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Bromovirus RNA Replication and Transcription Require Compatibility between the Polymerase- and Helicase-Like Viral RNA Synthesis Proteins

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The positive-strand RNA bromoviruses encode two nonstructural proteins, 1a and 2a, involved in RNA-dependent RNA replication. These proteins have extensive sequence similarities with methyltransferase, helicase, and polymerase proteins of other plant and animal viruses. 1a and 2a can also form a complex in vitro. To explore whether 1a-2a interaction is required for RNA replication in vivo, we reassorted the 1a and 2a genes from two different bromoviruses, brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV). 1a and 2a were expressed independently of viral replication by using RNA- or DNA-based transient expression, and their in vivo RNA replication activities were tested in protoplasts with BMV and CCMV RNA3 templates. RNA-based transient expression confirmed prior indications that bromovirus RNA replication is more sensitive to reductions in 1a expression than to reductions in 2a expression. DNA-based expression of the homologous combinations of 1a and 2a supported high levels of RNA synthesis, but both 1a-2a heterologous combinations exhibited RNA synthesis defects. The combination of CCMV 1a and BMV 2a did not support detectable synthesis of negative-strand, positive-strand, or subgenomic RNA. The converse combination of BMV 1a and CCMV 2a was preferentially defective in positive-strand and subgenomic RNA accumulation, showing that 1a-2a interaction is involved in these processes in ways distinct from negative-strand RNA synthesis, which was only slightly affected. These results indicate that at least some functions of 1a and 2a operate in a mutually dependent manner in vivo and that the mechanisms of positive- and negative-strand RNA synthesis are differentiated in part by features of such interactions.

Many positive-strand RNA viruses of plants and animals have important similarities in the replication and transcription of their genomes. In addition to the likely participation of some host factors (21, 37, 38), these RNA-dependent RNA synthesis events require viral proteins implicated in polymerase and helicase functions and, for members of the alphavirus-like superfamily, RNA capping functions (16). For different alphavirus-like viruses, conserved domains associated with these three functions may be combined on a single protein or divided among two or more separate proteins (3, 19). Nevertheless, despite these varied organizations, the high sequence conservation of these domains suggests that they have similar individual and collective functions in different viruses. A complete description of this collective function, however, is still being sought.

One system currently being used to study positive-strand RNA virus replication is the plant bromovirus group (1). The bromoviruses are icosahedral, positive-strand, tripartite RNA viruses in the alphavirus-like superfamily. The two bromovirus proteins required for RNA replication, 1a and 2a, are translated from genomic RNA1 and RNA2, respectively, while proteins required for infection spread are translated from genomic RNA3 and a subgenomic mRNA, RNA4, transcribed from negative-strand RNA3 (Fig. 1). Protein 1a (109 kDa) contains an N-terminal m^7G methyltransferase-like domain thought to be involved in capping viral RNA (32, 41) and a C-terminal helicase-like domain (17). Protein 2a (94 kDa) contains a central polymerase-like domain (6, 19, 23). Site-specific mutagenesis and other studies show that all three conserved domains in 1a and 2a are required for viral RNA synthesis (27, 28, 45, 46).

Bromovirus RNA synthesis can be divided into three distinct steps: negative-strand synthesis, positive-strand synthesis, and subgenomic mRNA transcription. Each of these steps is differentially regulated. For example, negative-strand RNA accumulation plateaus by 8 h postinoculation, while positive-strand genomic RNA and subgenomic mRNA continue to accumulate until or beyond 20 h postinoculation (28). In addition, both *cis*- and *trans*-acting mutations that alter the ratio of subgenomic to genomic RNA synthesis or positive-strand to negative-strand RNA synthesis have been constructed (12, 27, 31, 36). These differences suggest either that each step involves a different set of virus and/or host factors or that a common set of proteins interacts with positive-strand, negative-strand, and subgenomic initiation sites in different ways. However, although 1a and 2a are both required for complete RNA replication and transcription, the possible involvement or specific roles of 1a and 2a in each step remain unclear.

In addition, it has been unclear whether bromovirus RNA synthesis requires any direct in vivo interaction between 1a and 2a or whether, alternatively, 1a and 2a function separately or by indirect association through another factor, without the need for direct 1a-2a compatibility. A possibility that direct 1a-2a interaction might be required for bromovirus RNA synthesis in vivo has been suggested by genetic studies with two closely related bromoviruses, brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV), whose genomic RNAs 1 to 3 are herein designated B1 to -3 and C1 to -3, respectively. BMV and CCMV each replicates in barley protoplasts. By contrast, in the same cells, the heterologous RNA1-RNA2

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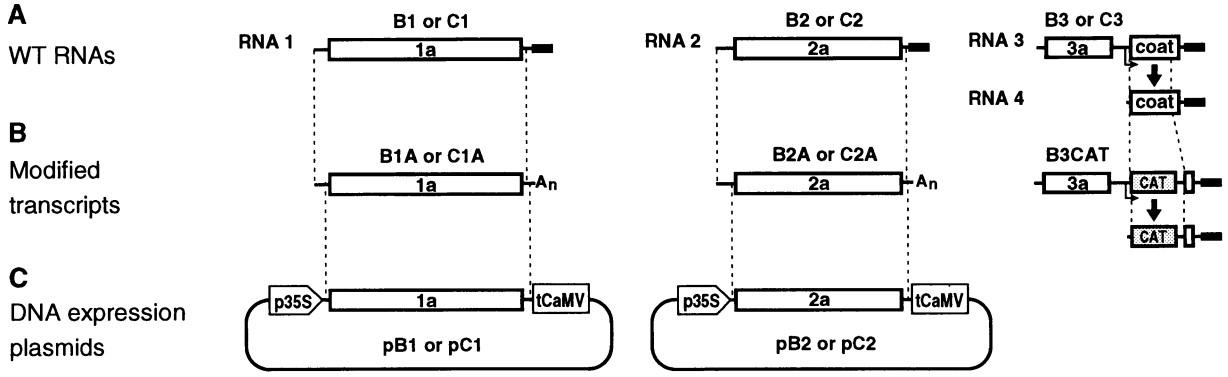

FIG. 1. Structures of the BMV and CCMV genomes and their derivatives used in this study. (A) wt RNAs. BMV or CCMV RNA1, RNA2, and RNA3 transcripts are designated B1 or C1, B2 or C2, and B3 or C3, respectively. RNA4, the 3′-coterminal subgenomic mRNA synthesized from the negative-strand RNA3, is shown below RNA3. Open boxes, the coding regions of the 1a, 2a, 3a, and coat proteins; thin lines, the flanking noncoding regions; solid boxes, the conserved 3′-terminal 200-base tRNA-like region. The bent arrow 5′ of the coat protein gene on RNA3 marks the position complementary to the RNA4 transcription start site. (B) Modified transcripts. In the B1A or C1A and B2A or C2A RNA derivatives, 3′-terminal noncoding sequences, including the negative-strand initiation signals, were deleted and replaced by poly(A) tracts (see Results and Table 1). In the B3CAT derivative of BMV RNA3, all but the 3′ portion of the coat protein (small open box) was deleted and replaced by the complete bacterial CAT gene plus a small segment of a 3′ noncoding sequence derived from the CAT gene. (C) DNA expression plasmids. In plasmids pB1 or pC1 and pB2 or pC2, the BMV and CCMV 1a and 2a coding regions and small portions of their flanking noncoding sequences were inserted between the 35S promoter (p35S) and terminator (tCaMV) from CaMV.

pairs B1-C2 and C1-B2 fail to support detectable replication of any RNA, including B3, which is replicated to high levels when coinoculated with either homologous pair, B1-B2 or C1-C2 (4). This failure of the heterologous RNA1-RNA2 combinations to support RNA replication might reflect functional incompatibility between the BMV and the CCMV 1a and 2a proteins, implying that interaction of 1a and 2a is required for RNA synthesis. However, this failure might also result from an inability of 1a or 2a to interact with the heterologous RNA1 or RNA2, blocking replication of that RNA and thus interfering with 1a or 2a gene expression. Thus, further in vivo experiments were needed to determine whether 1a and 2a must actually interact for successful RNA replication to occur.

In the experiments reported in this article, we examined the possible requirements for 1a-2a compatibility in vivo by transiently expressing BMV and CCMV 1a and 2a proteins in protoplasts, independently of RNA1 and RNA2 replication. While either homologous 1a-2a combination directed replication and transcription of B3 and C3, both heterologous protein combinations showed defects in RNA synthesis, demonstrating that functional compatibility between 1a and 2a is required in vivo for RNA synthesis. The nature of these defects indicates that at least positive-strand RNA synthesis and subgenomic mRNA transcription require 1a-2a compatibility and that aspects of 1a-2a interaction in these processes are separate from any requirements for negative-strand RNA synthesis. The results are discussed in relation to a recently demonstrated in vitro interaction between 1a and 2a (24, 25).

MATERIALS AND METHODS

Plasmids. Plasmids containing full-length cDNAs of each BMV and CCMV genomic RNA adjacent to a T7 promoter sequence (B1, pB1TP3; B2, pB2TP5; B3, pB3TP7 or pB3TP8; C1, pCC1TP1; C2, pCC2TP2; C3, pCC3TP4) were described previously (4, 22). The following additional plasmids will be referred to by brief descriptive names, with laboratory designations in parentheses where appropriate.

(i) pB3CAT. To allow the chloramphenicol acetyltransferase (CAT) gene to be expressed from the intercistronic promoter of BMV RNA3, the 1.7-kb *Cla*I-*Eco*RI partial digest fragment containing the complete CAT gene and surrounding BMV RNA3 sequences from pB3CA42 (13) was cloned between the *Cla*I and *Hin*dIII sites of pB3TP7, generating pB3CAT (pB3CA81). To simplify linearization of the final plasmid with *Pst*I prior to in vitro transcription (13), an unwanted *Pst*I site adjacent to the T7 promoter in pB3TP7 was first removed by cleaving with *Pst*I, treating with T4 DNA polymerase, and religating.

(ii) pB1A, pB2A, pC1A, and pC2A. As explained further in Results, plasmids pB1A, pB2A, pC1A, and pC2A provide templates for in vitro synthesis of 3′-truncated, nonreplicatable derivatives of BMV and CCMV RNAs 1 and 2. For pB1A, the 3-kb *Pst*I-*Bam*HI fragment of wild-type (wt) B1 cDNA clone pB1PK8 (28) was inserted into the polylinker site of plasmid pSP64-poly(A) (Promega). For pB2A, the 2.7-kb *Hin*dIII fragment of wt B2 cDNA clone pB2TP5 (22) was inserted into the polylinker sites of plasmid pSP64-poly(A) (Promega). For pC1A (pCC1MJ1), a cloned *Hin*dIII-*Xba*I fragment containing the 3′-terminal 41 heteropolymeric bases and poly(A) tract of human rhinovirus 14 cDNA from the clone pW2 (30) was substituted for the 3′-terminal, 198-base *Xmn*I-*Xba*I fragment of wt C1 cDNA in pCC1TP1 (4). For pC2A (pCC2MJ2), the same human rhinovirus 14 fragment was substituted for the 3′-terminal, 177-base *Dra*I-*Xba*I fragment of wt C2 cDNA in pCC2TP2 (4). Sequencing verified the expected CCMV-human rhinovirus 14 cDNA junctions in both final plasmids.

(iii) pB1, pB2, pC1, and pC2. These plasmids direct in vivo synthesis of 5′- and 3′-truncated, nonreplicatable derivatives of the indicated RNAs 1 and 2 in *Nicotiana benthamiana* and other dicot cells. In all four plasmids, the 1a and 2a genes were correctly oriented for expression from the cauliflower mosaic virus (CaMV) 35S promoter of pRT101 (44). For pB1 (pB1PA17), a 2.9-kb *Sal*I-*Xho*I fragment from pB1TP3 was inserted in the *Xho*I site of pRT101. For pB2 (pB2PA17), a 2.6-kb *Fsp*I-*Hin*dIII fragment from pB2TP5 was inserted in the *Sma*I site of pRT101. For pC1 (pCC1SD3), a 3.1-kb *Sal*I-*Eco*RI fragment from pCC1TP1 was inserted in the *Xho*I-

*Eco*RI site of pRT101. For pC2 (pCC2SD3), a 2.7-kb *Sal*I-*Eco*RI fragment from pCC2TP2 was inserted in the *Xho*I-*Eco*RI site of pRT101.

In vitro transcription and transfection of barley and *N. benthamiana* protoplasts. Prior to in vitro transcription, pB3CAT was linearized by cleavage with *Pst*I; pB1A, pB2A, and the three wt BMV cDNA clones were linearized with *Eco*RI; and pC1A, pC2A, and the three wt CCMV cDNA clones were linearized with *Xba*I. The linearized plasmids were transcribed with T7 RNA polymerase as previously described (22).

Isolation and inoculation of barley protoplasts (*Hordeum vulgare* L. cv. Morex) were performed as previously described (26, 27). *N. benthamiana* protoplasts were isolated essentially as described by Chupeau et al. (8), with the following modifications. Protoplasts were isolated from leaves of 3-month-old *N. benthamiana* grown in a growth chamber at 26°C with 16 h of light per day. After disinfection by immersion in a solution containing 20% bleach (Clorox) and 0.05% Tween 20, leaves were floated on the T0 medium (8) containing 0.5% Macerozyme R10 (Yakult Honsha Co.) and 2% Cellulase Onozuka R10 (Yakult Honsha Co.) overnight at 30°C. After elimination of large debris by filtration through cheesecloth, protoplasts were washed twice with two centrifugations in MKC medium (3.5 mM MES [morpholineethanesulfonic acid] [pH 5.8], 2.5% KCl, 0.2% $CaCl_2$), suspended in 10% (wt/vol) mannitol, and counted. We routinely obtained 3×10^6 protoplasts per leaf by this procedure. Inoculation of *N. benthamiana* protoplasts with RNA transcripts or plasmid DNA was performed essentially as previously described (26), with the following modifications: before transfection protoplasts were resuspended in 100 μl of 10% mannitol, and after transfection protoplasts were washed twice in MKC medium and incubated for 24 h in 1 ml of T0 medium in a 1.5-ml Microfuge tube at 30°C. Typically, 10^5 barley protoplasts or 2×10^5 *N. benthamiana* protoplasts were used for inoculation with the transcript produced from 0.2 μg of each DNA template (approximately 2 μg of each RNA) or with 5 μg of CsCl-purified plasmid DNA.

CAT assays. Twenty-four hours after transfection, protoplasts were centrifuged, and pellets were resuspended in 50 μl of 250 mM Tris-HCl, pH 7.4, and incubated for 10 min at 60°C. To 25 μl of the protoplast extract was added 4 mM acetyl coenzyme A–2 mM leupeptin–250 mM Tris-HCl (pH 7.5)–0.1 μCi of ^{14}C-chloramphenicol (Amersham). The reaction was performed at 37°C for 1 h and terminated by extraction with 10 volumes of ethyl acetate. The ethyl acetate phase was recovered and then evaporated under vacuum for 45 min. Dry pellets were dissolved in 15 μl of ethyl acetate, spotted on thin-layer chromatography plates, and submitted to ascending chromatography in chloroform-methanol (24:1, vol/vol). Separated spots of chloramphenicol and its acetylated forms were visualized by autoradiography.

RNA analysis. RNA extraction, glyoxylation, and Northern (RNA) blot analysis were performed as previously described (26, 27). For the DNA-based transient expression experiments, Northern blot analysis was slightly modified in that RNA was transferred to nylon membranes (Hybond N; Amersham) and fixed by UV cross-linking (Stratalinker 2400; Stratagene). Positive- and negative-strand viral RNAs were detected by hybridization with ^{32}P-labeled, strand-specific RNA probes derived from the conserved 3′-terminal 200 nucleotides of BMV and CCMV virion RNAs, as described elsewhere (5, 11). Specific signals were quantified by scanning blots directly with a digital radioactive imaging detector (Betagen) or, in some early experiments, by scanning autoradiograms with a Zeineh SLR-504-XL soft laser scanning densitometer.

RESULTS

To determine whether the functional incompatibility of heterologous combinations of BMV and CCMV RNA1 and RNA2 (4) reflected compatibility requirements between the encoded 1a and 2a proteins, rather than simply insufficient 1a or 2a expression due to failure to amplify RNA1 or RNA2, we used RNA- and DNA-mediated transient expression to uncouple 1a and 2a expression from virus-directed amplification of RNA1 and RNA2. The functional activities of homologous and heterologous protein combinations were then compared in protoplasts by cotransfection with B3, C3, or their derivatives, as described below. All experiments were repeated four or more times with consistent results.

Nonreplicatable 1a and 2a mRNAs support B3-directed reporter gene expression in barley protoplasts. For RNA-based transient expression of 1a and 2a, we used 3′-truncated RNA transcripts. Rao and Hall (39) have previously shown that 3′-truncated BMV RNA2 can support RNA replication. Plasmid templates for in vitro synthesis of 3′-truncated transcripts were generated by removing 3′-proximal noncoding segments from wt B1, B2, C1, and C2 cDNA clones and replacing them with poly(A) tracts (Fig. 1 and Table 1). Linearization of the resulting plasmids and transcription with T7 RNA polymerase produced 3′-truncated, polyadenylated transcripts designated B1A, B2A, C1A, and C2A (Fig. 1B). Each transcript contains the 5′ noncoding region and complete 1a or 2a protein coding region of its parental RNA. The viral 3′ noncoding sequences that were deleted contain essential *cis*-acting signals required for initiation of negative-strand synthesis in vitro (33) and genomic RNA replication in vivo (7, 11, 35). Thus, the resulting truncated RNAs are nonreplicatable but are still able to serve as mRNAs for transient in vivo expression of the 1a and 2a proteins. In addition to directing negative-strand RNA synthesis, the 3′ tRNA-like regions that were deleted have been shown to contribute significantly to the in vivo translatability and stability of viral RNAs (15). The addition of 3′ poly(A) tracts, which have similarly been shown to improve expression from mRNAs exogenously introduced into plant cells (15), should at least partially compensate the deletion of the 3′ tRNA-like regions. For similar reasons, all RNA1 and RNA2 transcripts and the RNA3 transcripts discussed below were capped by including synthetic GpppG in the transcription reaction.

To determine whether 1a and 2a proteins expressed from the replication-defective transcripts could direct RNA synthesis, we first used a replicatable B3 derivative, B3CAT, in which most of the natural coat gene sequence was replaced by the CAT gene (Fig. 1B). The CAT gene in B3CAT, like the natural coat gene in wt B3, cannot be translated directly from RNA3 but is dependent on synthesis of negative-strand RNA3 and subsequent transcription of subgenomic RNA4, which serves as a CAT mRNA (13, 21). B3CAT transcripts were cotransfected with B1A-B2A or C1A-C2A transcripts into barley protoplasts, which support the replication of both BMV and CCMV (4). Both of these homologous transcript combinations reproducibly led to expression of CAT activity, as evidenced by the appearance of the acetylated forms of chloramphenicol (Fig. 2, lanes 1 and 4). As expected, no CAT activity was expressed when B3CAT was transfected into cells alone (Fig. 2, lane 5) or when B3CAT was transfected with B1A or C1A alone or with B2A or C2A alone (data not shown). Thus, B3CAT expression is dependent on both 1a and 2a, and the 1a and 2a proteins encoded by the 3′-truncated, nonreplicatable transcripts are active and synthesized in sufficient amounts to

TABLE 1. Structures of 3′-truncated, polyadenylated BMV and CCMV RNA1 and RNA2 derivatives

RNA	wt starting RNA	wt RNA length (bases)	Coding region[a]	3′ truncation site[b]	No. of 3′ bases deleted[c]	3′ bases added[d]
B1A	BMV RNA1	3,234	75–2957	2990	244	GGAUCCCCGGGCGAGCUCCC(A)$_{30}$CCGAAUU$_{35}$
B2A	BMV RNA2	2,865	104–2569	2670	195	GGGCUGCAGGUCGACUCUAGAGGAUCCCCGGGCGAGCUCCC(A)$_{30}$CCGAAUU$_{33}$
C1A	CCMV RNA1	3,171	71–2944	2973	198	AACAAUAUAGACACUUAAUUUGAGUAGAAGUAGGAGUUUAU(A)$_{82}$GACUCUAG$_{91}$
C2A	CCMV RNA2	2,774	110–2533	2597	177	AACAAUAUAGACACUUAAUUUGAGUAGAAGUAGGAGUUUAU(A)$_{82}$GACUCUAG$_{91}$

[a] The indicated bases encompass the complete 1a protein (RNA1) or 2a protein (RNA2) coding region, not including the termination codon.
[b] The last wt BMV or CCMV base present in the modified transcript.
[c] The number of wt BMV or CCMV bases deleted from the 3′ end of the modified transcript.
[d] The novel bases and poly(A) tail added to the 3′ end of each of the modified transcripts (see the text).

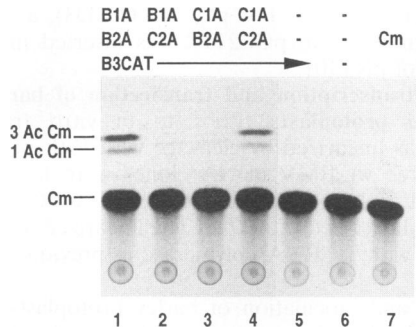

FIG. 2. CAT expression directed by truncated RNA1 and RNA2 transcripts in barley protoplasts. Barley protoplasts were transfected with the indicated combinations of nonreplicatable B1A, B2A, C1A, and C2A transcripts together with replicatable B3CAT transcripts. The protoplasts were harvested 24 h after transfection, and appropriate extracts were assayed for CAT activity. The transcript combinations inoculated are shown at the top, with a dash indicating the absence of a component from the inoculum. Lanes 5 and 6, assays of extracts from protoplasts transfected with B3CAT alone and mock transfected with no added transcripts, respectively. Lane 7, a control assay without any added protoplast extract, defining the pattern derived from unreacted ^{14}C-chloramphenicol (Cm). The migration positions of the acetylated forms (1 AcCm and 3 AcCm) and nonacetylated form of chloramphenicol are noted on the left.

support at least negative-strand and subgenomic mRNA synthesis.

Heterologous combinations of 1a and 2a proteins are defective in RNA synthesis. In contrast to the CAT expression directed by B1A-B2A and C1A-C2A, cotransfection of the B3CAT transcript with the heterologous combination B1A-C2A or C1A-B2A did not produce detectable CAT activity (Fig. 2, lanes 2 and 3). Since the transiently expressed BMV and CCMV 1a and 2a proteins supported CAT expression when used in either homologous combination, the failure of these heterologous combinations shows that some functional incompatibility exists between the heterologously paired proteins.

The failure of heterologous 1a-2a combinations to direct detectable CAT expression could be due to defective synthesis of negative-strand B3CAT RNA, subgenomic CAT mRNA, or even positive-strand B3CAT RNA, if positive-strand RNA synthesis is necessary to amplify negative-strand RNA production. As an initial approach to exploring which steps of RNA synthesis were affected by the heterologous 1a-2a combinations, viral RNA accumulation was analyzed by Northern blotting after barley protoplasts were cotransfected with various combinations of the truncated 1a and 2a transcripts and wt B3 transcripts (Fig. 3). wt B3 RNA was used for these experiments since it accumulates to higher levels than B3CAT in BMV-infected cells (13).

In keeping with the CAT results, no detectable accumulation of positive-strand B3 genomic RNA or B4 subgenomic mRNA was observed after B3 was coinoculated with the heterologous transcript combination B1A-C2A or C1A-B2A (Fig. 3A, lanes 5 and 6). Faint B3 signals were observed in these lanes after even more prolonged exposure, but these faint signals were no greater than the residual background level of surviving B3 inoculum in equivalent extracts of protoplasts inoculated with B3 alone or B3 in the presence of only RNA1 or RNA2 (Fig. 3A, lanes 10 and 11, and data not shown). Synthesis and accumulation of positive-strand B3 and also B4, which is not present in the inoculum, were supported by the homologous

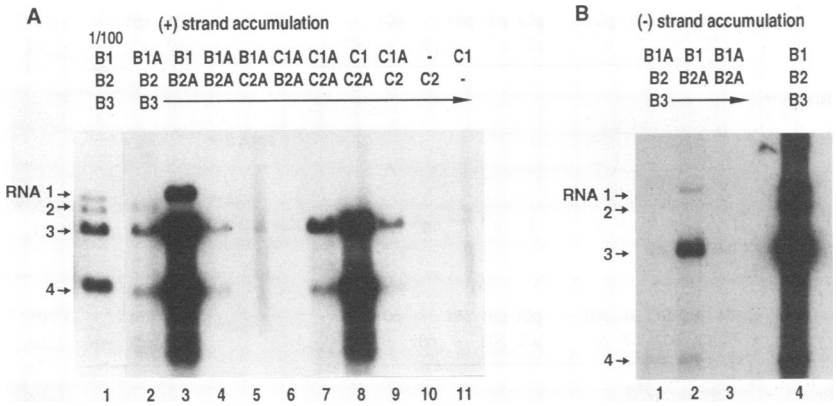

FIG. 3. Northern blot analysis of progeny RNA in barley protoplasts transfected with various combinations of wt and truncated RNA1 and RNA2 transcripts. Equal aliquots of barley protoplasts were transfected in parallel with wt B3 transcripts plus the indicated combinations of nonreplicatable transcripts (B1A, B2A, C1A, and C2A) and wt transcripts (B1, B2, C1, and C2), as shown at the top. Total protoplast RNA was extracted 24 h after transfection, denatured with glyoxal, electrophoresed in an agarose-phosphate gel, transferred to a nylon membrane, and hybridized with strand-specific probes of uniform specific activity to detect positive-strand (A) or negative-strand (B) BMV RNA sequences. As described in Materials and Methods, the positive-strand probe was a ^{32}P-labeled in vitro transcript complementary to the conserved 3′-terminal 200 nucleotides of BMV virion RNAs (Fig. 1A). The negative-strand probe was a ^{32}P-labeled, positive-sense in vitro transcript of the same 200-nucleotide region and, thus, was complementary to the conserved 5′ ends of the negative-strand RNAs. In panel A, lane 1 was loaded with RNA from protoplasts inoculated with all three wt BMV transcripts, but to facilitate comparison with other lanes, the fraction of the total protoplast RNA extract that was loaded in lane 1 was only 1/100 of that used for the samples in lanes 2 to 11. The dashes above lanes 10 and 11 indicate the absence of a component from the inoculum; since these incomplete combinations are unable to support RNA replication (4, 13), these lanes indicate the level of residual input RNA 24 h after transfection. The positions of BMV and CCMV RNAs 1 to 4 are indicated to the left of each panel. Autoradiograms for positive-strand RNAs were exposed with screens for 2 1/2 days, and autoradiograms for negative-strand RNAs were exposed for 8 days. In panel A, note that the smear observed on the right side of lane 5 has repetitively spaced counterparts in lanes 2, 8, and 11 and apparently corresponds to a background artifact inherent in the membrane. Visualization of this artifact was enhanced by the long exposure required for this autoradiograph.

transcript combinations B1A-B2A and particularly C1A-C2A (Fig. 3A, lanes 4 and 7). Even in these cases, however, B3 and B4 RNA accumulations were 100- to 500-fold lower than in protoplasts inoculated with wt B1, B2, and B3 transcripts (Fig. 3A; compare lanes 4 and 7 with lane 1 [100-fold-diluted wt sample]).

Blocking RNA1 amplification severely inhibits RNA replication and transcription. To determine whether the inhibition of RNA accumulation resulted more from blocking RNA1 synthesis or from blocking RNA2 synthesis, mixed combinations of wt transcripts and truncated polyadenylated transcripts were tested. When cells were inoculated with B1-B2A-B3 transcripts (Fig. 3A, lane 3), the levels of B1, B3, and B4 accumulation were consistently 10% of that in cells inoculated with B1-B2-B3 transcripts. Similar levels of RNA accumulation were observed when cells were transfected with C1-C2A-B3 (Fig. 3A, lane 8). However, when protoplasts were inoculated with B1A-B2-B3 or C1A-C2-B3, B3 and especially B4 accumulated only to around 1% of wt levels (Fig. 3A, lanes 2 and 9, respectively), which was similar to the results of inoculation with B1A-B2A-B3 or C1A-C2A-B3 (Fig. 3A, lanes 4 and 7, respectively). Thus, interfering with RNA1 amplification inhibited bromovirus RNA replication significantly more than interfering with RNA2 amplification, a finding which is consistent with some prior observations (28, 40).

For all inoculum combinations involving transient expression of 1a or 2a, negative-strand RNA3 accumulation was reduced in a manner similar to the effects on positive-strand accumulation (Fig. 3B and data not shown). For example, in cells inoculated with B1-B2A-B3 (Fig. 3B, lane 2), accumulation of B1 and B3 negative-strand RNA was approximately 10% of that for the wt (Fig. 3B, lane 4). As in wt infections (28), the level of negative-strand B4 was much lower than that of negative-strand B3. Negative-strand B3 RNA must also be present in cells inoculated with B1A plus B2 or B2A or with C1A plus C2 or C2A, since they support RNA-dependent synthesis of B3 and B4 positive-strand RNAs (Fig. 3A). However, in all of these cases, negative-strand B3 accumulation was at or below the limits of detection (Fig. 3B, lanes 1 and 3, and data not shown). This is not surprising, since bromovirus negative-strand RNA accumulates to approximately 100-fold lower levels than positive-strand RNA (11, 31), which was itself reduced 100-fold or more in these cases. Negative-strand B3 and B4 RNAs were also not detected in cells inoculated with heterologous transcript combinations B1A-C2A-B3 and C1A-B2A-B3 (data not shown). However, the inability to detect negative-strand RNA in cells inoculated with the homologous 1a-2a combinations illustrated that a system capable of higher-level RNA synthesis was needed to explore the functional limits of the heterologous 1a-2a combinations.

DNA-based transient expression of 1a and 2a. To increase the level and/or duration of 1a and 2a expression in the absence of RNA1 and RNA2 replication, we explored DNA-based transient expression. After testing several promoter-cell combinations for their ability to direct reporter gene expression (data not shown), by far the best results were obtained by using the 35S RNA promoter of CaMV in dicot protoplasts, including protoplasts from the dicot *N. benthamiana*, which is a systemic host for both BMV and CCMV (29, 29a, 34). Accordingly, cDNA copies of the BMV and CCMV 1a and 2a genes, with deleted 5′- and 3′-terminal noncoding sequences (Fig. 1), were inserted between the CaMV 35S RNA promoter and polyadenylation site in the expression vector pRT101 (44). The resulting plasmids, designated pB1, pB2, pC1, and pC2 (Fig. 1C), should thus direct in vivo synthesis of mRNAs encoding the full-length 1a and 2a proteins.

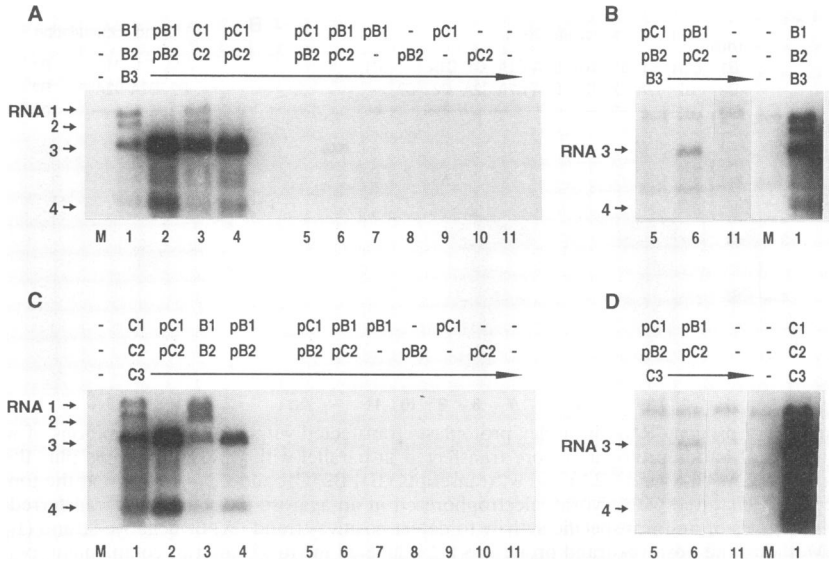

FIG. 4. Representative Northern blot analysis of positive-strand RNA accumulation in *N. benthamiana* protoplasts transfected with 1a and 2a expression plasmids. As shown above each lane, *N. benthamiana* protoplasts were transfected with wt B3 (A and B) or wt C3 (C and D) transcripts plus the indicated combinations of wt RNA1 and RNA2 transcripts (B1, B2, C1, and C2) or 1a and 2a expression plasmids (pB1, pB2, pC1, and pC2). Dashes indicate the absence of a component from the inoculum. Total protoplast RNA was extracted 24 h after transfection, denatured with glyoxal, electrophoresed in an agarose-phosphate gel, transferred to a nylon membrane, and hybridized with a mixture of both BMV and CCMV strand-specific probes of uniform specific activity to detect positive-strand RNA sequences. The probe for each virus, as described in Materials and Methods, was a ^{32}P-labeled in vitro transcript complementary to the conserved 3′-terminal 200 nucleotides of the positive-strand RNAs (Fig. 1A). Lanes 7 to 11, control assays in which one or both of the 1a and 2a genes were missing, indicating the level of residual input RNA3 persisting 24 h after transfection. In each panel, lane M shows total RNA extracted from protoplasts mock inoculated with buffer. (B and D) Fourfold-longer exposures of selected lanes in panels A and C, respectively. The positions of RNAs 1 to 4 are indicated. Autoradiograms for panels A and C were exposed for 4 h, and autoradiograms for panels B and D were exposed for 16 h.

In preliminary experiments, we first confirmed that B3 RNA was replicated in *N. benthamiana* protoplasts inoculated with B3 plus either wt B1-B2 or wt C1-C2 in vitro transcripts (Fig. 4A, lanes 1 and 3). Similar results were also obtained for C3 RNA after *N. benthamiana* protoplasts were inoculated with C3 plus B1-B2 or C1-C2 transcripts (Fig. 4C, lanes 1 and 3). The level of subgenomic RNA4 accumulation relative to RNA3 accumulation was usually lower in *N. benthamiana* cells (Fig. 4A and C, lanes 1 and 3) than in barley protoplasts (Fig. 3A, lane 1). Nevertheless, RNA4 bands were always apparent in the appropriate lanes. Moreover, substituting B3CAT for wt B3 produced the expected expression of CAT activity, confirming the synthesis of functional subgenomic mRNA (data not shown).

N. benthamiana protoplasts were next inoculated with the homologous DNA plasmid combination pB1-pB2 or pC1-pC2, together with wt B3 or C3 transcripts, by using the same polyethylene glycol-$CaCl_2$ transfection procedure as that used for transcripts alone. Such inoculations (Fig. 4A and C, lanes 2 and 4) not only directed B3 and C3 replication and transcription but consistently led to higher accumulation of B3-B4 and C3-C4 than inoculations involving wt RNA1 and RNA2 transcripts (Fig. 4A and C, lanes 1 and 3). Quantification of four independent experiments with a digital radioactive imaging system determined that RNA3 and RNA4 accumulation directed in *N. benthamiana* by pB1-pB2 or pC1-pC2 was consistently two to three times as high as that directed by B1-B2 or C1-C2 transcripts. As expected from prior results (13), no accumulation of positive-strand RNA3 or RNA4 occurred when cells were transfected with RNA3 plus any of the individual 1a or 2a expression plasmids (Fig. 4A and C, lanes 7 to 11).

Heterologous 1a and 2a combinations have different abilities to support positive-strand, negative-strand, and subgenomic RNA accumulation. Although high levels of RNA3 and RNA4 accumulation were directed by homologous combinations of the BMV and CCMV 1a and 2a expression plasmids, comparable RNA3 or RNA4 accumulation was not observed after transfection of *N. benthamiana* protoplasts with B3 or C3 RNA and the heterologous plasmid combination pB1-pC2 or pC1-pB2 (Fig. 4A and C, lanes 5 and 6). Thus, DNA-based transient expression and RNA-based transient expression both confirm that 1a-2a compatibility influences one or more steps of bromovirus RNA synthesis.

Even after prolonged exposure of the Northern blots in Fig. 4 and those in similar experiments, no accumulation of RNA3 or RNA4 was detected in cells coinoculated with pC1-pB2 (Fig. 4B and D, lanes 5); a few weak bands visible after such overexposure were common to mock-inoculated samples (Fig. 4B and D, lanes M). However, in repeated experiments, low but significant accumulation of RNA3 was observed after inoculation of either B3 or C3 with pB1-pC2 (Fig. 4B and D, lanes 6). RNA3 accumulation in these cases was about 3% of that in protoplasts inoculated with homologous combinations of the 1a and 2a plasmids and was clearly higher than any residual B3 or C3 present in the original inoculum (Fig. 4B and D, lanes 11).

Northern blotting with strand-specific BMV and CCMV probes was also used to assay negative-strand RNA accumulation in these cells (Fig. 5). None of the individual 1a and 2a proteins directed detectable negative-strand RNA3 accumulation (Fig. 5, lanes 7 to 10). The homologous 1a and 2a protein combinations directed negative-strand RNA3 accumulation, and in keeping with the positive-strand results, the level of

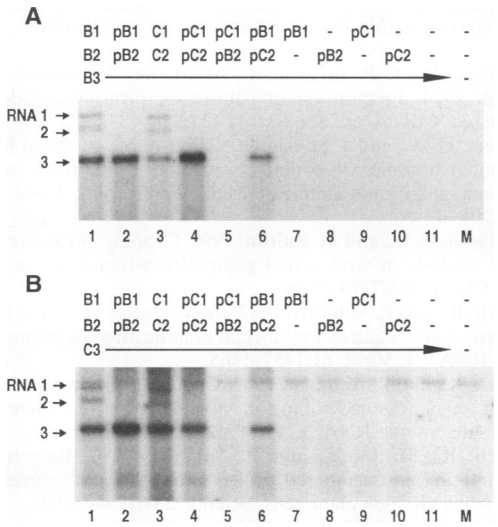

FIG. 5. Representative Northern blot analysis of negative-strand RNA accumulation in *N. benthamiana* protoplasts transfected with 1a and 2a expression plasmids. The same RNA samples shown in Fig. 4 were denatured with glyoxal, electrophoresed in an agarose-phosphate gel, transferred to a nylon membrane, and hybridized with a mixture of both BMV and CCMV strand-specific probes to detect negative-strand RNA sequences. The probe for each virus was a ^{32}P-labeled in vitro transcript derived from the conserved 3′-terminal sequences of the positive-strand RNAs and, thus, was complementary to the conserved 5′ ends of the negative-strand RNAs (see Fig. 1A and Materials and Methods). Lane designations and other details are as described in the Fig. 4 legend. The sizes and the specific activities of the BMV and CCMV negative-strand probes used were equal to those of the corresponding positive-strand probes of Fig. 4, and the autoradiograms were exposed for 4 days.

negative-strand RNA3 was higher on average when 1a and 2a were expressed from DNA plasmids rather than from wt RNA1 and RNA2 transcripts (Fig. 5, lanes 2 and 4 versus lanes 1 and 3). The heterologous plasmid combination pC1-pB2, which did not direct positive-strand RNA3 accumulation, also did not direct any detectable negative-strand RNA3 accumulation (Fig. 5, lanes 5). However, when RNA3 was cotransfected into cells with pB1-pC2, negative-strand RNA3 accumulation in repeated experiments averaged 50% of that with pB1-pB2 or pC1-pC2 (Fig. 5, lanes 6). Thus, the heterologous combination pB1-pC2 has a much greater defect in accumulation of positive-strand RNA3 and subgenomic RNA4 than negative-strand RNA3.

DISCUSSION

In the experiments reported here, we have used genetic approaches to examine the functional interdependence or independence of the 1a and 2a proteins involved in bromovirus RNA synthesis. Since prior experiments with replicating RNAs (4, 46) were unable to resolve this issue for reasons described in the introduction, a key feature of these experiments was the expression of 1a and 2a by approaches that were not dependent on viral replication of 1a and 2a mRNAs. Although transient expression of 1a and 2a from nonreplicatable mRNAs supported only a low level of RNA synthesis, plasmid-based expression of homologous 1a and 2a combinations via the CaMV 35S promoter in *N. benthamiana* supported high RNA3 and RNA4 accumulation, allowing conclusive testing of the various 1a-2a combinations.

1a-2a compatibility requirements in RNA replication and transcription. The results presented above show that successful bromovirus RNA replication in vivo depends in part on some form of compatible interaction between the 1a and 2a proteins. When used in homologous pairs, either the BMV or the CCMV 1a and 2a proteins directed replication and transcription of the B3 and C3 RNAs. Thus, each of these four proteins is competent for any necessary individual interactions with the RNA templates. Therefore, if 1a and 2a functioned in completely separate and dissociable subprocesses of viral RNA synthesis, RNA replication would occur in an unimpaired fashion when the BMV and CCMV 1a and 2a proteins were reassorted into heterologous pairs. However, the heterologous combinations of 1a and 2a were unable to direct normal replication or transcription of the same B3 and C3 RNAs. Thus, in the functional homologous combinations, 1a and 2a must interact or communicate in some fashion that is obligatory for or greatly facilitates one or more steps of RNA synthesis, while in the heterologous 1a-2a combinations the inappropriately paired proteins must either fail to interact or interact in an impaired fashion. Nevertheless, while these experiments show that some function(s) of 1a and 2a requires their mutual compatibility, they do not rule out the possibility that either 1a or 2a may have additional, independent functions.

Recently Kao et al. (25) and Kao and Ahlquist (24) reported that 1a and 2a will interact specifically and directly in vitro and showed that this interaction occurred between the helicase-like C-terminal domain of 1a and a segment near the N terminus of 2a. This interaction is likely to be responsible for at least a part of the in vivo compatibility requirement between 1a and 2a, as shown by correlations between the effect of certain 1a and 2a mutations on this interaction in vitro and on RNA replication in vivo (24) and by previous tests of BMV-CCMV 2a gene hybrids (45), which can be more definitively interpreted by virtue of the results reported here. The observed 1a-2a complex has fundamental similarities to the tobacco mosaic virus (TMV) 183-kDa protein, which is produced when the gene for the TMV 126-kDa protein, a homolog of the bromovirus 1a protein, undergoes occasional translational readthrough of its termination codon, leading to the covalent addition of a 2a-like polymerase domain. Both the 126- and the 183-kDa proteins are required for normal TMV RNA replication (20), but the 126-kDa protein is expressed in considerable excess over the 183-kDa protein. This preferential expression by TMV of a 1a-like protein over the adjacent 2a-like readthrough domain might be functionally related to the greater sensitivity of BMV replication to reduction of 1a rather than 2a expression (Fig. 3). The apparent need for higher-level expression of the bromovirus 1a and TMV 126-kDa proteins might reflect protein instability, multiple functional roles (1), multimer formation, or other requirements.

Distinct requirements for positive-strand versus negative-strand RNA accumulation. Relative to the homologous 1a-2a pairs, the BMV 1a and CCMV 2a combination showed a 30-fold reduction in positive-strand genomic RNA3 accumulation and no detectable subgenomic RNA4 accumulation (Fig. 4). However, there was only a twofold reduction in the accumulation of negative-strand RNA3, from which both positive-strand RNA3 and positive-strand RNA4 are synthesized (Fig. 5). Since a primary defect in accumulation of positive-strand RNA templates might be expected to reduce the subsequent yield of negative-strand RNA, even the twofold reduction may exaggerate any defect in negative-strand synthesis.

These asymmetric effects show that the reassorted BMV 1a

and CCMV 2a combination has preferential defects in positive-strand RNA synthesis and subgenomic mRNA transcription. This observation has two implications. First, some form or forms of 1a-2a interaction are involved in positive-strand genomic RNA synthesis and subgenomic mRNA transcription. Second, at least some aspects of 1a-2a interaction in positive-strand genomic and subgenomic RNA synthesis are distinct from the roles of 1a and 2a in negative-strand RNA synthesis.

The nature of the positive-strand-specific function(s) affected in the heterologous pB1-pC2 combination is not yet known. As previously discussed (1, 24, 28), 1a and 2a may interact to allow the 1a helicase-like and 2a polymerase-like domains to function in a jointly coordinated manner that might be disrupted in the heterologous combination. Alternatively, since negative-strand RNAs of alphavirus-like viruses are not capped (9, 42) while positive-strand RNAs lacking their usual 5′ caps are unstable in vivo (2, 14, 18), impairment of capping activities (10, 32, 41, 43) within the BMV 1a-CCMV 2a complex might cause the positive-strand-specific RNA accumulation defect.

For the CCMV 1a and BMV 2a combination, no detectable synthesis of any viral RNA, including negative-strand RNA, was observed (Fig. 4 and 5). This might reflect a primary defect in negative-strand RNA synthesis, which would imply that some form of 1a-2a interaction is involved in negative-strand RNA synthesis, in addition to the distinguishable 1a-2a interaction required for positive-strand genomic RNA synthesis and subgenomic mRNA transcription. However, the absence of detectable negative-strand RNA might also reflect a primary defect in positive-strand RNA synthesis, since the yield of negative-strand RNA synthesized only from the input inoculum might be insufficient for detection by Northern blotting as in Fig. 5. Further experiments are being pursued to distinguish these possibilities.

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