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Requirement for a Viral *trans*-Acting Factor Encoded by Brome Mosaic Virus RNA-2 Provides Strong Selection In Vivo for Functional Recombinants

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Interaction of specific nucleotide sequences with *trans*-acting proteins is intrinsic to replication of viral as well as eucaryotic genomes. Brome mosaic virus RNA-2 encodes one of the two viral proteins known to be essential for replication. (R. French, M. Janda, and P. Ahlquist, Science 231:1294–1297, 1986; P. A. Kiberstis, L. S. Loesch-Fries, and T. C. Hall, Virology 112:804–808, 1981). Transfection of barley protoplasts with wild-type transcripts of brome mosaic virus RNA-1 and RNA-3 and serial dilutions of RNA-2 transcripts possessing unaltered coding sequences but bearing mutations that greatly incapacitated replication of RNA-2 revealed that trace amounts of RNA-2 are sufficient to support replication of the viral genome. In six replicate experiments containing RNA-2 transcripts devoid of the 3′ 200 nucleotides that encompass the tRNA-like structure containing the minus-strand promoter, detectable levels of progeny RNA-1 and RNA-3 and subgenomic RNA-4 were present. This showed that viral p2 protein translated from the supplied RNA-2 functioned in *trans* to support replication of RNA-1 and RNA-3. However, in two similar experiments, progeny RNA-2 with electrophoretic mobility indistinguishable from that of wild-type RNA-2 was seen at 24 h postinoculation. Northern hybridization (RNA blot) analysis confirmed the presence of the tRNA-like 3′ terminus on these progeny RNAs, indicating that recombinational restoration of the deleted sequence had occurred. This suggests that, under certain circumstances, RNA recombination may be a rapid and frequent phenomenon.

Considerable insight into the replication processes of brome mosaic virus (BMV) RNA has been obtained through functional analyses in vitro of transcripts of cDNA clones containing defined mutations in the 3′ sequence common to each of the viral RNA components (3, 4). BMV RNA-1 and RNA-2 encode proteins 1a and 2a, respectively, and are known to be necessary for viral replication (8, 9). Consequently, incorporation of mutations debilitating one or more of the tRNA-like functions (aminoacylation, nucleotidyl transferase, minus-strand promoter activity) into the RNA-2 sequence was thought likely to have drastic effects on replication of this component but unlikely to affect its ability to be translated upon entry into the host cells. Using this approach, we substantiated the ability of RNA-2 to be translated prior to replication and showed that trace amounts of RNA-2 are effective in supporting viral replication.

Expression of replication-deficient mutants of BMV RNA-2 in barley protoplasts. Capped full-length transcripts corresponding to each of the three genomic RNAs of BMV were synthesized in vitro by using T7 RNA polymerase (6). Three mutations (5′PsK, 5′+3′PsK, and Δknob) (Fig. 1) present on the cDNA clone of BMV RNA-3 [pT7B3(-Tth)] (6) were introduced into a full-length cDNA clone of BMV RNA-2 (pT7B2) (6) as *Hin*dIII-*Bam*HI fragments (6, 17). Protoplasts were isolated from 6-day-old barley plants and transfected in the presence of polyethylene glycol (11) with mixtures of transcripts containing wild-type RNA-1 and RNA-3 and wild-type or mutant RNA-2. The relative accumulation of plus- and minus-sense progeny RNAs was studied by Northern (RNA) hybridization, using duplicate blots and RNA probes of the desired specificity (6, 17). Transfection of barley protoplasts with wild-type BMV genomic RNA-1, RNA-2, and RNA-3 transcripts resulted in the accumulation of progeny plus- and minus-sense RNAs (Fig. 2a and b, lanes 1). Inoculations containing RNA transcribed from 2/3t RNA-2, a clone bearing the 200 3′-terminal nucleotides from RNA-3, showed wild-type levels of infectivity, confirming that the presence of A instead of G at position 44 (1) in the 2/3t RNA-2 sequence had no detectable effect (Fig. 2a and b, lanes 2). In previous studies (3, 4) Dreher and Hall have characterized tyrosylation, adenylation, and minus-strand promoter functions in vitro of the three mutations (Fig. 1) that were introduced into RNA-2. (i) Mutation 5′PsK disrupts base pairing of the 3′ amino acid acceptor stem; in vitro tyrosylation, adenylation, and minus-strand promoter activities were 0, <1, and 8% of the wild-type activities, respectively. (ii) Mutation 5′+3′PsK restores base pairing but introduces a transversion of three bases; in vitro activities were 54, 282, and 71% of the wild-type activities, respectively. (iii) The Δknob mutation deletes a small (4-base) loop that is common to the putative anticodon arm of several viral tRNA-like structures (3); in vitro activities were 98, 8, and 6% of the wild-type activities, respectively. Barley protoplasts transfected with mixtures of RNA transcripts containing these RNA-2 mutants yielded levels of plus- and minus-sense RNA-2 that were less than 5% of the wild-type levels (Fig. 2a and b, lanes 3 through 5).

The coding region of RNA-2 was not altered by these mutations, and sufficient viral protein 2a was translated in the protoplasts to function in *trans* to permit the accumulation of progeny RNA-1, RNA-3, and RNA-4 at nearly wild-type levels (Fig. 2). However, in the absence of RNA-2 no replication of inoculum containing RNA-1 and RNA-3 occurred (Fig. 2a and b, lanes 6).

To determine the minimal amount of RNA-2 required to permit replication, protoplasts were transfected with inocula containing serial dilutions of RNA-2 transcripts bearing the 3′ mutations. The profile obtained for mutant 5′PsK (Fig. 2c)

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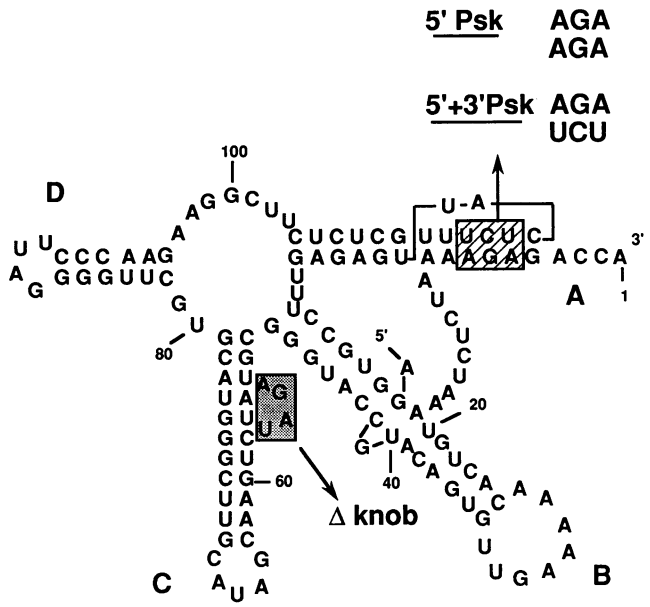

FIG. 1. Secondary structure of the 3′ region conserved in each of the four BMV RNAs. The sequence shown is that of RNA-3 into which the mutations we studied have been introduced. The nucleotides are numbered from the 3′ end, and the arms are identified by the letters A to D. The sequence substitutions for the 5′PsK and 5′+3′PsK mutations are indicated. For the Δknob mutation, the stippled area indicates the deletion of 4 nucleotides from arm C (between bases 52 and 57).

was similar to the profiles obtained for mutants 5′+3′PsK and Δknob (data not shown). Even at the lowest level used (1.9 ng/2 × 10^5 protoplasts), the detection of subgenomic RNA-4 (Fig. 2c, lane 7) showed that replication of RNAs occurred. These data suggest that the amount of protein 2a produced when RNA-2 mutant transcripts were supplied at a concentration $<6 \times 10^3$ molecules per protoplast was sufficient to support replication of RNA-1 and RNA-3. They also show that amplification of RNA-2 is not necessary for BMV replication. In all likelihood, only a small fraction of the supplied RNA-2 molecules entered the protoplasts and survived nuclease degradation prior to translation.

BMV RNA-4 is generated from the internal subgenomic promoter on RNA-3 (7, 13, 14) and is a very efficient messenger for viral coat protein in vitro (16). However, no viral proteins are translated from BMV RNA-4 or from BMV RNA-3 (which also encodes protein 3a, which is thought to be involved in cell-to-cell transport [5]) when the RNA is supplied alone as an inoculum to barley protoplasts, even at concentrations of up to 200 μg/ml (9). Consequently, the detection of viral coat protein in protoplasts transfected by inocula containing the 5′PsK RNA-2 dilution series (Fig. 2d) provides strong supporting evidence that very low levels of protein 2a are capable of mediating in *trans* the replication of fully functional BMV RNA molecules.

Expression in barley protoplasts of RNA-2 mutant lacking 3′ tRNA-like region. Although the data described above and data from previous experiments (8, 9) strongly imply that protein 2a is translated from BMV RNA-2 supplied as an inoculum to barley protoplasts, the low residual capacity for replication of the mutants tested does not rigorously exclude the possibility that the input RNA-2 is replicated prior to translation. To directly address this, a cDNA clone of RNA-2 (Δt RNA-2) completely incapacitated in replication was constructed by deleting the 3′ 200 nucleotides (containing the minus-strand promoter) from pT7B2 as a *Hin*dIII-*Bam*HI fragment (6) and was treated with mung bean nuclease prior to ligation and transformation into *Escherichia coli* JM101. The absence of the 3′ region was confirmed by agarose gel electrophoresis (Fig. 3a) and Northern hybridization analysis (Fig. 3b).

In six of eight independent experiments (Fig. 3c), transfection of barley protoplasts with RNA-1, RNA-3, and Δt RNA-2 yielded progeny RNA-1 and RNA-3 but no RNA-2. The level of minus-sense RNAs corresponding to RNA-1 and RNA-3 remained very low and required extended autoradiographic exposure for visualization (data not shown). Although a portion of the detected plus-sense RNAs might represent input inoculum (see below), the presence of subgenomic RNA-4 (Fig. 3c) indicated that the RNAs detected resulted from de novo synthesis. These data unequivocally demonstrated the ability of Δt RNA-2 to be translated without replication. The levels of RNA-1 and (especially) RNA-4 were lower at all times examined for experiments in which we used the Δt RNA-2 mutant than for all experiments that included the replication-defective mutants (Fig. 1). Presumably, whereas the low level of replication of the debilitated RNA-2 mutants provided sufficient protein 2a template to sustain the production of RNA-1, RNA-3, and RNA-4 at near-wild-type levels, the complete absence of Δt RNA-2 replication led to rapid depletion of protein 2a and consequently much lower levels of replication of the other RNAs.

In the two other experiments, when a probe specific for the viral 3′ tRNA-like structure was used, hybridization to RNA migrating at the position of wild-type RNA-2 was observed for progeny arising after 24 h postinoculation (p.i.) (Fig. 3d). Probing the same blot with labeled RNA complementary to the coding region of RNA-2 revealed traces of input Δt RNA-2 transcript up to 10 h p.i. (Fig. 3e). Both this band and a band that comigrated with wild-type RNA-2 were visible in the progeny of 24-, 48-, and 72-h-p.i. samples (Fig. 3e). Support for the belief that the more slowly migrating band represents a functionally restored RNA-2 sequence was provided by detection of elevated levels of progeny RNA-4 sequences at 24, 48, and 72 h p.i. (Fig. 3d), presumably because of the translation of the newly replicated RNA-2.

Thus far, we have not been successful in obtaining direct sequence information for the putative recombinant RNA-2 generated in these experiments. A trivial explanation of our results would be the presence of trace levels of wild-type RNA-2 in our incubation mixtures. We believe this to be highly unlikely for the reasons described below. (i) When appropriate primers were used, amplification of the DNA sequence corresponding to 450 bases upstream of the 3′ terminus of both the cDNA clone of RNA-2 (pT7B2) and the Δt RNA-2 construction by the polymerase chain reaction (19) technique yielded DNA of the expected size from the wild-type clone but not from the mutant construction (data not shown). (ii) Trace levels of wild-type RNA-2 contamination in the reagents or supplied transcripts would be expected to result in progeny virus containing all three genomic RNAs; however, no progeny RNAs were detected when protoplasts were inoculated with RNA-1 and RNA-3 (Fig. 2a and b, lanes 6). (iii) No infection was discerned after inoculation of *Chenopodium hybridum*, a sensitive local lesion host for BMV (18), with RNA transcripts of Δt RNA-2 and wild-type RNA-1 and RNA-3 at concentrations exceeding 300 μg/ml.

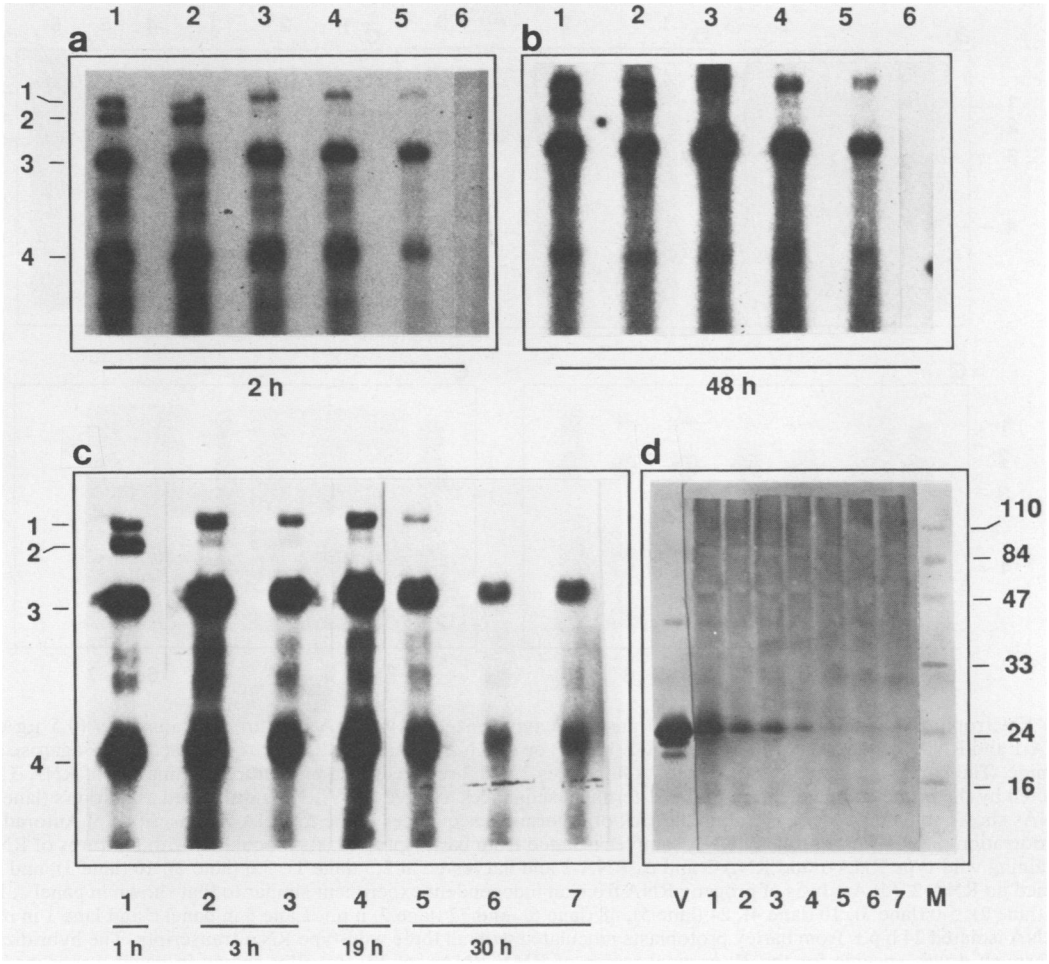

FIG. 2. Analysis of progeny RNA and protein in barley protoplasts. A Northern blot analysis of progeny plus-sense (a) and minus-sense (b) RNAs was conducted following inoculation of barley protoplasts with RNA transcript mixtures (2 μg each) containing wild-type RNA-1 and RNA-3 and wild-type RNA-2 (lanes 1), 2/3t RNA-2 (lanes 2), 5′PsK RNA-2 (lanes 3), 5′+3′PsK RNA-2 (lanes 4), Δknob RNA-2 (lanes 5), or no RNA-2 (lanes 6). (c) Plus-sense progeny RNA isolated from protoplasts inoculated with mixtures containing 2 μg each of wild-type RNA-1 and RNA-3 and 2 μg of wild-type RNA-2 (lane 1) or 5′PsK RNA-2 at a level of 2 μg (lane 2), 0.5 μg (lane 3), 125 ng (lane 4), 30 ng (lane 5), 7.8 ng (lane 6), or 1.9 ng (lane 7). The times below the gels indicate the autoradiographic exposures used to detect plus- and minus-sense RNAs. (d) Western (immunoblot) analysis of viral coat protein synthesized in the experiments described for lanes 1 through 7 of panel c. Lane V contained purified viral coat protein (~200 ng), and lane M contained standards (in kilodaltons) having the M_r values indicated on the right. The positions of the four wild-type BMV RNAs are shown on the left. RNAs extracted from barley protoplasts (11) were electrophoresed in 1% agarose gels after denaturation with glyoxal and transferred to nylon membranes (6, 17). The blots were then hybridized with ^{32}P-labeled minus- and plus-sense RNA probes (6, 17) representing the homologous 3′ region present on each of the four BMV RNAs. Protein samples from protoplasts were isolated by precipitation with 13 volumes of acetone and subjected to Western analysis (20) by using antiserum raised against BMV coat protein.

In the case of Qβ (15), generation of defective interfering particles by ligation of a host cell tRNA to the viral RNA has been documented. The occurrence of analogous events as an explanation for our observations seems most unlikely since it would invoke efficient viral promoter functions for the cellular tRNA and the presence in the plant protoplasts of an efficient RNA ligase. Moreover, the hybridization probe used, for example, for the data shown in Fig. 3b and d is highly specific for the viral 3′ terminus and would not detect tRNAs of host origin.

In our previous experiments (6), no recombinational restoration of function was observed when barley plants were inoculated with wild-type transcripts of RNA-1 and RNA-2 plus RNA-3 bearing either the 5′PsK mutation or mutation ψGG. Replication in protoplasts of RNA-3 bearing these mutations was reduced to 20 and 40%, respectively, compared with that of the wild-type control. Replication in barley plants was even more severely debilitated (6), a situation which should lead to strong selection for recombinants with wild-type infectivity. The lack of recombination observed in such experiments suggested that the frequency of this phenomenon is lower than was inferred from the experiments of Bujarski and Kaesberg (2). Since no evidence exists for cell-to-cell spread of virus in protoplast systems, the similar intensities for RNA-1 and the restored RNA-2 in lanes 5 through 7 of Fig. 3d and e indicate that recombinational restoration of the apparently wild-type sequence occurred in a high proportion of the protoplasts during the relatively short incubation periods used.

The contrast between the apparent frequency of RNA

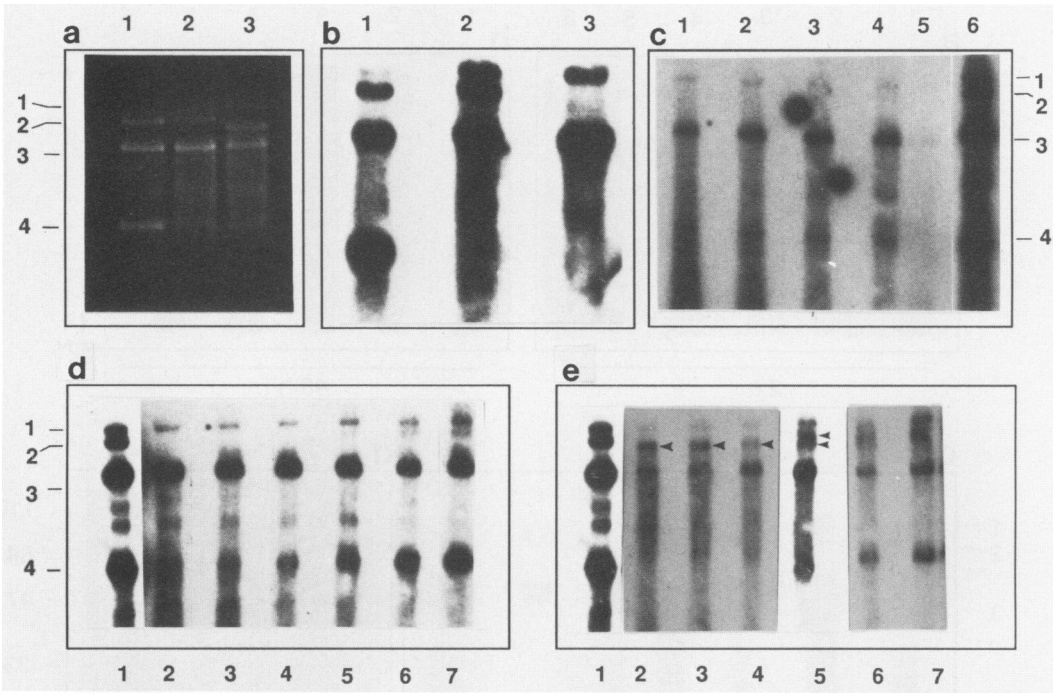

FIG. 3. (a) Electrophoretic analysis of Δt RNA-2 transcripts synthesized in vitro. A mixture of transcripts (0.5 μg each) containing wild-type RNA-1 and RNA-3 and either wild-type RNA-2 (lane 2) or Δt RNA-2 (lane 3) was electrophoresed in 1% agarose and stained with ethidium bromide. The bands that comigrated with RNA-4 in lanes 2 and 3 resulted from premature termination of RNA-3 transcripts; they were not detected by the probe complementary to the 3′-terminal sequences. Native BMV RNAs were used as markers (lane 1). (b) Northern blot of the RNAs shown in panel a, demonstrating the lack of 3′-terminal sequences in the Δt RNA-2 transcript. (c) Autoradiograph showing Northern hybridization of plus-sense progeny RNA samples isolated from barley protoplasts inoculated with mixtures of RNA transcripts (2 μg each) containing wild-type RNA-1 and RNA-3 and Δt RNA-2 and harvested at 2.5 (lane 1), 5.0 (lane 2), 10 (lane 3), and 24 (lane 4) h p.i. Lane 5 contained no RNA-2. (d) Analysis of progeny RNA from an independent experiment similar to that shown in panel c. Protoplasts were harvested 2.5 (lane 2), 5.0 (lane 3), 10 (lane 4), 24 (lane 5), 48 (lane 6), and 72 (lane 7) h p.i. Lane 6 in panel c and lane 1 in panel d contained the progeny RNA isolated 24 h p.i. from barley protoplasts inoculated with all three wild-type RNA transcripts. The hybridization probe used for panels b through d was specific for the 3′-terminal region of BMV RNAs (6, 17). (e) Blot shown in panel d reprobed with sequences representing the coding region of RNA-2. The arrowheads in lanes 2 through 4 indicate the position of Δt RNA-2; in lane 5, the lower arrowhead indicates the position of Δt RNA-2, and the upper arrowhead indicates the position of recombinant RNA-2 bearing the restored 3′-terminal sequence. The Northern blot analysis was performed as described in the legend to Fig. 2. An RNA probe specific for BMV RNA-2 was transcribed from plasmid pT7T3B2 by using T7 RNA polymerase. This plasmid is a derivative of pT7/T3 and contains 1,124 nucleotides obtained as a *Kpn*I-*Sac*I fragment from the coding region of pT7B2 (6).

recombination in some experiments and the complete absence of recombination in other, similar experiments is puzzling. The physiological condition of the host cells or protoplasts may be an important parameter influencing the frequency of recombination. Although the molecular processes involved in RNA recombination are not known, copy choice, in which the viral RNA polymerase switches templates during minus-strand synthesis (10, 12), has been invoked. Further characterization of recombination in the protoplast system, in which markers on RNA-1 and RNA-3 are used to help map crossover sites, appears to be a promising approach for elucidating the mechanisms and factors involved. Such studies should permit evaluation of whether RNA recombination contributes to the interchange of genetic information between viruses infecting animals, insects, and plants.

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