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Defined Mutations in a Small Region of the Brome Mosaic Virus 2a Gene Cause Diverse Temperature-Sensitive RNA Replication Phenotypes

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The central portion of the brome mosaic virus (BMV) 2a protein represents the most conserved element among the related RNA replication components of a large group of positive-strand RNA viruses of humans, animals, and plants. To characterize the functions of the 2a protein, mutations were targeted to a conserved portion of the 2a gene, resulting in substitutions between amino acids 451 and 484. After the temperature profile of wild-type BMV RNA replication was defined, RNA replication by nine selected mutants was tested in barley protoplasts at permissive (24°C) and nonpermissive (34°C) temperatures. Four mutants did not direct RNA synthesis at either temperature. Various levels of temperature-sensitive (*ts*) replication occurred in the remaining five mutants. For two *ts* mutants, no viral RNA synthesis was detected at 34°C, while for two others, an equivalent reduction in positive- and negative-strand RNA accumulation was observed. For one mutant, positive-strand accumulation was preferentially reduced over negative-strand accumulation at 34°C. Moreover, this mutant and another displayed preferential suppression of genomic over subgenomic RNA accumulation at both 24 and 34°C. The combination of phenotypes observed suggests that the 2a protein may play a role in the differential initiation of specific classes of viral RNA in addition to a previously suggested role in RNA elongation.

Though varying dramatically in virion morphology and genetic organization, many positive-strand RNA viruses of animals and plants share fundamental features of RNA replication (18). One set of such viruses, which includes the plant bromoviruses and the animal alphaviruses, produces RNAs with 5′ caps, expresses genes via subgenomic RNAs, and contains three similar domains in the nonstructural proteins required for replication. Despite continuing effort, however, the mechanisms of RNA replication in these positive-strand RNA viruses of eucaryotes remain largely uncharacterized.

One virus which has been extensively used as a model for studying RNA replication is the bromovirus brome mosaic virus (BMV) (1, 15). The BMV genome is divided among three RNAs designated RNA1 (3.2 kilobases), RNA2 (2.9 kilobases), and RNA3 (2.1 kilobases) (3, 6). These genomic RNAs serve as mRNAs for nonstructural proteins 1a (104 kilodaltons [kDa]), 2a (94 kDa), and 3a (32 kDa), respectively. The 20-kDa coat protein encoded by RNA3 is translated from RNA4, a subgenomic mRNA. BMV can be genetically manipulated in vitro by using cDNA clones, and high-efficiency RNA inoculation techniques allow the study of early replication events in plant protoplasts (4, 5, 28). In addition, a template-specific BMV RNA polymerase extract that initiates negative-strand and subgenomic RNA synthesis in vitro can be isolated from infected plants (32, 33). With these systems, studies have defined the *cis*-acting BMV RNA signals required for negative-strand initiation in vitro (2, 11, 12, 15), for viral RNA amplification in vivo (16), and for subgenomic RNA synthesis both in vitro and in vivo (17, 30).

Protoplast studies show that only BMV RNA1 and RNA2 are required for viral RNA replication (16, 25). The 1a and 2a proteins encoded by these RNAs display extensive similarity with nonstructural proteins now implicated in the replication of alphaviruses and other capped RNA viruses (7, 20). The most conserved motif, Gly-Asp-Asp flanked by hydrophobic residues, resides within the 2a protein and is shared by a large group of known or suspected RNA polymerases from a wide variety of positive-strand RNA viruses (8, 24). While the function of this segment is not known, Inokuchi and Hirashima (22) reported that five different amino acid substitutions for Gly in this core sequence blocked RNA polymerase activity in the bacteriophage Qβ RNA replicase.

Despite the requirement for the 2a gene in BMV replication, direct biochemical data on the function of the 2a protein have proven difficult to obtain, and the putative role of the BMV 2a protein as a polymerase has not yet been substantiated. In fact, although both the 1a and 2a proteins are present in BMV RNA polymerase extracts, only antibodies directed against the 1a protein blocked BMV negative-strand synthesis in vitro, while antibody binding to the C terminus of 2a failed to do so (21, 37).

To further examine 2a protein function, we have introduced amino acid substitutions into a region of the BMV 2a protein that is distinct from the highly conserved Gly-Asp-Asp segment but similar to regions in other positive-strand virus replication proteins. All nine of the targeted mutations tested produced clear *trans*-acting effects on viral RNA synthesis in barley protoplasts. Four mutants were identified with unconditional blocks to RNA synthesis, while five others displayed temperature-sensitive (*ts*) defects in replication. For one mutant, positive-strand RNA synthesis was preferentially inhibited over negative-strand synthesis at the nonpermissive temperature. Two *ts* mutants also showed a preferential reduction in the synthesis of genomic RNA relative to subgenomic RNA at permissive (24°C) and nonpermissive (34°C) temperatures.

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MATERIALS AND METHODS

Materials. Plasmids pB1TP3, pB2TP3, and pB3TP8 contain complete cDNA copies of wild-type (wt) BMV RNA1, RNA2, and RNA3, respectively, and are capable of producing infectious transcripts upon T7 RNA polymerase transcription (23). Cellulysin was purchased from Calbiochem-Behring, La Jolla, Calif., and macerozyme was purchased from Yakault Honsha Ltd., Nishinomiya, Japan. Barley seeds (*Hordeum vulgare* L. cv. Morex) were a gift from Anheuser-Busch, Inc., St. Louis, Mo.

Mutant construction. A nested set of BMV RNA2 primers was prepared by incubating 10 μg of *Eco*RV-linearized pB2TP3 in 50 μl of T4 DNA polymerase buffer (29) with 11 U of T4 DNA polymerase (Promega Biotec, Madison, Wis.) in the absence of nucleotides. Samples were removed after 2.5, 5, 10, and 15 min at 37°C, and the reaction was terminated by the addition of EDTA. After extraction with phenol and precipitation with ethanol, the DNA was pooled and cleaved with *Eco*RI. The smaller of the two resulting sets of DNA fragments, corresponding to approximately the 3′ half of BMV RNA2 cDNA, was isolated after electrophoresis in 1% low-melting-temperature agarose. A 0.5-μg sample of this primer preparation was mixed with 0.5 μg of dUTP-containing single-stranded pB2TP3 DNA (26) in 10 μl of T4 DNA polymerase buffer, denatured at 100°C for 3 min, and then annealed at 65°C for 30 min. The hybrids formed were subjected to reverse transcriptase-mediated single nucleotide misincorporation of either dATP, dCTP, or dGTP in separate reactions by procedures similar to those of Kunkel (26). Phage from the resulting transformations of *ung*$^+$ *Escherichia coli* JM101 cells were screened by dideoxy sequencing, using a primer complementary to BMV RNA2 from bases 1707 to 1723. The 240-base-pair *Eco*RV-*Mlu*I fragment from clones containing mutations was exchanged with the corresponding fragment of pB2TP3 for further testing. Transcripts produced from the mutant plasmids were translated in vitro in the presence of [^{35}S]methionine by using a reticulocyte lysate extract (Promega) following the protocol of the manufacturer. The products were separated by electrophoresis in 12.5% polyacrylamide gels (36) and visualized by autoradiography after drying at 80°C under vacuum.

In vitro transcription and preparation of radioactive probes. GpppG (Pharmacia, Inc., Piscataway, N.J.) capped transcripts were synthesized from *Eco*RI-linearized plasmids as described by French and Ahlquist (16). For positive-strand Northern (RNA) blot analyses, ^{32}P-labeled RNA probes were synthesized in vitro from plasmid pB3HE1 (16) in the presence of [α-^{32}P]CTP (100 μCi; 3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), using the conditions described above except that GpppG was omitted and ATP, GTP, and TTP at 500 μM and CTP at 10 μM were used. The final reaction volume of 25 μl contained 1 μg of DNA and typically yielded 10^8 cpm of radioactive RNA. Negative-strand viral RNA was detected with 5′ ^{32}P-end-labeled BMV virion RNA. Before end labeling, virion RNA was hydrolyzed essentially as described by Nassuth and Bol (35). RNA (20 μg) was treated for 30 min at room temperature in 200 μl of 50 mM NaOH. The solution was neutralized by adding 100 μl of 1.0 M Tris hydrochloride (pH 8.0) and 80 μl of 1 N HCl. Hydrolyzed RNA was precipitated in the presence of 0.3 M sodium acetate (pH 6.5) by adding 0.65 volumes of isopropanol. RNA was recovered by centrifugation and suspended in TE (10 mM Tris, 0.1 mM EDTA [pH 7.5]). Hydrolyzed BMV RNA (1.5 μg) was end labeled with [γ-^{32}P]ATP (200 μCi; 5,000 Ci/mmol; Amersham) at 37°C for 30 min in the presence of 10 U of T4 polynucleotide kinase (New England BioLabs, Inc., Beverly, Mass.) in a 30-μl reaction containing 70 mM Tris hydrochloride (pH 7.5), 10 mM dithiothreitol, and 10 mM $MgCl_2$. The reaction was terminated by the addition of 3 μl of 0.5 M EDTA and typically yielded 10^8 cpm/μg of RNA. Unincorporated nucleotides were removed from reaction mixtures by following the G-50 Sephadex spun column procedure of Maniatis et al. (29).

Protoplast isolation and inoculation. Protoplasts were prepared from 6-day-old barley plants as described by Loesch-Fries and Hall (28), except that after the first centrifugation protoplasts were suspended in 10% mannitol and underlaid with 20% sucrose. After centrifugation at 50 × *g* for 8.5 min, protoplasts were collected from the top of the sucrose pad and suspended in 10% mannitol. Approximately 10^5 protoplasts were inoculated with transcripts produced from 0.5 μg of each cDNA plasmid by following the polyethylene glycol procedure described by Samac et al. (39). Transfected protoplasts were incubated in 500 μl of medium (28) in 1.5-ml Eppendorf tubes at the given temperature under constant illumination for 20 h unless otherwise specified.

RNA isolation and Northern blot analysis. Total nucleic acids were isolated from transfected protoplasts by following the procedures described by Loesch-Fries and Hall (28), except that protoplasts were not pelleted before the addition of 200 μl of extraction buffer (0.33 M glycine [pH 9.5]. 0.33 M NaCl, 33 mM EDTA, 3.3% sodium dodecyl sulfate [SDS], 16.6 mg of bentonite per ml). The aqueous phase was extracted twice with phenol-choloroform (1:1) before ethanol precipitation. Nucleic acids were collected by centrifugation and suspended in 30 μl of water. For analysis of positive-strand RNA, nucleic acids were separated by electrophoresis in nondenaturing 1% agarose gels in Tris-borate buffer (29). For analysis of negative-strand RNA, nucleic acids were denatured in the presence of glyoxal (Aldrich Chemical Co., Inc., Milwaukee, Wis.) by the method of McMaster and Carmichael (31), except that the denaturing mix contained 20 mM sodium phosphate (pH 7.0). Denatured RNA was separated by electrophoresis in 1% agarose gels in 10 mM sodium phosphate buffer (pH 6.5) with constant buffer recirculation. After electrophoresis, RNA was transferred overnight onto a Zeta-Probe (Bio-Rad Laboratories, Richmond, Calif.) hybridization membrane in the presence of 10× SSC (pH 7.0) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by capillary blotting, and nucleic acids were fixed on the membrane by baking for 2 h at 80°C. Membranes were prehybridized for at least 1 h at 60°C in a solution containing 50% formamide, 50 mM sodium phosphate, 0.8 M NaCl, 1 mM EDTA, 10× Denhardt solution (29), 0.25 mg of sheared, denatured salmon sperm DNA per ml, 0.5 mg of yeast RNA per ml, and 0.5% SDS. Hybridization to radioactive RNA was in the same buffer for 12 to 24 h at 60°C. BMV positive strands were detected after hybridization to 2.0×10^7 cpm of ^{32}P-labeled RNA transcribed from plasmid pB3HE1. BMV negative strands were detected after hybridization with 2.0×10^7 cpm of ^{32}P-labeled BMV RNA. Nonspecific hybridization was removed by washing the filters three times for 10 min each time at room temperature in 2× SSC–0.2% SDS, then twice for 15 min each time at 60°C in 0.2× SSC–0.2% SDS. The filters were dried, and hybridized RNA was visualized after exposure to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −80°C by using intensifying screens. Quantification of autoradiographs

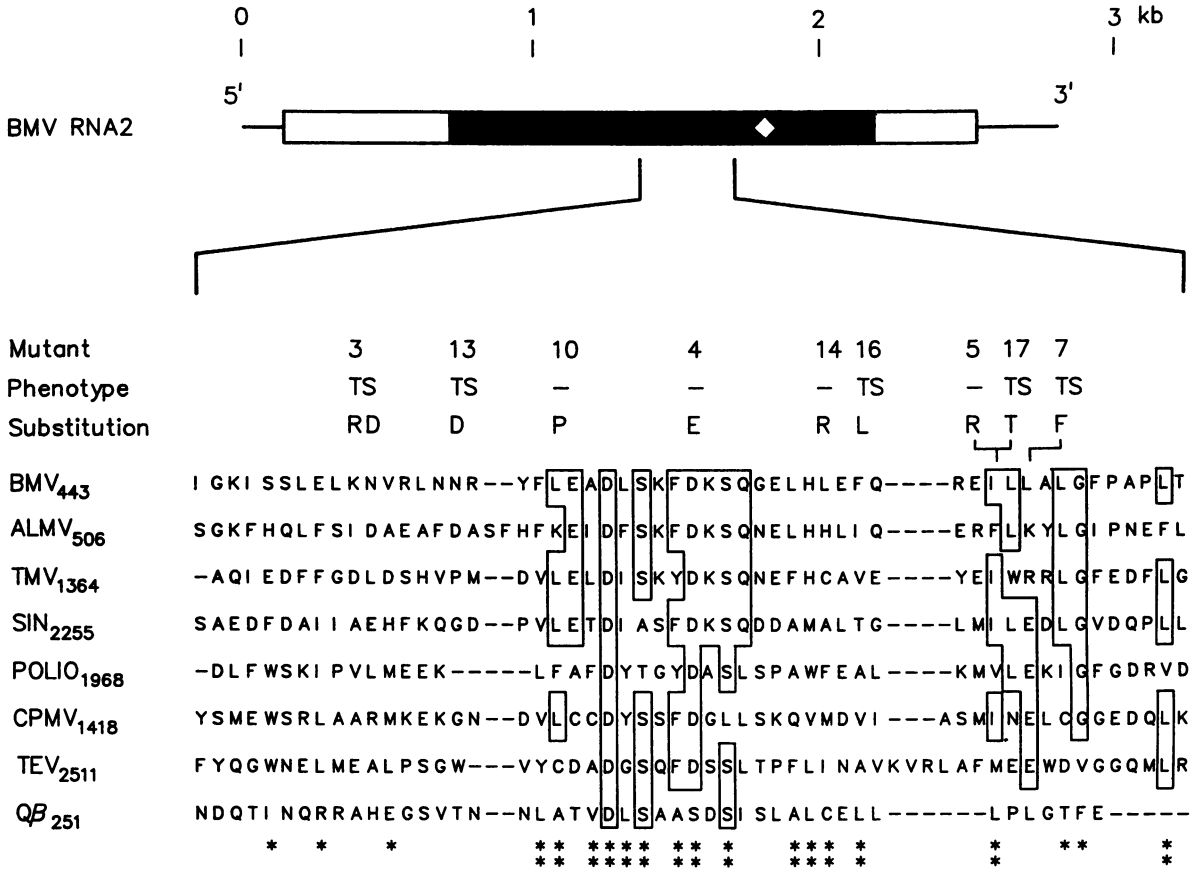

FIG. 1. Locations and phenotypes of BMV 2a amino acid substitution mutants and relationships to interviral 2a protein similarities. The schematic at top represents BMV RNA2, with the open reading frame encoding the 2a protein boxed. The shaded portion of this box shows the 2a protein similarity to proteins of other plant and animal viruses (20). The position of the near invariant GDD segment is shown by an open diamond (24). The expanded view shows the mutagenesis target region within the BMV 2a amino acid sequence, depicting the location and phenotypes of the 2a substitution mutants and their relationship to similar segments of other viral replication proteins. The identification numbers and phenotypes of the BMV substitution mutants are as described in Table 1. Boxes identify positions where at least four viral proteins share identical amino acids. Asterisks indicate positions where the aligned sequences also show similarity within amino acid groups as defined by Kamer and Argos (24) (strong turn formers, D, G, N, and P; acidic and polar, D, E, N, and Q; basic, K and R; hydrophobic, A, C, F, H, I, L, M, V, W, and Y; polar, T and S). Two asterisks indicate positions where at least seven of eight viral proteins share a combination of identical or similar amino acids, and one asterisk indicates positions where at least six positions contain identical or similar amino acids. Subscripts identify the position of the first amino acid of each segment within each viral protein. ALMV, alfalfa mosaic virus, 90-kDa protein; TMV, tobacco mosaic virus, 180-kDa protein; SIN, Sindbis virus, nonstructural polyprotein (nsP4 segment); POLIO, poliovirus, polyprotein (3D segment); CPMV, cowpea mosaic virus, nonstructural polyprotein; TEV, tobacco etch virus, polyprotein; Qβ, Qβ bacteriophage replicase, β-subunit. The specific alignment shown is taken from an alignment of a larger group of viral proteins kindly provided by E. V. Koonin.

was performed by using a Zeineh SLR-504-XL soft laser scanning densitometer.

RESULTS

Construction and mapping of substitution mutations in the BMV 2a gene. The goal of targeted mutagenesis in the BMV 2a gene was to explore 2a protein function by isolating mutants with potentially informative phenotypes such as partial activity loss or *ts* RNA replication. To enhance the recovery of nonlethal phenotypes from BMV RNA2 mutants, we selected a target region of the 2a protein distinct from the conserved Gly-Asp-Asp sequence, since substitutions in the corresponding segment of bacteriophage Qβ replicase β-subunit blocked detectable RNA polymerase function in vivo (22). The region mutated lies between amino acids 451 to 487 and contains features conserved among replication proteins from a diverse collection of positive-strand RNA viruses (Fig. 1). However, as this is not the most rigidly conserved segment among these viral proteins, it was hoped to be sufficiently flexible to retain at least partial function after mutagenesis.

Base substitutions were introduced into the selected region of the 2a gene by forced reverse transcriptase misincorporation in the presence of a single nucleotide. To enhance mutant yields, misincorporation was carried out on single-stranded DNA templates containing dUTP (26). Substitutions were targeted with a set of nested primers generated by treating an appropriate restriction fragment with the 3′ to 5′ exonuclease of T4 DNA polymerase. Mutations were identified in progeny clones by sequencing, and nine distinct nonsilent mutations were selected for further study. The names, base substitutions, and locations of these mutations are given in Table 1, and the context of the amino acid

TABLE 1. Properties of BMV RNA2 mutants

Mutant	Base position	Base change	Amino acid change	Replication in barley protoplasts[a]
B2DR3	1458	AGA → GGG	Lys-452-Asn-453 → Arg-Asp	TS (equivalent positive- and negative-strand reduction)
B2DR4	1513	T → G	Asp-470 → Glu	–
B2DR5	1554	T → G	Ile-484 → Arg	–
B2DR7	1559	C → T	Leu-486 → Phe	TS (equivalent positive- and negative-strand reduction)
B2DR10	1486	TCTT → CCCC	Leu-462 → Pro	–
B2DR13	1475	A → G	Asn-458 → Asp	TS (equivalent positive- and negative-strand reduction)
B2DR14	1536	T → G	Leu-478 → Arg	–
B2DR16	1541	T → C	Phe-480 → Leu	TS (preferential positive-strand reduction; genomic/subgenomic ratio reduced)
B2DR17	1554	T → C	Ile-484 → Thr	TS (equivalent positive- and negative-strand reduction; genomic/subgenomic ratio reduced)

[a] Phenotypes were determined by Northern blot analysis as in Fig. 3. TS, Temperature-sensitive; –, no detectable replication. See text for full discussion of phenotypes.

changes with respect to related replication proteins is shown in Fig. 1. Seven mutants contain single base substitutions, while two mutants, B2DR3 and B2DR10, contain clustered substitutions of two and three nucleotides, respectively. Double substitutions such as those in B2DR3 resulted when the first misincorporation could be followed immediately by correct incorporation of the single nucleotide provided in the misincorporation reaction. The resulting base-paired 3′ end of the extended primer provided the opportunity for a second misincorporation. Mutant B2DR10 was the only triple substitution observed and apparently involved a rare instance of two misincorporations in direct succession.

A 240-base-pair *Eco*RV-*Mlu*I fragment (RNA2 nucleotides 1440 to 1680) containing the mutagenized 2a gene segment was removed from the selected mutant clones and substituted for the corresponding fragment of pB2TP3, which contains a complete expressible cDNA copy of BMV RNA2 (23). The entire *Eco*RV-*Mlu*I fragment in the resulting final plasmid clones was resequenced to verify that the base substitutions identified in the phage were the only alterations present. As an additional check, maintenance of the 2a gene open reading frame was verified by in vitro translation of transcripts from the final mutant plasmids (results not shown).

Temperature dependence of wt BMV RNA replication in vivo. To select permissive and nonpermissive conditions for identifying *ts* phenotypes among the 2a gene mutants, the replication of wt BMV RNA was tested in barley protoplasts incubated for 20 h at temperatures ranging from 15 to 36°C (Fig. 2). Experiments using either BMV virion RNA or transcripts from wt BMV cDNA clones as inoculum gave consistent results. Significant replication was seen from 20 to 34°C, with peak RNA accumulations at 30 and 32°C. Poor replication occurred below 20°C, and as the temperature increased above 34°C, replication quickly dropped below detectable levels. Gross discoloration of cells incubated at 36°C suggested that loss of protoplast viability was a major factor in the decline of BMV replication. For mutant screening 34°C was chosen as the nonpermissive temperature, since this was the highest temperature at which wt BMV RNA replication reliably occurred. For the permissive temperature, 24°C was chosen because replication at this temperature was equivalent to that seen at 34°C and because this has been a standard incubation temperature for many previous protoplast experiments with BMV (16, 38).

***ts* and unconditional defects in positive-strand RNA accumulation.** To test the effects of the 2a protein amino acid substitutions on BMV RNA replication, protoplasts were inoculated with transcripts derived from the individual mutant RNA2 plasmids, together with RNA transcribed from wt cDNA clones of RNA1 and RNA3. Each inoculated protoplast sample was then divided into two aliquots and incubated at either 24 or 34°C. After 20 h, total nucleic acids were isolated from the protoplasts and accumulation of viral positive-strand RNA was determined by Northern blot analysis. Representative results are presented in Fig. 3A. Though clustered in a relatively small region of the 2a gene, the amino acid substitutions resulted in a wide spectrum of altered replication behavior at the two temperatures. The single amino acid substitutions in B2DR4, B2DR5, B2DR10, and B2DR14 all blocked detectable accumulation of positive strands at either temperature, confirming the requirement for a functional 2a protein in the replication process. These mutants contained substitutions in some of the most highly conserved positions in the segment of the 2a protein shown in Fig. 1.

The five remaining mutants all showed various levels of temperature sensitivity in RNA replication. The levels of

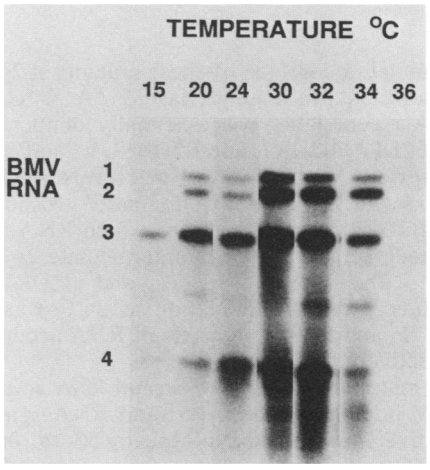

FIG. 2. Temperature profile of BMV replication in barley protoplasts. A total of 10^5 barley protoplasts were inoculated with 1 μg of BMV virion RNA and incubated at the indicated temperature for 24 h in the presence of [5,6-^{3}H]uridine (40 μCi; 48.6 Ci/mmol Amersham). Total nucleic acids were isolated and separated by electrophoresis on a 1% agarose gel. The gel was treated with 1% sodium salicylate for 15 min at room temperature, dried under vacuum at 80°C, and then exposed to X-ray film for 4.5 days at −80°C with an intensifying screen. Each lane contains the nucleic acids from approximately 17,000 protoplasts.

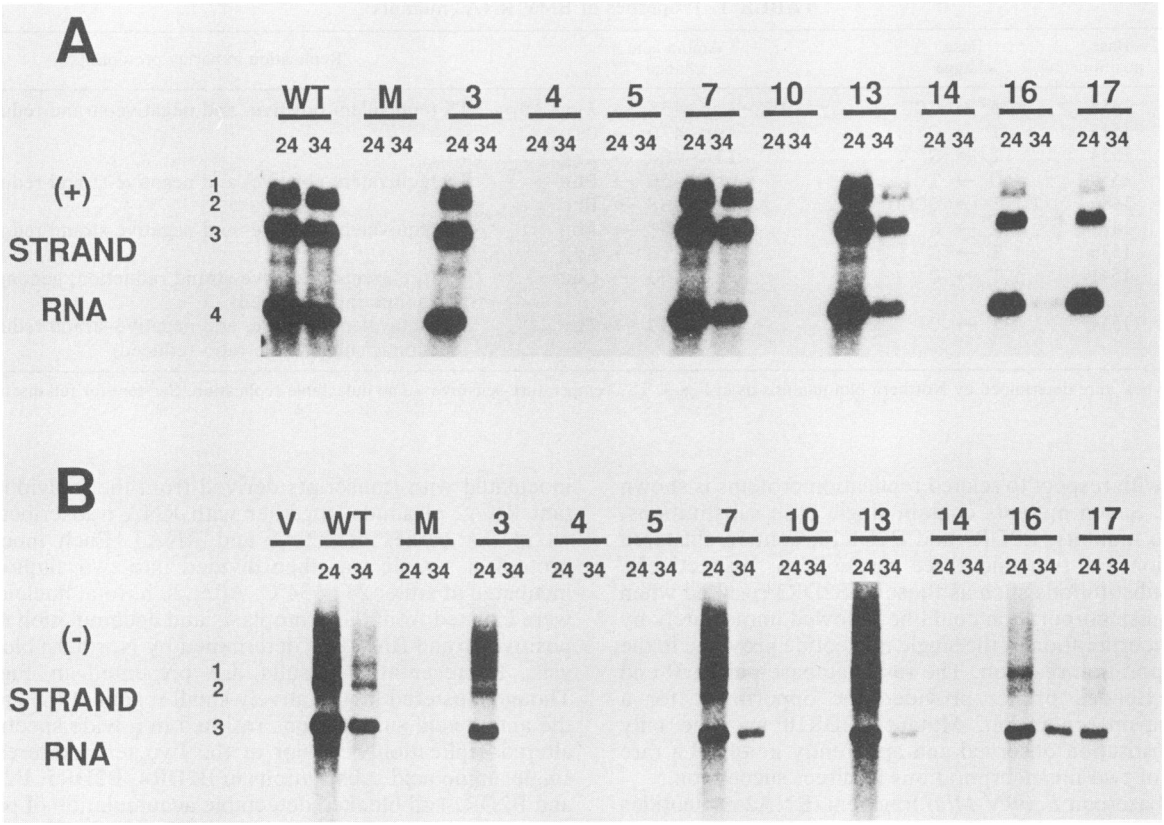

FIG. 3. Northern blot analysis of BMV positive-strand (A) and negative-strand (B) RNA levels in protoplast infections containing wt or mutant BMV RNA2 transcripts. RNA2 substitution mutants are designated by number as in Fig. 1. A total of 2×10^5 protoplasts were inoculated with BMV RNA transcripts 1 and 3 and either mutant or wt RNA2 transcripts, divided into two aliquots, and incubated at 24 and 34°C, respectively, for 20 h. Total nucleic acids were isolated and separated by electrophoresis on 1% agarose gels. After transfer to nylon membranes, RNA levels were determined by hybridization to radioactive probes specific for positive- or negative-strand BMV RNA. Autoradiographic exposure for positive and negative strands was 3.5 and 48 h, respectively, at −80°C with an intensifying screen. Lane M represents nucleic acids from mock-inoculated protoplasts. Positions of BMV RNAs are indicated to the left of each panel. Lane V in the negative-strand panel represents 0.1 μg of BMV virion RNA. Each of the remaining lanes contains nucleic acids from approximately 17,000 protoplasts.

RNA accumulation directed by these mutants at 24 and 34°C were not correlated in a simple fashion. At 24°C, positive-strand RNA accumulation was essentially identical to that of the wt for B2DR3, B2DR7, and B2DR13, while B2DR16 and B2DR17 showed 15 to 20% of wt RNA levels, as determined by laser densitometry. The most dramatic *ts* responses were seen with B2DR3 and B2DR17, for which RNA accumulation was undetectable at 34°C. Intermediate *ts* responses were seen with B2DR7, B2DR13, and B2DR16, whose positive-strand RNA accumulation at 34°C was approximately 75, 25, and 5% of the levels of RNA accumulated at 24°C, respectively.

Effect of mutations on negative-strand RNA accumulation. In wt BMV infections, positive-strand RNA synthesis exceeds negative-strand RNA synthesis by 50- to 100-fold (16, 28). To see whether the observed 2a mutant defects were specific to positive-strand synthesis or otherwise affected the ratio of positive to negative strands, the level of negative-strand RNA was determined in protoplast infections (Fig. 3B). Radioactive BMV virion RNA fragments were used as a probe to detect negative-strand RNA. The specificity of this probe for negative-strand RNA is demonstrated in Fig. 3B, lane V, which shows that no signal was detected in the presence of 0.1 μg of BMV virion RNA, the amount of positive-strand viral RNA synthesized in a typical protoplast infection (WT 24°C lanes in the positive-strand panel [Fig. 3A]). In addition, no negative-strand signal was detected for RNA4 in either wt or mutant lanes, confirming that full-length negative-strand RNA3 is the template for the synthesis of RNA3 and RNA4 (17, 30, 32).

Figure 3B shows that many, but not all, of the negative-strand RNA results paralleled those seen with positive-strand RNA. For example, B2DR4, B2DR5, B2DR10, and B2DR14, which failed to produce detectable positive-strand RNA, also failed to produce negative-strand RNA. The relative order of negative-strand RNA levels at 24°C for the remaining mutants directly followed their positive-strand levels at the same temperature. Mutants B2DR3 and B2DR17, which were strongly *ts* in positive-strand accumulation, also failed to produce detectable negative-strand RNA at 34°C.

Other than for the strongly *ts* mutants, however, the ratio of positive to negative strands differed between 24 and 34°C. For wt BMV, positive-strand RNA levels varied only slightly between 24 and 34°C, while negative-strand RNA levels at 34°C were fourfold lower than those at 24°C. The absence of a complementary drop in positive strands suggests that positive RNA synthesis in normal 24°C BMV infections is probably not limited by negative-strand templates but by some other factor. For B2DR7 and B2DR13,

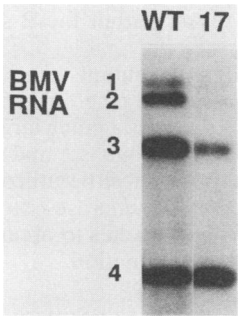

FIG. 4. Northern blot analysis illustrating the altered balance of genomic and subgenomic positive-strand RNA synthesis in protoplast infection with B2DR17. Protoplasts were infected with BMV transcripts 1 and 3 and either wt BMV RNA2 or B2DR17. After incubation at 24°C for 20 h, total RNA was isolated and analyzed as described in Fig. 3. To allow direct comparison between genomic RNA/subgenomic RNA ratios in the two cases, the mutant 17 lane was overexposed relative to the wt lane to equalize intensity of the subgenomic RNA4 band. Each lane contains nucleic acids from approximately 17,000 protoplasts.

negative-strand levels at 34°C were reduced approximately 7- and 20-fold below 24°C levels, respectively. After allowing for the fourfold reduction seen even in wt, these negative-strand reductions closely parallel the *ts* inhibition of positive-strand synthesis of each mutant.

The above results show that negative-strand synthesis is more strongly inhibited at 34°C than is positive-strand synthesis for wt BMV, B2DR7, and B2DR13. Interestingly, the opposite behavior is shown by B2DR16. When 34°C levels are compared with 24°C levels, positive-strand levels for B2DR16 were reduced 20-fold, while negative-strand levels were reduced only 3-fold. The distinct nature of B2DR16 behavior is readily seen by comparisons with other lanes in Fig. 3. For example, while B2DR16 actually showed a higher level of negative-strand accumulation at 34°C than did B2DR13, the accumulation of positive strands at 34°C was dramatically lower for B2DR16 than for B2DR13. Mutant B2DR16 thus shows preferential inhibition of positive-strand accumulation at 34°C, in direct contrast to both wt BMV and the other intermediate *ts* mutants.

Effect of mutations on the ratio of genomic to subgenomic RNA. wt BMV infections maintain a characteristic balance between the levels of the genomic RNAs and subgenomic RNA4 (27). Genomic RNA3 and its subgenomic RNA4 are normally produced in nearly equimolar amounts and are encapsidated together in a single virion. Comparison of positive-strand RNA profiles at 24°C reveals that for *ts* mutants B2DR16 and B2DR17 the level of all three genomic RNAs was preferentially suppressed relative to subgenomic RNA4 (Fig. 3A). For further clarity, Fig. 4 shows a direct comparison of the relative levels of genomic and subgenomic RNA accumulation directed by wt RNA2 and B2DR17. Densitometry of autoradiographs from separate protoplast infections indicates that the ratio of genomic to subgenomic RNA accumulation by B2DR16 and B2DR17 is consistently reduced about twofold and threefold, respectively, relative to wt BMV infections. Mutants B2DR3, B2DR7, and B2DR13 showed normal ratios of genomic to subgenomic RNA.

For all replicating mutants, the balance of genomic RNA1, RNA2, and RNA3 relative to each other appeared normal, although the absolute levels of the RNAs varied. None of these mutants showed preferential reduction in RNA2 accumulation over other genomic RNAs, which would have been characteristic of a *cis*-acting defect in replication or stability of RNA2. In addition, all mutant RNA2 transcripts were as active as wt RNA2 during translation in vitro (data not shown).

DISCUSSION

To explore the role of the BMV 2a gene in viral RNA replication, we tested the effects of substitution mutations in a moderately conserved, suspected functional domain of its encoded protein. This mutagenesis strategy was highly successful, in that the nine mutants analyzed yielded a surprising diversity of RNA replication phenotypes, including both *ts* and non-*ts* defects. The relationship of these phenotypes to the putative role of the 2a protein in RNA elongation is discussed below.

The altered RNA levels in protoplast infections containing the mutant RNA2 transcripts appear to be due to defects in *trans*-acting functions caused by amino acid substitutions in the 2a protein and not to *cis*-acting effects on RNA2 stability or replication. We did not observe a preferential reduction in RNA2 levels for any mutant in these experiments. In other experiments in our laboratory, a number of translationally silent nucleotide substitutions have been introduced into the BMV RNAs, and in no case to date has the replication of the mutant RNA been less than that of wt (P. Traynor, R. Allison, and P. Ahlquist, unpublished results). Also, deletion mutants in RNA2 spanning the region containing the amino acid substitutions reported here are replicated at or near wt levels when coinoculated with a complete wt BMV genome, showing that *cis*-acting replication signals are not located in this region in RNA2 (P. Traynor and P. Ahlquist, unpublished results).

Of nine mutants tested, four failed to produce any detectable viral RNA at either 24 or 34°C. Further analysis of these lethal mutations may yield additional information concerning 2a function. For example, mutant Qβ RNA replicase β-subunits which are unable to support replication also interfere with wt Qβ replication in vivo, possibly because they bind RNA templates but cannot initiate RNA chain elongation (22). Similar interference experiments might characterize analogous 2a protein functions and identify potential antiviral agents.

All mutants in this study, whether displaying conditional or nonconditional phenotypes, showed concomitant alteration of positive- and negative-strand RNA accumulation. Most interesting in this respect were *ts* mutants B2DR7 and B2DR13, for which positive- and negative-strand accumulation at 34°C was jointly reduced by a similar fraction. Although other explanations are possible, these phenotypes are consistent with a defect in elongation. The possible function of the 2a protein as an RNA polymerase in BMV infections is strongly suggested by the similarity displayed by the mutagenized segment and surrounding portions of the 2a protein to poliovirus RNA-dependent RNA polymerase protein 3D and to the β-subunit of Qβ replicase (Fig. 1) (10, 24, 42). In addition, recent analysis also shows that Sindbis virus mutant *ts6*, which is defective in RNA elongation in vitro (9), maps to a change in protein nsP4, a homolog of the BMV 2a protein (Fig. 1) (19, 20). The *ts6* mutation, however, is located in the N-terminal portion of nsP4, far removed from the region corresponding to the mutagenesis target of this study (Fig. 1).

Other mutant phenotypes observed in this study suggest that the 2a gene product may function in replication steps

distinct from, or in addition to, RNA elongation. While wt BMV and weakly *ts* B2DR7 and B2DR13 showed greater inhibition of negative-strand synthesis at 34°C, B2DR16 showed preferential suppression of positive-strand synthesis. This shift in the balance of RNA synthesis suggests that B2DR16 could have a selective defect in initiation of positive-strand synthesis. Similarly, an in vitro BMV RNA polymerase extract can initiate negative-strand synthesis from positive-strand templates but does not initiate positive-strand genomic RNA from full-length negative-strand templates (15). This is consistent with analyses of alphaviruses and other viruses related to BMV, in which it was shown that the syntheses of positive- and negative-strand RNAs are genetically separable events (14, 40, 41).

In addition to their *ts* behavior, B2DR16 and B2DR17 show reduced synthesis of the genomic RNAs compared with subgenomic RNA4 at 24°C, and this altered ratio is maintained by B2DR16 at 34°C. These mutants might have a defect in initiation of genomic over subgenomic RNA synthesis. Other studies have shown that the normally equimolar synthesis of RNA3 and RNA4 can be uncoupled in vivo by manipulating distinct *cis*-acting regulatory sequences on RNA3 (16, 17). In addition, the ability of a BMV RNA polymerase extract to synthesize subgenomic but not genomic RNA from full-length negative-strand RNA3 templates in vitro suggests that initiation of genomic RNA might require some distinct factor(s) (15, 32). Alternatively, the altered genomic to subgenomic RNA ratio might result if an elongation defect led to premature termination of genomic RNA synthesis, thus enhancing relative accumulation of the smaller subgenomic RNA4. Such a defect, however, would necessarily be distinct from any elongation defect of the other mutants, which show normal genomic and subgenomic RNA ratios. Moreover, the degree of premature termination could not simply be length dependent, since relative levels of the genomic RNAs do not appear to be altered in positive or negative strands.

It is not clear whether the *ts* defects in B2DR16 and B2DR17 result from the same biochemical defect responsible for the alteration in the ratio of genomic to subgenomic RNA. Separate functions might be disrupted if the region depicted in Fig. 1 were multifunctional or if an individual mutation both affected local function and altered 2a protein folding to interfere with the function of a distal region. Such factors may contribute to the number of distinguishable phenotypes produced by mutations in this small region of the 2a gene.

The phenotypes observed in this study are similar to two classes of replication-defective mutants isolated after chemical mutagenesis of tobacco mosaic virus (13, 14). Tobacco mosaic virus mutant III_2-35 failed to synthesize positive- and negative-strand viral RNA after the shift of infected leaves from 25°C to the restrictive temperature (35°C), while mutant IV-35 showed a specific defect in the synthesis of positive-strand genomic RNA at 35°C. In contrast, *ts* mutants in both RNA1 and RNA2 of alfalfa mosaic virus showed specific inhibition of negative-strand synthesis at the nonpermissive temperature (40). The particular lesions responsible for the replication defects in these tobacco mosaic virus and alfalfa mosaic virus mutants have not been reported.

Recently, Mills et al. (34) reported the construction and analysis of 37 amino acid insertion mutants in the β-subunit of Qβ replicase. While all mutations in the central portion of the protein resulted in lethal phenotypes, nearly all mutations in the N- and C-terminal regions resulted in the production of a functional replicase. None of the mutations reported, however, reside within the β-subunit region depicted in Fig. 1.

In addition to the diverse mutants generated in this study, we have recently constructed a set of in-frame linker insertion mutants distributed across a much larger region of the 2a gene. Selected mutants from this set and from the substitution mutants described here will be utilized in further tests with in vitro BMV RNA polymerase extracts (15), temperature shifts, and other approaches to obtain a more comprehensive view of 2a protein function.

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