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De Novo Generation of Defective Interfering-like RNAs in Broad Bean Mottle Bromovirus

JUDIT POGANY,* JAVIER ROMERO,† QI HUANG,*,[1] JEAN-YVES SGRO,‡
HONGZHONG SHANG,*,[1] and JOZEF J. BUJARSKI*,[2]

*Plant Molecular Biology Center and the Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois 60115; †CIT-INIA, Department of Plant Protection, 28080 Madrid, Spain; ‡Institute for Molecular Virology, University of Wisconsin, Madison, Wisconsin 53706

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Broad bean mottle virus (BBMV) is the only member of the bromoviruses that is known to accumulate defective-interfering (DI) RNAs (Romero *et al., Virology* 194, 576–584, 1993). *De novo* generation of DI-like RNAs was demonstrated during serial passages of BBMV in broad bean using either DI RNA-free virion RNA preparations or transcribed genomic RNA inocula. As for previously described DI RNAs, all but one of the characterized *de novo* generated DI-like RNAs were derived by a single in-frame deletion from the RNA2 component. The sole exception was derived by two shorter in-frame deletions from RNA2. The maintenance of an open reading frame by all DI-like RNAs suggests the importance of coding capacity and/or the shortened 2a protein in the accumulation of these RNAs during infection. The deletion junction sites were between nucleotides 1152 and 2366, suggesting that the retained regions are essential for the efficient accumulation of BBMV DI-like RNAs *in planta*. Short regions of sequence similarity and/or complementarity were revealed at the 5′ and 3′ junction borders. We speculate that these regions can facilitate DI (DI-like) RNA formation. In addition to DI-like RNAs, the full-length nucleotide sequences of RNA2 components of the Type and Morocco strains of BBMV are presented.

INTRODUCTION

Defective (D) or defective-interfering (DI) RNAs are deletion and/or rearrangement mutants of the viral genome which are unable to replicate in the absence of the helper virus (Holland, 1991). The DI RNAs that have been found in association with plant virus infections are basically of two types. In one type, the DI RNAs consist of a mosaic of the parental viral RNA genome. Such DI RNAs were first found in tomato bushy stunt tombusvirus (TBSV) (Hillman *et al.*, 1987) and thereafter in cymbidium ringspot (CymRSV) (Burgyan *et al.*, 1989) and cucumber necrosis (CNV) (Finnen and Rochon, 1993) tombusviruses, as well as in the closely related turnip crinkle virus carmovirus (TCV) (Li *et al.*, 1989). Another type of defective RNAs, i.e., with single internal deletions, was identified for clover yellow mosaic potexvirus (CYMV) (White *et al.*, 1991), tomato spotted wilt tospovirus (TSWV) (Resende *et al.*, 1991), and in three furoviruses: soil-borne wheat mosaic virus (SBWMV) (Chen *et al.*, 1994), beet necrotic yellow vein virus (Bouzoubaa *et al.*, 1991), and peanut clump virus (Manohar *et al.*, 1993). DI RNAs associated with plant viruses can exert various effects on plant disease symptoms, causing reduction of symptoms (e.g., CymRSV on *Nicotiana clevelandii*) (Burgyan *et al.*, 1991), no effects on symptoms (e.g., CYMV on broad bean) (White *et al.*, 1991), or mild to profound intensification of disease severity, e.g., TCV on turnip plants (Li *et al.*, 1989) and SBWMV on wheat plants (Chen *et al.*, 1994).

De novo generation of DI RNAs has been demonstrated in the laboratory for TBSV (Knorr *et al.*, 1991), CymRSV (Burgyan *et al.*, 1991), TCV (Li *et al.*, 1989), SBWMV (Bouzoubaa *et al.*, 1991), and TSWV (Resende *et al.*, 1991) during serial passages at high multiplicity of infection (m.o.i.). For SBWMV, the RNA2 component undergoes frequent spontaneous deletions *in vivo* when mechanically transmitted. Depending on the time of the year, the isolates of SBWMV show a range of RNA2 sizes between that of wild type and a stable defective form (Chen *et al.*, 1994). In contrast to the above viruses, a mutant of CNV that did not express the 20-kDa nonstructural protein produced high levels of DI RNAs without serial passages in *N. clevelandii* plants (Rochon, 1991). The DI RNAs of CNV did not require coat protein for efficient accumulation and spread *in planta* (Rochon *et al.*, 1994). For CYMV, attempts to generate *de novo* D RNAs by 11 passages at high m.o.i. were unsuccessful in broad bean (White *et al.*, 1991).

Different mechanisms have been postulated for the formation of DI (D) RNAs in different viral systems. In particular, the presence of complementary sequences at the deletion sites was previously described by us in two preexisting (naturally occurring) broad bean mottle virus (BBMV) DI RNAs (Romero *et al.*, 1993). We suggested that the resulting double-stranded structures can bring

[1] Present address: Department of Plant Pathology, University of Wisconsin, Madison, WI 53706.

[2] To whom correspondence and reprint requests should be addressed.

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the remote parts of the RNA template together, permitting the viral replicase to loop out the region in between. In contrast, sequence similarities were found at the deletion sites in TBSV DI RNAs (White and Morris, 1994). The authors suggested that this facilitates the selection of deletion sites through base pairing between the incomplete nascent strand and the acceptor RNA template. Short sequence similarities were also observed at the deletion junctions of the engineered TMV constructs that spontaneously deleted their duplicated sequences during infection (Raffo and Dawson, 1991). For SBWMV, an analysis of 32 independent viral isolates obtained at different stages after serial mechanical transfers revealed a conserved region for the upstream deletion borders, while the downstream borders were much more dispersed (Chen *et al.*, 1994). Most deletion sites in SBWMV DI RNAs were located in the unpaired part of stem–loop structures, indicating that the deletion may occur during synthesis of plus RNA strands.

BBMV is a member of the bromovirus group (Lane, 1981) which has a tripartite, positive-sense single-stranded RNA genome. BBMV RNA1 and RNA2 components encode for two putative replicase proteins, 1a and 2a, respectively, while BBMV RNA3 encodes for putative movement protein and for coat protein (Dzianott and Bujarski, 1991; Romero *et al.*, 1992). We have reported the characterization of internal deletion-type DI RNAs in Morocco and Tu strains of BBMV (Romero *et al.*, 1993). These DI RNAs exacerbated the severity of disease symptoms on pea. They were derived by single in-frame deletions in the RNA2 segment. The translational activity of the DI RNAs was confirmed by *in vitro* assays and by an analysis of the polyribosomal RNA fractions (Romero *et al.*, 1993). To date, the RNA2-derived BBMV DI RNAs are the only DI RNAs described in bromovirus infections.

In order to gain more insight into the mechanism of generation of defective RNAs, BBMV DI-like RNAs were induced in broad bean plants by serial passages at high m.o.i. Here we report the molecular characterization of 13 (11 *de novo* generated and 2 preexisting) DI-like (DI) RNAs. Analysis of the nucleotide sequences near junction sites suggests potential mechanisms which may operate to generate BBMV DI-like (DI) RNAs.

MATERIALS AND METHODS

Virus strains, serial passages, and RNA extraction

The Type strain of BBMV was acquired from P. Kaesberg (Institute for Molecular Virology, Madison, WI), whereas the Morocco (Mo) and Sudan (Su) strains were obtained from K. M. Makkouk (ICARDA, Syria). BBMV strains were maintained in broad bean (*Vicia faba* cv. Windsor).

A DI RNA-free culture of BBMV was isolated by a passage from broad bean to bean and back to broad bean, as described by Romero *et al.* (1993). These isolates were determined to be free of DI RNAs by Northern blot hybridization and reverse transcriptase–polymerase chain reactions (RT–PCR). Another source of DI-free infectious BBMV RNAs was those synthesized *in vitro* from full-length cDNA clones, using T7 RNA polymerase (BRL, Gaithersburg, MD) as described previously (Pogany *et al.*, 1994). Six-day-old broad bean seedlings (at the three to four-leaf stage) were inoculated mechanically with a mixture of three transcribed genomic BBMV RNAs in a buffer containing 10 m*M* Tris, pH 7.4, 1 m*M* EDTA, 0.2% Bentonite, and 0.2% Cellite. Serial passage experiments were initiated on broad bean using either BBMV RNAs extracted from virion preparations after passaging through bean plants or *in vitro* transcribed BBMV RNAs. Systemically infected, upper leaves from plants inoculated 2 weeks earlier were harvested and ground in the presence of 1% Cellite and the homogenate was used to inoculate new broad bean seedlings.

For RNA analysis, the upper, noninoculated broad bean leaves were harvested 2 weeks after inoculation. Total RNAs were isolated from the tissue according to White and Kaper (1989). Virions were isolated as described by Lane (1986). Virion RNA was prepared by a proteinase K/SDS/phenol procedure, according to Romero *et al.* (1993).

Construction of full-length cDNA clones of DI-like RNAs

Full-length transcribable cDNA clones of DI-like RNAs were obtained by RT–PCR according to Romero *et al.* (1993). The following deoxyoligonucleotide primer (synthesized in an Applied Biochemicals DNA synthesizer) was used to initiate first-strand cDNA synthesis: 5′CGCGGATCCTGGTCTCCCCTAAGAG3′ (primer 223), complementary to nucleotides 2906 to 2921 of RNA2 (see Fig. 1) and containing a *Bam*HI restriction site (underlined). Second-strand synthesis primer contained upstream T7 RNA polymerase promoter sequence (underlined) and downstream RNA2-specific sequence: 5′ATTAATACGACTCACTATAGATAAGTGTAGAATCGAGG3′ (primer 319), representing nucleotide positions 1 to 18 of Fig. 1. The first-strand cDNA was synthesized with maloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD) as described by Romero *et al.* (1993), except that RNAs were not denatured with methyl mercuric hydroxide. One microliter from the first-strand reaction was used directly for the PCR amplification (using VENT DNA polymerase, New England Biolabs) reaction in 25 thermocycles (92°, 1 min; 57°, 2 min; 72°, 3 min), performed in a Perkin–Elmer thermocycler Model 480. The reaction mixture was as described by Romero *et al.* (1993). The full-length cDNA products were purified by electrophoresis in a low-melting-point agarose gel. DNA was recovered from the gel by incubation at 65° for 10 min followed by SDS/phenol/chloroform extraction, as described by Sambrook *et al.* (1989). After digestion with

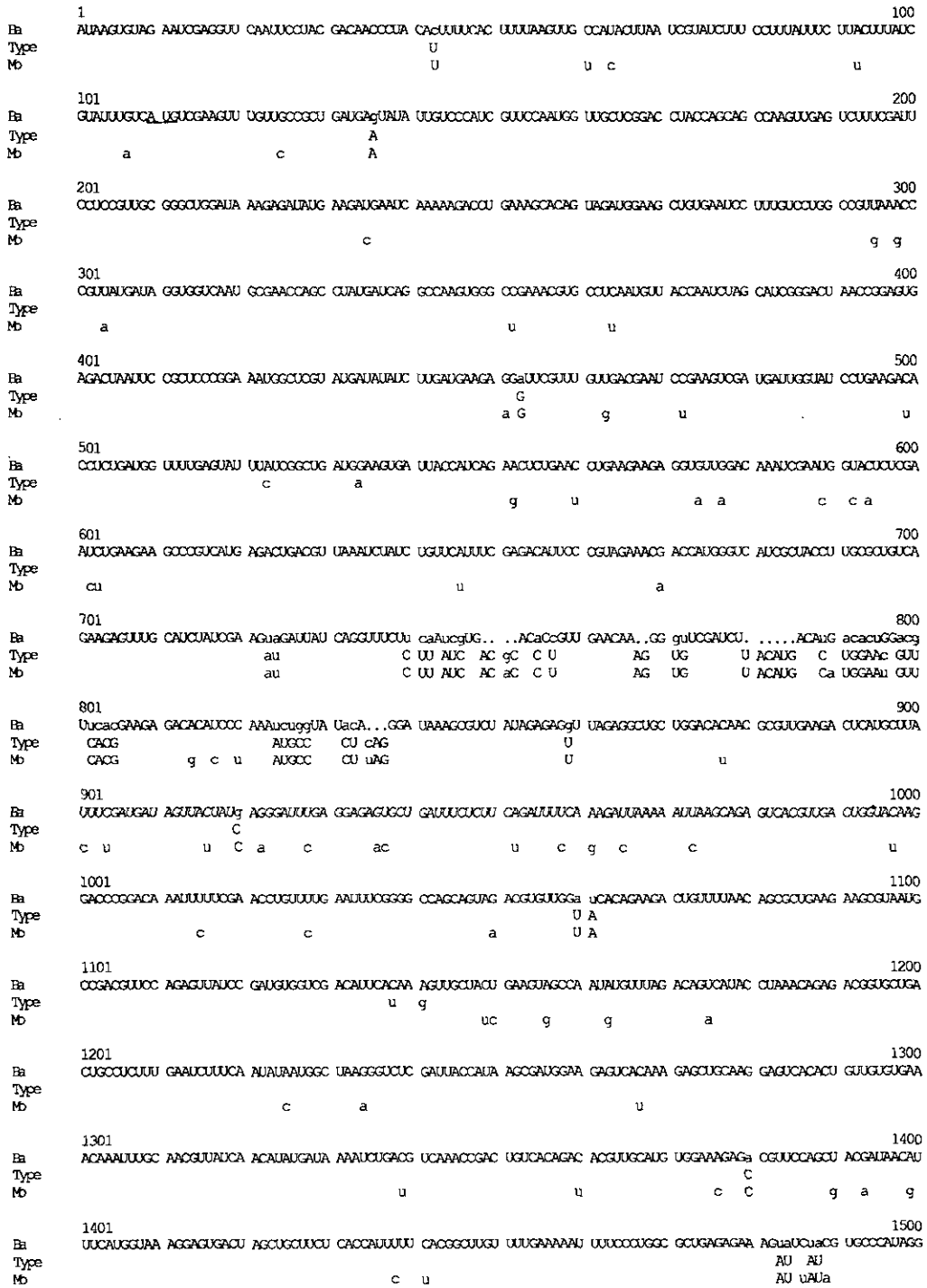


FIG. 1. The complete nucleotide sequences of BBMV RNA2 of Type and Mo strains (second and third lines), as compared to each other and to previously determined (Romero *et al.*, 1992) RNA2 sequence of Ba isolate (first line), using the program PILEUP. The first line shows the complete sequence, whereas the second and third lines display only the differences. The Type and Mo sequences were obtained from single full-length transcribable cDNA clones (described by Pogany *et al.*, 1994) on both cDNA strands using a set of synthetic oligonucleotide primers. The uppercase letters mark nucleotides common between at least two RNAs. The initiation and termination codons are underlined. The 84-nt imperfect repeat in Mo RNA2 3′ noncoding region is indicated with italics. GenBank Accession Nos. for Type and Mo RNA2 sequences are U24495 and U24496, respectively. Dots indicate nucleotides that are not present in other strains.

*Bam*HI the cDNA was ligated into a pUC19 vector between *Bam*HI and *Sma*I sites. In order to confirm that no RNA1 or RNA3-derived defective molecules were present in the virion RNA preparations, RT–PCR reactions were also performed using RNA1- and RNA3-specific primers, as described previously (Pogany *et al.*, 1994).

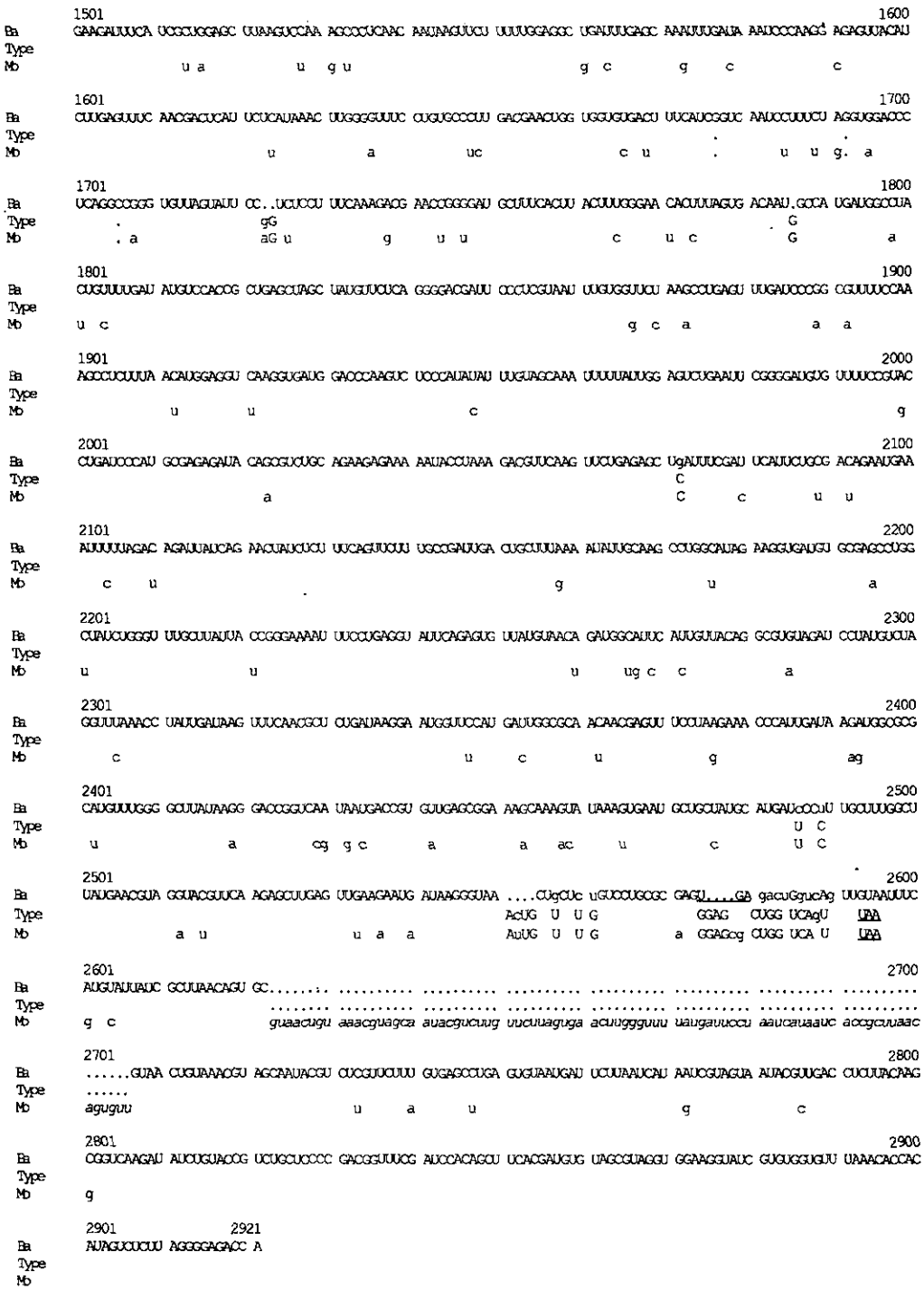


FIG. 1—*Continued*

In vitro transcription

The plasmids containing the *Bam*HI-linearized cDNA clones (10 μg) of DI-like RNAs were transcribed *in vitro* according to a procedure described by Janda *et al.* (1987). After removal of the DNA template by incubation with RQ1 DNase (Promega) for 15 min, the synthesized RNA was purified by phenol–chloroform extraction and ethanol precipitation. The transcribed RNAs were used to inoculate plants, as described above.

In vitro translation

The *in vitro* translation reactions were done in rabbit reticulocyte extracts, using 1 μg of transcribed RNA, a translation kit from Promega, and [^{35}S]methionine. The

translation products were analyzed in a 12.5% polyacrylamide gel containing SDS (Laemmli, 1970).

Northern hybridization

Northern blot hybridization was performed as described by Kroner *et al.* (1989), except that Hybond N^+ (Amersham Corp., Arlington Heights, IL) nylon membrane was used. To detect BBMV RNA2-specific sequences, an [α-^{32}P]rCTP (Amersham Corp.)-labeled RNA probe, complementary to nucleotides 39–461 of BBMV RNA2, was synthesized by run-off transcription from an *Eco*RI-linearized pBB2-21 plasmid (Romero *et al.*, 1993).

DNA sequencing

Plasmid DNA was denatured by an alkaline treatment (Sambrook *et al.*, 1989). Standard sequencing reaction was performed with the Sequenase enzyme (United States Biochemicals, Cleveland, OH) and α-^{35}S-dATP (Amersham Corp.), according to the manufacturers' specifications.

Computer-assisted sequence analysis

BBMV RNA sequences were analyzed using programs BESTFIT, GAP, PILEUP, and DISTANCES from the GCG sequence analysis package (Devereux *et al.*, 1984). RNA secondary structure was predicted using the program MFOLD 2.2 from M. Zuker (Zuker, 1989) on a Silicon Graphics workstation.

RESULTS

Comparison of nucleotide and amino acid sequences in RNA2 components of three BBMV isolates

The complete nucleotide sequence of the RNA2 component of a BBMV isolate (designated Ba strain and obtained from Roger Hull, Norwich, England) that did not contain DI RNAs and was maintained on *N. clevelandii* has been determined before by Romero *et al.* (1992). Since the DI-like RNAs described in this work were generated in broad bean plants using two other BBMV isolates (Type strain and Mo strain), the complete nucleotide sequences of full-length cDNA clones of RNA2 components (from which infectious RNA transcripts could be synthesized) for both isolates were determined in order to facilitate comparison of the sequences at junction sites. Both Type and Mo strain sequences were determined from a single infectious clone. Figure 1 shows a computer-assisted alignment of the three BBMV RNA2 sequences. RNA2 of Type strain consists of 2834 nucleotides (nt), while that of Mo has 2917 nt. As such they are 23 and 106 nt longer than the previously determined Ba sequence. There is 95 and 93% sequence similarity between the RNA2 sequence of Ba and that of Type and Mo, respectively. In total, there are 150 single nucleotide differences between Type and Mo sequences which showed 92% identity. More than half of the changes are silent since there are only 24 amino acid differences in the corresponding 2a proteins (Fig. 2). The most profound differences with the published Ba sequence are at two locations within the 2a protein open reading frame (positions 749 to 785 and 1677 to 1786) as they lead to local frame changes from amino acids 211 to 232 and from 524 to 560, respectively. The majority of the remaining amino acid changes is clustered within the C-terminal domain of 2a (between amino acids 757 and 829). These data imply a higher tolerance for modifications within the C-terminus of 2a, which is in agreement with previously published data for the 2a protein of brome mosaic virus (BMV) (Traynor *et al.*, 1991).

There is an imperfect 84-nt repeat within the 3′ noncoding region of the Mo RNA2 as compared to Ba and Type RNA2 (Fig. 1, in italics). The first Mo RNA2 repeat has 18 nt that are different as compared to the homologous Type sequence, while the second Mo repeat has only 5 such nucleotides. This sequence is not found in BMV RNA2, cowpea chlorotic mottle virus (CCMV) RNA2, or BBMV Ba RNA1, but it is present in BBMV Ba RNA3 (although not repeated). Using the GCG program DISTANCES we found that the first Mo repeat is most similar to the second Mo repeat but least similar to the Ba RNA3 sequence (data not shown). This suggests that the repeat in the RNA2 of Mo could have arisen by a snap-back mechanism or by recombination between two RNA2 molecules rather than being acquired from the RNA3 component.

Spontaneous generation of BBMV DI-like RNAs

The two previously described (Romero *et al.*, 1993) preexisting DI RNAs from Mo and Tu strains (the latter from Tunisia; see Makkouk *et al.*, 1988, for description of BBMV strains) are shown in Fig. 3 as variants DI2 and DI4, respectively. We have characterized additional preexisting DI RNA species (designated DI1 and DI3 in Fig. 3) in the Type and Su strains, respectively, by cloning and sequencing of the RT–PCR-amplified cDNA preparations, as specified under Materials and Methods. All four DI RNA variants were derived by single deletions from their corresponding BBMV RNA2 component.

The above studies demonstrated that each natural BBMV isolate accumulated only one type of preexisting DI RNA in detectable quantities. To analyze additional deletion junction sites, new DI-like RNAs were generated *de novo* by serial passages of DI RNA-free Type or Mo strains through broad bean, at high m.o.i. In one approach, the DI RNAs were first removed by passing the Mo strain through bean plants (the progeny determined to be DI RNA-free by Northern blot analysis and RT–PCR) followed by up to 10 serial passages through broad bean. Virion RNAs were isolated from systemically infected broad bean leaves after the 1st, 2nd, 3rd, and 10th

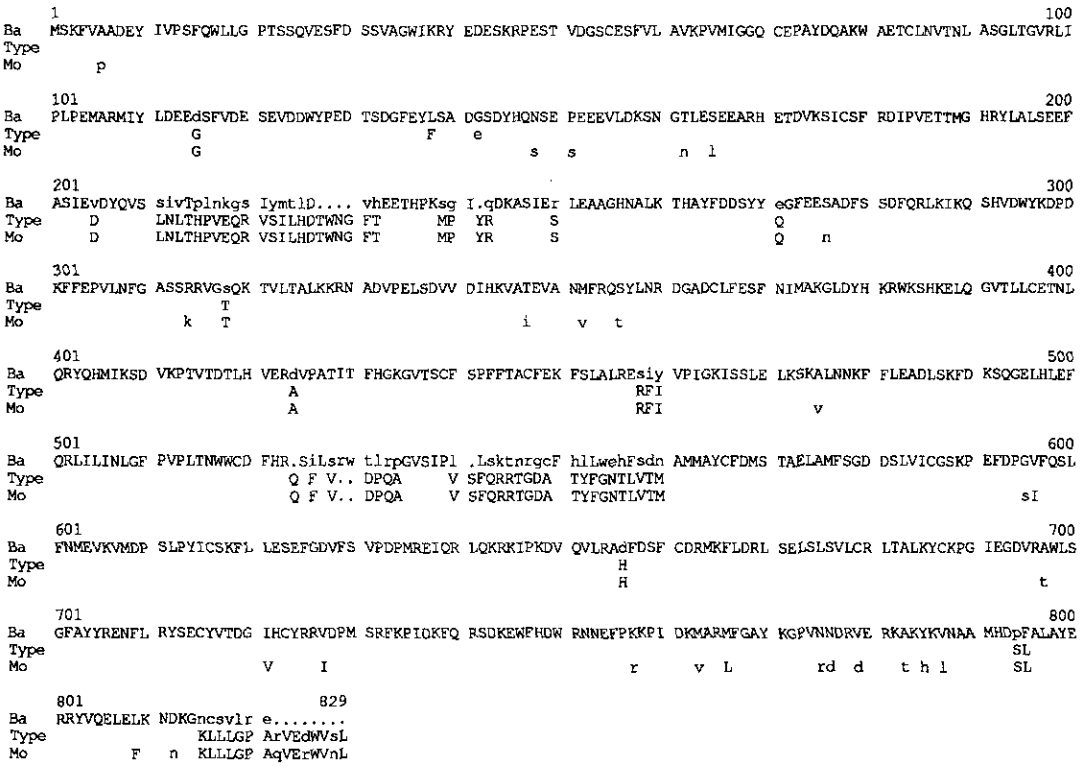


FIG. 2. Computer-assisted comparisons among amino acid sequences of 2a protein encoded by the RNA2 components of isolates Ba (first line), Type (second line), and Mo (third line). The uppercase letters indicate amino acids that are common between at least two sequences.

passages and analyzed for the presence of DI-like RNAs. Seven DI-like variants (DI5 through DI9 and DI11 in Fig. 3 and DI10 not shown) were identified using this procedure.

Since the above procedure may not guarantee a complete removal of preexisting DI RNAs from the BBMV inoculum, another approach was used in which viral infections were initiated using *in vitro* transcribed BBMV Type or Mo RNAs 1, 2, and 3 (Pogany *et al.*, 1994) and serially passaged. This allowed for identification of 4 further DI-like RNA variants (DI4 and DI15 in Fig. 3 and DI12 and DI13 not shown). Virion RNAs were extracted after each passage and the RNA composition was analyzed by electrophoresis in agarose gels, followed by Northern blot analyses (see Materials and Methods).

Electrophoresis in agarose gels (Fig. 4A) demonstrated that after 10 serial passages using either BBMV passaged through bean (lanes 5 to 10) or BBMV RNA transcripts (lanes 3 and 4) to initiate the passages, single RNA bands of variable size often appeared in addition to genomic viral RNAs. Northern blot hybridization (e.g., Fig. 4B) of progeny genomic RNAs of all passage lines with an RNA2-specific probe revealed the presence of smaller than full-length RNA2 molecules in 10 of 18 independent passage experiments. Comparison of Figs. 4A and 4B shows that, apparently, in some infections the defective RNAs comigrated with the genomic RNA3 molecules.

In all but one case, only one type of DI-like RNA was isolated at the end of each independent serial passage. The only exception was the isolation of variants DI12 and DI13 from the same passage experiment. DI12 appeared (not shown) after the 8th passage of transcript-induced BBMV infection. During the next passage, another DI-like RNA (designated DI13) accumulated to a detectable level, in addition to DI12. After the 10th passage DI12 and DI13 were present approximately in 1:1 ratio in the infected plants (not shown). Thus, DI13 has either been derived from DI12 or arisen independently from the RNA2 component.

Cloning and sequencing of *de novo* generated DI-like RNAs

To characterize the accumulated DI-like RNA components, RNA2-related molecules were amplified by RT–PCR from virion RNA preparations using RNA2-specific primers (see Materials and Methods). As an example, Fig. 4C shows cDNA products amplified by RT–PCR from Type (after 8 passages, lane 2) and Mo (after 10 passages of transcript-derived infection, lane 5) virion RNAs. The presence of additional cDNA products shorter than RNA2 demonstrates that both BBMV strains accumulated DI-like RNAs. In contrast, only a single cDNA band, corresponding to the full-length BBMV RNA2 segment, was amplified from initial progeny (from broad bean) of each

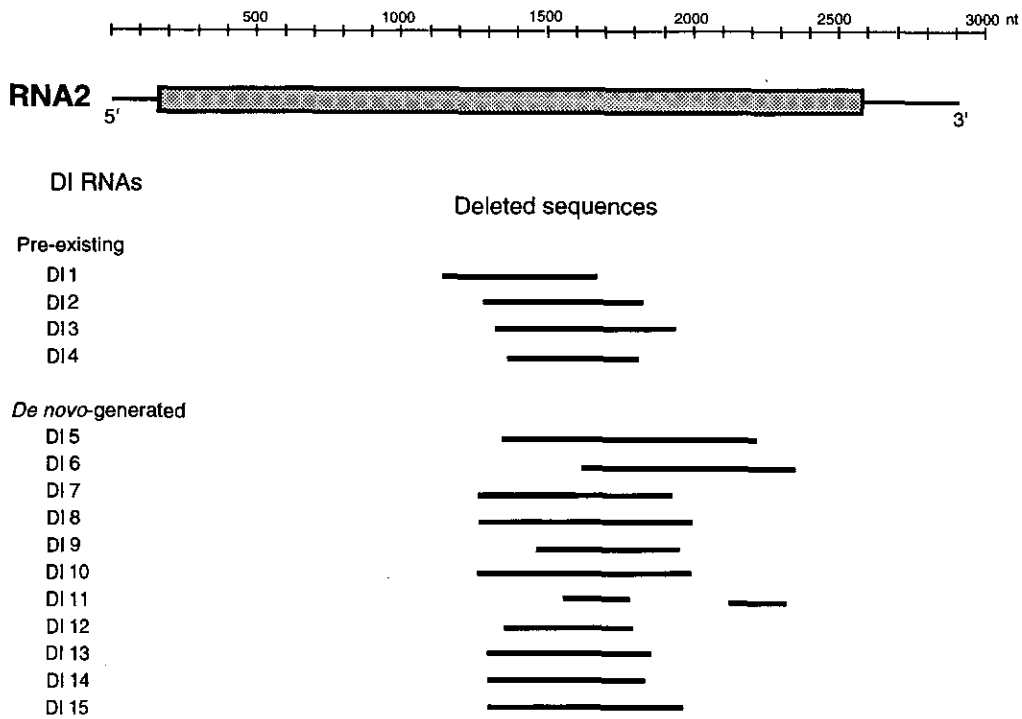


FIG. 3. Diagrammatic representation of the location of deletions in 13 newly characterized BBMV DI-like RNAs and the two previously published Mo and Tu DI RNAs (DI2 and DI4, respectively). The DI or DI-like RNA variants are named by a prefix DI followed by a serial number, indicating when they were identified as related. DIs 1 to 4 represent naturally occurring variants; DIs 5 to 11, those induced using bean-passaged BBMV inocula; and DIs 12 to 15, those obtained using *in vitro* transcribed BBMV RNA inocula. The RNA2-specific sequences were amplified from virion RNAs (of Type, Mo, and Su strains) using RT–PCR, and the cDNA products were ligated into pUC19 cloning vector, as described under Materials and Methods. The individual clones of interest were sequenced using a set of primers that covered the deletion region. The black bars represent the deleted sequences. The nucleotide positions use the coordinates of the corresponding wt RNA2 component and are indicated at the top of the figure. For the exact location of junction sites, please refer to Fig. 6. The 2a ORF is depicted by an open shaded box.

of the three DI RNA-free Type or Mo virion RNAs [Fig. 4C, lane 1 (transcript-derived Type strain), lane 3 (bean-purified Mo strain), and lane 4 (transcript-derived Mo strain)]. Similar analyses of other virion RNA samples revealed that the sizes of BBMV DI-like RNAs were variable and were smaller than the full-length RNA2 cDNA by approximately 0.4 to 0.8 kb (data not shown).

To establish the exact location of the deletion junction sites, the PCR cDNA products were digested with *Bam*HI and cloned into pUC19 between *Sma*I and *Bam*HI sites. The clones were initially mapped using RNA2-specific restriction enzymes (data not shown), followed by sequencing. One representative clone was sequenced for each experiment. These analyses confirmed that all but two DI-like RNAs contained unique junction sites. One DI-like RNA (DI10) had the same junction site as DI8, while DI12 had the junction point at the same location as the previously published Tu DI RNA (DI4). The total length of the deleted sequences varied between 441 and 864 nt, with deletions restricted to a region between nucleotide positions 1152 and 2366 (Fig. 3). Junction sites appeared to cluster at certain regions on the parental RNA2 molecule (Fig. 3): most of the 5′ junctions were between nucleotide positions 1275 and 1379 (exceptions being DIs 1, 6, 9, and 11), whereas the majority of 3′ junctions were between nucleotide positions 1816 and 1998 (exceptions being DIs 1, 5, 6, and 11).

The activity of DI-like RNAs in translation

Sequencing through junction sites revealed that deletions occurred in-frame for all 15 characterized DI-like (DI) RNAs. To confirm the possible activity of these molecules in translation, 7 of the 15 DI-like RNA transcripts were subjected to *in vitro* translation reactions using the rabbit reticulocyte translation system. Figure 5 shows that all of the tested RNA templates generated protein products with electric mobilities proportional to those expected from the size of the open reading frame (ORF) of the DI-like RNAs.

Primary sequences and secondary structures near the junction sites

To study putative signal sequences responsible for the occurrence of deletions, two features, the similarity and complementarity of sequences surrounding the 5′ and 3′ deletion junction sites, were analyzed. Stretches of identity could potentially facilitate reannealing of nascent strands during viral RNA replication while complementary regions could bring the remote parts of the RNAs

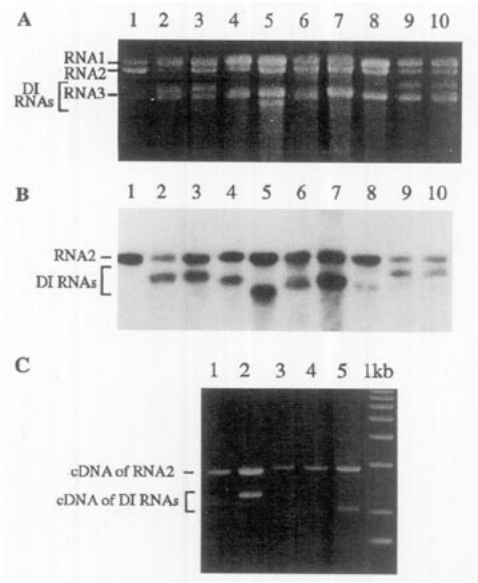


FIG. 4. Detection of *de novo* generated BBMV DI-like RNAs after serial passages. (A) Electrophoretic analysis of virion RNAs that were isolated from the following BBMV-infected broad bean plants: after inoculation with transcribed Mo genomic RNAs (lane 1); after inoculation with virion RNA extracted from Mo strain containing a natural DI RNA (lane 2); the 9th passage sap of two independent passage lines originating from transcribed Mo genomic RNAs (lanes 3 and 4); and the 9th passage sap of six independent passage lines originating from Mo virion RNA preparations after passaging through bean plants (lanes 5–10). The RNA molecules were separated in 1% agarose gel and stained with ethidium bromide. The positions of RNA1, RNA2, RNA3, and DI-like RNAs are indicated at the left. (B) Northern blot analysis of the gel shown in A. The blot was probed with a ^{32}P-labeled RNA probe specific for RNA2 sequences, as described by Romero *et al.* (1993). The positions of RNA2 and DI RNAs are indicated at the left. (C) Electrophoretic analysis of RNA2-specific cDNA products obtained by RT–PCR using primers 319 and 223 (as specified under Materials and Methods) from virion RNA preparations extracted as described under Materials and Methods. Lanes 1, 3, and 4 show the inoculum RNAs for the passage lines, while lanes 2 and 5 are examples showing 2 of 13 *de novo* DI-like RNAs generated in this study. Lane 1, cDNA of transcript-derived Type virion RNA (not passaged); lane 3, cDNA of Mo RNA2 that has been passed through bean plants followed by propagation in broad bean (the latter to assess the DI RNA-free nature of the inoculum); lane 4, cDNA of transcript-derived Mo virion RNA2 (not passaged); lanes 2 and 5, cDNA from the 8th and 10th passages through broad bean of transcript-induced infection of Type and Mo strains, respectively, using RNA2-specific primers. RT–PCR products were separated in a 1% agarose gel and stained with ethidium bromide.

into close proximity. Figure 6 depicts the nucleotide sequences surrounding the 5′ and 3′ deletion borders in 15 BBMV DI (DI-like) RNAs. The computer-calculated level of highest sequence similarity was observed over the 6- to 7-nt stretches of AAUUUG, CAAAUUU, UUUCCU, CAAAUUU, AAAUUUU, AAUUUG, and CAAAUUU sequences in clones 2, 3, 6, 7, 9, 13, and 15, respectively. In other clones, sequence identities were less perfect. The similar sequences occurred within either retained or deleted areas (or both) on the parental RNA2 molecule.

Some of the above similar stretches contained the palindromic motif AAAUUU. In addition to similarity, this motif can act as complementary sequences, when present at two separate locations within the parental RNA2 molecule. The extent of complementarity between 3′ and 5′ border sequences was further examined using the BESTFIT computer program. The longest complementary sequences were found in clones 1, 2, 3, 5, 6, 7, 9, 11A, 11B, and 13, as shown in Fig. 6. Other DI-like RNAs contained shorter complementary sequences within the border regions.

DISCUSSION

In this work we demonstrate that BBMV DI-like RNAs are generated spontaneously (*de novo*) from BBMV RNA2 components during serial passages at high m.o.i. in broad bean. All of the *de novo* BBMV DI-like RNAs strikingly resemble the previously characterized naturally occurring DI RNAs in this virus, since (i) both DI and DI-like RNAs are exclusively derived by deletions in the RNA2 component, (ii) the deletions are restricted to between nucleotide positions 1152 and 2366, and (iii) all DI (DI-like) RNAs encode a shortened 2a protein. These characteristics might reflect features related to both the mechanism of DI (DI-like) RNA generation and postevent selection requirements. The interfering properties of *de novo* generated DI-like RNAs remain to be demonstrated. More detailed aspects of the deletion formation and selection, as well as the role of translation in DI-like RNA accumulation, are discussed below.

Comparison of nucleotide sequences of the RNA2 component in three different strains of BBMV, together with a previously observed 5′ repeat in Ba RNA3 (Pogany *et al.*, 1994) and the formation of DI RNAs (Romero *et al.*, 1993; this paper) all confirm the plasticity of BBMV RNAs within both coding and noncoding sequences.

Possible mechanisms leading to internal deletions in BBMV RNA2

It is believed that DI RNAs arise as a result of template switching by the viral replicase during viral RNA synthe-

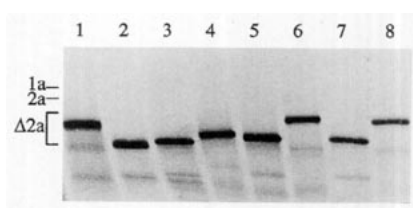
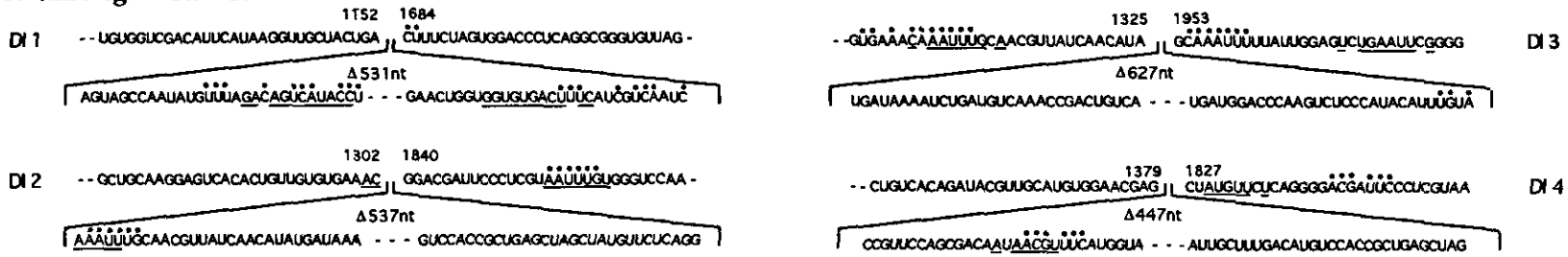
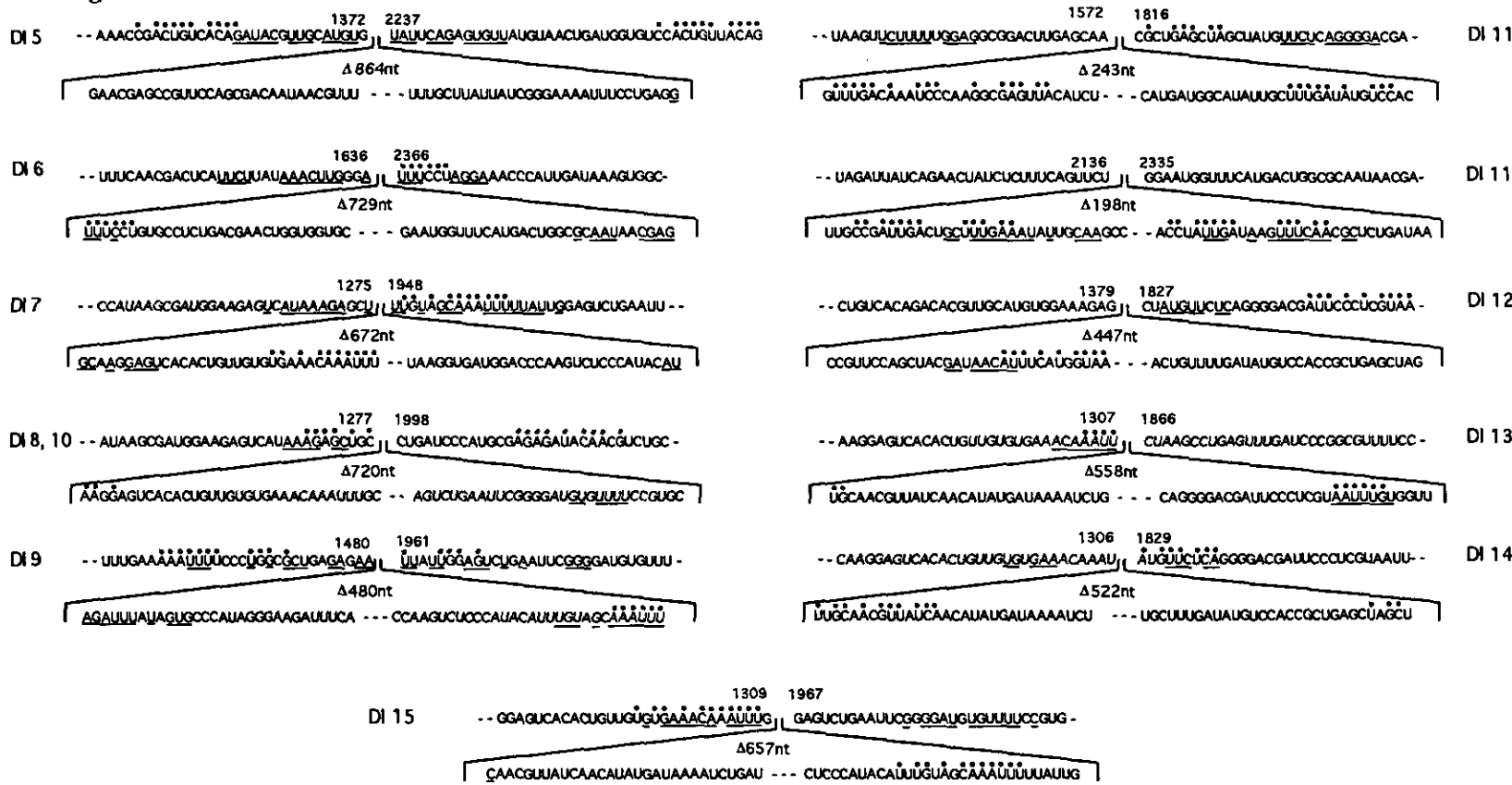


FIG. 5. Translational activity of BBMV DI-like RNAs *in vitro*. The figure shows an electrophoretic analysis (in a 12.5% polyacrylamide/SDS gel) of *in vitro* translation products synthesized in rabbit reticulocyte extracts using *in vitro* transcribed DI-like RNAs obtained from full-length cDNA clones of individual DI-like RNAs. As a control, lane 1 shows the translational products from *in vitro* transcribed preexisting DI2 (Romero *et al.*, 1993). Lanes 2–8 show products of translation from *in vitro* transcribed *de novo* generated DI-like RNAs corresponding to the following DI-like RNAs: DI5, DI6, DI7, DI8, DI9, DI10, and DI11 (described in Fig. 3). The positions of 1a, 2a, and Δ2a proteins are indicated at the left. Shorter translational products reflect either premature termination of translation or processes of degradation.

Pre-existing DI RNAs:



De novo-generated DI RNAs:



sis (Lazzarini *et al.*, 1981; Cascone *et al.*, 1990; Burgyan *et al.*, 1991). Increasing evidence suggest a copy-choice model involved in RNA recombination (reviewed by Bujarski *et al.*, 1994). As described above, similar and/or complementary sequences are present at the 5′ and 3′ borders of the junction sites in the BBMV DI-like RNAs. Regions of similarity might guide the reassociation of the nascent complementary RNA after the RNA polymerase falls off the primary template. Models based on discontinuous action of the RNA polymerase have been proposed to explain the generation of DI RNAs in mouse hepatitis coronavirus (MHV) (Makino *et al.*, 1988), TCV (Cascone *et al.*, 1990), and CymRSV (Burgyan *et al.*, 1991). Specific 3′ terminal sequences are required for initiation of RNA synthesis in BMV (Ahlquist *et al.*, 1985) and CCMV (Pacha *et al.*, 1990). Thus, one can speculate that the resumption of the RNA synthesis by BBMV RNA polymerase at internal locations requires certain signal motifs, e.g., the (A)AAUUU motif that was found near deletion borders in 6 of 15 DI-like RNAs.

Distant complementary regions may bring the remote parts of RNA2 molecule together (as proposed by Romero *et al.*, 1993). To gain more insight into the role of internal double-stranded structures in facilitating sequence deletions, minimum-energy foldings of the positive-strand sequence of the entire BBMV Type and Mo RNA2 molecules were obtained. As shown in Fig. 7, the Mo RNA2 can be folded to form extensive secondary structures with the 3′ and 5′ parts interacting along a central section. Similar folding was obtained for the Type strain RNA2 (not shown). The majority of crossover sites are located within the highly structured RNA "domains." Such extensive folding may be more difficult to unwind by the polymerase and thus such structures are occasionally deleted. Since the corresponding 5′ and 3′ deletion borders did not occur at the reciprocal positions, the replicase would have to jump over spatially proximal locations. Because various conformations may occur in a dynamic population of folded RNA structures, the replicase might jump between temporarily closer positions.

An unresolved question is why are the DI-like RNAs in BBMV system derived exclusively from the RNA 2 component? Since the (A)AAUUU sequences are also present in BBMV RNAs 1 and 3 (not shown), this motif cannot by itself be responsible for the RNA2 derivation of BBMV DI-like RNAs. One possibility is that the DI-like RNA is derived from RNA2 because the RNA polymerase-encoding RNA2 component has some preferences in being replicated in *cis* as regards RNA translation. *Cis* correlation between translation and replication has been observed for poliovirus (Johnson and Sarnow, 1991) and for turnip yellow mosaic virus (Weiland and Dreher, 1993). Also, one cannot exclude that BBMV DI-like RNAs are derived from genomic RNAs 1 or 3, but they might not be favored during selection (see below). Interestingly, RNA3-derived defective RNA components were recently characterized in cucumber mosaic virus (M. Graves and M. Roossinck, personal communication).

The role of open reading frames

All of the characterized DI (DI-like) RNAs code for internally deleted 2a proteins (Δ2a). It was found by Traynor *et al.* (1991) that BMV RNA2 mutants having deletions in similar locations did not support RNA replication *in trans.* The conserved GDD motif, characteristic of RNA polymerases, was deleted in 10 of 15 BBMV DI-like RNAs. Thus, the presence of this motif may not confer evolutionary advantage to DI-like RNAs. In contrast, all of the shortened BBMV 2a proteins retained the N-terminal portion analogous to the one involved in the interaction between 1a and 2a of BMV (Kao and Ahlquist, 1992). Therefore, Δ2a could compete with wt 2a for binding with BBMV 1a protein, thus decreasing the concentration of active replication complexes.

The apparent preservation of open reading frames suggests their role in the accumulation of BBMV DI-like RNAs *in planta.* The most important function of the coding capacity can be in stabilization of BBMV DI-like RNAs. The importance of translation to the stability of plant mRNAs has been described by Vancanneyt *et al.* (1990). White *et al.* (1992) demonstrated that coding capacity determines the accumulation of DI-like RNAs associated with CYMV *in planta.* Studies on DI RNAs of MHV revealed that in cell culture the lack of an active ORF markedly decreased the fitness of DI RNAs (de Groot *et al.*, 1992). An artificial DI-like RNA of BMV having a mutation that prevented translation did accumulate in barley protoplasts (Marsh *et al.*, 1991). The above observations suggest that, although not necessary for replication, an active ORF may increase the fitness of BBMV DI-like RNAs. Along these lines, shortened 2a proteins might possess an increased affinity to DI-like RNAs, hence contributing to RNA stabilization.

Factors affecting the selection of BBMV DI-like RNAs

It is possible that active postrecombinational processes may be involved in the selection of the observed

FIG. 6. Characterization of sequences surrounding the deletion junction sites in BBMV DI-like RNAs. In each panel the top line shows sequences that are present in the DI-like RNAs upstream of the 5′-proximal junction and downstream of the 3′-proximal junction, whereas the bottom line shows the sequences that are absent in the DI RNAs but are present in the corresponding parental RNA2 molecules. The most similar sequences near both junction sites are depicted by the dots above the nucleotides, while the complementary regions are underlined. G:U pairs are allowed in the selection of complementary sequences. The names of individual DI-like RNAs are shown at the left or right of each panel and correspond to the names used in Fig. 3. The nucleotide coordinates of the junction sites are shown by numbers above the center of the top lines and the number of deleted nucleotides is shown below each top line by a Δ followed by the number.

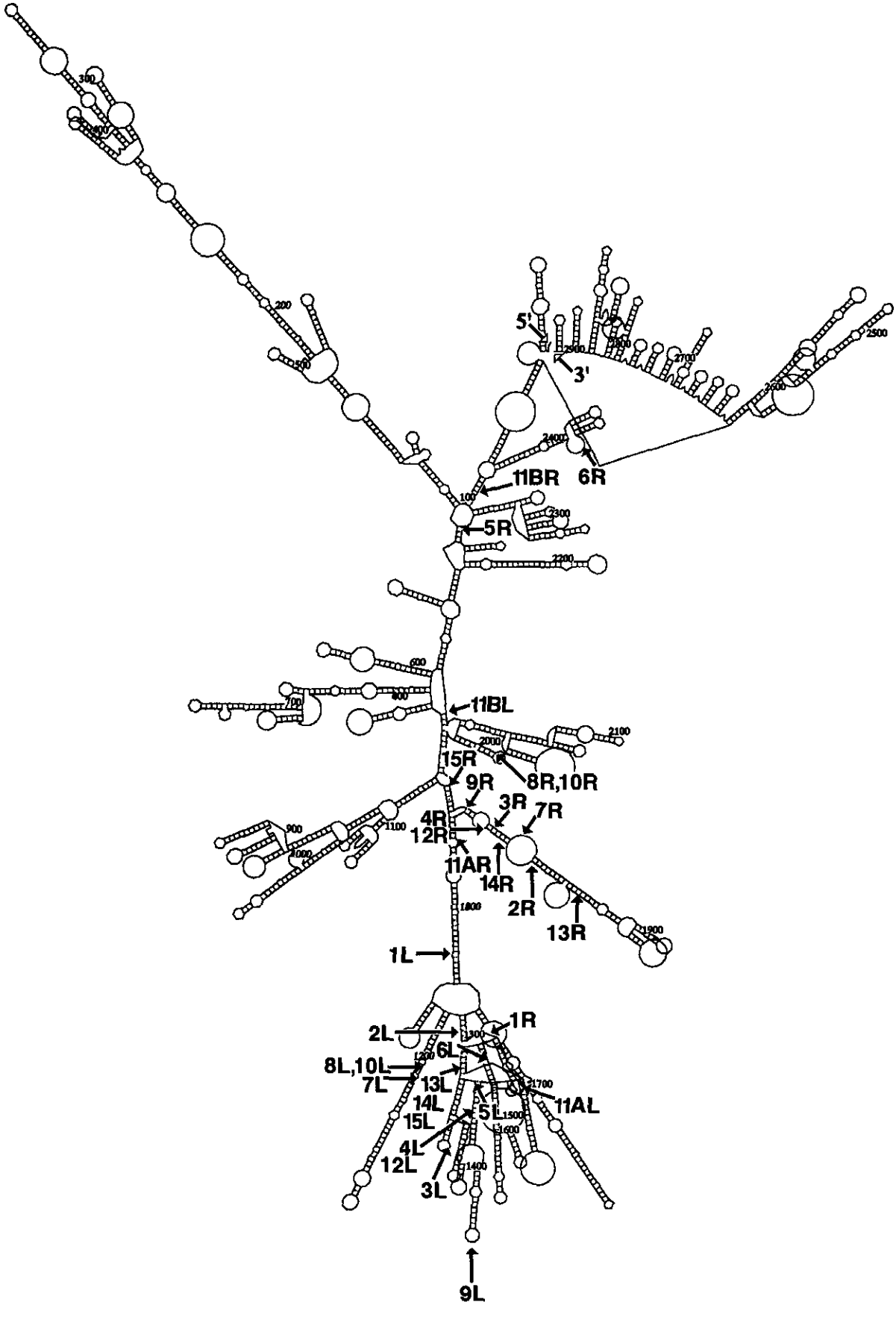


FIG. 7. Computer-generated minimal energy folding of RNA2 of BBMV Mo strain, using the RNA folding program of Zuker (1989) run on a Silicon Graphics workstation. The nucleotide coordinates (based on Fig. 1) are shown by numbers, while the positions of crossovers are depicted by solid arrows with the name of the DI-like RNA (taken from Fig. 3) and an indication of whether it is the left (L) or the right (R) side of the junction. The 5′ and 3′ ends of Mo RNA2 are depicted with open arrows.

BBMV DI-like RNA variants. For instance, the overall size of the DI-like RNAs might affect their encapsidation and/or the stability of BBMV virions. This, in turn, might influence the cell-to-cell and/or long-distance movement of a particular BBMV RNA variant. Our work has been done on encapsidated DI-like RNAs. The comparison of DI-like RNA profiles in virion and in total cellular RNA preparations could serve as a useful method for revealing the role of encapsidation in BBMV DI RNA selection.

Certain sequences in the RNA2 molecule might serve as signals for RNA replication, RNA encapsidation, or for other unknown *cis*- or *trans*-acting functions. Based on our data, one can envision that the *cis*-acting functions in DI (DI-like) RNAs are contained within the first 1152 and the last 468 retained nucleotides. In BMV, *cis* elements for RNA2 replication reside at both noncoding regions and within a large 5′ portion of the 2a ORF (Traynor *et al.*, 1991).

Comparison of deletion sites revealed that a sequence between nucleotide positions 1636 and 1684 was missing from all DI-like RNAs. This might be coincidental, or this region might interfere with the replicative ability and/or might decrease the fitness of DI-like RNAs. A region that interferes with viral RNA replication in a *trans*-complementation experiment was identified at the 5′ terminal half of the 30K protein gene of tobacco mosaic virus (TMV) (Ogawa *et al.*, 1992). The authors proposed that TMV polymerase components may bind to this part and that there might be a competition between this region and the 3′ and/or 5′ regions of the viral RNA involved in minus- and/or plus-strand RNA synthesis.

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