or no effect on RNA3 accumulation.

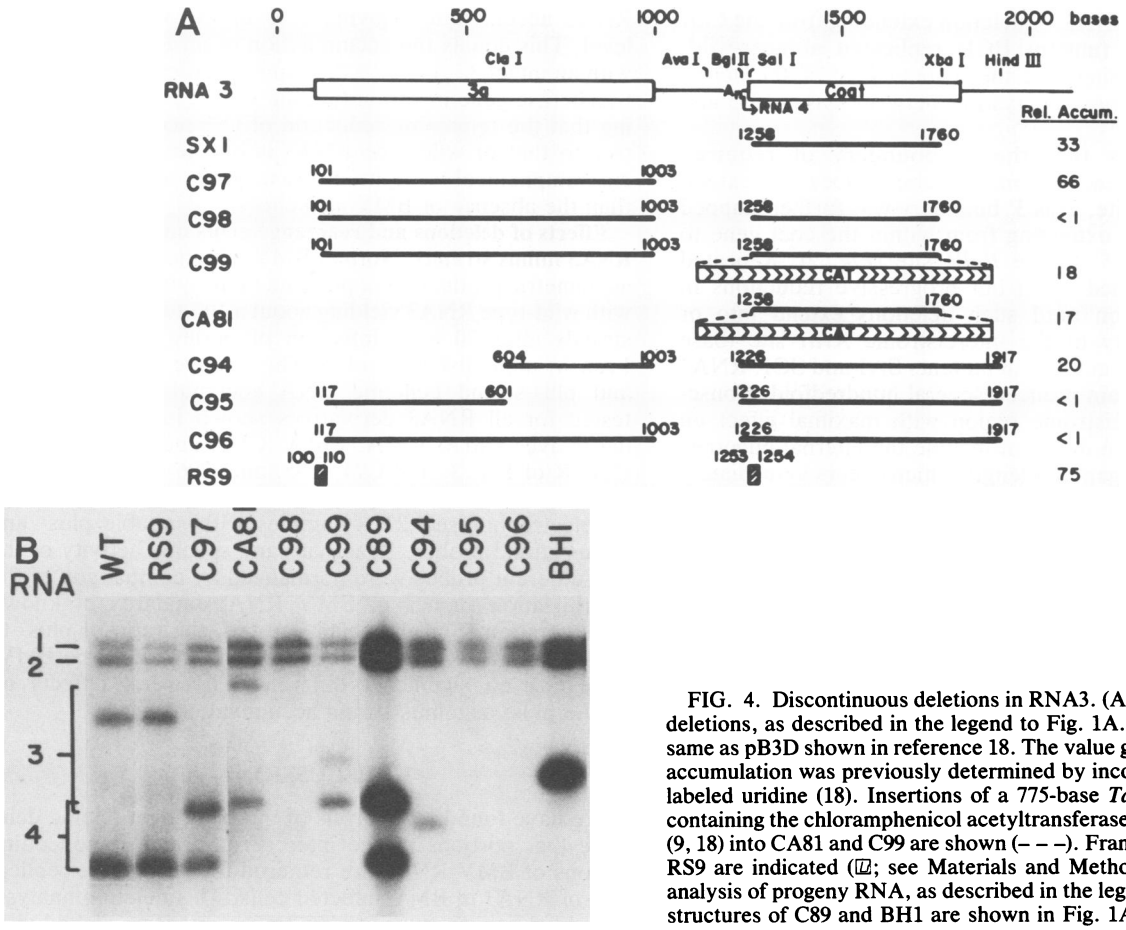

FIG. 4. Discontinuous deletions in RNA3. (A) Schematic map of deletions, as described in the legend to Fig. 1A. Mutant SX1 is the same as pB3D shown in reference 18. The value given for its relative accumulation was previously determined by incorporation of radiolabeled uridine (18). Insertions of a 775-base *Taq*I fragment (▭) containing the chloramphenicol acetyltransferase gene from pBR325 (9, 18) into CA81 and C99 are shown (– – –). Frameshift insertions in RS9 are indicated (◪; see Materials and Methods). (B) RNA blot analysis of progeny RNA, as described in the legend to Fig. 1B. The structures of C89 and BH1 are shown in Fig. 1A.

Deletions from the central *Cla*I site to within 25 bases of the 3a gene 5′ end (variant C84), to the exact 3a gene 3′ end (variant C61A), or between both extremes (variant C89) (Fig. 1A) all directed progeny RNA accumulation nearly as efficiently as did wild-type RNA3 (Fig. 1B). RNA3 replication thus has no specific requirement for either the RNA sequences or protein product of the 3a gene. RNA1 and RNA2 replication is independent of that of RNA3 (18, 26), and we have shown previously that neither RNA3 nor RNA4 synthesis requires coat protein (18). Taken together, these results indicate that synthesis of the four major species of BMV RNA does not require known RNA3 gene products. Preliminary results from a finer-scale deletion mapping of sequences extending into the 5′ noncoding region show that RNA3 accumulation is unaffected until deletions enter the noncoding region itself (bases 1 to 91), with a decline in replicative ability of the mutants as the deletion endpoint moves closer to the 5′ end (J. Bujarski and P. Ahlquist, unpublished results).

Deletions in the coat gene and 3′ noncoding region. We have demonstrated previously that deletion of a major segment of the coat gene, extending from the *Sal*I to *Xba*I sites (Fig. 1), has only a moderate (three-fold) effect on either RNA3 or RNA4 production during infection (18). In this study, additional deletions extending further in both directions were made and tested in barley cells. Three of these (SH1, SN1, and S1; Fig. 1) have increasing amounts of the 3′ noncoding sequence removed, retaining 200, 162, and 100 bases, respectively, of the 3′ terminus. Of these, SH1 progeny accumulated to 40% of the wild-type level, SN1 progeny accumulated to levels several-hundred-fold lower than those of the wild-type RNA3, and S1 progeny were undetectable (Fig. 1). Similar to previously tested deletions within the coat gene (18), SH1 also generated a small subgenomic RNA with a predicted length of only 216 bases. Extending the 5′ boundary of coat gene deletions from the *Sal*I site just within the coat gene to the *Bgl*II site 20 bases 5′ of the subgenomic mRNA initiation site, as in variants BX1 and BH1 (Fig. 1), had two notable and possibly related effects. Presumably because the subgenomic initiation site was removed, no detectable subgenomic RNA was produced. In addition, genomic RNA3 accumulation for *Bgl*II-bounded deletions was severalfold above that for the analogous *Sal*I-bounded deletions, such as SH1.

Deletion of the intercistronic region seriously inhibits RNA3 replication. Although the results above show that some sequences of the RNA3 intercistronic region may be deleted without affecting RNA3 production, any of a variety of deletions into or across the intercistronic region dramatically reduces RNA3 accumulation by up to several hundredfold. This effect is illustrated in Fig. 2 by, e.g., variants C67A, CV1, SC1, and SA1. Progressive deletions from either side of the intercistronic region were used to localize the area responsible for this behavior (Fig. 2). When deletions extended from 48 to 140 bases into the 5′ side of the intercistronic region, RNA3 accumulation was progressively reduced. In each case, the level of RNA4 was also reduced so that the molar ratio of RNA4/RNA3 remained near the 1:1

ratio of wild-type RNA3. A deletion extending from the *Cla*I site to base 1015 (mutant BC1) replicated at near-wild-type levels, indicating that the 5′ boundary of the active region defined by these assays lies between bases 1015 and 1051.

Figure 1 shows that the 3′ boundary of required intercistronic sequences identified above does not extend beyond the *Bgl*II site. This 3′ boundary was further mapped by using deletions extending from within the coat gene to various endpoints 5′ to the *Bgl*II site (Fig. 2). Repeated experiments revealed small but progressive reductions in RNA3 accumulation until such deletions extend into or beyond the vicinity of the intercistronic *Ava*I site (base 1147). When this occurs, as in mutants SA1 and SC1, RNA3 accumulation is again reduced several hundredfold. Consequently, the intercistronic region with maximal effect on RNA3 accumulation does not include the internal oligo(rA) sequence of heterogeneous length which occurs immediately 5′ to the *Bgl*II site (6).

While these results indicate that a subset of the intercistronic region contributes to RNA3 accumulation, all deletions in Fig. 2 have at least one endpoint well inside adjoining coding regions. To confirm that the effect was entirely due to deletion of intercistronic sequences, three additional mutants with smaller deletions were constructed (Fig. 3). Two of these, HV1 and HB1, have only intercistronic sequences deleted, and the deletion in HS1 extends only to the beginning of the coat cistron. Even the smallest deletion in this region (HV1, 130 bases deleted) reduced the level of RNA3 to nearly the same extent as did the larger deletions, confirming that intercistronic sequences upstream of the oligo(rA) sequence are important for RNA3 accumulation (Fig. 3). In addition, a 221-base intercistronic segment encompassing the entire region implicated above was inverted in the strongly replicating variant C61A to generate C61AR (Fig. 3). Similar to deletion of the entire intercistronic region, accumulation of not only plus (Fig. 3) but also minus RNA strands (see below) was severely inhibited with C61AR compared with C61A, showing that this intercistronic segment functions only in appropriate orientation relative to other elements of BMV RNA3.

Simultaneous deletions in the 3a and coat genes. As noted above, nearly complete or complete deletion of either the 3a or coat gene had only small effects on the production of RNA3 or RNA4 (Fig. 1 and 4). However, when major deletions in both coding regions occur simultaneously in the same molecule (e.g., mutants C95, C96, and C98; Fig. 4), RNA3 levels were again reduced over 100-fold. Among tested mutants with deletions in both genes, only C94, which retains the 5′ half of the 3a gene and has the least total amount of sequence removed, accumulated to readily visible amounts but was still depressed compared with wild-type RNA3. A possible but unlikely explanation for these results was that expression of either the coat protein or the N-terminal domain of the 3a protein, although not both, was required for efficient RNA3 amplification. To test this, an RNA3 derivative (mutant RS9) with frameshift mutations just within the 5′ boundaries of both the 3a and coat cistrons was constructed (Fig. 4). This mutant both replicated and generated subgenomic RNA at levels comparable to those of wild-type RNA3, confirming that neither RNA3 nor RNA4 synthesis depends on the known RNA3 gene products.

Mutant C98 was further modified by inserting the bacterial chloramphenicol acetyltransferase gene sequences (9) in place of the deleted coat gene (mutant C99; Fig. 4). Despite its heterologous nature, this insertion dramatically improved RNA3 accumulation, giving 17% of the wild-type RNA3 level. This equals the accumulation of an RNA3 derivative with an intact 3a gene and the same chloramphenicol acetyltransferase gene insertion (mutant CA81; Fig. 4B), suggesting that the remaining reduction of C99 accumulation relative to that of wild-type RNA3 is due to the presence of chloramphenicol acetyltransferase gene sequences rather than the absence of BMV sequences.

Effects of deletions and rearrangements on accumulation of RNA3 minus strands. Normal BMV infections induce highly asymmetric production of plus and minus viral RNA strands, with wild-type RNA3 yielding about a 100-fold excess of plus strands after 20 h of infection of barley protoplasts (R. French, unpublished results). The relative levels of minus- and plus-strand genomic RNA accumulation were also tested for all RNA3 derivatives shown in Fig. 1 and for derivatives C61A, C63A, C65A, CV1, and CS1 of Fig. 2; C61AR of Fig. 3; and C94, C95, and C96 of Fig. 4. These levels were tested by probing parallel blots of the same gel-electrophoresed RNA samples with suitable plus- and minus-strand probes. Reactivity and specific activity of the two different probes were normalized by comparison of the hybridization signals of BMV RNA standards of known concentration. For all variants tested, the ratio of plus- to minus-strand RNA3 was similar to that of wild-type BMV, indicating no significant difference in overall effects on RNA3 plus- or minus-strand accumulation.

DISCUSSION

We have found by analysis of multiple, overlapping deletions that portions of the intercistronic and 3′ noncoding regions of BMV RNA3 are required for productive replication of RNA3 in BMV-infected cells. This deletion analysis depended on infectious in vitro transcripts from cloned viral cDNA (4) to allow construction of RNA deletion mutants through standard recombinant DNA techniques. In addition, high-efficiency protoplast inoculation methods (40) routinely infected 50 to 100% of the treated protoplasts, allowing viral RNA accumulation to be followed in initially infected cells, without the need for secondary infection cycles. The amplification assay was thus independent of RNA packaging requirements or other factors which might be involved in intercell spread. The behavior of each RNA3 mutant was confirmed by replicate tests, and moreover, the importance of each region of RNA3 for productive RNA accumulation was determined by not just one, but a series of constructs. Similar experiments have also demonstrated a requirement for 5′-terminal noncoding sequences in RNA3 amplification (J. Bujarski and P. Ahlquist, unpublished results).

Levis et al. (29) recently reported a deletion study with a defective interfering RNA of the Sindbis virus, an animal virus whose nonstructural proteins show considerable amino acid homology with those of BMV and which is believed to also share fundamental similarities in RNA replication mechanism with BMV (7). That study, which did not distinguish between replication and packaging constraints, found specific requirements for 5′- and 3′-proximal sequences for successful defective interfering RNA amplification (29). However, unlike BMV RNA3, no requirement for specific internal sequences was noted.

The experiments described above do not define the mechanistic roles of the sequence segments implicated in BMV RNA3 amplification. Since the known coding regions of RNA3 may be disrupted by deletions and frameshift mutations without significant impact on RNA3 accumulation,

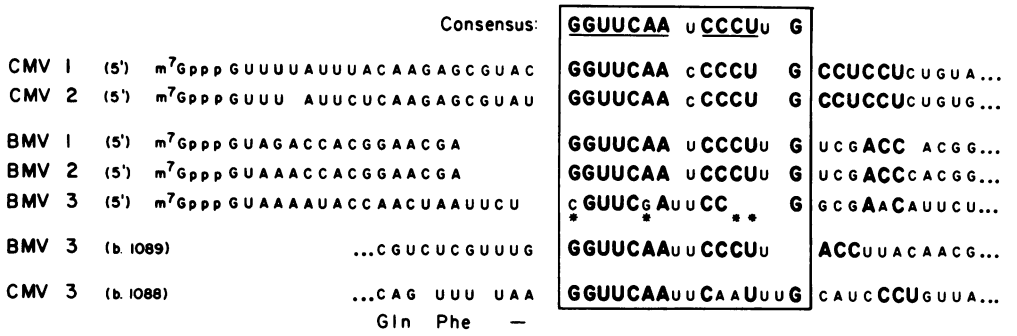

FIG. 5. Homologous sequence elements found in RNAs of BMV and CMV. The source of each sequence is shown at the left, with its starting position shown in parentheses. Relevant portions of BMV RNA1 and RNA2 (3), BMV RNA3 (6), CMV RNA1 (39), CMV RNA2 (38), and CMV RNA3 (20) sequences are positioned to align their common homology (boxed) with the consensus sequence at the top. Gaps have been introduced into some sequences to align intraviral homologies among adjacent sequences and also to separate the terminal codons of the CMV 3a gene. Within the box, bases which are conserved among the originally defined members of this set (thus excluding the 5' end of BMV RNA3) are written in boldface type. Asterisks indicate mismatches between the 5' BMV RNA3 sequence and nucleotides conserved at the homologous sites in BMV RNA1 and RNA2 and the intercistronic region of BMV RNA3. Intravirally conserved triplets, CCU in CMV and ACC in BMV, after the intervirally homologous boxed region are also shown in boldface type.

however, the observed effects appear to be mediated by the RNA itself and not by RNA3-encoded protein products. Possibilities include action of the required sequences in *cis* either as recognition sites for *trans*-acting replication factors or as regulatory elements. In addition, the stability of many cellular RNAs depends on internal sequence elements (19, 27, 36), and sequences required for RNA3 accumulation might similarly influence RNA stability after replication. It is also conceivable that portions of the viral RNA, perhaps assisted by other viral or host factors, might directly participate in catalyzing a reaction step such as initiation of RNA synthesis (15, 21, 41). These alternative roles are not mutually exclusive, and some specific possibilities are considered below.

In contrast to the three specifically required regions within the noncoding sequences, either of two large blocks on RNA3, each encompassing one of the two coding regions, can be removed without major reduction of RNA3 accumulation. Sequences of the coat gene can also be replaced with nonviral coding sequences (18), and similar insertions may be made in the 3a gene (R. French and P. Ahlquist, unpublished results). However, despite the individual dispensability of these regions, simultaneous large deletions in both genes drastically reduced RNA3 accumulation (Fig. 4). This could reflect a minimum length limit for normal RNA3 accumulation, perhaps owing to a need for spacing or flexibility between particular domains of the RNA. Consistent with this view, the insertion of a segment of foreign sequence, which evolved in a procaryotic DNA environment and is itself somewhat detrimental to RNA3 accumulation, substantially restores the replicative ability of the double-deletion mutant C98.

Requirements for 3'-terminal noncoding sequences. The last 134 bases of RNA3 are necessary and sufficient in vitro for effective template recognition and initiation of minus-strand synthesis on plus-strand RNA templates by BMV RNA polymerase preparations (1, 11, 33) and for interaction of BMV RNA with 3' tRNA nucleotidyl transferase and aminoacyl-tRNA synthetase (10, 25). These latter two enzymes covalently modify the 3'-OH group of the viral RNA, which could confer resistance to exonuclease or mediate regulation of viral RNA function by interaction with host factors or other means (3, 17, 22, 23). As shown above, however, a larger 3'-terminal sequence of 163 to 200 bases is required for normal RNA3 accumulation in vivo. This is consistent with the nearly exact conservation of the 3'-terminal 193 bases in all BMV RNAs (2). It remains unclear whether the additional sequence required in vivo contributes to an unidentified function(s) of the 3' end or modulates the activity of known functions.

Although deletions extending within 162 bases or less of the 3' end inhibit RNA3 accumulation, not all of the last 162 bases are required for RNA3 accumulation. Deletion of bases 81 to 100 from the 3' end gives an RNA3 mutant which is viable (10), although at a selective disadvantage compared with wild-type RNA3 (13). Similarly, other sequences conserved among the BMV genomic RNAs 5' to the last 200 bases, including at least one additional hairpin stem and loop (2, 3), are not required for efficient RNA3 accumulation (Fig. 1). Conserved sequences 5' to the last 200 bases, and possibly sequences within the last 200 bases, may thus have functions not related directly to RNA production. The most obvious possibility is the provision of signals for viral RNA encapsidation, analogous to the assembly origin of tobacco mosaic virus RNA (14).

Requirement for intercistronic sequences. An unexpected result of this study was the observation that an approximately 150-base region within the intercistronic sequence is required in its natural orientation for efficient amplification of BMV RNA3 in an infection. Deletion analysis did not reveal a sharp 3' boundary to this region, but rather a gradual decrease in RNA3 accumulation as deletions enter the 3'-proximal portion of the intercistronic region (Fig. 2). One possible explanation is that the function of this region is dependent on a higher-order RNA structure whose stability is gradually eroded by progressive deletions.

The requirement for intercistronic sequences in productive replication of BMV RNA3 suggests that sequences of similar function, and possibly similar primary sequence, might exist in BMV RNA1 and RNA2 (3). As noted by Rezaian et al. (39), a 12- to 14-base element is conserved between the required intercistronic sequence of BMV RNA3 and the 5' noncoding sequences of BMV RNA1 and RNA2, and closely homologous sequences exist in the analogous regions of RNA1, -2, and -3 of the related cucumber mosaic virus (CMV). In all six RNAs (Fig. 5), an invariant GGUUCAA occurs, followed by a run of pyrimidines which usually contains CCCU and is usually followed by a G. In

addition, we note that a sequence with a weaker homology to this consensus, violating two of the seven consecutive residues invariant in the other copies, exists at the 5′ end of BMV but not CMV RNA3 (Fig. 5). Except for the occurrence of the subset GGUUCAA (not followed by a run of pyrimidines) at base 355 of BMV RNA2, we have not detected recognizable homology with these elements at any other sites in either strand of the BMV or CMV genomes.

The requirement for the intercistronic region in efficient RNA3 amplification must relate to either RNA3 replication or stability. The required intercistronic region is not present in RNA4, suggesting that it is not necessary for BMV RNA stability. If this segment contributes to RNA3 replication, the independent competence of 3′-terminal sequences in minus-strand initiation in vitro (1, 33) suggests that its role may be in RNA3 plus-strand initiation. The internal location of this segment in RNA3 and its minus strands does not preclude such a role, since a precedent for an internal viral polymerase recognition site, separated from its functional initiation site, exists in the single-stranded RNA bacteriophage Qβ (32). Participation of the intercistronic region in RNA3 plus-strand synthesis might involve the consensus element shown in Fig. 5, whose conservation at the 5′ ends of BMV and CMV RNA1 and RNA2 is consistent with a role in initiation of plus-strand RNA synthesis. However, the possible relation between the conserved element shown in Fig. 5 and the observed requirement for the intercistronic sequence in RNA3 amplification presently remains uncertain. Preliminary results show that this element is not required in close linkage to the RNA4 initiation site to direct subgenomic RNA synthesis, another function of the intercistronic region (R. French, unpublished results).

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