Use of Bromovirus RNA3 Hybrids To Study Template Specificity in Viral RNA Amplification

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Brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV) are related positive-strand RNA viruses with genomes divided among RNAs 1, 2, and 3. RNAs 1 and 2 encode the viral RNA replication factors, which share extensive conservation with proteins encoded by the animal alphaviruses and diverse plant viruses. In barley protoplasts, CCMV RNAs 1 and 2 support high but distinguishable amplification of either BMV RNA3 (B3) or CCMV RNA3 (C3), while BMV RNAs 1 and 2 show even greater discrimination, amplifying C3 poorly relative to B3. To identify the *cis*-acting determinants of these template-specific and virus-specific differences in RNA3 accumulation, we constructed and tested a series of B3/C3 hybrids that exchange in turn the 5', 3', and intercistronic noncoding regions, which contain all sequences required in *cis* for efficient B3 and C3 amplification. Despite suggestive prior *in vitro* results, the 3' noncoding regions were not the major determinant of the differences in amplification of B3 and C3 *in vivo*. Rather, 3' exchanges had relatively modest effects and did not transfer the distinctive asymmetry of amplification between B3 and C3. Intercistronic exchanges produced larger effects on RNA3 accumulation and transferred some of the polarized characteristics of the wild-type B3 and C3 behaviors. 5' exchanges revealed context-specific effects showing that the contribution of the B3 5' region to RNA3 amplification is dependent on some other B3 segment or segments. Together with previous results implicating the BMV and CCMV 1a genes in *trans*-acting discrimination between B3 and C3 (P. Traynor and P. Ahlquist, *J. Virol.* 64:69–77, 1990), these observations should help to guide studies of protein-RNA interactions governing template specificity in bromovirus RNA replication.

Brome mosaic virus (BMV) is an isometric plant virus whose genome consists of three capped, messenger-sense RNAs denoted RNA1 (3.2 kb), RNA2 (2.9 kb), and RNA3 (2.1 kb) (3, 6, 15). RNAs 1 and 2 encode nonstructural proteins 1a (109 kDa) and 2a (94 kDa), respectively. These proteins are required *trans*-acting factors for viral RNA replication (19, 25, 28, 29, 42, 43) and share extensive sequence similarity with proteins encoded by many other viruses, including the animal alphaviruses (7, 20, 21, 24). RNA3 encodes nonstructural protein 3a (32 kDa), which is required for systemic movement of infection (11), and the coat protein (20 kDa), which is translated from a subgenomic mRNA denoted RNA4 (0.9 kb). Because RNA3 is dispensable for viral RNA replication, it has been used to determine *cis*-acting sequences required for genomic RNA amplification (17, 34) and subgenomic RNA synthesis (18, 32, 35), to study viral RNA recombination (11, 14), and to express foreign genes (19, 39).

Biologically active cDNA clones and complete genomic sequences have been generated for BMV and a second member of the bromovirus group, cowpea chlorotic mottle virus (CCMV) (4, 8, 9). Studies utilizing these clones show that BMV and CCMV possess altered specificities at several levels of RNA replication, including functional incompatibility between heterologous combinations of the 1a and 2a genes and differences in RNA template specificity (8, 27, 42). Among other differences, BMV and CCMV RNA replications respond asymmetrically to BMV RNA3 (B3) and CCMV RNA3 (C3) templates (8, 42). CCMV RNAs 1 and 2

support high-level amplification of either B3 or C3 in barley protoplasts, with the heterologous B3 template accumulating to levels as high or higher than the natural template, C3. However, when B3 or C3 is inoculated into barley protoplasts together with BMV RNAs 1 and 2, marked discrimination is evident, with the heterologous C3 being amplified to low levels relative to B3. The 1a protein has been implicated as the major *trans*-acting factor discriminating B3 and C3 templates (42).

Prior experiments showed that the *cis*-acting signals directing B3 and C3 replication *in vivo* are confined to various noncoding regions (17, 38) and suggested several possible models for the basis of discrimination between B3 and C3. For example, the tRNA-like 3' terminal noncoding regions of BMV and CCMV RNAs (2), which contain signals directing negative-strand RNA synthesis (1, 16, 17, 34, 38), might be major determinants of B3/C3 discrimination; in keeping with the observed *in vivo* asymmetry, a BMV RNA-dependent RNA polymerase extract synthesizes negative-strand RNA from exogenously added BMV RNAs but discriminates against CCMV and other RNAs, whereas the equivalent CCMV extract efficiently utilizes BMV as well as CCMV RNAs for negative-strand RNA synthesis (36, 37).

Differences in the B3 and C3 intercistronic and 5' noncoding regions may also contribute to their differential template activities. Deletion of a 100- to 150-base segment of the B3 intercistronic region significantly inhibits B3 amplification in BMV-infected cells, while the entire C3 intercistronic region can be deleted with little or no effect on C3 amplification in CCMV-infected cells (17, 38). The 5' noncoding regions of B3 and C3 also contain *cis*-acting sequences required for RNA3 amplification (17, 38) and differ markedly in length and sequence (9).

To determine how the various *cis*-acting regions contrib-

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ute to virus-specific differences in RNA3 accumulation, we analyzed the in vivo response of BMV and CCMV replicases to hybrid RNA3 molecules constructed by precisely exchanging individual noncoding regions between B3 and C3. The amplification of these hybrids demonstrates that all three noncoding regions influence virus-specific discrimination between B3 and C3 templates, though to differing extents. The survival of wild-type (wt) and hybrid RNA3s in vivo in the absence of replication suggested that differences in RNA3 accumulation were mainly due to differences in RNA synthesis rather than stability.

MATERIALS AND METHODS

Starting plasmids. Plasmids pB1TP3, pB2TP5, and pB3TP8, when linearized with *EcoRI* and transcribed in vitro with T7 RNA polymerase, give infectious transcripts corresponding to BMV RNAs 1, 2, and 3, respectively (23). *XbaI*-linearized CCMV plasmids pCC1TP1, pCC2TP2, and pCC3TP4, when transcribed in vitro with T7 RNA polymerase, yield infectious transcripts corresponding to CCMV RNAs 1, 2, and 3, respectively (8). pCC3TP8 (11) is a pCC3TP4 derivative in which the C3 3a gene is flanked by a *BamHI* site (created by two site-directed substitutions) and by a naturally occurring *BglII* site as follows: . . . [GGATCC]CGATG . . . TAGCT [AGATCT] . . . , where the start and stop codons of the 3a gene are shown in bold italics, the *BamHI* (5') and *BglII* (3') sites are bracketed, and the bases changed from the wt sequence to create the *BamHI* site are underlined.

The following plasmids were kindly provided by R. Allison and M. Janda (10; see also Fig. 1). pB3TP10 is a pB3TP8 derivative bearing four oligonucleotide-directed base substitutions that create *BamHI* and *BglII* sites flanking the 3a gene in a manner similar to that of pCC3TP8, viz., . . . [GGATCC]CGATG . . . TAGGT[AGATCT] . . . pB3TP13 is a pB3TP8 derivative bearing only the two substitutions creating the indicated 3'-proximal *BglII* site. pCC3TP10 is a pCC3TP4 derivative in which a *Sall* site (introduced by a single silent substitution) and a naturally occurring *Bsp1286I* site overlap the start and stop codons, respectively, of C3 coat gene: . . . AT[GTGCGAC] . . . TA[GTGCCC] Identical sequences and sites occur naturally at the ends of the wt B3 coat gene in pB3TP8, pB3TP10, etc.

pBC3AJ8 is a derivative of pB3TP10 in which the C3 coat gene (taken from pCC3TP10) has been substituted for the B3 coat gene. pBC3AJ10, a derivative of pCC3TP8 and pBC3AJ8, contains both the 3a and coat genes from C3 flanked by the 5', intercistronic, and 3' noncoding regions from B3. pBC3AJ12, a derivative of pCC3TP8, pCC3TP10, and pB3TP10, contains the 3a and coat protein-coding sequences from BMV RNA3 flanked by the 5', intercistronic, and 3' noncoding regions from CCMV RNA3.

Construction of RNA3 hybrids exchanging individual noncoding regions. As described below in detail, six major hybrids were constructed that exchange each noncoding region in turn between B3 and C3. Standard procedures for plasmid constructions were used, with minor modifications (31). The structures of the final hybrids are shown schematically in Fig. 2 through 5.

Hybrids RP18 and RP19, which exchange the 5' noncoding segments, were constructed as follows. Plasmid pB3TP10 was cleaved at a *PstI* site external to the T7 promoter and the 5' end of the BMV cDNA insert, blunted with the Klenow fragment of DNA polymerase I (Boehringer Mannheim), and cleaved at the *BamHI* site adjacent to the 3a start codon, and the resulting 130-bp and 5.15-kb fragments were isolated.

Clone pCC3TP8 was cleaved at a *SmaI* site external to the T7 promoter and the 5' end of the CCMV cDNA insert and at the *BamHI* site adjacent to the 3a start, and the resulting 280-bp and 5.1-kb fragments were isolated. The 280-bp pCC3TP8 fragment was ligated to the 5.15-kb pB3TP10 fragment, yielding pBC3RP18 (hereafter called RP18). The 130-bp pB3TP10 fragment was ligated to the 5.1-kb pCC3TP8 fragment, yielding pBC3RP19 (RP19). The additional hybrid pBC3RP20 (RP20) was made by ligating the 280-bp *SmaI*-*BamHI* pCC3TP8 fragment with the 4.0-kb *PstI*-*ClaI* fragment of pB3HV1, an intercistronic deletion mutant of B3 (17), after all fragment ends were reduced to bluntness with T4 DNA polymerase.

Hybrids AJ14 and AJ15, which exchange the intercistronic segments between B3 and C3, were constructed as follows. pBC3AJ14 (AJ14) was made by ligating the 215-bp *BglII*-*Sall* fragment of pCC3TP10 (containing the entire C3 intercistronic region) with the 5.0-kb *BglII*-*Sall* fragment of pB3TP13. pBC3AJ15 (AJ15) was made by ligating the 1.6-kbp *BamHI*-*SacII* fragment from pBC3AJ10 (containing the C3 3a gene, B3 intercistronic region, and a portion of the C3 coat gene) with the 3.8-kb *BamHI*-*SacII* fragment from pCC3TP8.

Hybrids AJ13 and AJ16, in which the 3' noncoding regions were exchanged, were constructed as follows. pBC3AJ13 (AJ13) was made by ligating the 820-bp *Sall*-*HindIII* fragment from pBC3AJ12 (containing the B3 coat gene linked to the C3 3' noncoding region) with the 4.4-kb *Sall*-*EcoRI* fragment from pB3TP13. pBC3AJ16 (AJ16) was made by ligating the 870-bp *Sall*-*EcoRI* fragment of pBC3AJ8 (containing the C3 coat gene linked to the B3 3' noncoding region) with the 4.45-kb *Sall*-*XbaI* fragment of pCC3TP10. The pBC3AJ8 *EcoRI* site, pCC3TP10 *XbaI* site, pBC3AJ12 *HindIII* site, and pB3TP13 *EcoRI* site, which all occur 3' to the cDNA inserts in these plasmids, were blunted with the Klenow fragment of DNA polymerase I before cleavage with *Sall*.

In vitro transcription and protoplast inoculations. Wild-type C3 plasmid pCC3TP4 and hybrid clones having a 3' end from CCMV were linearized with *XbaI* (8), except for plasmid pBC3AJ13, which was linearized at an immediately downstream *SphI* site since an *XbaI* site exists within the B3 coat protein gene that it contains (see Fig. 2A). Wild-type BMV plasmids and hybrid clones bearing a 3' end from BMV were linearized with *EcoRI* (5). In vitro transcription, isolation of barley protoplasts, RNA inoculations, and protoplast incubations were carried out essentially as described previously except that the inoculated protoplasts were incubated at 30°C for 22 h (8, 17, 28).

RNA isolation and Northern blot analysis. Total nucleic acids were extracted from transfected protoplasts as described previously (17, 28). For Northern (RNA) analysis, DNA was removed with DNase I treatment (31), and 1/10 of the total RNA isolated from approximately 100,000 protoplasts was glyoxalated (33) and separated by electrophoresis on 1.0% agarose gels in 10 mM sodium phosphate (pH 7.0) with constant buffer recirculation. After electrophoresis, the RNAs were transferred overnight onto Hybond N hybridization membrane (Amersham) in 10 mM sodium phosphate (pH 7.0) by using the capillary transfer method (41). Baking, prehybridization, hybridization, and washing of the membrane were carried out as described previously (38). ³²P-labeled RNA probes complementary to the 3' ends of BMV and CCMV positive-strand RNAs were transcribed in vitro as described previously (28) from *HindIII*-linearized pB3HE1 (17) and *EcoRI*-linearized pCC3RA518 (11), respectively. To

FIG. 1. Schematic diagram of relevant restriction sites and *cis*-acting regions in BMV RNA3 (top) and CCMV RNA3 (bottom). The 3a and coat protein (cp) genes are indicated by open or shaded boxes, the surrounding noncoding regions are indicated by lines centered on the boxes, and the oligo(A) tracts within the intercistronic (IC) regions are indicated by solid boxes. The three hatched boxes above the B3 map mark the locations of sequences required in *cis* for efficient B3 amplification in vivo (17), while the two hatched boxes below the C3 map show the regions required in *cis* for C3 amplification (38). As described in the text, B3 and C3 derivatives exist in which the indicated *Bam*HI, *Bgl*II, *Sal*I, and *Bsp*1286I sites immediately flank or overlap the ends of the coding regions, and these sites were used to create a series of hybrids that exchange the 5', intercistronic, and 3' noncoding segments between BMV and CCMV RNA3. Some of the sites shown occur naturally in the wt B3 or C3 sequence and others were engineered by selected, translationally silent, oligonucleotide-directed substitutions (see text).

detect BMV and CCMV negative-strand RNA, 32 P-labeled hybridization probes were prepared by transcription of the opposite strands of *Eco*RI-linearized pB3HE1 and *Xba*I-linearized pCC3RA518, respectively. RNA3 hybrids having the 3' noncoding region from BMV were detected with probes from pB3HE1, while RNA3 hybrids having CCMV 3' ends were detected with probes from pCC3RA518. Autoradiography was performed with Kodak X-Omat AR film and an intensifying screen (Cronex Lightning-Plus; Du Pont) at -80°C .

The amount of RNA3 and RNA4 produced during infection was quantitated by direct scintillation counting of radioactive bands excised from hybridization filters. In each Northern blotting experiment, samples containing known amounts of BMV and CCMV virion RNAs were included and counted to empirically compare the specific activities of the separate BMV and CCMV probes used, and minor data corrections were made as appropriate to allow proper comparison of the various hybrid signals with those of wt B3 and C3. Some of the quantitative data obtained by scintillation counting was rechecked by laser densitometry of autoradiographs, and consistent results were obtained. Each hybrid RNA3 derivative was tested in four or more separate protoplast experiments, and the average value with the standard deviation is reported.

RESULTS

To determine whether the observed virus-specific differences in RNA3 accumulation segregated with one or more particular segments of RNA3, hybrids were constructed between BMV RNA3 (B3) and CCMV RNA3 (C3) by using biologically active cDNA clones from which infectious in vitro transcripts can be produced. Since all sequences required in *cis* for efficient B3 or C3 replication and accumulation in vivo reside in their various noncoding regions (Fig. 1), a series of six hybrid clones was produced that precisely

exchanged each of the three noncoding segments in turn. As described in Materials and Methods, the 3' noncoding regions were exchanged by using naturally occurring, common *Bsp*1286I sites overlapping the 3' ends of the BMV and CCMV coat genes. The 5' and intercistronic noncoding segments were exchanged by using restriction sites previously introduced into equivalent positions in B3 and C3, immediately flanking both ends of the 3a gene and overlapping the start of the coat gene (Fig. 1). For minimal sequence disruption, each of these sites was created by only one or two oligonucleotide-directed, translationally silent base substitutions (see Materials and Methods), and B3 and C3 derivatives bearing one or more of these sites all displayed wt levels of RNA3 accumulation when assayed in protoplasts with the homologous RNAs 1 and 2 (10, 11). The names and schematic structures of the individual hybrids are given in Fig. 2 through 5.

The accumulation of each chimeric RNA3 was examined in barley protoplasts coinoculated with RNAs 1 and 2 of BMV or CCMV (hereafter designated B1+B2 and C1+C2, respectively). For each hybrid, accumulation of RNA3 and RNA4 was measured by Northern blot analysis as described in Materials and Methods and was averaged over four to eight independent experiments. To account for occasionally observed variations in inoculation efficiency between independent inoculations of the same RNA combination, RNA3 levels were normalized to the internal standard of wt RNAs 1 and 2 in each infection. These normalized RNA3 results are summarized in histograms in Fig. 2 through 5, together with representative Northern blot primary results. RNA4 levels were normalized to those of RNA3 because both are synthesized from the same full-length, negative-strand RNA3 template (35). The few cases of significant alteration in RNA4:RNA3 ratios are discussed in the text, and the total set of RNA4 values measured are presented in Table 1.

C3-like accumulation with B1+B2 segregates with C3 intercistronic region. The effects of B3/C3 sequence exchanges on RNA3 amplification were first examined in cells coinoculated with B1+B2. To determine whether any C3 noncoding segment(s) confers a C3-like low-accumulation phenotype on B3, we began by testing hybrids RP18 and AJ13, which have the B3 5' and 3' noncoding regions, respectively, replaced with the corresponding regions from C3 (Fig. 2A). Northern blots showed that both RP18 and AJ13 were well amplified by B1+B2 (Fig. 2B). When the blot results were quantitated and averaged over a number of experiments, RP18 and AJ13 showed approximately twofold reductions from wt B3 in the RNA3:RNA1+2 ratio, although the statistical significance of the difference between AJ13 and wt B3 was small (Fig. 2A). The effects of the two exchanges on subgenomic RNA4 accumulation differed (Fig. 2B and Table 1). For RP18, there was an over threefold increase in the RNA4:RNA3 ratio. For AJ13, the RNA4:RNA3 ratio was mildly depressed.

As illustrated by the Northern blot of Fig. 2B, much greater inhibition of RNA3 accumulation occurred with hybrid AJ14, in which the B3 intercistronic region was replaced with the corresponding C3 region. In this case, the relative accumulation of RNA3 and RNA4 decreased fivefold compared with that of wt B3 (Fig. 2A). The low RNA3 accumulation obtained with AJ14 was very similar to that of wt C3 and also to that of B3 intercistronic deletion mutant HV1 (17), which lacks a 130-base segment encompassing the *cis*-acting B3 intercistronic region mapped in Fig. 1. Figure 2B confirms that the HV1 intercistronic deletion induces a marked inhibition of RNA3 accumulation relative to that of

FIG. 2. In vivo amplification of predominantly B3-derived hybrids after coinoculation with B1+B2 transcripts. (A) The table on the left depicts the structures of wt B3, wt C3, and the relevant RNA3 hybrids. The five columns headed 5', 3A, IC, cp, and 3' correspond to the RNA3 segments defined in Fig. 1. BMV-derived segments are denoted by B, and CCMV-derived segments are denoted by C. CCMV-derived segments in the BMV-like RNA3 recombinants are underlined, and a hyphen in the IC column indicates the deletion of that segment in the corresponding RNA3. To the right of each entry in the table is a bar graph showing, for that hybrid, the level of in vivo RNA3 accumulation relative to the internal standard of coinoculated RNA1 and RNA2. The length of the solid bar represents the mean value from four or more independent protoplast experiments, such as that shown in panel B, and the length of the thin line denotes the standard deviation. The value for each experiment was determined by taking the ratio of the ^{32}P activity in the RNA3 band excised from the Northern blot to the ^{32}P activity in the RNA1 and RNA2 bands jointly excised from the same lane of the blot. Minor corrections for activity differences between the BMV and CCMV probes were made as indicated in Materials and Methods. (B) Northern blot analysis showing amplification of viral RNAs in protoplasts inoculated with in vitro transcripts from wt BMV RNA1 and RNA2 cDNA clones (B1+B2) and with transcripts from cDNA clones of the indicated RNA3 hybrids. The lane headed with a minus sign contains RNA from protoplasts inoculated with RNA1 and RNA2 transcripts but no RNA3 transcript, and lane M (mock) contains RNA from protoplasts inoculated with water only. Total protoplast RNA was glyoxalated before electrophoresis, and the final membrane was hybridized with a mixture of BMV and CCMV probes to detect positive-strand viral RNA as described in Materials and Methods. The positions of progeny viral RNAs are indicated at left. Each lane contains 1/10 of the RNA extracted from 100,000 inoculated protoplasts. Here and in the remaining figures, equal exposures of all wt and recombinant RNA3 lanes are shown unless indicated otherwise.

wt B3, although the specific level of inhibition measured in our experiments (Fig. 2A) was somewhat less than that seen in the earlier study. This quantitative difference may reflect the higher protoplast incubation temperature used in the experiments reported here or other unidentified differences in protoplast inoculation or RNA isolation.

The region of C3 with the greatest sequence similarity to

the *cis*-acting RNA amplification element within the B3 intercistronic region is a subset of the 5' noncoding sequences required for C3 amplification (9). Conceivably, this similarity might reflect the presence in the C3 5' noncoding region of elements functionally related to both the 5' and intercistronic regions of B3, which would explain the observed differences in the organization of B3 and C3 *cis*-acting

TABLE 1. Ratios of subgenomic RNA4 to genomic RNA3 for various hybrid RNAs

RNA3 ^a	RNA4:RNA3 ^b with:	
	B1+B2	C1+C2
wt B3	1.2	0.7
wt C3	1.3	0.8
RP18	3.9	1.8
AJ14	1.4	1.1
AJ13	0.7	0.5
HV1	1.0	1.0
RP20	1.5	0.8
RP19	1.2	1.2
AJ15	1.3	0.6
AJ16	1.1	1.0

^a Hybrid RNA3 structures are given in Fig. 2 and 3.

^b Ratio of ³²P counts in the RNA4 band to ³²P counts in the RNA3 band of suitable Northern blots as shown in Fig. 2 through 5. Each table entry is the average of values from four to eight experiments.

sequences (Fig. 1). If so, then the C3 5' noncoding region might at least partially compensate for the accumulation defect in B3 intercistronic deletion mutants such as HV1. To test this possibility, we constructed and tested hybrid RP20, in which the 5' noncoding region of HV1 was replaced by the C3 5' noncoding region (Fig. 2A). Because of the plasmid construction approach used, hybrid RP20 also lacks a 5' portion of the 3a gene, which was shown previously to have little or no effect on the accumulation of either B3 or C3 (17, 38). In cells coinoculated with RP20 and B1+B2, accumulation of RNA3 and RNA4 was even lower than that seen with HV1 or AJ14 (Fig. 2B).

B3-like accumulation with B1+B2 does not segregate with any individual B3 segment. Because the most obvious differences between B3 and C3 templates occur in the intercistronic region, we next asked whether C3 accumulation could be enhanced in BMV-infected cells by replacing the intercistronic region with that of B3, as in hybrid AJ15. AJ15 accumulation relative to B1+B2 was not significantly different from that of wt C3 (Fig. 3A and B). The RNA4:RNA3 ratio was also essentially unchanged (Table 1). Nevertheless, while the relative RNA levels were unchanged, the absolute level of all viral RNAs in cells inoculated with B1+B2+AJ15 was enhanced over cells inoculated with B1+B2+C3 for all experiments conducted (Fig. 3B). The possible significance of this observation is currently unclear.

We then tested the behavior of hybrids RP19 and AJ16, in which the B3 5' and 3' noncoding regions, respectively, were transferred to C3. RP19 accumulation was consistently difficult to visualize on Northern blots (Fig. 3B), with measurements indicating an average drop of at least twofold compared with the already low accumulation of C3 with B1+B2. Compared with C3, AJ16 showed a 30% reduction in the average RNA3:RNA1+2 ratio. The RNA4:RNA3 ratio remained near that of wt C3 in both cases (Table 1). Thus, none of the individual B3 noncoding segments notably improved the relative accumulation of C3 in BMV-infected cells.

The intercistronic region contributes to B3 accumulation in C1+C2 infected protoplasts. Having examined the results of coinoculating the hybrid and mutant RNAs with B1+B2, we next coinoculated them with C1+C2 (Fig. 4 and 5). The average C3:C1+C2 ratio is not significantly different from the B3:B1+B2 ratio (note the higher scale of Fig. 4A and 5A

relative to that of Fig. 2A and 3A), while the B3:C1+C2 ratio is even higher, due in part to an apparently competitive decrease in C1+C2 levels in the presence of B3 (8, 42). Since the intercistronic region makes no apparent contribution to the amplification of C3 by C1+C2 (38), it was not clear whether the efficient amplification of B3 by C1+C2 was dependent on or independent of the *cis*-acting intercistronic sequences that are important for B3 amplification by B1+B2 (17). Conceivably, CCMV replicase might differ from BMV replicase in being insensitive to the presence or nature of the intercistronic region in B3 templates. To investigate this issue, we tested hybrid AJ14, which substitutes the C3 intercistronic region for that of B3, and B3 intercistronic deletion mutant HV1. Similar to their behavior with B1+B2, CCMV-directed accumulation of both derivatives was suppressed 10-fold or greater relative to that of wt B3 (Fig. 4A and B). Hybrid RP20, which substitutes the C3 5' noncoding region into HV1, did not significantly improve RNA3 accumulation over HV1 (Fig. 4).

We then examined the contribution of 5' and 3' noncoding regions to the high amplification of B3 in CCMV-infected cells. The effects of 5' and 3' substitutions on B3 amplification by B1+B2. As shown in Fig. 4, these changes induced only 30 to 50% average reductions in the RNA3:RNA1+2 ratio, still leaving strongly visible RNA3 signals. Substitution of the C3 5' end (hybrid RP18) increased the RNA4:RNA3 ratio by over twofold compared with that of B3, while substitution of the C3 3' end (hybrid AJ13) lowered the RNA4:RNA3 ratio (Fig. 4B; Table 1).

High amplification of B3 relative to C1+C2 segregates with B3 intercistronic region. Figure 5 shows the results of inoculating C1+C2 with those hybrids having predominantly C3-derived sequences. Just as had been observed with B1+B2, substituting the B3 3' noncoding region for its counterpart in C3 (hybrid AJ16) decreased RNA3 accumulation by approximately 50% compared with that of wt C3, while transferring the B3 5' noncoding region onto C3 (hybrid RP19) produced an even more substantial decline in RNA3 levels (Fig. 5B). In the presence of C1+C2, transfer of the B3 intercistronic region into C3 (hybrid AJ15) increased the RNA3:RNA1+2 ratio 3.5-fold over that of wt C3, yielding a ratio similar to that of wt B3 (Fig. 5A). This was a significant difference from the results for AJ15 coinoculation with B1+B2, for which the corresponding ratio was not significantly higher than that of wt C3 (Fig. 3).

Effects of noncoding segment exchanges on negative-strand RNA accumulation. To see if the hybrid-induced variations in positive-strand RNA3 and RNA4 accumulation were associated with altered negative-strand RNA accumulation, the *in vivo* levels of negative-strand RNA were determined. In wt BMV infections, negative-strand RNA accumulation is 50- to 100-fold lower than that of positive-strand RNA (17, 30). As shown in Fig. 6, the levels of negative-strand RNA in wt CCMV infections are somewhat lower than for BMV. Figures 6A and B also show that for most hybrids negative-strand RNA results paralleled the positive-strand RNA results described above. Most infections showing reduced levels of positive-strand RNA3 accumulation, for example, also showed similarly reduced levels of negative-strand RNA3. These include C3, HV1, RP20, AJ14, RP19, AJ15, and AJ16 infections with B1+B2 and HV1, RP20, RP18, AJ14, and RP19 infections with C1+C2. One deviation was found with AJ16 in the presence of C1+C2, for which the RNA3:RNA1+2 ratio appeared higher for nega-

FIG. 3. In vivo amplification of predominantly C3-derived hybrids after coinoculation with B1+B2 transcripts. (A) The structures of the relevant hybrids are represented in tabular form as described in the legend to Fig. 2A. BMV-derived segments in the CCMV-like RNA3 recombinants are underlined. (B) Northern blot analysis showing amplification of the indicated RNA3 hybrids after coinoculation with C1+C2 transcripts. Lane labels and other features are as described in the legend to Fig. 2B. The lane marked B3 was exposed threefold less than the remaining lanes because, in the experiment shown, the overall signal in this lane was unusually high relative to that of the C3 lane and other inoculations (see Results regarding occasional variations in inoculation efficiency). See lanes B3 and C3 of Fig. 2B (and similar figures in references 8 and 42) to confirm that the resulting image approximates the typical relative intensity of B3 and C3 in these inoculum combinations.

tive-strand RNA (Fig. 6B) than for positive-strand RNA (Fig. 5).

Recently it has been observed that negative-strand B1+B2 accumulate to higher levels in the absence of B3 (26). Figure 6 confirms this and shows that B1+B2 and C1+C2 accumulations are also enhanced when an RNA3 is present but amplified to low levels, as for C3, HV1, RP20, AJ14, RP19, and AJ16 infections with B1+B2 and AJ14, RP19, HV1, and RP20 infections with C1+C2.

Figure 6 also confirms previous observations (12, 30) that the amount of negative-strand RNA migrating at the position of subgenomic RNA4 is small compared with the level of negative-strand genomic RNA3 for B3 and C3, as well as for all hybrids save AJ13. Although the positive-strand RNA4 level was mildly depressed in AJ13 infections with either

B1+B2 or C1+C2 (Fig. 2B and 4B; Table 1), AJ13 was exceptional in showing negative-strand RNA4 signals at least as intense as those for RNA3 (Fig. 6A). AJ13 was also the only hybrid in this study for which in vitro transcripts were produced from DNA templates with 3' protruding ends (see Materials and Methods). This can lead to the synthesis of additional RNA complementary to the desired runoff transcription product (40), and for AJ13 two higher-molecular-weight in vitro transcripts were consistently observed in addition to the expected RNA3-sized product (results not shown). Negative-strand transcripts or consequent double-stranded RNA (dsRNA) formation in the AJ13 inoculum may have contributed artifactually to the high RNA4 negative-strand signal (see next section and Discussion).

In vivo stability of the hybrid RNAs. Most of the RNA3

FIG. 4. In vivo amplification of predominantly B3-derived hybrids after coinoculation with C1+C2 transcripts. (A) The structures of the relevant hybrids are represented in tabular form as described in the legend to Fig. 2A. Note that the scale of the histograms in this panel and in Fig. 5A are expanded compared with that of the histograms of Fig. 2A and 3A. (B) Northern blot analysis showing amplification of the indicated RNA3 hybrids after coinoculation with C1+C2 transcripts. Lane labels and other features are as described in the legend to Fig. 2B.

hybrids were amplified and accumulated to significant levels after coinoculation with either B1+B2 or C1+C2 or with both. However, some hybrids, such as RP19, AJ14, and HV1, did not accumulate to significant levels with either B1+B2 or C1+C2. The low accumulation of these hybrids might result from intrinsically low RNA stability rather than low activity as a replication template. To compare in vivo RNA stabilities, in vitro transcripts of the wt B3, wt C3, and hybrid RNA3 cDNA clones were inoculated into separate protoplast samples in the absence of RNAs 1 and 2 to preclude RNA replication. The level of surviving RNA was examined at 2 and 20 h postinoculation by long exposures of Northern blots of total protoplast RNA, and the survival of most hybrid RNA3 transcripts paralleled or exceeded the survival of wt B3 and C3 transcripts (Fig. 7). The unusual behavior of AJ13 formed a special case, which is considered below. Among the remaining hybrids, high levels of HV1 and RP19 and a lower level of AJ14 were particularly notable in Fig. 7, since RNA stability was a specific issue for these hybrids. However, while such differences were consistent

between time points in the same experiment, they were not consistent between experiments, suggesting that they reflect variations in inoculation efficiency. Upon independent repetition of the experiment, the AJ14, HV1, RP19, and wt B3 bands were all of equal intensity.

AJ13 produced unexpectedly complex results in these assays (Fig. 7). The level of full-length AJ13 RNA3 transcript recovered was less than that of the other hybrid RNA3s, and two major lower-molecular-weight bands were seen. The lower of these bands migrates at the position of RNA4 and is markedly stronger than any other band in the experiment. Because such a band is not visible in the initial AJ13 in vitro transcripts (results not shown) and because the lack of RNAs 1 and 2 prevents in vivo viral RNA synthesis, the RNA4-like band must arise from processing of the input transcripts in the cell. As considered in Discussion, this RNA4-like band and the high AJ13 negative-strand RNA4 signal of Fig. 6 might be related artifacts arising from the processing of unwanted dsRNA present in AJ13 in vitro transcripts.

FIG. 5. In vivo amplification of predominantly C3-derived hybrids after coinoculation with C1+C2 transcripts. (A) The structures of the relevant hybrids are represented in tabular form as described in the legend to Fig. 2A. (B) Northern blot analysis showing amplification of the indicated RNA3 hybrids after coinoculation with C1+C2 transcripts. Lane labels and other features are as described in the legend to Fig. 2B.

DISCUSSION

To define which sequences contribute *in cis* to the observed BMV- and CCMV-specific differences in RNA3 accumulation, we exchanged each of the 5', 3', and intercistronic sequences between B3 and C3 and determined the effects of these exchanges on genomic RNA3 and subgenomic RNA4 levels in cells coinoculated with either BMV or CCMV RNAs 1 and 2. Exchanges in all three noncoding regions affected B3 and C3 accumulation, but to varying degrees. Symmetric exchanges between B3 and C3 in the 5' or intercistronic regions, moreover, produced some significantly asymmetric effects. Upon inoculation in the absence of RNAs 1 and 2, the persistence of the input hybrid RNAs relative to the persistence of wt B3 and C3 suggested that the changes in RNA3 accumulation were mainly due to differences in RNA synthesis rather than RNA stability, although it is possible that the surviving RNA inoculum followed in the stability assays does not have the same intracellular distribution as actively replicating viral RNA.

Effects of 3' exchanges. While 3' exchanges had some definite effects, the 3' noncoding regions were found to be neither the sole nor the major determinants of the observed differences between B3 and C3 amplification *in vivo*. Transfer of the B3 3' end onto C3, for example, failed to improve the poor amplification of C3 in BMV-infected cells, producing instead a modest reduction. Converse transfer of the C3 3' end onto B3 had relatively little effect on the *in vivo* replicative advantage of B3, particularly with B1+B2. On average, a small reduction in RNA3 accumulation was seen with this change, but the level of this reduction was not of great significance in statistical terms and was clearly much less than the effect of transferring the C3 intercistronic region into B3 (hybrid AJ14, Fig. 2B and 4B). Thus, the effects induced by 3' exchanges were relatively small compared with those of other exchanges and were not polarized as expected for elements controlling wt B3 and C3 behavior.

Significant levels of *in vivo* replication have also been found for chimeras constructed by replacing the 3' noncod-

FIG. 6. Northern blot analysis of negative-strand viral RNA accumulation in protoplasts inoculated with chimeric RNA3s and wt B1+B2 or C1+C2 transcripts. (A and B) Protoplasts were inoculated with transcripts of the indicated wt or hybrid RNA3 cDNA clones in combination with transcripts of RNAs 1 and 2 from wt BMV (B1+B2) or wt CCMV (C1+C2) cDNA clones. Lanes marked with a minus sign represent inoculation with RNAs 1 and 2 in the absence of RNA3, while lanes marked M show the results of mock inoculation with water. Total protoplast RNA was glyoxalated before electrophoresis, and the final membrane was hybridized with a mixture of BMV and CCMV probes to detect negative-strand viral RNA as described in Materials and Methods. The positions of the various negative-strand viral RNAs are indicated at left. Each lane contains 1/10 of the total RNA extracted from 100,000 inoculated protoplasts. The structures of the hybrids are shown in Fig. 2 and 3. For ease of comparison, the lanes are grouped in the same patterns used for positive-strand analysis in the B panels of Fig. 2 through 5.

ing region of tobacco mosaic virus strain L with diverged 3' regions from other tobacco mosaic virus strains (22). Both bromovirus and tobamovirus RNAs have tRNA-like, aminoacylatable 3' ends, which for BMV are known to interact with tRNA nucleotidyl transferase, aminoacyl tRNA synthetase, and a translation elongation factor (13). The interaction of these 3' ends with a variety of host factors may constrain their structural and functional plasticity, leading to the observed conservation of 3'-proximal RNA secondary and tertiary structure within each group (2, 44) and perhaps in consequence favoring functional recognition by replicases from other viruses in the same group. Alternatively, replicase interaction with these tRNA-like 3' ends might be mediated or assisted by one or more of the host factors that interact with these ends.

Effects of intercistronic exchanges and deletions on B3 and

C3 amplification. The highest levels of RNA3 amplification relative to either B1+B2 or C1+C2 were associated with RNA3 derivatives containing the B3 intercistronic region. Moreover, deleting the intercistronic region of B3 or replacing it with that of C3 greatly decreased RNA3 amplification with both viral replicases. The intercistronic region thus appears to make a significant contribution to the differential behavior of B3 and C3 in both BMV- and CCMV-infected cells.

These results show that even though CCMV replicase is insensitive to the presence or absence of the intercistronic region of its cognate template, C3 (38), it responds to features present in the intercistronic region of B3. The different primary sequence organization of *cis*-acting amplification signals in B3 and C3 (Fig. 1; 17, 38) thus does not necessarily imply a significant difference in the structures or

FIG. 7. Persistence of wt B3, wt C3, and RNA3 hybrids following transfection in the absence of RNAs 1 and 2. Protoplasts were inoculated with individual *in vitro* transcripts from cDNA clones of the indicated wt and hybrid RNA3s. After incubation, total protoplast RNA was extracted, glyoxal denatured, electrophoresed, and transferred to a membrane that was hybridized with a mixture of BMV and CCMV probes to detect positive-strand viral RNA3. Arrows at right indicate the positions in the AJ13 lane of the expected full-length AJ13 RNA3 band (top) and the two unexpected lower-molecular-weight bands (x; see text). The figure shows protoplast RNA isolated at 2 h postinoculation; RNA isolated at 20 h postinoculation gave the same relative band intensities but a much fainter overall pattern in which some bands were difficult to reproduce photographically. The autoradiograph was exposed for 3 days at -80°C with an intensifying screen. The length of this exposure is approximately 72-fold greater than those of the exposures shown in Fig. 2 through 5 but comparable to that of the exposure shown in Fig. 6. Each lane contains 1/6 of the RNA extracted from 100,000 inoculated protoplasts. The structures of the hybrids are shown in Fig. 2 and 3.

the processes by which BMV and CCMV replicases interact with their templates. RNA looping mediated by interactions between RNA-bound proteins might allow some bromovirus regulatory sequences to function in a relatively position-independent manner (18).

Asymmetric effects of 5' exchanges. The B3 and C3 5' noncoding regions show significant differences in length (91 versus 238 bases) and sequence, suggesting possible specialized interactions with their respective replicases (9). Transferring the C3 5' end onto wt B3 had little effect on RNA3 accumulation, whereas the converse transfer of the B3 5' end onto C3 substantially decreased RNA3 accumulation, particularly with C1+C2 (hybrid RP19 in Fig. 5B). This latter result was not readily predictable, since wt B3 achieves higher RNA3:RNA1+2 ratios than C3 with both B1+B2 and C1+C2.

The inability of the B3 5' noncoding segment to support significant RNA3 amplification in an otherwise C3 context shows that its contribution is dependent on or strongly influenced by another segment(s) of RNA3. For example, the B3 5' end might encode only some of the functions found in the C3 5' end, with the remainder provided by the *cis*-acting B3 intercistronic region (Fig. 1). The alternate possibility, that the adjacent CCMV 3a gene sequence in RP19 creates an inhibitory local sequence or structural context for the B3 5' end, appears unlikely since B3 derivatives in which the 3a gene has been replaced with that of CCMV are amplified in protoplasts to much higher levels than RP19, although they contain the same B3 5'/C3 3a gene junction (10). The B3 5' noncoding sequence also supports efficient RNA3 accumulation when fused to arbitrary internal sites in the BMV 3a gene (17).

Effects of exchanges on relative levels of subgenomic RNA4 and genomic RNA3. wt B3 and C3 gave RNA4:RNA3 ratios of 1.2 and 1.3, respectively, when inoculated with B1+B2 and ratios of 0.7 and 0.8 when inoculated with C1+C2 (Table 1). Thus, although B1+B2 directs higher RNA4 production than C1+C2, the BMV and CCMV subgenomic promoters appear functionally equivalent in both systems. This is consistent with the highly similar core promoter sequences in the two viruses (18), although an oligo(A) tract and upstream activating elements that are also important to the level of B3-directed subgenomic RNA synthesis are altered in C3 (9).

Most of the RNA3 hybrids produced RNA4:RNA3 ratios similar to those of wt B3 and C3, with a few exceptions (Table 1). AJ13, for example, showed reduced RNA4:RNA3 ratios. The most notable difference was shown by hybrid RP18, which consistently produced an unusually high RNA4:RNA3 ratio, particularly in cells coinfecting with B1+B2 (Fig. 2B; Table 1). Because RNA3 and RNA4 are both copied from negative-strand RNA3 (35), the altered balance with RP18 might result from uneven competition between the presumably normal efficiency of RNA4 initiation from the B3 subgenomic promoter in its wt context versus the potentially reduced efficiency of genomic RNA3 initiation at the heterologous C3 5' end.

High RNA4 signals in AJ13 RNA stability and negative-strand assays. In both RNA3 stability assays and negative-strand RNA assays, hybrid AJ13 showed unexpected major bands comigrating with RNA4. The similar migration and absolute intensities of these bands (see Fig. 7 legend) and other considerations suggest that they may be related artifacts arising from the *in vitro* transcripts used as AJ13 inoculum. As noted above, the RNA4-like band seen in AJ13 inoculum survival experiments (Fig. 7) must result from *in vivo* processing of some fraction of the AJ13 *in vitro* transcripts, which are likely to contain some unwanted AJ13 negative-strand transcripts and dsRNA because of the use of AJ13 DNA templates with 3' protruding ends (40; see also Materials and Methods). The most weakly base-paired region in AJ13 dsRNA would be the 18-base oligo(A) tract just upstream of the RNA4 start site (Fig. 1; 6, 18). This region may open sufficiently for attack by intracellular nucleases, which would produce a dsRNA4-like product that may be more stable than single-stranded RNA *in vivo* and would explain the high RNA4 signal intensity in Fig. 7. After RNA extraction from protoplasts, the glyoxal denaturation used prior to electrophoresis would release positive- and negative-strand RNA4-like species, which would be detected with the strand-specific 3'-terminal probes used in the blots in Fig. 6 and 7. An additional band migrating between AJ13 RNA3 and the RNA4-like species in Fig. 6 and 7 has a mobility consistent with residual dsRNA4.

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