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Infectious In Vitro Transcripts from Cowpea Chlorotic Mottle Virus cDNA Clones and Exchange of Individual RNA Components with Brome Mosaic Virus

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Complete cDNA copies of genomic RNA1, RNA2, and RNA3 of cowpea chlorotic mottle virus (CCMV) were cloned 1 base downstream from a T7 RNA polymerase promoter. The mixture of capped in vitro transcripts from all three clones produced normal CCMV infections in barley protoplasts and cowpea plants. By using transcripts from these clones and from a similar set of biologically active clones of the related brome mosaic virus (BMV), all possible single component exchanges between the BMV and CCMV tripartite genomes were tested. Viral RNA replication was not observed with any heterologous combination of RNA1 and RNA2, which encode *trans*-acting viral RNA replication factors. However, substitution of the heterologous RNA3 into either genome produced viable hybrid viruses, both of which replicated in barley protoplasts and produced lesions on *Chenopodium hybridum*, a local lesion host for both parent viruses. In hybrid infections, BMV and CCMV coat proteins each readily packaged RNAs from the heterologous virus, but BMV RNAs were replicated to a higher level than CCMV RNAs, even when *trans*-acting RNA replication factors were provided by CCMV genes. Neither hybrid systemically infected the natural host of either parent virus, suggesting that host specificity determinants in BMV and CCMV are encoded by RNA3 and at least one other genomic RNA.

The bromoviruses are a group of icosahedral plant viruses whose genomes are divided among three separately encapsidated, messenger-sense RNAs designated RNA1, RNA2, and RNA3 (ca. 3.2, 2.9, and 2.1 kilobases, respectively) (29). Each of these genomic RNAs serves as an mRNA for a separate noncapsid protein. In addition, the 3′ portion of RNA3 encodes the coat protein, which is translated from a 0.9-kilobase subgenomic mRNA, RNA4. The best-studied bromovirus is the type member brome mosaic virus (BMV). All three BMV genomic RNAs have been sequenced (2, 5) and cloned as full-length cDNAs from which infectious transcripts can be synthesized in vitro (3, 4). RNA1 and RNA2, which are both required and together are sufficient to direct RNA replication in protoplasts (19, 26), encode proteins that share extensive amino acid similarity with noncapsid proteins encoded by the animal alphaviruses and a variety of other plant viruses (6, 12, 20). *cis*-Acting sequences directing BMV genomic RNA replication and subgenomic RNA production have also been identified (10, 11, 17, 18).

Sequence similarities and antigenic cross-reactivity indicate that the closest relative to BMV among known bromoviruses is cowpea chlorotic mottle virus (CCMV) (13, 39). The highly conserved 3′-terminal 200 bases of the CCMV RNAs are strikingly similar in predicted secondary structure to the corresponding BMV RNA region, which directs both viral RNA aminoacylation and initiation of negative-strand RNA synthesis (1, 10). Template-specific RNA-dependent RNA polymerase extracts from CCMV-infected cells reject other viral RNAs but readily copy either BMV or CCMV virion RNAs (35). Moreover, a hybrid virus containing BMV RNA1 and RNA2 and CCMV RNA3 replicates in *Chenopodium hybridum*, a local lesion host common to the two viruses (8). Despite close genetic, morphological, and functional similarities, the systemic host ranges of CCMV and BMV are markedly distinct. BMV systemically infects monocotyledonous plants, including cereals, whereas systemic hosts of CCMV are dicotyledonous plants, principally legumes.

Because these viruses possess fundamental genetic similarities and yet have distinct biological properties, combined studies of CCMV and BMV should assist in the understanding of virus replication and host interaction. We report here the construction of complete cDNA clones from which infectious transcripts of the three CCMV genomic RNAs can be synthesized in vitro and the use of these clones to test all possible genomic RNA exchanges between BMV and CCMV. Significantly, heterologous combinations of RNA1 and RNA2 were unable to direct viral RNA replication. Examination of other replicating hybrids in protoplasts, local lesion hosts, and systemic hosts of the parent viruses suggests that factors encoded by RNA3 and at least one other RNA must be independently or coordinately adapted for successful systemic infection.

MATERIALS AND METHODS

Preparation and sequencing of intermediate cDNA clones. Oligonucleotides d(TATGGTACTCAAGTC) and d(TGTG CACGAGTTAAC), complementary to interior sites of CCMV RNA2 and RNA3, respectively, were annealed to total virion RNA of CCMV type strain (28), extended with reverse transcriptase (Life Sciences, Inc.), and made double stranded (21). RNA2 cDNA and *Sau*3A-I-cut RNA3 cDNA were ligated into the *Sma*I and *Bam*HI sites, respectively, of M13mp18 (44) by using standard techniques (31). Selected clones from the resultant transformants were sequenced (9) to determine the terminal sequence of the cDNA inserts.

Primer extension on RNA templates. Oligonucleotide primers for 5′ end sequencing, and the nucleotide positions of their annealing sites within each RNA, were d(TTCGAG

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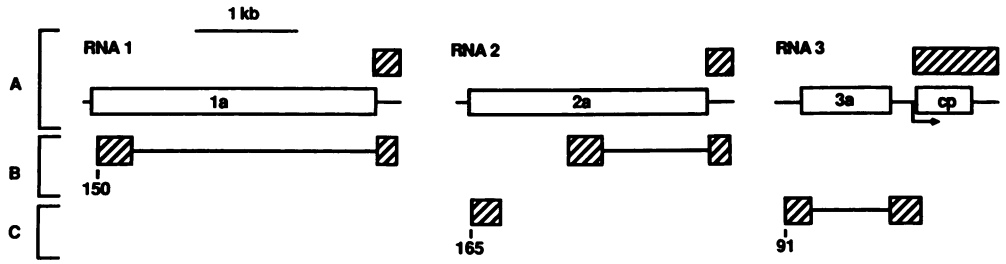

FIG. 1. Map of partial CCMV cDNA clones involved in determining the 5'-terminal sequences. (A) Schematic of CCMV RNA1, RNA2, and RNA3, showing known coding regions (▭) and noncoding regions (——). The beginning of the subgenomic RNA4 sequence (↳) and the extent of previously determined CCMV sequences (1, 13) (▨) are also shown. (B) Partial CCMV cDNA clones generated by J. Bujarski (unpublished results); sequenced portions are indicated (▨). (C) Additional partial cDNA clones generated by extending oligonucleotide primers complementary to regions of known sequence. The extent of terminal sequences determined for each clone is shown (▨); the position of the 5'-most nucleotide cloned for each RNA is indicated on the appropriate clones.

TAGTCAACTTGCTC) (bases 173 to 192) for RNA1, d(ATCCATTGGAATGAG) (bases 153 to 167) for RNA2, and d(ACTATTCCGTGAATGT) (bases 182 to 197) and d(GCTGCTCACGGGAAT) (bases 52 to 66) for RNA3. The oligo(A) region of RNA3 was sequenced with the primer d(TGTGCACGAGTTAAC), complementary to bases 34 to 48 of CCMV RNA4 (13). Sequencing reactions were performed with [α-^{32}P]dATP as described (1). In addition (see Fig. 2A), 5′ ^{32}P-d(GCTGCTCACGGGAAT) was extended on CCMV RNA3 in 4-μl reaction mixtures containing 0.5 μg of total CCMV virion RNA, 0.4 ng of 5′ ^{32}P-labeled primer, 1 U of reverse transcriptase, 25 μM each deoxynucleotide, 50 μM selected dideoxynucleotide, 25 mM Tris hydrochloride (pH 8.3), 5 mM dithiothreitol, and $MgCl_2$ at either 4 or 0.5 mM. After 15 min at 42°C, half of each reaction mixture was heated for 5 min at 100°C, cooled to room temperature, and incubated for 60 min at 37°C after addition of 2 μl containing 50 mM Tris hydrochloride (pH 7.5), 2.5 mM dithiothreitol, 625 μM each deoxynucleotide, and 2 U of terminal deoxynucleotidyltransferase (Bethesda Research Laboratories, Inc.).

Synthesis and cloning of full-length cDNA. Complete cDNAs were synthesized as described (4), except that T4 DNA polymerase (New England BioLabs, Inc.) was used for second-strand synthesis. The first-strand cDNA primer was d(CAGTCTAGATGGTCTCCTTAGAGAT) for all three CCMV RNAs. Second-strand cDNA primers were d(TAATCCACGAGA(A/G)CG) for RNA1 and RNA2 and d(TAATCTTTACCAAAC) for RNA3. Full-length double-strand cDNA was isolated from 0.8% low-melting-point agarose gels, cut with *Xba*I (Boehringer Mannheim Biochemicals), and ligated to *Stu*I- and *Xba*I-cut pMJ5. The sequence of pMJ5 (see Results) between the *Sma*I and *Bam*HI sites of pUC118 (43) is CCCGGGGATCT CGATCCCGCGAAATTAATACGACTCACTATA/GGCCT GGATCC, where the /marks the start of sequences transcribed by T7 RNA polymerase and the underlined sequences are *Sma*I, *Stu*I, and *Bam*HI sites, respectively.

In vitro transcription, protoplast inoculation, and RNA analysis. In vitro transcription of *Xba*I-linearized plasmids, protoplast inoculation and incubation, and protoplast RNA extraction and analysis were similar to those previously described for BMV (17). To isolate encapsidated viral RNA, protoplasts were sonicated for 30 s with a Braun Sonic 2000 microprobe at 35% power. The solution was cleared by centrifugation for 5 min at 12,000 × *g*, and virions were precipitated from the supernatant with polyethylene glycol (PEG) (30). RNA was purified from virions by phenol-chloroform extraction and ethanol precipitation. Probes for CCMV RNAs were ^{32}P-labeled in vitro transcripts from pCC3RA514, a BlueScribe M13$^+$ plasmid (Vector Cloning Systems) containing the conserved 179-base 3′-proximal *Dra*I-*Xba*I fragment of CCMV RNA3. Probes specific for BMV RNAs were in vitro transcripts from pB3HE1, which contains an analogous 3′ region from BMV (17).

Infectivity testing. Primary leaves of 10-day-old cowpeas (*Vigna sinensis* (Torner) Savi. cv. Queen Anne black-eye) were dusted with carborundum and inoculated with 4 to 8 μg of each transcript RNA in 25 μl of 10 mM Tris hydrochloride (pH 8.0)–0.1 mM EDTA. At 10 days following inoculation, virus was isolated from secondary leaves by the method of Lane (30), and RNA was isolated directly from ground leaf tissue by phenol-chloroform extraction and ethanol precipitation. Leaves of 30-day-old *C. hybridum* L. were inoculated similarly, and local lesions were counted 8 days postinoculation.

RESULTS

5′-Terminal sequences of CCMV RNA1, RNA2, and RNA3. High specific infectivity of in vitro transcripts from BMV cDNA clones depends on close coupling of the 5′ end of the cDNA to a suitable promoter (24). To efficiently clone complete cDNA copies of the CCMV genomic RNAs and to effectively link these cDNAs to a suitable promoter, we determined the 5′-terminal RNA sequences through a series of primer extensions. Starting materials were partial RNA1 and RNA2 cDNA clones (kindly provided by Jozef Bujarski; unpublished results) and the known sequence of RNA4 (13) (Fig. 1). Determination of the terminal sequences of the partial RNA1 and RNA2 clones and comparison with the known BMV sequence (2) suggested that the RNA1 clone was nearly complete, whereas the RNA2 clone represented the 3′ half of CCMV RNA2 (Fig. 1B). Oligonucleotides complementary to the terminal sequence of the partial RNA2 clone and to the 5′ end of the subgenomic RNA4 were used to produce cDNA clones which extended to within 165 and 91 bases, respectively, of the 5′ ends of RNA2 and RNA3 (Fig. 1C). The 5′-proximal sequences of these clones were then established and used to design oligonucleotide primers from which the extreme 5′ nucleotides of RNA1, RNA2, and RNA3 were determined by dideoxy chain termination reactions on the RNA template.

Primer extension on CCMV RNA1 and RNA2 produced two major "strong-stop" reverse transcription runoff products, as has previously been observed for other capped RNAs (4, 22). Under standard primer extension reaction

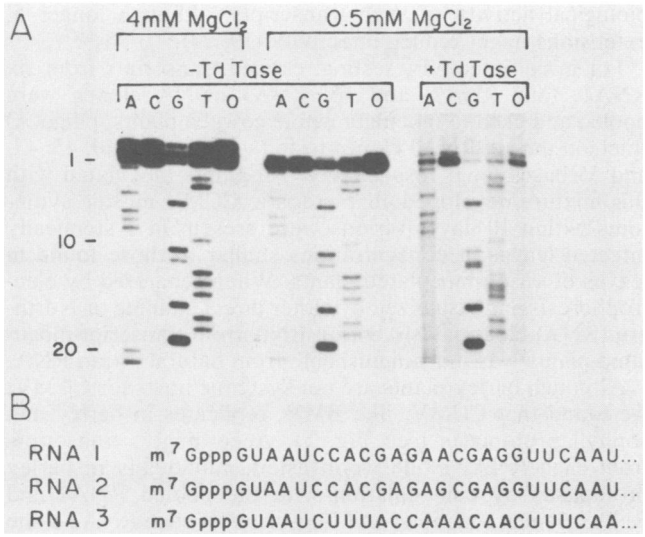

FIG. 2. (A) Autoradiograph of an 8% polyacrylamide sequencing gel showing 5′-end analysis of CCMV RNA3. A 5′ ^{32}P-labeled oligonucleotide complementary to RNA3 bases 52 to 66 was used to prime cDNA synthesis in 4 mM or 0.5 mM $MgCl_2$, as shown. Individual reaction mixtures contain the indicated dideoxynucleotides (A, C, G, and T) or no dideoxynucleotide (0). Numbering at the left corresponds to the position of the complementary base in CCMV RNA3. After cDNA synthesis, portions of the 0.5 mM $MgCl_2$ reaction mixtures were further treated with terminal deoxynucleotidyltransferase before fractionation in the rightmost set of lanes (+TdTase). Additional background in these lanes results from tailing of excess, unannealed 5′ ^{32}P-labeled primer. (B) 5′ sequences of CCMV RNA1, RNA2, and RNA3.

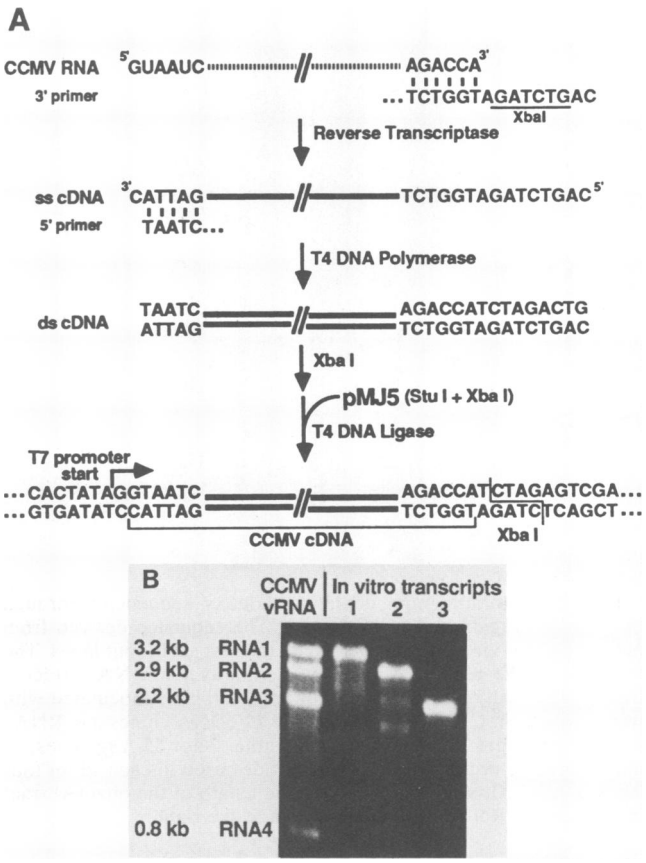

FIG. 3. (A) Schematic diagram of CCMV cDNA cloning strategy. -----, RNA; ▬, cDNA strands. (B) Ethidium bromide-stained 0.8% agarose gel comparing CCMV virion RNA (left lane) with in vitro transcripts from *Xba*I-linearized cDNA clones pCC1TP1, pCC2TP2, and pCC3TP4 (lanes 1 to 3, respectively). Approximate sizes of virion RNAs are indicated at the left.

conditions, CCMV RNA3 unexpectedly gave a third major strong stop, but this band disappeared if the Mg^{2+} concentration was reduced (Fig. 2A), the reaction temperature was raised to 50°C, or the reaction time was shortened to 5 min (results not shown). To identify the 5′-terminal nucleotide in RNA3, we treated primer extension products with terminal deoxynucleotidyl transferase and excess deoxynucleotides to elongate any cDNAs with 3′ hydroxyl ends. This treatment preferentially elongated all strong-stop bands except the lower band terminating with a dideoxy in the ddCTP lane, indicating that this band corresponds to the 5′-terminal nucleotide of RNA3 and that the 5′ residue is a G (Fig. 2A). This result was expected from comparison with primer extension results of BMV RNA3 (4). The 5′-terminal nucleotides of CCMV RNA1 and RNA2 were not determined directly but were provisionally assigned as G residues because of the evident similarity of their 5′-terminal sequences to BMV RNA1 and RNA2 (2). This assignment agrees with recent direct RNA sequencing of the 5′ ends of these CCMV RNAs (J. Bujarski, personal communication). The 5′-terminal sequence of all three RNAs is shown in Fig. 2B. The total sequence of the CCMV genome is now being completed by sequencing the M1 set of biologically active cDNA clones described below. The complete sequence and its analysis will be presented elsewhere (R. Allison, M. Janda, and P. Ahlquist, unpublished results).

Cloning complete cDNA copies of CCMV RNA1, RNA2, and RNA3. To expedite construction, analysis, and expression of complete CCMV cDNA clones, we constructed plasmid pMJ5 (Fig. 3A) by inserting a 42-base-pair *Bam*HI-*Bgl*II fragment containing the T7 promoter-*Stu*I fusion from pAR2463 (42) in the *Bam*HI site of pUC118 (43).

Using the newly derived 5′ sequences and known 3′ sequences of CCMV RNAs (1), we designed synthetic oligonucleotides to prime first- and second-strand synthesis of full-length cDNA copies of CCMV RNA1, RNA2, and RNA3. The first-strand primer included a flanking *Xba*I site (Fig. 3A) to provide a unique restriction site for efficient cloning and subsequent plasmid linearization prior to in vitro transcription. *Xba*I was chosen because analysis of the partial cDNA clones described above and inspection of the 5′- and 3′-terminal sequences indicated that no *Xba*I sites occur in the CCMV genome. The second-strand primers lacked the initial G residue of CCMV RNAs, which was restored by ligation of the cDNA to the *Stu*I site of pMJ5 (Fig. 3A). This strategy reduced the nonviral sequence between the T7 promoter initiation site and CCMV cDNA to a single G (see below).

Since the 42-bp T7 promoter insertion of pMJ5 preserves the pUC118 *lacZ* reading frame, cDNA-containing transformants from the above strategy were selected as colorless colonies on ampicillin plates containing isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-Gal). The full-length character of the cDNA inserts was verified by sequencing both cDNA ends, using the standard *lacZ* primer for the 3′ ends and the 5′-proximal primer appropriate for each RNA. Multiple full-length cDNA clones of the three CCMV genomic RNAs

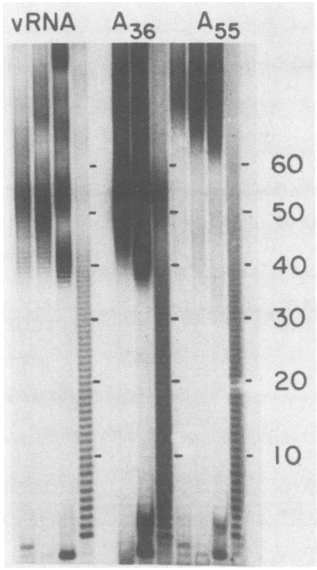

FIG. 4. Autoradiograph depicting dideoxy sequencing through the oligo(A) region of CCMV RNA3. The sequence derived from natural CCMV virion RNA is shown in the first set of four lanes. The template for the next two sets of reactions was total RNA extracted from systemically infected leaves of cowpea plants inoculated with transcripts from CCMV RNA1 and RNA2 cDNA clones and RNA3 clones with oligo(A) tracts containing either 36 or 55 A residues, as indicated. The order of dideoxynucleotides used in each set of four lanes is A, C, G, and T. A scale for the length of the oligo(A) tract in the various RNA3 samples is shown at the right.

were obtained. For RNA3, approximately 100 full-length clones were produced from 30 ng of total CCMV RNA.

Location and length heterogeneity of intercistronic oligo(A) in CCMV RNA3 cDNA clones. Previously we have shown that the intercistronic regions of RNA3 from three bromoviruses, BMV, CCMV, and broad bean mottle virus, each contains an oligo(A) tract (1). In BMV this tract occurs 20 bases upstream of the start of sequences encoding subgenomic RNA4, varies from around 16 to 22 residues in length in the virion RNA population (5), and is required for efficient RNA4 production during infection (18). If the CCMV RNA3 oligo(A) tract has a similar function, variation in its length might influence the biological activity of RNA3 cDNA clones. To examine the location and length variation of the oligo(A) in CCMV RNA3, we sequenced 22 randomly selected full-length cDNA clones with a primer complementary to the start of the coat protein gene. The CCMV oligo(A) began 18 bases 5′ to the start of RNA4 sequences (13) and varied from 34 to 55 residues in length in different clones. Sequencing with the same primer revealed that the oligo(A) ranged primarily between 35 and 45 residues in length in natural virion RNA populations (Fig. 4). Thus, although the position of the oligo(A) in CCMV is similar to that in BMV, its average length is about twice that of the BMV oligo(A).

Infectivity in transcripts from complete CCMV clones in cowpea plants. Transcripts from *Xba*I-linearized CCMV cDNA clones comigrated with the appropriate virion RNAs (Fig. 3B). Relative to CCMV virion RNAs, these transcripts have one additional 5′ G, arising from the T7 polymerase start site, and five extra 3′ bases, arising from the added *Xba*I site (Fig. 3A). Previous experience with BMV suggests that these extensions should not significantly inhibit the biological activity of such transcripts, although longer 5′ extensions might reduce infectivity (19, 24).

For initial infectivity testing, capped transcripts from six RNA1, four RNA2, and six RNA3 cDNA clones were pooled and used to inoculate whole cowpea plants. Oligo(A) tracts in the six RNA3 clones tested were 34, 36, 40, 45, 47, and 55 bases long, respectively. All plants inoculated with this mixture developed characteristic CCMV mosaic symptoms within 10 days. Virions were present in systemically infected leaves at concentrations similar to those found in leaves of virion-inoculated plants. When separated by electrophoresis and visualized by either direct staining or Northern (RNA) blot analysis, virion RNA from transcript-inoculated plants was indistinguishable from natural virion RNA.

Although barley plants are not systemic hosts for CCMV, we found that CCMV, like BMV, replicates in barley mesophyll protoplasts (see Fig. 5). Accordingly, transcripts from each RNA1 clone were tested individually in barley protoplasts by coinoculation with the pooled RNA2 and RNA3 clone transcripts, and transcripts of each RNA2 clone were similarly tested with pooled RNA1 and RNA3 clone transcripts. Transcripts from each clone directed viral RNA replication at similar levels, as assayed by electrophoresis and fluorography of total protoplast RNA labeled with [^{3}H]uridine (19). From this group, clones pCC1TP1 and pCC2TP2, containing RNA1 and RNA2 cDNA, respectively, were chosen for further infectivity tests.

When inoculated onto whole cowpea plants with RNA1 and RNA2 transcripts from pCC1TP1 and pCC2TP2, transcripts of each of the six RNA3 clones induced typical CCMV symptoms, with no observed variation in severity or time of onset. To see whether oligo(A) length differences were preserved in progeny viruses, virion RNAs were isolated from plants inoculated with transcripts from the clones containing either the A_{36} or A_{55} tracts and sequenced with the primer complementary to the start of the coat gene. Oligo(A) lengths in progeny from the two infections were distinct (Fig. 4). In both cases, the most common lengths were somewhat greater than those of the oligo(A) tract of the parent cDNA clone, which may result from variations in oligo(A) lengths introduced during in vitro transcription of the original inoculum. However, in addition to a major class with oligo(A) lengths around 60 bases, the RNA3 population derived from the A_{55} clone also contained a small subpopulation with oligo(A) lengths similar to those of the uncloned starting virus (Fig. 4). Thus, further passages in whole plants might reveal selection toward oligo(A) lengths observed in the wild-type virus population.

To provide a reference CCMV genome for further study, a set of three biologically active plasmids, pCC1TP1, pCC2TP2 and pCC3TP4, was selected from those tested. The first digit in each name corresponds to the CCMV RNA component cloned, and the final digit represents an arbitrary isolation number. The length of the intercistronic oligo(A) tract in pCC3TP4, 40 residues, is near the average in the virus population used for cDNA cloning. When the procedures outlined in Materials and Methods were used, all cowpea plants inoculated to date with capped RNA transcripts from these three *Xba*I-linearized clones became infected systemically. No infected plants were observed when the inoculum consisted of uncapped RNA transcripts or of untranscribed plasmid DNA in either the closed-circular or the linearized form. As a genetically defined isolate, this set of viral cDNA clones and the progeny virus derived from them were designated the Madison 1 or M1 strain of CCMV. For brevity, transcripts from these clones

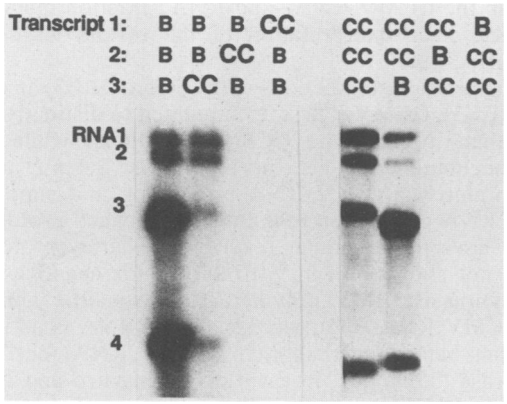

FIG. 5. Protoplast replication assays of CCMV transcripts and CCMV-BMV reassortant genomes. The autoradiograph shows blots of total protoplast RNA hybridized to mixed probes that recognize the 3'-terminal sequences of BMV or CCMV RNAs. BMV (B) and CCMV (CC) transcript inoculation combinations are indicated above each lane, and genomic RNA bands are identified at the left.

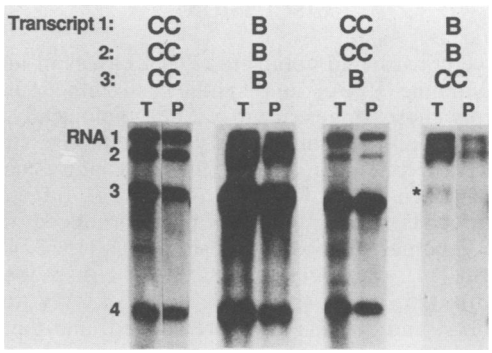

FIG. 6. Encapsidation of viral RNA in protoplasts. [^{3}H]uridine-labeled RNA is shown in autoradiographs of agarose gels of either total (T) protoplast RNA or PEG-precipitated (P) virion RNA isolated from protoplasts. BMV (B) and CCMV (CC) transcript inoculation combinations are indicated above each lane, and genomic RNA bands are identified at the left. An asterisk indicates the position of RNA3 bands visible in the original autoradiograph of the two rightmost lanes. For comparison purposes, P lanes were exposed four times longer than T lanes for both wild-type and hybrid infections, since only a portion of the total RNA was recovered in the virion fraction from these microscale extractions.

will be referred to as CC1, CC2, and CC3. Transcripts from clones pB1TP3, pB2TP5, and pB3TP8 (24), which represent the M1 strain of BMV (3), will be referred to as B1, B2, and B3.

Replication and packaging of reassorted bromovirus transcripts in barley protoplasts. Since the tripartite nature of bromovirus genomes permits the mapping of some virus functions by reassorting RNA components (8, 37), we tested all possible single-component exchanges between the BMV and CCMV genomes in protoplasts. Notably, none of the heterologous combinations of RNA1 and RNA2 supported detectable viral RNA replication (Fig. 5). However, RNA3 from either virus was replicated when substituted into the heterologous genome, showing that the replication signals on RNA3 were conserved between the two viruses (Fig. 5). The level of RNA3 replication differed considerably between the two exchanges. The combination (B1,B2,CC3) induced substantial replication of B1 and B2, while accumulation of CC3 was barely detectable. In contrast, with the combination (CC1,CC2,B3), B3 was not only replicated to a significant level, but was also associated with suppressed CC1 and CC2 replication relative to normal CCMV infection (Fig. 5). BMV genomic RNAs thus displayed a replicative advantage in both of the hybrid combinations.

To examine the spectrum of encapsidated RNAs in wild-type and hybrid infections, we precipitated virions from lysed protoplasts with PEG and recovered encapsidated RNA. Three observations confirmed that the [^{3}H]uridine-labeled RNA in this PEG-precipitated fraction represented encapsidated RNA. First, host RNA bands visible in total RNA extracts were selectively lost during this fractionation. Such host bands are visible in the (CC1,CC2,CC3) and (CC1,CC2,B3) total RNA lanes of Fig. 6 and were much more pronounced in several other experiments. Second, the RNA in this PEG-precipitated fraction was unaffected by RNase treatments which destroyed unencapsidated viral RNA (results not shown). Third, a BMV coat protein deletion-frameshift mutant, incapable of coat protein synthesis, completely blocked viral RNA recovery by this assay (R. Sacher, M. Janda, and P. Ahlquist, unpublished results). For both viable hybrid combinations, RNA1 and RNA2 were encapsidated by the coat protein encoded by heterologous RNA3 (Fig. 6). Moreover, as in wild-type BMV and CCMV infections, the relative abundance of packaged RNAs reflected the profile of viral RNAs in the total protoplast RNA fraction. Thus, RNA1 and RNA2 from either BMV or CCMV appeared to be similarly active substrates for encapsidation by the coat protein of either virus.

Inoculation of local lesion and systemic plant hosts of BMV and CCMV with reassortant genomes. *C. hybridum* is a local lesion host for both BMV and CCMV; i.e., each virus produces necrotic lesions of distinct morphology at primary infection sites on inoculated leaves (8). In our experiments BMV induced large lesions with necrotic areas 2 mm in diameter, surrounded by 2-mm chlorotic borders, whereas CCMV induced smaller lesions with necrotic areas of less than 1 mm in diameter and with 0.5-mm chlorotic borders. These lesion morphologies were duplicated by inoculation with the in vitro transcripts from the BMV and CCMV cDNA clones. Local lesions were also produced reproducibly by inoculating *C. hybridum* with (CC1, CC2, B3) and (B1, B2, CC3), the two hybrid transcript combinations with demonstrated RNA replication and packaging in protoplasts. Morphology of the lesions induced by these reassortant genomes was similar to those induced by (CC1, CC2, CC3) inoculation.

Barley and cowpea plants are selective hosts for systemic infection by BMV and CCMV, respectively. Accordingly, barley and cowpea plants were inoculated with all possible RNA component exchanges between the BMV and CCMV genomes. Although wild-type genome combinations produced normal systemic infection of their natural hosts, plants inoculated with all heterologous RNA combinations remained symptomless, and hybridization with virus-specific probes failed to detect viral RNA in total RNA extracts from uninoculated leaves. Thus, although hybrid viruses in which RNA3 is exchanged between BMV and CCMV are competent for RNA replication and packaging in protoplasts and localized cell-to-cell spread in *C. hybridum* they are not competent for systemic infection in the natural host of either virus.

DISCUSSION

We have constructed complete cDNA clones of all three CCMV genomic RNAs and synthesized infectious transcripts from these clones in vitro. Such biologically active cDNA clones should considerably facilitate the study of CCMV RNA replication, gene expression, and gene function, as has been shown for BMV (10, 17–19). Infectious in vitro transcripts have now also been produced for the complete genomes of tobacco mosaic virus (15, 32), black beetle virus (14), and several animal RNA viruses including Sindbis virus (25, 36, 38, 42). Virion RNAs of BMV, tobacco mosaic virus, and Sindbis virus are capped, and their nonstructural protein sequences have extensive regions of similarity (6). For all three of these viruses, transcript infectivity was markedly stimulated by capping (3, 32, 38) and reduced by addition of only five to seven nonviral 5′ bases (15, 24, 38). The infectivity of CCMV RNAs is also highly dependent on capping, and limiting nonviral 5′ extensions to a single additional G produced highly infectious transcripts. Similar considerations may facilitate effective expression of cDNAs from other RNA viruses which share common characteristics with bromoviruses, tobacco mosaic virus, and Sindbis virus (20).

Compatibility requirements of RNA1 and RNA2 for successful RNA replication. Bromovirus RNA replication requires functions encoded by both RNA1 and RNA2 but not RNA3 (17, 19, 26). RNA1 and RNA2 are monocistronic mRNAs for the viral 1a and 2a proteins, respectively, and various mutations in either of these genes render BMV RNA replication temperature sensitive (P. Kroner, P. Traynor, D. Richards, and P. Ahlquist, unpublished results). Heterologous combinations of BMV and CCMV RNA1 and RNA2 failed to direct detectable viral RNA replication. This may reflect the failure of 1a or 2a protein to recognize *cis*-acting signals for plus- or minus-strand RNA synthesis on heterologous RNA1 or RNA2, thereby blocking continued production of the 1a or 2a mRNA. Alternatively, one or more steps of RNA replication may require the formation of a complex between the 1a and 2a proteins, and the heterologous proteins may be unable to interact effectively. Such a complex might be functionally similar to the 183-kilodalton readthrough protein synthesized by tobacco mosaic virus, which contains domains with substantial sequence similarity to both the bromovirus 1a and 2a proteins (23). Experiments are now in progress to determine whether incompatibility of the heterologous RNA1 and RNA2 combinations is due principally to *cis*-acting features of the RNAs or to *trans*-acting properties of their encoded proteins.

RNA replication, packaging, and local lesion responses induced by BMV-CCMV hybrids with heterologous RNA3. For both BMV and CCMV, Bancroft previously separated virion RNA into an RNA3 fraction and a fraction containing both RNA1 and RNA2 and showed that BMV RNA1 and RNA2 and CCMV RNA3 replicated together to produce lesions on *C. hybridum* (8). We have confirmed this result by using BMV and CCMV in vitro transcripts and have shown that the converse combination of CCMV RNA1 and RNA2 with BMV RNA3 also produces local lesions. Additionally, we have examined RNA replication and packaging with these hybrid combinations in protoplasts. The failure to demonstrate infection with CCMV RNA1 and RNA2 and BMV RNA3 in the virion RNA experiments of Bancroft might have resulted from unidentified differences in the virus strains or *C. hybridum* varieties used. Alternatively, an observed high background of lesions due to residual CCMV RNA3 in the CCMV RNA1 and RNA2 fraction might have prevented clear interpretation of the results with added BMV RNA3 (8).

During protoplast infections with (CC1,CC2,B3) as well as (B1,B2,CC3), CCMV RNAs accumulated to distinctly lower levels than in wild-type CCMV infections, while BMV RNAs accumulated to near normal levels. Sakai et al. (41) found preferential BMV RNA production and suppressed CCMV RNA production in coinfected tobacco protoplasts, and we have found similar results with barley protoplasts (results not shown). In in vitro assays for negative-strand RNA synthesis, BMV polymerase shows little ability to copy CCMV RNAs (34), whereas BMV RNA is actually a somewhat better template than CCMV RNA for CCMV polymerase (35). Thus, in a variety of in vitro and in vivo situations, BMV RNAs display a replicative advantage over CCMV RNAs. In contrast, BMV and CCMV RNA1 and RNA2 were encapsidated with similar efficiency by coat protein from either virus, as indicated by similar relative levels of viral RNAs in total and packaged RNA samples (Fig. 6).

Determinants of virus adaptation for successful systemic infection of specific hosts. Although both of the BMV-CCMV hybrids derived by RNA3 exchange direct in vitro RNA replication, encapsidation, and sufficient cell-to-cell spread to form macroscopic lesions in *C. hybridum*, they are unable to systemically infect either barley or cowpea, natural hosts of the parent viruses. Thus, appropriate adaptation of some factor or factors encoded by RNA3 must be required for successful systemic infection. However, since RNA3 substitution is not sufficient to exchange the host range of the two bromoviruses, systemic infection must also require proper adaptation of factors encoded by RNA1 and/or RNA2, either for direct compatibility with the host or for functional compatibility with RNA3 genes.

Two nonexclusive mechanisms have been implicated in virus adaptation to systemically infect specific plant hosts (7). Active, host-adapted virus functions may be required for transport of infection between cells. Present evidence indicates that the 30-kilodalton protein encoded by tobacco mosaic virus is such a transport factor (16, 33, 45). The 32-kilodalton 3a proteins encoded by the 5′ portions of BMV and CCMV RNA3 may be analogs of this gene (23), and the reassortment results are consistent with the possibility that the 3a gene is one determinant of bromovirus host specificity. In addition to possible transport factors, adaptation of other virus characteristics may be required to avoid induction of host defense responses. Depending on the host genotype, induction of such responses may involve either capsid or noncapsid determinants (27, 40), possibly including nonstructural proteins such as those encoded by bromovirus RNA1 and RNA2.

The establishment of readily manipulated, biologically active cDNA clones for both CCMV and BMV provides a means of exchanging or altering any selected portion of the two genomes. By this approach, the involvement of each bromovirus gene in virus replication, host specialization, and other processes can be examined directly. Comparisons, mutagenesis, and exchange of noncoding sequences in these related but distinct viruses also enhance prospects for characterizing the role of *cis*-acting regulatory elements in specific steps of RNA translation, replication, and packaging.

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