JOURNAL OF VIROLOGY, Dec. 1992, p. 7293–7302
0022-538X/92/127293-10$02.00/0

Vol. 66, No. 12

Identification of the Domains Required for Direct Interaction of the Helicase-Like and Polymerase-Like RNA Replication Proteins of Brome Mosaic Virus

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Received 9 July 1992/Accepted 8 September 1992

Brome mosaic virus is a positive-strand RNA virus whose RNA replication requires viral protein 1a, which has putative helicase and capping functions, and 2a, which has putative polymerase function. Since domains of related sequence are conserved in a wide range of plus-strand RNA viruses, analysis of 1a and 2a function should have applicability to many other viruses. We have recently demonstrated that 1a and 2a form a complex in vivo and in vitro. Using immune coprecipitation and mutant polypeptides made in reticulocyte lysates, we have now mapped both the 1a and 2a domains necessary for complex formation. The sequences needed to bind 2a map to the carboxy-terminal helicase-like domain of 1a. Truncated polypeptides containing this domain were able to bind to 2a, while several small insertions in the helicase-like domain disrupted binding. The sequence required for binding 1a lies within a 115-residue subset of the 2a N-terminal segment preceding the polymerase-like domain. Truncations or fusion polypeptides containing this segment can bind 1a. We also determined that highly purified 2a protein made in insect cells can form a complex with highly purified 1a helicase-like domain made in *Escherichia coli*, suggesting that no other factor is required to mediate 1a-2a interaction. Previous genetic analyses of 1a and 2a are consistent with this mapping and show that the newly defined 1a and 2a binding regions are required for RNA synthesis. The locations of these interacting regions are discussed with regard to models of viral replication and the evolution of positive-strand RNA virus genomes.

The replication of positive-strand RNA virus genomes requires an RNA-dependent RNA polymerase (RdRp) that is composed of viral and apparently also cellular polypeptides (19, 35). Understanding how the various polypeptides interact in this enzyme complex will be useful in reconstituting RdRp activity in vitro and in elucidating the mechanism of RNA replication. One positive-strand RNA virus whose replication has been intensively studied is brome mosaic virus (BMV) (for reviews, see references 1 and 31). BMV infects members of the family Graminae such as barley and wheat. It has an RNA genome composed of three molecules, called RNA1, RNA2, and RNA3, that are each packaged into separate virions. During infection, a subgenomic transcript, RNA4, which encodes a 23-kDa capsid protein, is synthesized from a promoter present in the minus strand of RNA3. The other three proteins encoded by BMV are the 110-, 95-, and 32-kDa products of RNA1, RNA2, and RNA3, respectively. The 32-kDa protein, designated 3a, is required for the movement of the virus from the infection site (4, 8). The 109-kDa (1a) and 94-kDa (2a) polypeptides are required for RNA replication (13, 26) and are components of the BMV RdRp complex (35). The RdRp complex is thought to recognize sequences at both the 3′ and 5′ ends of BMV genomic RNAs and to initiate the synthesis of the minus- and plus-strand RNAs and the transcription of the subgenomic RNA4 (10–12, 34).

Analyses of BMV polypeptide sequences have been undertaken in an effort to understand their activities and functional domains (1). The N-terminal half of 1a has homology to the Sindbis virus nsP1 protein, which has methyltransferase and possibly guanylyltransferase activity for RNA capping (2, 32, 37), and the C-terminal half contains sequence homology to viral and cellular helicases (16, 29). Between the two domains is a region that is not as well conserved in length or sequence as are the putative methyltransferase and helicase sequences. Kroner et al. (29) showed that mutations distributed throughout the 1a polypeptide abolished or perturbed RNA replication in transfected protoplasts. Furthermore, defects in the methyltransferase-like domain cannot be complemented in *trans* by 1a proteins mutant in the helicase-like domain, suggesting that the two domains must work together in one molecule (29).

The 2a protein contains three segments: a central portion that is highly conserved with viral RNA polymerases (5, 18, 23), and less conserved flanking N- and C-terminal regions. Deletions which remove the C-terminal 125 residues do not significantly affect RNA replication and transcription (40). However, deletions within either the polymerase-like domain or the N-terminal region can result in a loss or drastic decrease of BMV RNA replication (40).

The replication of tobacco mosaic virus (TMV) requires two TMV-encoded polypeptides of 126 and 183 kDa (21). The larger polypeptide, p183, contains all three conserved domains found in BMV 1a and 2a (18). This observation suggests that 1a and 2a may need to physically interact and function as a complex for at least some steps in RNA replication (2). Genetic support for this possibility came from the observation that the 1a and 2a genes from a closely related bromovirus, cowpea chlorotic mottle virus (CCMV), cannot be heterologously exchanged with their BMV homologs and allow RNA replication (3, 27). We have recently presented three lines of biochemical evidence that BMV 1a and 2a form a specific complex: (i) the two proteins cofractionated with enzymatically active RdRp through several chromatography steps; (ii) immunoprecipitations with antisera to either 1a or 2a precipitated both proteins even though they do not share epitopes; and (iii) in vitro-translated 1a bound to 2a that was fixed on a membrane (25).

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TABLE 1. Summary of 1a plasmids and 1a protein derivatives

Plasmid[a]	Encoded polypeptide	Amino acids inserted[b]	Amino acids deleted	In vivo phenotype[c]	In vitro binding to 2a[d]
1a insertion mutants[e]					
pB1TP3	wt 1a	None		WT	+
pB1PK2	PK2	D-P(507)		WT	+
pB1PK3	PK3	G-S(5)		–	+
pB1PK4	PK4	G-S(492)		TS	+
pB1PK5	PK5	G-S(207)		WT	+
pB1PK6	PK6	G-S(95)		–	+
pB1PK7	PK7	G-S(239)		–	+
pB1PK9	PK9	G-S(154)		WT	+
pB1PK10	PK10	G-P(198)		–	+
pB1PK11	PK11	G-P(203)		–	+
pB1PK12	PK12	W-A-H(311)		–	+
pB1PK13	PK13	W-A-H(403)		–	+
pB1PK14	PK14	G-P(556)		WT	+
pB1PK15	PK15	W-A-H(651)		–	+
pB1PK16	PK16	G-P-T(464)		–	–
pB1PK17	PK17	D-P(869)		–	–
pB1PK18	PK18	G-S(912)		–	–
pB1PK19	PK19	G-S(670)		TS	TS
pB1PK20	PK20	D-P(905)		–	–
pB1PK21	PK21	D-P(542)		WT	+
1a deletion mutants					
p1a-Δ303	1a-Δ303		2–303		+
p1a-Δ502	1a-Δ502		2–502		+
p1a-Δ599	1a-Δ599		2–599		–
p1a-Δ700	1a-Δ700		2–700		–
p1a-Δ502ET	1a-Δ502ET		2–502		+
pPK20/*Bam*HI	PK20/*Bam*HI		905–961		–
pPK18/*Bam*HI	PK18/*Bam*HI		912–961		–

[a] pB1TP3 was described by Janda et al. (22); all other plasmids in the PK insertion mutant series were described by Kroner et al. (29). The N-terminal 1a deletion mutants were constructed as described in Materials and Methods (see also Fig. 2). The truncations pPK18/*Bam*HI and pPK20/*Bam*HI were pPK18 and pPK20 digested with *Bam*HI. Their encoded products were made by in vitro translation of the resultant runoff transcripts.

[b] The amino acids inserted are indicated by the standard one-letter code. The insertion occurs directly after the indicated amino acid number (in parentheses) in the 1a sequence.

[c] Ability of the mutant 1a gene to support BMV RNA replication in vivo in barley protoplasts inoculated with mutant RNA1 and wt RNA2 and RNA3, as determined by Kroner et al. (29). WT, RNA accumulation at 18, 24, and 35°C was equivalent to that of a wt infection; TS, RNA accumulation at 35°C was markedly reduced relative to that at 24°C; –, no detectable RNA accumulation at 18, 24, or 35°C.

[d] Ability of the mutant 1a protein to bind to wt 2a protein. +, binding; –, no detectable binding; TS, temperature-sensitive binding.

[e] See also Fig. 1.

In this study, we have extended the analysis of the 1a-2a complex by mapping the domains required for their interaction. After defining the necessary domains, we correlated the effects of these mutations on binding with previously published results on their effects on in vivo replication (29, 40). These correlations suggest that the 1a-2a interaction is a vital step in BMV RNA replication. We also present evidence that the 1a-2a interaction is by direct protein-protein contact.

MATERIALS AND METHODS

1a and 2a mutant plasmids and general recombinant DNA protocols. The 1a and 2a plasmids used and the names of their encoded products are described in Tables 1 and 2, respectively. DNA ligations, purification of restriction fragments for cloning, and transformation were performed as previously described (6). Plasmids were purified through either CsCl gradients or Qiagen-500 columns (Qiagen Inc.). The plasmids purified through the Qiagen columns were further extracted with a 1:1 mixture of phenol and chloroform prior to use in transcription reactions. In vitro transcription reactions were performed as previously described (25). In vitro translations were performed with rabbit reticulocyte lysates (Novagen) in the presence of [^{35}S]methionine (Amersham). Polymerase chain reaction (PCR) was performed in a thermocycler (Coy) for 35 cycles, usually at 94°C for 1 min, 42 to 48°C for 0.5 min, and 72°C for 2 min.

Construction of 1a N-terminal deletions. To construct N-terminal deletions of the 1a polypeptide, a plasmid vector containing a T7 RNA polymerase promoter 5′ of *Eco*RI, *Spe*I, and *Bam*HI restriction sites was constructed from the pBS+ plasmid (Promega). DNA fragments, flanked by *Spe*I and *Bam*HI sites, that would encode a series of N-terminally truncated 1a polypeptides from an engineered translation start site were generated by PCR, using pB1TP3 as the template. The 5′ primers used, with the *Spe*I sites underlined, were 1a-300 (5′-ATAACTAGTCATGGTATGGTTTG AAGACATA-3′), 1a-502 (5′-ATAACTAGTCATGGCCAA GACCAAGCGCTCG-3′), 1a-600 (5′-ATAACTAGTCATGG AGATCGCAAATAAGAGC-3′), and 1a-700 (5′-ATAACTA GTCATGGGAGAGGACCTAATT-3′). The 3′ primer, 1a3′ BHI (5′-ATAGGATCCTGGTCTCTTTTAGAGATT-3′), adds a *Bam*HI site to the sequence corresponding to the 3′ end of the RNA1 cDNA. All of the primers have three nucleotides at the 5′ end to facilitate the eventual restriction digestion of the PCR products.

Construction of in-frame fusions of 2a and 3a genes. Selected DNA fragments flanked by *Pst*I and *Bam*HI sites and encoding portions of the 2a N terminus were synthesized by PCR. The 5′ primer used for PCR was B1.2 (5′-GCGCTGC

TABLE 2. Summary of 2a plasmids and 2a protein derivatives

Plasmid[a]	Encoded polypeptide	Type of mutation[b]	Location of alteration	2a amino acids fused to 3A	In vivo phenotype[c]	In vitro binding[d]
2a deletion mutants[e]						
pB2TP5	wt 2a	None			+	+
pB2TP5/*Eco*RV	Δ*Eco*RV	Del	447–822		NA	+
pB2TP5/*Kpn*I	Δ*Kpn*I	Del	384–822		NA	+
pB2TP5/*Nco*I	Δ*Nco*I	Del	260–822		NA	+
pB2PT14	PT14	Del	3–24		+	+
pB2PT16	PT16	Del	3–51		−/+	−/+
pB2PT18	PT18	Del	3–101		−	−
pB2PT64	PT64	DD	3–24, 698–822		+	+
pB2PT66	PT66	DD	3–51, 698–822		−/+	−/+
pB2SB8	SB8	FS	600		−	+
pB2SB9	SB9	FS	673		−	+
pB2PT50	PT50	Del	698–822		+	+
Fusion of 2a segments to the N terminus of 3a						
p260-3a	F260			1–260		+
p200-3a	F200			1–200		+
p200-14-3a	F200-14			1–2, 25–200		+
p200-18-3a	F200-18			1–2, 102–200		−
p180-14-3a	F180-14			1–2, 25–180		+
p160-14-3a	F160-14			1–2, 25–160		+
p140-14-3a	F140-14			1–2, 25–140		+
p120-14-3a	F120-14			1–2, 25–120		−

[a] pB2TP5 was described by Janda et al. (22). The truncations pB2TP5/*Eco*RV, pB2TP5/*Kpn*I, and pB2TP5/*Nco*I represent pB2TP5 digested with the indicated restriction enzymes, which cleave within the 2a gene coding sequence. Their encoded products were made by in vitro translation of the resultant runoff transcripts. The plasmids in the PT series were described in Traynor et al. (40). The 2a-3a fusions were constructed as described in Materials and Methods (see also Fig. 4).

[b] Del, deletion of the amino acids shown in the next column; DD, deletions at both N and C termini of 2a; FS, frameshift after amino acid shown in the next column.

[c] The ability of the mutant 2a gene to support BMV RNA replication in protoplasts inoculated with mutant RNA2 and wt RNA1 and RNA3 as determined by Traynor et al. (40). NA, not applicable; +, competent for RNA synthesis; −, no detectable RNA synthesis; −/+, RNA synthesis severely reduced in comparison with the wt level.

[d] Ability of the mutant 2a protein to bind wt 1a protein in vitro. +, consistent binding; −/+, severely reduced binding; −, no detectable binding.

[e] See also Fig. 3.

AGTAATACGACTCACTATAGTARACCACGGAACG-3′, where R is purine), which contains a *Pst*I restriction site (underlined), a bacteriophage T7 promoter, and a sequence corresponding to the 5′ end of RNA2 cDNA. The 3′ oligonucleotides, 2a-260 (5′-TATGGATCCTGGAATAATCAC CATTTTC-3′), 2a-200 (5′-AGGATCCTAACCCTATGTTC AATAGG-3′), 2a-180 (5′-ATGGATCCTCTCTTCATCAAT GCCCAT-3′), 2a-160 (5′-ATGGATCCTAACACGAACTT CCTCAAT-3′), 2a-140 (5′-ATGGATCCTATGATCTTCAT CGGCGGC-3′), and 2a-120 (5′-ATGGATCCTGTCATCAA TTTCATCTTC-3′), all contained a *Bam*HI site (underlined) and sequence complementary to the plus strand of the 2a gene. The template DNA used for PCR was either pB2TP5, pB2PT14 (Table 1), which lacked nucleotides 109 to 174 (codons 3 to 24), or pB2PT18, which lacked nucleotides 110 to 406 (codons 3 to 101). The products of the expected sizes were first cloned into pCR1000 (InVitrogen), and then the insert was excised with *Pst*I and *Bam*HI for cloning into pB3TP10, which contains a unique *Pst*I site in front of the T7 polymerase binding site and a unique *Bam*HI site 6 bp upstream of the translation start site of the 3a gene (33). The fusion of the 2a sequence to 3a at the *Bam*HI site added three codons between the two genes. The first residue (Arg, Val, or Trp) was variable as a result of the fusion of 2a sequence to the *Bam*HI site in pB3TP10. The second and third residues were Ile and Pro, respectively.

Immune coprecipitation. Immune coprecipitation assays were performed as previously described (25). All experiments were performed at least twice and usually many more times. Recombinant 2a made by baculovirus expression in *Spodoptera frugiperda* Sf21 insect cells, designated Ac2a, and a control preparation made by equivalent extraction of Sf21 cells infected with a wild-type (wt) baculovirus, designated Acwt, were prepared as described previously (25) and generously provided by R. P. Hershberger. Usually the protein extracts (at approximately 0.1 mg/ml) were diluted 10-fold with L buffer (25), and 2 μl of the diluted material was mixed with previously programmed reticulocyte lysates for the immunoprecipitation assays.

Expression of an epitope-tagged 1a helicase-like domain. A DNA fragment that would express an epitope-tagged 1a helicase-like domain in *Escherichia coli* was synthesized by PCR. The 3′ oligonucleotide primer was 1a3′BHI (described above); the 5′ oligonucleotide primer (5′-ACACATATGG CTTACCCATACGATGTGCCAGATTACGCCAAGACC AAGCGCTCG-3′) contains an *Nde*I site (underlined), a sequence encoding an 11-residue epitope from influenza virus hemagglutinin, and residues 502 to 508 of 1a. The DNA fragment was digested with *Nde*I and *Bam*HI and cloned into the pET11a expression vector (Novagen). After initial transformation and screening, a plasmid with the correct insert was transformed into *E. coli* BL21(DE3) (F^- *ompT* r_K^- m_B^-::λDE3; Novagen). The cells were grown at 37°C to an optical density of 0.5 at 600 nm prior to induction for 2 h at 26°C with 0.5 mM (final concentration) isopropyl β-D-thiogalactopyranoside (IPTG; Sigma). The cells were harvested by centrifugation for 5 min at 6,000 × *g*, washed with sterile water, and pelleted again before suspension in HKEEG buffer *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (25 mM [HEPES; pH 7.5], 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol) amended with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 50 μM

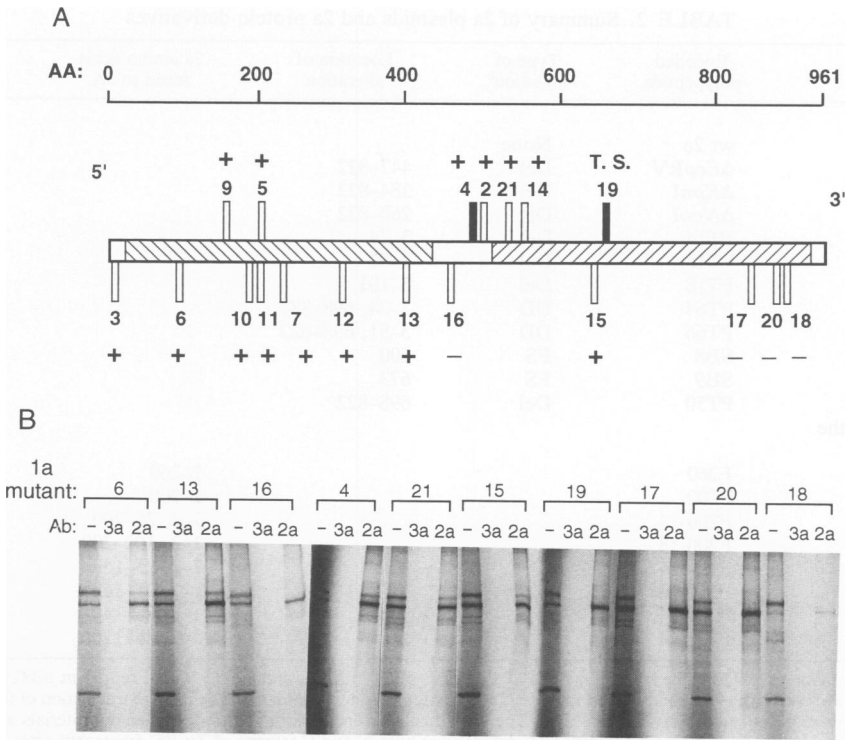

FIG. 1. Effects of two- and three-amino-acid insertions in 1a on coprecipitation with 2a. (A) Locations of insertion mutations in the PK series (Table 1) (29) and summary of their effects on in vitro coprecipitation of 1a with 2a. The scale at the top shows amino acid (AA) position within 1a. The methyltransferase- and helicase-like domains are represented by the left and right hatched areas, respectively. Blank areas represent regions that are less conserved relative to analogous proteins of other bromoviruses, tobamoviruses, alphaviruses, etc. (2). + or − indicates the ability of each mutant to be coprecipitated with 2a. The positions of insertion mutations that support viral RNA replication in vivo are shown above the bar representing the 1a polypeptide, while mutations that prevent detectable in vivo RNA replication (i.e., less than 0.2% of the wt level) are shown below the bar, as determined by the results of Kroner et al. (29). The solid markers indicate mutations (PK4 and PK19) conferring temperature-sensitive (T.S.) RNA replication in vivo. (B) Fluorograph of coprecipitation results for representative 1a insertion mutants. Wild-type 2a was cotranslated with each 1a insertion mutant, and 1 μl of the translation product was loaded in lanes marked −; 7 μl was then used for immunoprecipitation with antiserum (Ab) against 3a or 2a, as indicated above the relevant lanes. The positions of the 1a and 2a bands are indicated at the left. The asterisk marks an approximately 48-kDa product that is believed to arise from spurious in vitro transcription of the plasmid vector sequences (25). This product is produced by in vitro translation of transcripts from either the 1a or 2a plasmid, but interacts with neither the 1a nor 2a antiserum. The identity of the 1a mutant cotranslated with 2a is indicated above each set of lanes.

aprotinin, and 10 μM each leupeptin and pepstatin). The suspension was sonicated with four sets of 10 1-s bursts with a probe sonicator (Branson Instruments), each set interspersed with a 1-min incubation on ice. The lysate was centrifuged at 15,000 × *g* for 10 min, and the supernatant was used for further analyses.

Antibody-mediated purification of 1a-Δ502ET and Ac2a. *E. coli* lysate containing 1a-Δ502ET was purified through a DEAE–Bio-Gel A column to enrich for 1a-Δ502ET, which eluted in the flowthrough fraction. 1a-Δ502ET was further purified with monoclonal antibody (MAb) 12CA5, which recognizes the influenza virus hemagglutinin epitope tag at the N terminus (41). The MAb 12CA5 and 1a-Δ502ET were mixed together and incubated on a rotating platform in 1× PN buffer (0.25 M NaCl, 25 mM $NaPO_4$ [pH 7.0]) for 2 h at room temperature. Protein G-Sepharose was added, and the mixture was incubated for 2 h at room temperature before being washed twice each with 2× PN buffer, 1 M LiCl–20 mM Tris-Cl (pH 7.4), 2 M KCl, and L buffer (25). Ac2a, equilibrated in L buffer, was added to the beads and mixed for 3 h before being washed once with L buffer and twice each with 0.5 M LiCl–20 mM Tris-Cl (pH 7.4), 0.75 M LiCl–20 mM Tris (pH 7.4), and L buffer. The beads were then separated into two aliquots. Sample buffer (30) was added directly to one aliquot, and the other was treated with 20 bead volumes of 20 mM glycine-HCl (pH 2.5) to elute all proteins bound to MAb 12CA5. After this latter treatment, the supernatant was aliquoted and adjusted to 10% trichloroacetic acid. After a 10-min incubation on ice, the precipitates were pelleted by centrifugation for 10 min at 14,000 × *g*, rinsed with acetone, dried, and resuspended in sample buffer. Electrophoresis was performed in a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel, and the polypeptides were visualized by staining with silver nitrate (17). Western immunoblots were performed as described previously (25).

RESULTS

Effects of 1a insertion mutations on 1a-2a interaction. An immune coprecipitation assay (25) was used to determine the effects of various mutations on 1a-2a protein interaction. We first tested the series of 1a mutants of Kroner et al. (29), which contain insertions of two or three amino acids at defined positions throughout the 1a polypeptide (Fig. 1A and Table 1). Rabbit reticulocyte lysates were programmed with a 1a mutant transcript and the wt 2a transcript. Aliquots of the translation products from each reaction either were left untreated or were immunoprecipitated with anti-2a serum. As a control, an equal aliquot of the translation product was immunoprecipitated with anti-3a serum. The immunoprecip-

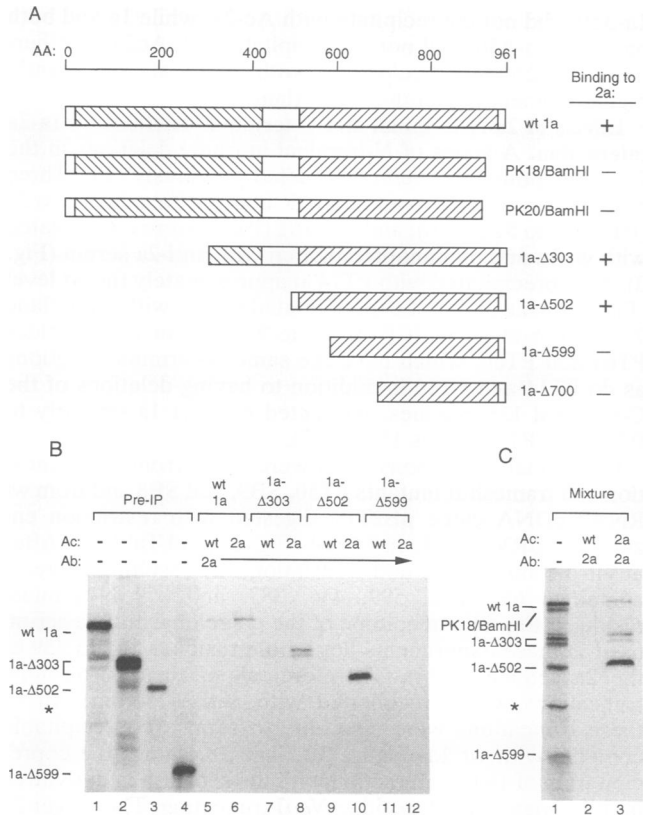

FIG. 2. Deletion mapping of the binding domain in protein 1a. (A) Summary of the 1a mutant constructs tested (see also Table 1). The 1a domains are represented by the markings described in the legend to Fig. 1A. + or − indicates the ability of the construct to coprecipitate with Ac2a. AA, amino acids. (B) Fluorograph of representative translation and immunoprecipitation results for wt 1a and N-terminal deletion mutants. For each of wt 1a and mutants 1a-Δ303, 1a-Δ502, and 1a-Δ599, 1 μl of the in vitro translation products was electrophoresed prior to immunoprecipitation (Pre-IP; lanes 1 to 4, respectively); 7 μl of the same lysates was incubated at 28°C for 40 min with unlabeled Ac2a or Acwt lysates (Ac), as indicated above the lanes, prior to the addition of anti-2a serum (Ab). The subsequently immunoprecipitated products are shown in lanes 5 to 12. The wt and mutant 1a constructs tested are listed above each pair of lanes. (C) wt 1a, the C-terminally truncated PK18/*Bam*HI polypeptide, 1a-Δ303, 1a-Δ502, and 1a-Δ599 were translated separately and mixed together, and an aliquot was electrophoresed in lane 1. Two equal aliquots were incubated for 40 min at 28°C with either Acwt or Ac2a (Ac), as indicated. The products obtained after immunoprecipitation with anti-2a serum (Ab) are shown in lanes 2 and 3. The protein bands are identified at the left. The mixing of the translation products enhanced the abundance of a spurious band (marked by an asterisk in lane 1) that was common to all translation reactions. As described in the legend to Fig. 1B, this polypeptide probably arose from transcripts originating from the plasmid vector and is not immunoprecipitated by antiserum to 2a.

itates were electrophoresed on an SDS-polyacrylamide gel and fluorographed, as shown for representative mutants in Fig. 1B. Previously, we had tested two of these mutants (25); in this work, we tested 19 mutants from this collection. Of these 19 1a mutant polypeptides, five (PK16, PK17, PK18, PK19, and PK20) reproducibly failed to be coprecipitated with 2a by the anti-2a serum. Of these mutations, PK16 mapped to the nonconserved segment within the central portion of 1a (25, 29), while the other four mutations mapped to the helicase-like domain at the C terminus (Fig. 1A). We did not identify any insertions in the methyltransferase-like domain that abolished coprecipitation with 2a, although PK3 and PK5 were decreased in the amount of 1a which coprecipitated (results not shown). These preliminary mapping results were confirmed with analyses of other 1a mutants (see below).

Effects of 1a N- and C-terminal deletions on 1a-2a interaction. Mutant 1a polypeptides with N-terminal deletions were constructed to determine the limits of 1a sequences required for 1a-2a interaction. The deletions contained translational start sites at RNA1 nucleotides 984, 1578, 1872, and 2166, corresponding to deletions of amino acids 2 to 303, 2 to 502, 2 to 599, and 2 to 700 of the wt 1a polypeptide (Fig. 2A and Table 1). The resultant genes encoding the truncated polypeptide products, designated 1a-Δ303, 1a-Δ502, 1a-Δ599, and 1a-Δ700, were cloned behind the bacteriophage T7 promoter to allow in vitro transcription and subsequent translation (Fig. 2B). The 1a-Δ502 polypeptide migrated more slowly than expected for its size (lane 3). Also, for unknown reasons, the 1a-Δ303 product usually migrated as a doublet band (lane 2). However, DNA sequencing confirmed that each plasmid contained the correct deletion, and as shown below, the resulting proteins were active in binding 2a.

A portion of the in vitro-translated product from each truncated 1a gene was mixed with an Sf21 cell extract highly enriched for 2a protein expressed by a recombinant *Autographa californica* nuclear polyhedrosis virus vector (20) (see Materials and Methods). We have previously shown that this baculovirus-expressed 2a, designated Ac2a, readily interacts with in vitro-translated wt 1a to form an immunoprecipitable complex (25). As a control, a second portion of each translation product was mixed with the similarly prepared. Acwt extract made from Sf21 cells infected with wt *A. californica* nuclear polyhedrosis virus. After incubation in conditions which facilitate complex formation between wt 1a and Ac2a (25), the reaction was immunoprecipitated with anti-2a serum. Wild-type 1a, both bands of 1a-Δ303, and 1a-Δ502 reproducibly coprecipitated with Ac2a (Fig. 2B, lanes 6, 8, and 10) at levels markedly higher than those for the minor, nonspecific precipitation seen in the control reactions with Acwt (lanes 5, 7, and 9). However, the 1a-Δ599 (lane 12) and 1a-Δ700 (not shown) polypeptides did not coprecipitate with Ac2a. Thus, the N-terminal half of 1a is not required for binding 2a, but deletions extending into the helicase-like domain blocked this interaction.

C-terminal deletions of 1a were also tested for coprecipitation with 2a. The PK20 and PK18 insertions introduce unique *Bam*HI restriction sites into the 1a gene after codons 905 and 912, respectively (Fig. 1A and Table 1). Plasmids pB1PK18 and pB1PK20 were digested with *Bam*HI, and transcripts were synthesized from the linearized templates. After translation in reticulocyte lysates, the resulting C-terminally deleted 1a derivatives (Fig. 2A) were incubated with Ac2a extract and treated with anti-2a serum. Neither truncated PK18 or PK20 coprecipitated with Ac2a (Fig. 2C and data not shown). Therefore, consistent with results from the insertion mutations and N-terminal deletions, C-terminal deletions impinging on the helicase-like domain also prevented interaction with 2a.

1a-derivative 1a-Δ502, which lacks 502 residues from the 1a N terminus, appeared to coprecipitate with 2a more efficiently than did wt 1a (Fig. 2B, lanes 6 and 10). To more directly compare the binding efficiencies of different 1a mutant polypeptides, the immune coprecipitation assay was performed on a mixture of full-length and truncated 1a polypeptides (Fig. 2C). Wild-type 1a, the C-terminally truncated PK18/*Bam*HI polypeptide, 1a-Δ303, 1a-Δ502, and 1a-

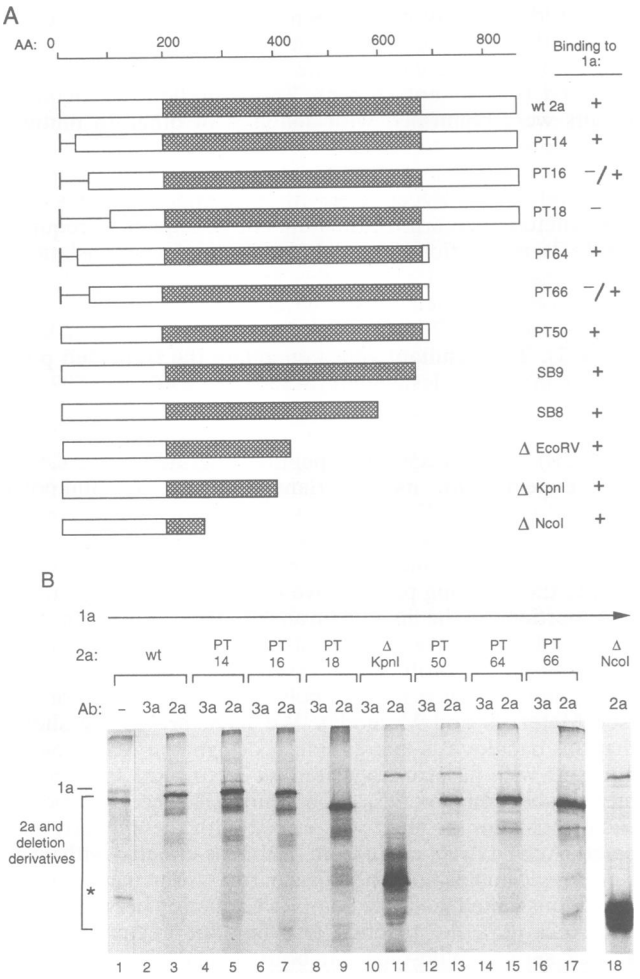

FIG. 3. Effects of 2a deletions on binding 1a. (A) Summary of deleted 2a polypeptides tested for binding 1a. In the schematics, the central RNA–polymerase-like domain is cross-hatched, while the N-terminal and C-terminal flanking segments are left blank. These flanking segments have no apparent counterparts in the proteins encoded by TMV, and the C-terminal flanking segment is dispensable for in vivo replication (40). For each deletion derivative (Table 2), only the areas expressed in reticulocytes are represented. ΔEcoRV, ΔKpnI, and ΔNcoI denote C-terminal 2a truncations produced by cleaving the wt 2a cDNA clone with the indicated restriction enzyme, followed by in vitro transcription and translation. + or − indicates whether the construct was able to bind 1a; −/+ indicates that the construct was able to coprecipitate 1a but at a greatly reduced level. AA, amino acids. (B) Representative autoradiograph of an immunoprecipitation assay using wt 1a and selected mutant 2a polypeptides. Lane 1 contained 1 μl of the in vitro-translated wt 1a and 2a. The identity of the 2a mutant cotranslated with wt 1a is noted above each lane. Immunoprecipitation assays used antiserum (Ab) against either 3a or 2a, as indicated. The result shown for the 2a truncation, Δ*Nco*I (lane 18), was obtained in a separate experiment. Control immunoprecipitations of Δ*Nco*I with anti-3a serum did not precipitate either wt 1a or the truncated 2a proteins (data not shown). A polypeptide that is believed to originate from the plasmid vector is marked by an asterisk (see the legend to Fig. 1B).

Δ599 were separately synthesized, treated with RNase A, and mixed together (Fig. 2C, lane 1). Equal aliquots were then incubated with either Acwt (lane 2) or Ac2a (lane 3) extracts before immunoprecipitation with anti-2a serum. In agreement with the previous results, truncated PK18 and 1a-Δ599 did not coprecipitate with Ac-2a, while 1a and both bands of 1a-Δ303 did not coprecipitate with Ac2a. Furthermore, 1a-Δ502 coprecipitated with Ac2a at significantly higher efficiency than did wt 1a (lane 3).

Effects of 2a N-terminal and C-terminal deletions on 1a-2a interaction. A series of N-terminal in-frame deletions in the 2a polypeptide has been constructed previously (40). Three mutant polypeptides containing deletions of residues 3 to 24 (PT14), 3 to 51 (PT16), and 3 to 101 (PT18) were cotranslated with wt 1a and immunoprecipitated with anti-2a serum (Fig. 3). 1a coprecipitated with PT14 at approximately the wt level (Fig. 3B, lane 3), but it coprecipitated poorly with PT16 (lane 7) and not at all with PT18 (lane 9). Mutant polypeptides PT64 and PT66, which have the same N-terminal deletions as do PT14 and PT16 in addition to having deletions of the C-terminal 125 residues, interacted with wt 1a similarly to PT14 and PT16 (lanes 15 and 17).

C-terminal truncations in 2a were made from the truncation and frameshift mutants PT50, SB9, and SB8 and from wt RNA2 cDNA clone pB2TP5 digested with restriction enzymes *Eco*RV, *Kpn*I, and *Nco*I (Fig. 3A and Table 2). After in vitro transcription and translation, the resulting proteins contained 697, 672, 599, 446, 383, and 259 N-terminal residues (Fig. 3). An epitope of the polyclonal anti-2a serum used in these experiments lies within residues 101 to 259 of the 2a sequence (24). Therefore, all of these C-terminal truncations were precipitated with anti-2a serum. All of these truncations were also able to form a coprecipitable complex with wt 1a. Figure 3B, lane 18, shows the coprecipitation of 1a by interaction with the shortest 2a derivative in this series, the 260-residue *Nco*I truncation. The longer 2a derivatives in this C-terminal truncation series gave similar results (Fig. 3 and results not shown). As expected, control immunoprecipitations with the anti-3a serum precipitated very little of either 1a or the truncated 2a products. Thus, these deletions show that the nonconserved C-terminal domain and most of the polymerase-like domain are dispensable for in vitro interaction with wt 1a.

Further analyses of 2a C-terminal deletions fused to BMV 3a. Since the N-terminal portion of 2a both is required to interact with 1a and contains an epitope for the anti-2a serum used in these experiments, we further dissected this region by using in-frame fusions to the BMV 3a polypeptide (Fig. 4 and Table 2). The fusion polypeptides were cotranslated with wt 1a and immunoprecipitated with anti-3a serum. Wild-type 1a and 2a were not specifically recognized by anti-3a serum (Fig. 4B, lane 12), but 3a and all 2a-3a fusion polypeptides were precipitated (lanes 13 to 19). In addition, fusions in which the 2a segment extended beyond the first 140 residues of 2a were able to coprecipitate 1a even when 2a residues 3 to 24 were deleted (lanes 15 to 18). However, fusions that contained only 2a residues 1 to 2 and 101 to 200 (F200-18) or residues 1 to 2 and 25 to 120 (F120-14) did not coprecipitate 1a (lanes 14 and 19). As expected, immunoprecipitations with a control antiserum raised against rabbit immunoglobulin G (IgG) did not precipitate either the 2a-3a fusion polypeptides or 1a (data not shown). Therefore, the domain in 2a required for interacting with 1a lies between amino acid residues 25 and 140.

The required binding domains mapped in 1a and 2a are sufficient for 1a-2a interaction. The mutational analyses described above suggested that a 115-amino-acid segment near the N terminus of 2a was required to bind the C-terminal helicase-like domain of 1a. We directly tested this prediction by cotranslating 1a-Δ502 (Fig. 2A) and F140-14 (Fig. 4A), which, respectively, contain the 1a helicase-like domain and the relevant segment from the 2a N terminus fused to 3a.

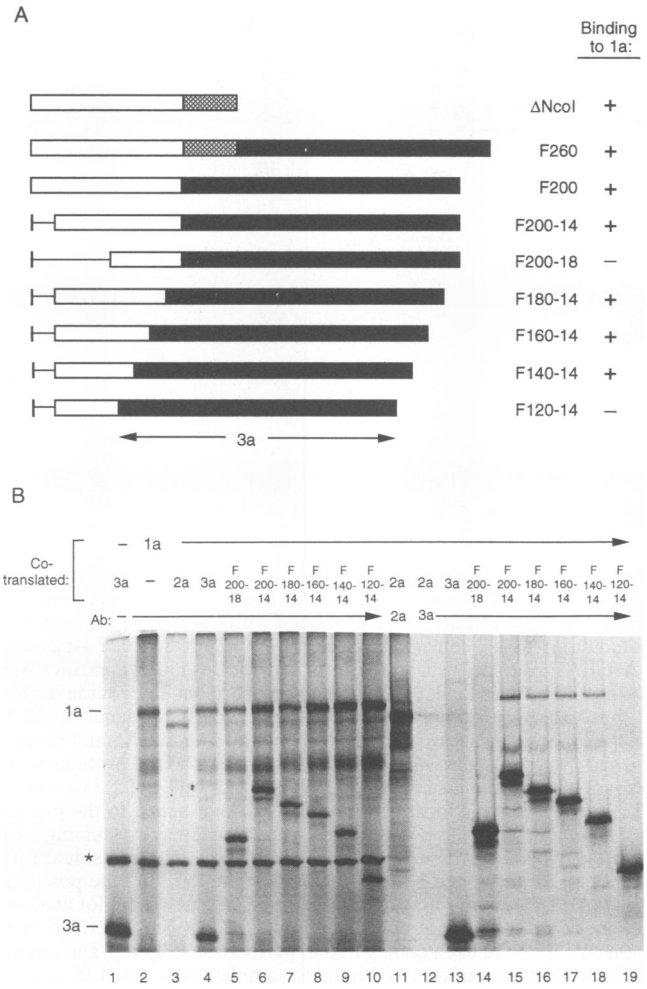

FIG. 4. Further mapping of the binding domain in 2a by using 2a-3a fusions. (A) Schematic of the constructs tested and summary of their binding to wt 1a. The fusion polypeptides contain a variable 2a N terminus (blank and cross-hatched areas) fused in frame to the entire 3a polypeptide (black). + or – indicates the ability of the construct to bind to 1a. (B) Autoradiograph of the immunoprecipitation assay with wt 1a and 2a-3a fusion polypeptides. The identity of the reticulocyte translation product is indicated above each lane. Lanes 1 to 11 contained 1 μl of the in vitro-translated samples before immunoprecipitation. The products of the immunoprecipitation assay using 7 μl of programmed reticulocyte lysates were loaded in lanes 12 to 19. The antiserum (Ab) used was against either 2a (lane 11) or 3a (lanes 13 to 19). The asterisk marks the position of a spurious band believed to originate from the plasmid vector.

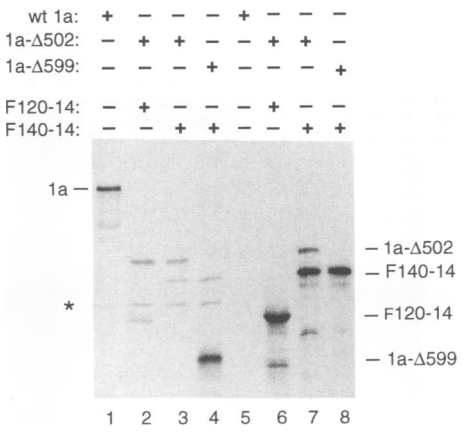

FIG. 5. Ability of the 2a N terminus fused to 3a to bind the 1a helicase-like domain. The indicated combinations of 1a N-terminal deletions (see Fig. 2A) and 2a-3a fusions (see Fig. 4A) were cotranslated in reticulocyte lysates. One microliter of the translation product from each reaction was loaded in lanes 1 to 4, and 15 μl was then subjected to immunoprecipitation with anti-3a serum (lanes 5 to 8). 1a-Δ502 was cotranslated with F120-14 (lanes 2 and 6) or with F140-14 (lanes 3 and 7). 1a-Δ599 was also cotranslated with F140-14 (lanes 4 and 8). The identities of the bands are marked beside the autoradiograph. The anti-3a serum does not cross-react with 1a (lanes 1 and 5).

Immunoprecipitations with anti-3a serum resulted in the precipitation of the 2a-3a fusion protein and significant amounts of 1a-Δ502 (Fig. 5, lane 7). Several negative controls were performed and yielded the expected results. First, anti-3a serum did not precipitate wt 1a (lanes 1 and 5). Second, F120-14, which was unable to interact with wt 1a (Fig. 4), did not form a coprecipitable complex with cotranslated 1a-Δ502 (lanes 2 and 6). Finally, 1a-Δ599, which was unable to interact with wt 2a (Fig. 2), did not form a coprecipitable complex with F140-14 (lanes 4 and 8).

1a and 2a interact by direct protein-protein contact. To determine whether cellular factors present in the reticulocyte extract are required to mediate the 1a-2a interaction, we expressed biochemically useful amounts of the 1a helicase-like domain in *E. coli*, using the IPTG-inducible pET system (36). For ease of purification, an 11-amino-acid epitope recognized by MAb 12CA5 (41) was added to the N terminus of the helicase-like domain of 1a-Δ502 (Fig. 2), creating polypeptide 1a-Δ502ET. *E. coli* BL21(DE3), when harboring this recombinant plasmid, synthesized a polypeptide of the expected size (approximately 60 kDa) after induction with IPTG (Fig. 6A, lane 5) but not before (lane 3). After sonication, approximately 25% of this polypeptide was in the soluble fraction and the remainder was in the insoluble pellet. Further analyses were done with the soluble fraction. In a Western blot, the 60-kDa polypeptide reacted with anti-1a serum (Fig. 6D, lanes 3, 6, and 7). This polypeptide was also able to preferentially bind 2a protein synthesized by reticulocyte lysates when it was fixed on a membrane in a far Western assay (data not shown). These results confirm the identity of 1a-Δ502ET and demonstrate that it is functional in binding 2a.

Before determination of whether 1a-Δ502ET can bind Ac2a in a specific manner, the polypeptide was first enriched by passage through a DEAE–Bio-Gel A resin (Bio-Rad), where it was located in the flowthrough pool. We then affinity purified 1a-Δ502ET by binding the N-terminal epitope tag with MAb 12CA5 and recovered the MAb/1a-Δ502ET complex through attachment of the MAb to protein G-Sepharose beads. The protein G-Sepharose beads and the immune complexes were incubated with Ac2a and washed extensively as described in Materials and Methods. The protein complex on the protein G-Sepharose beads was then eluted by two methods: (i) denaturing of the complex with Laemmli sample buffer (Fig. 6B, lane 6) or (ii) elution with 20 mM glycine-Cl (pH 2.5), which elutes fewer of the IgG molecules (lane 7). Either elution condition produced only five visible polypeptides after staining with silver: 1a-Δ502ET, Ac-2a, the two differently modified forms of IgG heavy chain, and IgG light chain. The bands migrated at approximately the expected positions in comparison with controls (lanes 2 to 5). In addition, the Ac2a band reacted

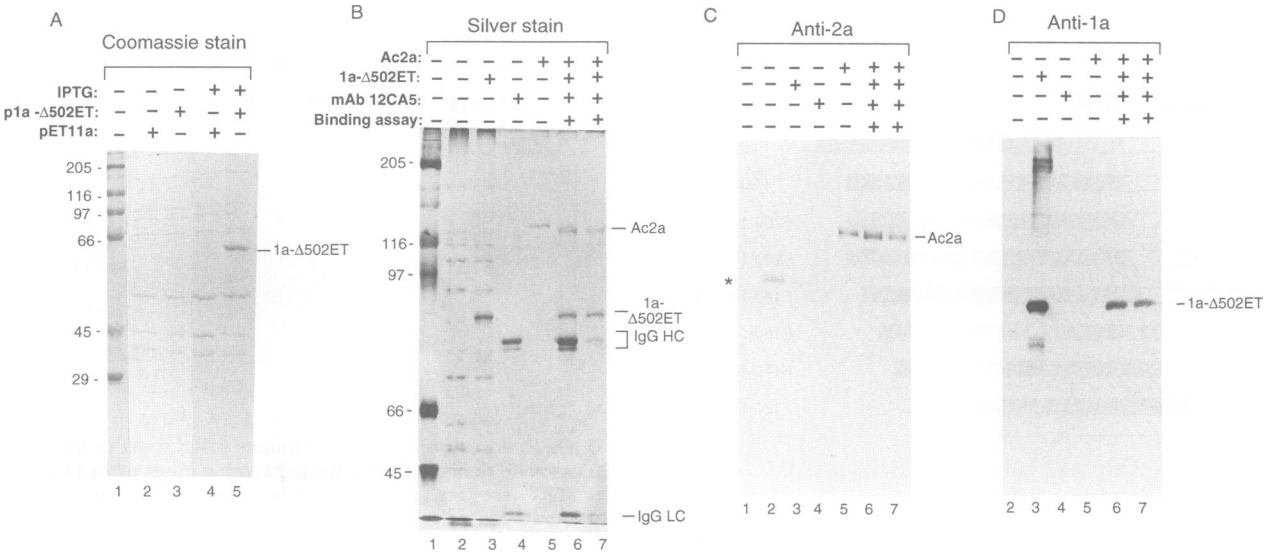

FIG. 6. Evidence that 1a and 2a interaction is via protein-protein interaction. (A) Expression of polypeptide 1a-Δ502ET (identified at the right) in *E. coli* BL21(DE3). Total *E. coli* lysates from a strain harboring pET11a (lanes 2 and 4) or p1aΔ502ET (lanes 3 and 5) were prepared with and without IPTG induction, as indicated, electrophoresed on an SDS–10% polyacrylamide gel, and stained with Coomassie brilliant blue R-250. Sizes of the molecular mass markers (lane 1) are indicated in kilodaltons at the left. (B) Evidence that highly purified Ac2a and 1a-Δ502ET complex does not contain any other cellular polypeptides. A DEAE–Bio-Gel A fraction enriched for 1a-Δ502ET extract was loaded in lane 3, and an equivalent extract from *E. coli* that did not express 1a-Δ502ET was loaded in lane 2 for comparison. To provide further markers, the other components of the binding assay, MAb 12CA5, and the Ac2a polypeptide (see Results) were loaded in lanes 4 and 5, respectively. For the binding assay, 1a-Δ502ET was affinity purified from the DEAE extract by complexing it with MAb 12CA5 and protein G-Sepharose. Ac2a was then added, and the complex was extensively washed (Materials and Methods). The remaining bound proteins were then eluted from the protein G-Sepharose either nonspecifically, by denaturation in Laemmli sample buffer (lane 6), or by a pH 2.5 treatment (lane 7), which appears to preferentially elute proteins bound by MAb 12CA5 while leaving most of the MAb 12CA5 attached to the protein G-Sepharose. The proteins were electrophoresed on an SDS–8% polyacrylamide gel, and the polypeptides were visualized by staining with silver (17). The slight difference in Ac2a migration before (lane 5) and after (lanes 6 and 7) immunoprecipitation may be due to residual LiCl from salt washes in the immunoprecipitations (25). Sizes of the molecular mass markers (lane 1) are indicated on the left, and the positions of Ac2a, 1a-Δ502ET, and the IgG heavy chain (HC) and light chain (LC) of MAb 12CA5 are indicated on the right. (C) Western blot analysis of duplicate samples from panel B. Antisera against 2a reacted with Ac2a (lanes 5 to 7) and an *E. coli* polypeptide (*) that is not derived from either BMV 1a or 2a. The secondary antibody was a goat anti-rabbit IgG molecule that had been conjugated to peroxidase (Sigma). The signals were visualized with a chemiluminescence detection system (Amersham). (D) Western analysis of duplicate samples from panel B, probed with anti-1a serum. The blot was processed in the same way as was the blot in panel C except that lane 1 (molecular weight markers) was omitted. The remaining lanes are numbered as in Fig. 6B and C.

with anti-2a serum in a Western blot (Fig. 6C, lanes 5 to 7), and the 1a-Δ502ET band reacted with anti-1a serum (Fig. 6D, lanes 6 and 7). When MAb 12CA5 was replaced by purified goat IgG molecules in the reaction or when 1a-Δ501ET was left out of the reaction, no Ac2a was detected (data not shown). If the reaction was heated to 45°C for 5 min, the amount of Ac2a present was reduced (data not shown), consistent with our previous result that the complex is labile at 45°C (25). Thus, no apparent factors from plants, reticulocyte lysates, *E. coli*, or the Sf21 insect cells used for production of Ac2a are required for 1a-2a interaction.

DISCUSSION

Recently we have shown that the BMV 1a and 2a RNA replication proteins form a complex in vivo and in vitro (25). In the experiments reported here, we have demonstrated that 1a and 2a interact directly and have mapped the regions required for this interaction. The mapping data from 1a insertion mutants, N- and C-terminal deletions of 1a and 2a, and 2a-3a fusions were all consistent. Together, these data show that sequences within the helicase-like domain of 1a bind to sequences within a 115-residue region in the N-terminal segment preceding the polymerase-like domain of 2a. These results are in agreement with other observations suggesting that the interaction between 1a and 2a may mediate the coordinated action of their polymerase-, helicase-, and methyltransferase-like domains (3, 27, 39), and recently we have discussed models for how coordination of these activities might facilitate RNA replication (1).

1a and 2a interact directly. Our previous results demonstrated that the 1a-2a interaction does not require BMV RNA or plant factors (25). However, we were not able to rule out the involvement of some factor(s) present in the reticulocyte lysates used to express 1a and 2a. We have now found that 2a purified from insect cells can bind to the affinity-purified, MAb-immobilized 1a helicase-like domain in the absence of any other detectable polypeptides (Fig. 6). It therefore appears that the 1a-2a interaction is direct and does not require additional factors. The final product of such binding reactions, when stained with silver nitrate, produced only the expected bands (1a-Δ502ET, Ac-2a, and the 12CA5 antibody; Fig. 6B). If any other factor is required for this in vitro 1a-2a interaction, it either is present at a much lower stoichiometry than is either 1a or 2a or migrates at the dye front of the gel.

Correlation between the effects of 1a and 2a mutations on in vitro interaction and in vivo RNA replication. A variety of results, including the effects of exchanging 1a and 2a genes between related bromoviruses (see below), suggest that

1a-2a interaction is essential for one or more steps of BMV RNA synthesis in vivo. The results reported here further elaborate and provide biochemical support for this hypothesis. Our mapping results correlated well with previous genetic analyses of 1a and 2a (28, 29, 39, 40). With the 2a polypeptide, we have determined that sequences between amino acids 25 and 140 of the N-terminal segment are necessary and sufficient for binding 1a in vitro (Fig. 3 and 4), although other domains may enhance the stability of the 1a-2a interaction in vivo. Traynor et al. (40) found the same 2a N-terminal region to be required for RNA replication in vivo. Moreover, in-frame N-terminal deletions PT14, PT16, and PT18, which were, respectively, wt, severely reduced, and abolished for binding 1a, were wt, severely reduced, and null for RNA replication in vivo (40). Thus, one required function of the 2a N terminus appears to be the ability to coordinate 1a-2a interaction in vivo.

This hypothesis is in good agreement with the results of Allison et al. (3) and Traynor and Ahlquist (39). CCMV is closely related to BMV. The two viruses are nearly identical in genome organization, share extensive homology in their amino acid and nucleotide sequences, and will both replicate BMV RNA3 templates in vivo (3, 39). However, the replication proteins of the two viruses will not support BMV RNA3 replication when paired in heterologous 1a/2a combinations (1, 3, 27). To determine the sequence(s) responsible for this specificity, Traynor and Ahlquist (39) exchanged segments between the BMV and CCMV 2a genes and found that a low level of RNA replication by the BMV 1a/CCMV 2a combination could be achieved by replacing the N terminus of CCMV 2a with the corresponding sequence of 358 amino acid residues from BMV 2a. Since this segment encompasses the 115 residues of the 2a N terminus required to bind 1a, perhaps the binding between 1a and 2a confers specificity in their interaction.

Results from assays with the 1a insertion mutants further contribute to the correlation between RNA replication and 1a-2a interaction. All seven mutants that were viable in vivo were still able to bind 2a in vitro (Fig. 1). In addition, 1a mutant PK19 was temperature sensitive for both RNA replication and interaction with 2a (25). Several 1a mutants that were not able to replicate in vivo were still able to bind 2a (Fig. 1), suggesting that protein-protein interaction can be separated from 1a enzymatic activities. Similar results were observed in the herpes simplex virus helicase-primase protein complex (42).

The central portion of the 1a polypeptide may be a molecular hinge that separates the methyltransferase- and helicase-like domains. Mutations in this region can affect 1a-2a interaction, as observed with PK16, which is also defective for replication (29). However, the intact putative hinge region is not required for 1a-2a interaction. 1a-Δ502, which is missing the methyltransferase-like domain and most of the putative hinge region, including the site of PK16 insertion, binds 2a better than does wt 1a. This result can be explained if the putative hinge is flexible enough, at least in vitro, to allow the methyltransferase-like domain to hinder 2a access to, or stable interaction with, the helicase-like domain. Removal of the methyltransferase-like domain would then enhance the 1a-2a interaction in vitro.

Thus, all 1a and 2a mutations identified to date that prevent stable 1a-2a interaction in vitro also affect RNA replication in vivo. These multiple, independent correlations strongly suggest that the binding regions in 1a and 2a are important for the function of the BMV RNA replicase.

Implications of BMV 1a-2a interaction for replication of other positive-strand RNA viruses. TMV encodes two overlapping proteins, p126 and p183, which are required for RNA replication (14, 21). p126 is an analog of BMV 1a, while p183 contains all three conserved domains found in BMV 1a and 2a (i.e., the putative methyltransferase, helicase, and polymerase domains; Fig. 7). This observation in part led to previous suggestions that the BMV replication proteins may have to interact to bring the three domains close together for functional reasons (1, 3) and was one of our original motives for examining the physical interaction between 1a and 2a. Since the helicase-like domain appears to interact directly with the N terminus of 2a, it is now possible to envision the two proteins as a complex diagrammed in Fig. 7. This alignment of 1a and 2a bears a striking resemblance to the linear organization of the three conserved domains in p183.

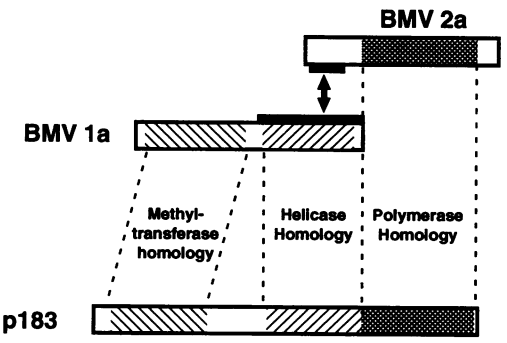

FIG. 7. Summary of interacting domains in BMV 1a and 2a and comparison with TMV p183. The polymerase-, helicase-, and methyltransferase-like domains of the BMV 1a and 2a polypeptides and TMV p183 are represented by different shadings, as indicated. Regions in BMV 1a and 2a containing sequences necessary and sufficient for their mutual interaction are indicated with solid black bars.

The diverse alphavirus-like viruses that share conserved helicase-, polymerase-, and methyltransferase-like domains (15, 18, 38) contain a number of viruses besides BMV that express their helicase- and polymerase-like domains in separate polypeptides. Such proteins may commonly interact, since for both BMV and Sindbis virus, the putative helicase and polymerase proteins have been extracted from infected cells as part of an immunoprecipitable complex (7, 25, 35). It is interesting to note that Sindbis virus and other animal alphaviruses (38), rubella virus (9), and a variety of tripartite RNA plant viruses (15) all contain a less conserved N-terminal segment in front of the polymerase-like polypeptide, just as in BMV 2a. The results presented here suggest that, as in BMV 2a, these N-terminal segments may have evolved in part to mediate noncovalent interaction with the cognate helicase-like protein. Consistent with this proposed role, no corresponding segment is present in TMV p183, in which the putative helicase and polymerase are covalently linked in a single translation unit with few or no intervening amino acids (Fig. 7).

Just as TMV RNA replication requires p126 as well as p183, several results suggest that the BMV RNA replication machinery may be more complex or dynamic than a simple 1a-2a dimer. Similar to the overexpression of TMV p126 relative to p183, for example, BMV RNA replication is more sensitive to reductions in 1a gene expression than to reductions in 2a gene expression (29, 39). Thus, further issues to be investigated include the stoichiometry of the helicase-like and polymerase-like proteins in the replication complexes of BMV, TMV, and other viruses, whether distinct replication complexes mediate different steps in RNA replication, whether BMV proteins 1a or 2a or their homologs have

independent functions outside of a complex, and the probable association of host proteins with viral RNA replication.

ACKNOWLEDGMENTS

We thank R. Hershberger and R. Quadt for critically reading the manuscript, R. Hershberger for generously providing recombinant Ac-2a and Ac-wt Sf21 lysates, and the Scripps Research Institute and Carol Eng for antiserum 12CA5.

This research was supported by the National Institutes of Health under Public Health Service grant GM35072. C.C.K. was supported by National Science Foundation Plant Biology postdoctoral fellowship DIR9104366.

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